

Project Report

On

**STATISTICAL STUDY ON YOUTUBE
USAGE DURING COVID-19**

Submitted

in partial fulfilment of the requirements for the degree of

BACHELOR OF SCIENCE

in

MATHEMATICS

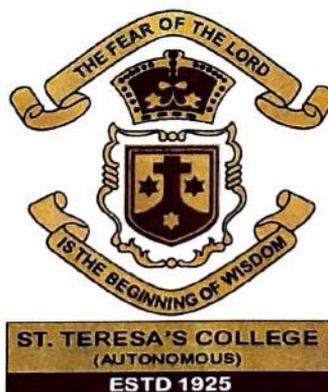
by

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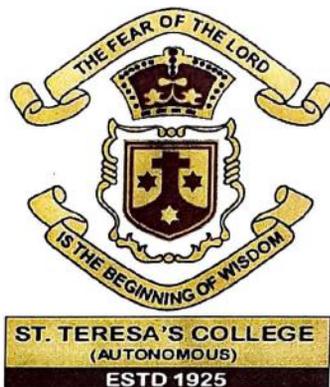


**DEPARTMENT OF MATHEMATICS
ST. TERESA'S COLLEGE (AUTONOMOUS)**

ERNAKULAM, KOCHI - 682011

MARCH 2022

ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM

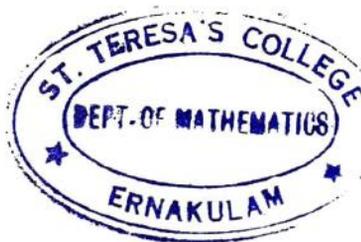


CERTIFICATE

This is to certify that the dissertation entitled, **STATISTICAL STUDY ON YOUTUBE USAGE DURING COVID-19** is a bonafide record of the work done by Ms. **NANDHITHA ANN MARY** under my guidance as partial fulfillment of the award of the degree of **Bachelor of Science in Mathematics** at St. Teresa's College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam. No part of this work has been submitted for any other degree elsewhere.

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DECLARATION

I hereby declare that the work presented in this project is based on the original work done by me under the guidance of Dr.Elizabeth Reshma M T, Assistant Professor, Department of Mathematics, St. Teresa's College(Autonomous), Ernakulam and has not been included in any other project submitted previously for the award of any degree.

Ernakulam.

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First of all, I would like to thank our Almighty God for giving us the determination to complete this project and to improve ourselves . The courage to make this work done with the strength, time, and effort that the proponents have.

Secondly, I want to convey my heartfelt appreciation and gratitude to our guide Dr. Elizabeth Reshma M T (Assistant Professor) and to Smt.Rosmin Raju for giving enough information and guiding through the project. In regard with this we would like to thank Dr.Ursala Paul (H.O.D of Mathematics) for the continuous support,patience and motivation.

I would like to thank my group members for not letting anyone lose hope and giving each of us determination,encouragement and for unity.I'm quite grateful to all my friends and family for their great support and encouragement.

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Chapter 1

INTRODUCTION

A year ago on 11 March 2020, WHO declared that the global COVID-19 epidemics had become so widespread that they constituted a pandemic. COVID-19 has affected our lives in many ways. The usage of social media has been drastically increased. So here we are going to find out the effect of COVID-19 on YouTube usage.

1.1 ABOUT STATISTICS AND COLLECTION OF DATA

Statistics is a mathematical science including methods of collecting, organizing and analyzing data in such a way that meaningful conclusions can be drawn from them.

In any field of scientific investigation, collection and analysis of numerical information concerning the problem under investigation is an essential part. Statistics has developed powerful tools which enable us to make valid inferences regarding characteristics of a population by studying only a representative part of it called a sample, with its reliability measured in terms of probability.

In general, its investigation and analysis fall into two broad categories called descriptive and inferential statistics. Statistics are used to describe the characteristics of group. These characteristics are referred as variables.

Data collection is the process of gathering and measuring information on targeted variables in an established system. There are two types of data 1) Primary Data and 2) Secondary Data. Data collected by an investigator for the purpose of investigation at hand is called primary data and that collected by other for some other purpose and used by the investigator is called secondary data.

The process of collecting the required information from the units or elements of the population is called enumeration. If the information is collected from every unit of the population the method of collection is known as complete enumeration or census. If the information is collected only from a representative part of it the method of collection is called sampling. Here we are collecting data from a representative part of a unit using questionnaire.

1.2 SIGNIFICANCE OF THE STUDY

Technology advancements and social media create opportunities to keep people safe, informed and connected. However, the same tools also enable and amplify the current infodemic that continues to undermine the global response and jeopardizes measures to control the pandemic. YouTube is the 2nd largest search engine next to google. It influence people in different ways.

This is a study to understand the impact of COVID-19 on YouTube usage

1.3 OBJECTIVES

- To examine the association between COVID-19 and youtube usage.
- To find the variables that has an impact on usage of youtube during COVID-19.
- To find whether youtube has become a profitable media.
- To create the rank list of preferred genres of videos in youtube.

Chapter 2

DATA DESCRIPTION

2.1 SOURCE OF DATA

Here we use primary data for studying the impact of COVID-19 on YouTube usage. We used google forms for collecting data. The target is the people of Ernakulam district. We have collected 334 samples for the survey. YouTube is used by every age group thus the survey is not concentrated in a special age group. Online survey made it more easy in collecting data and was cost-effective.

2.2 DATA DESCRIPTION

The variables are,

- **Age:**
The Age of the person filling the form was recorded.
- **Gender:**
Whether the person is Male Or Female.
- **Place of Residence:**
Whether He/she lives in Urban or Rural area.
- **Time Spent (Before and during COVID-19) :**
Number of hours spent by a person on YouTube each day (on an average basis) before and during COVID-19 is inquired. Data is

collected in form of class intervals such as 0hr,1-2 hrs,3-4 hrs,5-6 hrs,Above 7 hrs

- Preferred Genre of Videos:

To create a rank list of preferred genre of videos in YouTube. Common types of genres are asked in MCQ pattern.

Where options such as : Branded content(NBS,Buzz feed,etc.), Comedy, Cookery, Devotional, Sports, Gaming, Music Playlist/Videos, Original Films/Animation, Re-run of TV Shows, Tutorial/ Educational, Vlog, Sports, Others

- Network Connection :

Whether the person is using mobile data or WiFi or both.And about setting up a new network connection during COVID-19

- Income out of YouTube:

Whether the person has started earning with the help of YouTube : By Creating a new YouTube channel or By Starting a New business by learning from YouTube.

Age and Time Spent (Before and During COVID-19) are the quantitative variables. Gender,place of residence,preferred genre of videos,network connection and income out of YouTube are the qualitative variables.

Chapter 3

METHODOLOGY

Methodology simply refers to the method we use to conduct an investigation. Methodology of data includes sources of data, sampling design and presentation. Here we use regression analysis, chi square test and graphical representation to study the impact of COVID-19 on YouTube usage.

3.1 GRAPHICAL REPRESENTATION

Graphical representation is used for analysing numerical data using charts and graphs. In graph, statistical data is represented in the form of lines or curves. Representational graphics can quickly illustrate general behaviour and highlight phenomena and relationships between data points that may otherwise be overlooked and may contribute to predictions and better data driven decisions. There are different types of graphical representation. Some of them are bar graphs, line graphs, histograms, line plot, frequency table, stem and leaf plot, circle graph, etc.

3.2 CHI SQUARE TEST OF INDEPENDENCE

The chi square test of independence is used to determine the association between the categorical variables. It is considered as a non-parametric test. It is mostly used to test statistical independence. For this test, the data must meet the following requirements :

- Two categorical variables.
- Relatively large sample size.
- Categories of variables (two or more).
- Independence of observations.

Steps of chi square test :

- Specify the null and alternative hypothesis :
 1. The null hypothesis (H_0) is a statement of no effect or association between two or more variables.
 2. The alternative hypothesis (H_1) is the statement that there is an effect or association between variables.
- Specify the level of significance :

The significance level is generally set at 0.05. This means that there is a 5 percentage chance to reject the null hypothesis.
- Calculate the value of X^2 :
$$X^2 = \sum (O-E)^2/E$$

where O=Observed value and E=Expected value.

The degree of freedom = $(r-1)(c-1)$

where r is the number of rows and c is the number of column.
- Find the corresponding p value
- Draw a conclusion:

If the p value is numerically less than the significance level, we reject the null hypothesis. Otherwise we say, we fail to reject the null hypothesis.

3.3 REGRESSION ANALYSIS

Regression analysis is a statistical technique for investigating and modelling the relationship between variables. It is a method to discover the relationship between one dependent and more than one independent variables. The simplest model is multiple linear regression model. It is a model in which dependent variable y is influenced by p independent variables X_1, X_2, \dots, X_p .

The model is defined by,

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \dots + \beta_p X_p + \epsilon ,$$

where Y is the response value , X_1, X_2, \dots, X_p are independent variables $\beta_0, \beta_1, \dots, \beta_p$ are unknown parameters to be estimated and ϵ is the standard error.

Steps of Regression Analysis :

- Multiple regression using the Data Analysis Add-in in Excel:
 1. Select the range for independent variables under Input X Range
 2. Select the range for dependent variable under Input Y Range
- Interpreting the ANOVA table:

In Excel's ANOVA table, Significance F is the p-value for the F-test of overall significance.

If this p-value for the overall F-test is less than significance level, we can conclude that the regression model as a whole is statistically significant.
- Interpreting the Regression coefficients table:
 1. The p-values for the coefficients indicate whether the dependent variable is statistically significant. If the p-value is less than the significance level, we reject the null hypothesis that indicates no relationship.

2. Write the regression equation.
3. Check whether the coefficients of independent variables is positive or negative.
4. The positive sign indicates that there is a positive association between the two variables.
5. The negative sign indicates that there is a negative association between the two variables.

Chapter 4

DATA ANALYSIS

4.1 GRAPHICAL REPRESENTATION

Graphical representation refers to the use of charts and graphs to visually display, analyze, clarify, and interpret numerical data and other qualitative structures.

In this study we have received 334 responses out of which 104 responses are from males and 230 from females.

4.1.1 AGE

The answers were collected from people of different age groups. given below is the histogram showing the ages of people who have responded to our questions

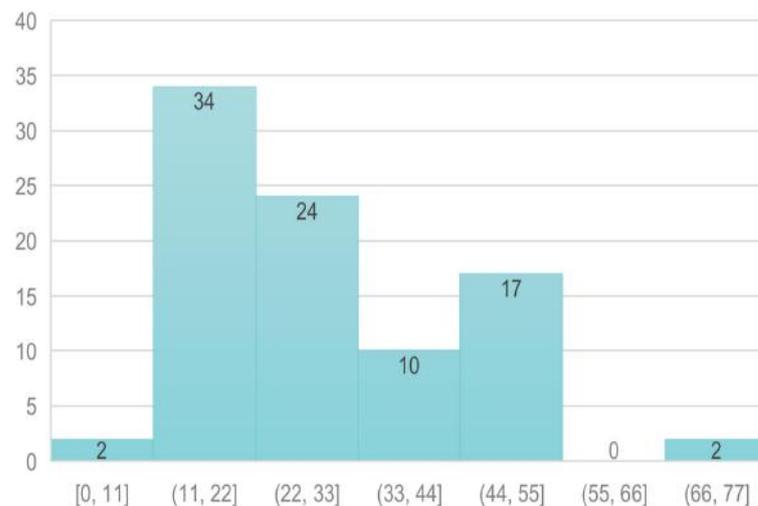


Figure 4.1: No.of people who have filled the questionnaire in different age groups.

4.1.2 TIME SPENT ON YOUTUBE

The time spent by watching videos in YouTube were also collected. Given below is the double bar diagram showing the hours spent by people before and during Covid-19.

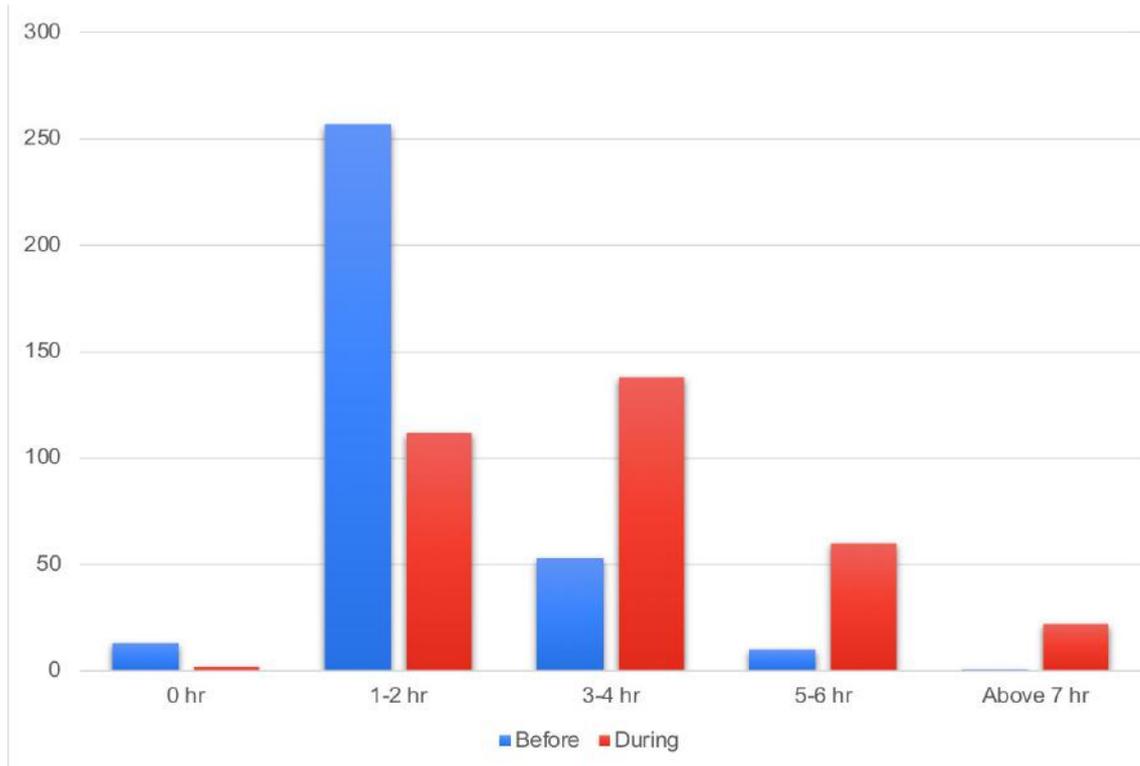


Figure 4.2: Time spent on YouTube per day before and during covid 19

As we can see, the time spent on YouTube before Covid-19 was only 1-2 hr for most people, whereas during Covid 19 it increased to 3-4 hr for a huge population. Thus there is an impact on time spent by people in YouTube by Covid 19 .

4.1.3 PREFERRED GENRES

While YouTube is being widely used by many, we wanted to know which genre/type of videos were preferred by them. Given below is the bar diagram showing the the genres preferred by the people of Ernakulam district.

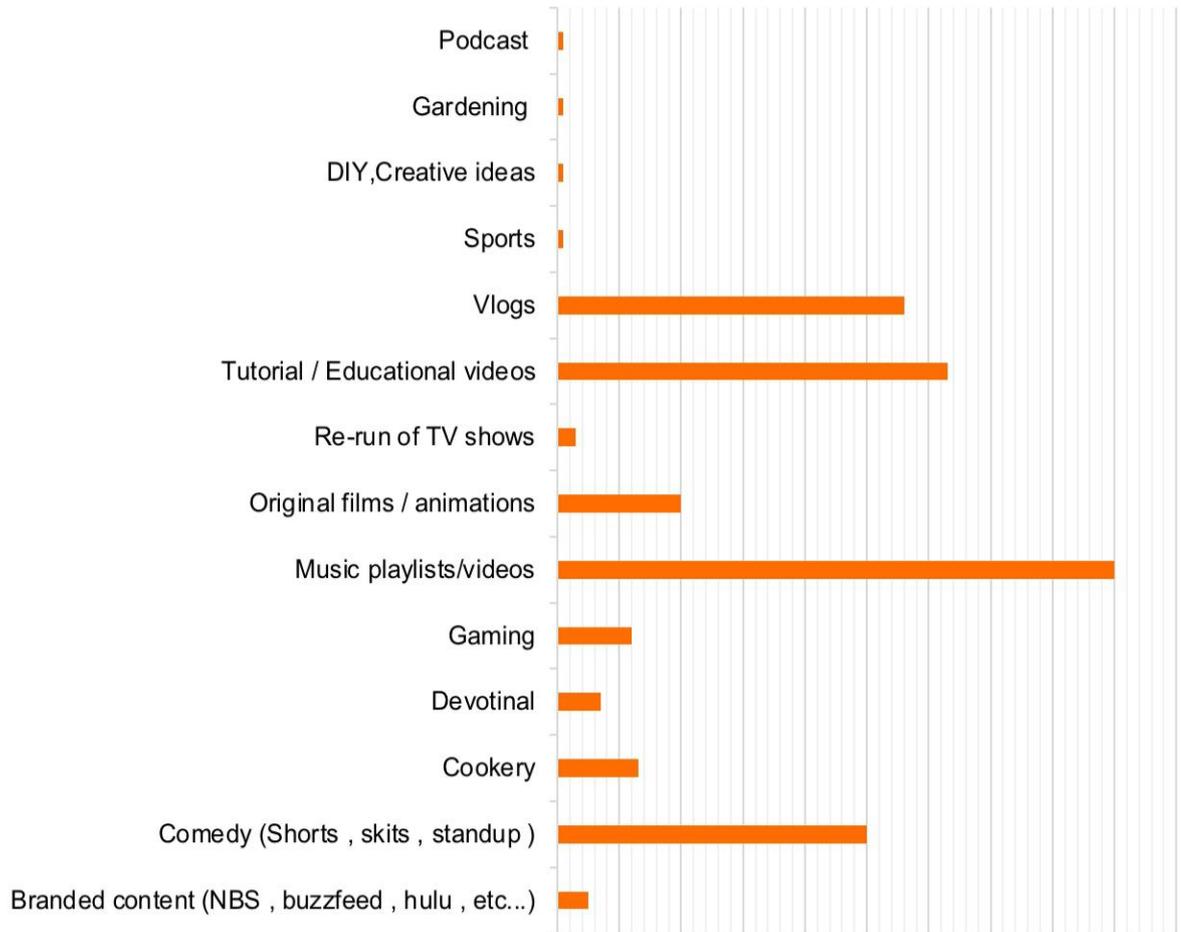


Figure 4.3: Types of videos preferred by people in the Ernakulam district

It is clear from the table above that the genre that ranks First is music playlists/videos and the genres that ranks Second and Third are tutorial/educational videos and vlogs respectively.

We found out that the genres preferred by males and females were quite different. Given below is the bar diagram showing the genres preferred by males and females. As we can see the genre that is preferred the most by both males and females is Music playlists/videos. The second most preferred genre among males are tutorial/educational videos whereas among females it is Vlogs.

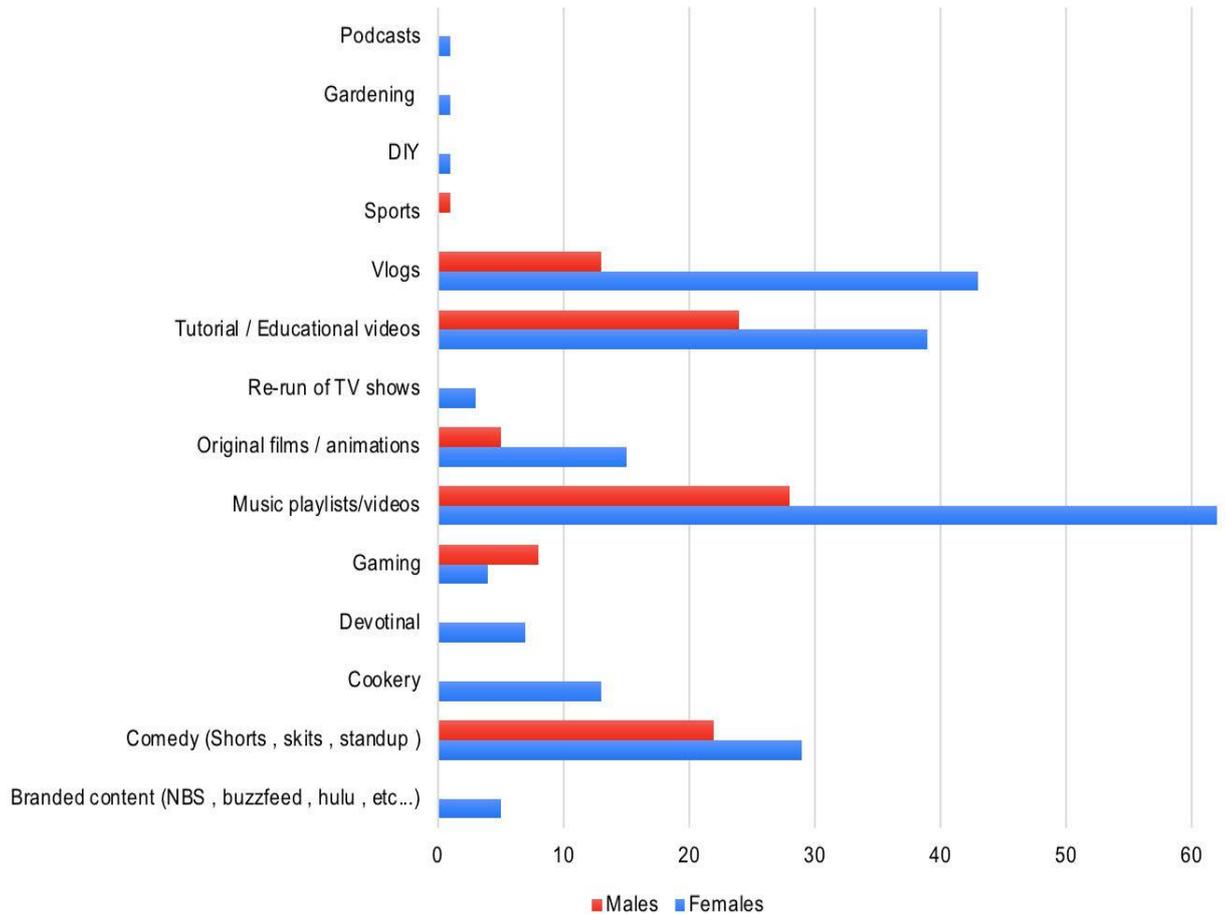


Figure 4.4: Types of videos preferred by males and females in the district of Ernakulam.

4.1.4 NETWORK CONNECTION

While collecting responses we have also inquired the type of network connection used by them. Given below is the Pie chart showing the type of network connection used by people in the Ernakulam district.

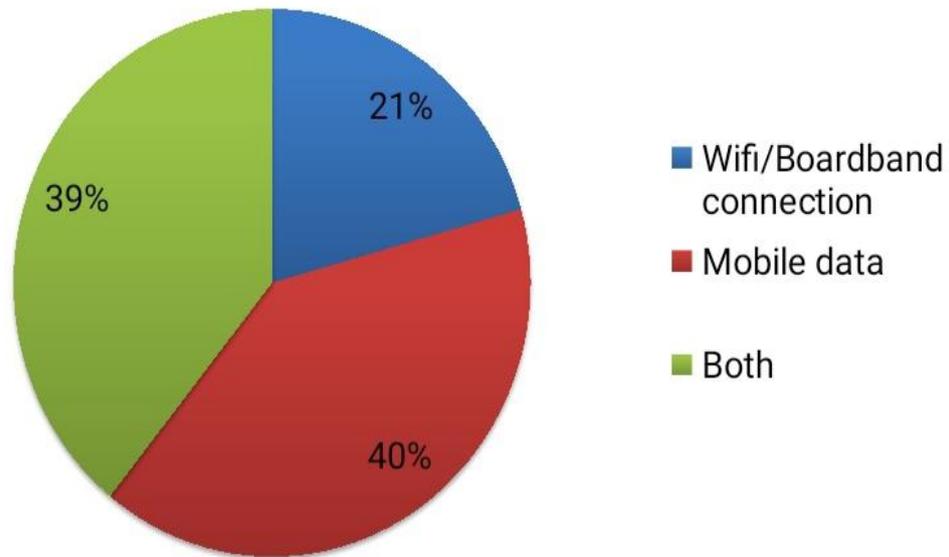


Figure 4.5: The types of network connection used by the people in Ernakulam district.

Besides inquiring the type of network connection used by them we have also inquired about their locality i.e., whether the respondent lives in urban area or rural area. Given below is the double bar diagram showing the type of network connection used by urban people and rural people

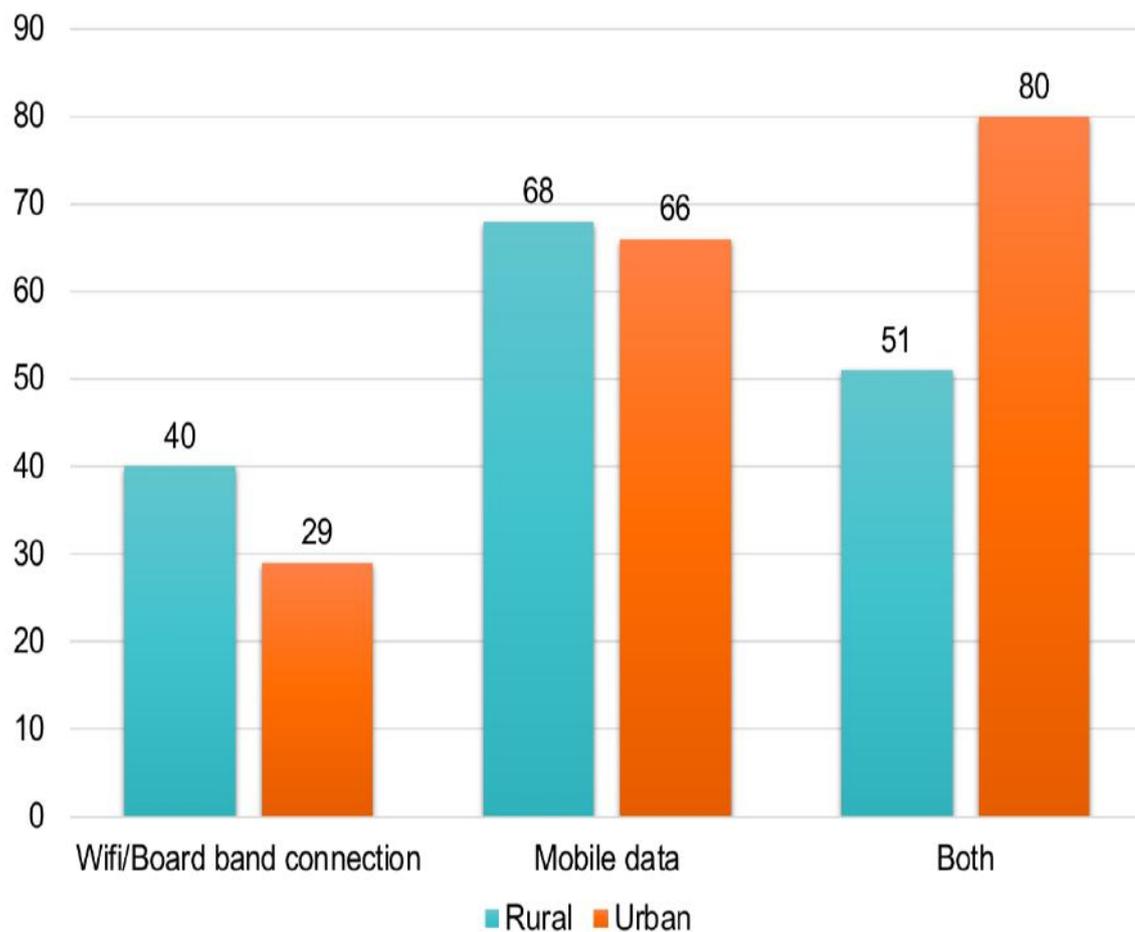


Figure 4.6: Type of network connection used by urban people and rural people

The graph clearly shows us that most of the people living in urban area use both types of connection i.e, WiFi Mobile data whereas the people living in rural area mostly use Mobile data alone.

We have also collected the data regarding setting up of new network connection during the pandemic. The results are as shown below.

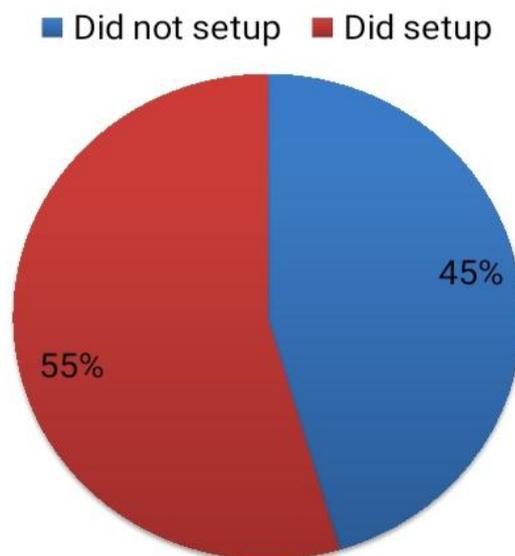


Figure 4.7: Pie chart showing the percentage of people who have set up a new network connection during Covid 19 versus those who have not.

It is evident that only 45 percentage of people have set up a new network whereas majority of the population has continued using the network they had before.

4.1.5 INCOME FROM YOUTUBE

We inquired whether the respondent makes any income from YouTube (either through their own channel or by starting a new business by learning from YouTube) to which we have obtained the results as shown below.

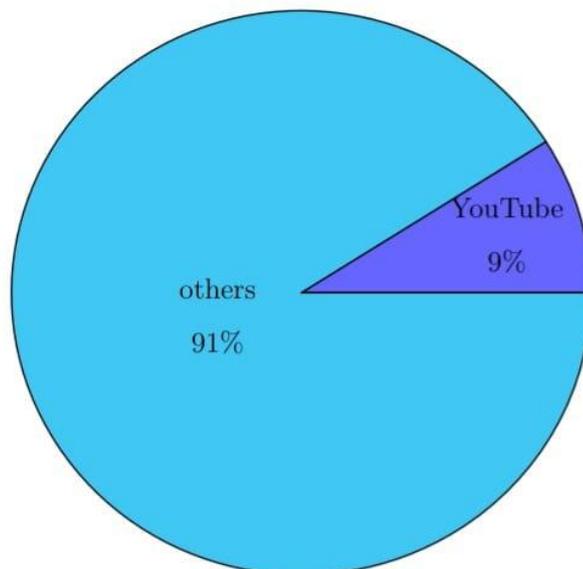


Figure 4.8: Pie chart showing the percentage of respondents who makes an income out of YouTube.

As the Pie chart shows only 9 percentage of the population makes money out of YouTube now, from this 9 percentage of the population, we observed the result (given in the Graph below) showing how many makes money using YouTube channel and how many makes money by running business by learning from YouTube.

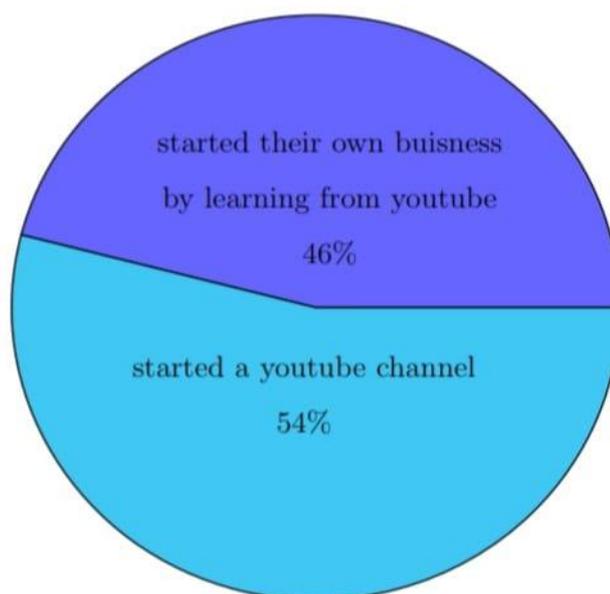


Figure 4.9: Pie chart showing the percentage of people using different means to make money with YouTube.

About 54 percentage of people out of the 9 percentage of total population has started their own YouTube channel to make an income. Whereas the rest i.e, 46 percent out of the 9 percent of population has started doing their own business by learning from YouTube

4.2 CHI-SQUARE TEST

Our objective is to 'Compare the use of YouTube among the viewers before and during COVID-19' So we fix,

H_0 : There is no association between two attributes viz., COVID-19 and YouTube usage

H_1 : There is association between two attributes viz., COVID-19 and YouTube usage

	Observations(O)					
	0hr	1-2 hr	3-4 hr	5-6 hr	Above 7 hr	Total
Before COVID-19	13	257	53	10	1	334
During COVID-19	2	112	138	60	22	334
Total	15	369	191	70	23	668

	Expected(E)					
	0hr	1-2 hr	3-4 hr	5-6 hr	Above 7 hr	Total
Before COVID-19	7.5	184.5	95.5	35	11.5	334
During COVID-19	7.5	184.5	95.5	35	11.5	334
Total	15	369	191	70	23	668

	$(O-E)^2/E$					
	0hr	1-2 hr	3-4 hr	5-6 hr	Above 7 hr	
Before COVID-19	4.033333	28.2892	18.913612	17.857142	9.5869565	
During COVID-19	4.033333	28.2892	18.913612	17.857142	9.5869565	

χ^2 Value	157.76041
Degree of freedom	4
p value	4.4178E-33

Since p value is less than alpha value, we reject H_0 .

Therefore there is association between two attributes viz., COVID 19 and YouTube usage

4.3 REGRESSION ANALYSIS

- We performed regression analysis to estimate the relationship between a dependent variables and more than one independent variables. The variables in this analysis are,
 - Dependent variable: Time spend on YouTube during COVID 19
 - Independent variables: Age, Income Gender, Place of residence, network connection, Change in data recharging.

Regression Statistics	
multiple R	0.23662252
R square	0.055990217
Adjusted R square	0.038615804
Standard Error	0.878687435
Observations	333

ANOVA

	df	SS	MS	F	Significance F
Regression	6	14.9290431	2.488173849	3.222567359	0.004339123
Residual	326	251.7075935	0.772109183		
Total	332	266.6366366			

	Coefficients	Standard Error	t Stat	P-value
Intercept	2.895538532	0.2534348	11.42518128	1.18537E-25
Age	-0.011427623	0.003885235	-2.941295504	0.003502309
Gender	-0.067476899	0.108057552	-0.624453335	0.532766675
Place of residence	0.039866673	0.098715772	0.403853119	0.686585518
Network connection	-0.069934192	0.054486029	-1.283525232	0.200219723
Change in data recharging	-0.113466051	0.089125793	-1.273100041	0.20388964
Income	-0.410705604	0.178774783	-2.297335214	0.022232848

- The p values of the variables, Gender, Place of residence, network connection, Change in data recharging are greater than significance level=0.05. So we excluded these variables, because it doesn't matter in predicting the outcome.
- Then we rerun the regression analysis on the remaining independent variables ,i.e, age and income.

Regression Statistics	
multiple R	0.211564548
R square	0.044759558
Adjusted R square	0.038970222
Standard Error	0.878535453
Observations	333

ANOVA

	df	SS	MS	F	Significance F
Regression	2	11.93453794	5.967268972	7.731380193	0.000523113
Residual	330	254.7020987	0.771824541		
Total	332	266.6366366			

	Coefficients	Standard Error	t Stat	P-value
Intercept	2.67971036	0.195087969	13.73590784	2.86244E-34
Age	-0.01202698	0.00385586	-3.119143107	0.001973616
Income	-0.41361141	0.168187789	-2.459223785	0.014436452

- In Anova table, 'Significance F' value tells how statistically significant the results are. It is the p-value of F. Our p-value is 0.000523113, and is less than the significance level=0.05. So we can conclude that our regression model is statistically significant.
- For our two independent variables, the the p values are less than significance level=0.05. So we can reject the null hypothesis, that indicates no relationship. Therefore age and income are both statistically significant.

Here the regression equation is,

$$Y = 2.67971036 + -0.01202698X_1 + -0.41361141X_2$$

- In coefficients table, the coefficient for age is -0.01202698. There is a negative association between these two variables. That is, as age increases, the YouTube usage tends to decrease. The coefficient of income is -0.41361141. There is a negative association between these two variables. That is, as income increases, the YouTube usage tends to decrease.

Chapter 5

CONCLUSION

We performed regression analysis to find the relationship between a dependent variable and more than one independent variable and from that we came to find a relationship between them. We excluded the variables : gender, place of residence, network connection and change in data recharging because it doesn't matter in predicting the outcome. After excluding those and considering the remaining variables : age and income ,we rerun regression and found that our regression model is statistically significant. In coefficients table, the coefficient for age and income is negative , i.e , as age and income increases the YouTube usage tends to decrease. Thus there is a negative association between those two variables .

Our null hypothesis was that there is no association between COVID-19 and YouTube usage and the alternative hypothesis is that there is an association. Using Chi-Square Test we got a p-value less than alpha value thus we rejected the null hypothesis and accepted the alternative hypothesis. Therefore there exists an association between COVID - 19 and YouTube usage.

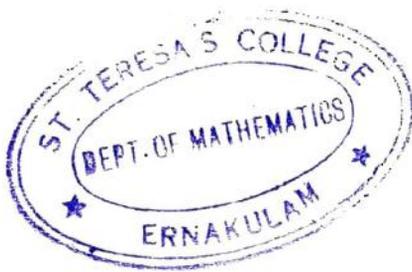
From the survey, we can conclude that the most preferred genres is music playlist/videos. The number of peoples who opted this genres are 90. The second rank of the preferred genres is goes to tutorial/educational videos. Between 60-70 number of people preferred

it. Among 50-60 number of peoples has chosen Vlogs as the preferred genres so third rank is goes to it. Number of peoples who had chosen comedy (shorts, skit, stand up) is 50 so forth rank is goes to it. Fifth rank is goes to original films / animation . The number of peoples that opted this genres are twenty. Cookery , devotional and gaming are other preferred genres with the sixth , seventh and eighth rank respectively.

Mainly it became profitable media in two ways . some peoples have their own YouTube channel and on other hand some had their own business by learning from YouTube. From the survey conducted 9% of the peoples make their own income from YouTube. Among that 9% people 54.2% peoples make income from their own YouTube channel . Remaining 47.5% peoples make income by starting their own business by learning from YouTube.

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Project Report

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**THE EFFECTS OF COVID-19 LOCKDOWN
ON SOCIAL INTERACTION**

Submitted

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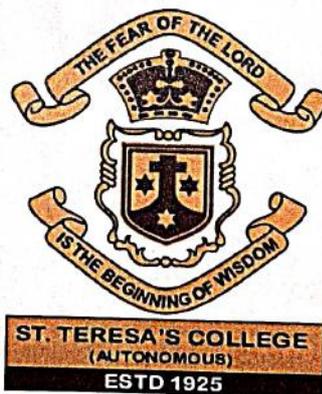
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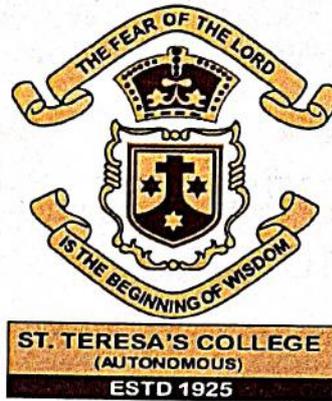


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APRIL 2022

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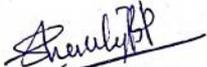


CERTIFICATE

This is to certify that the dissertation entitled, **THE EFFECTS OF COVID-19 LOCKDOWN ON SOCIAL INTERACTION** is a bonafide record of the work done by Ms. SONA BINU under my guidance as partial fulfillment of the award of the degree of Bachelor of Science in Mathematics at St. Teresa's College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam. No part of this work has been submitted for any other degree elsewhere.

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DECLARATION

I hereby declare that the work presented in this project is based on the original work done by me under the guidance of Smt. Shanty B P. Assistant Professor and Head, Department of Statistics, St. Teresa's College(Autonomous), Ernakulam and has not been included in any other project submitted previously for the award of any degree.

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Date: 06-05-2022



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Chapter 1

INTRODUCTION

Social interactions are greatly affected by the COVID-19 pandemic. In order to understand how the usage of protective equipment and social distancing affect the ability to communicate the emotions of others, thus, in order to effectively interact with others, we conducted online research on the crowd after the lockdown. The social distance with community members is adjusted in the opposite direction that is, people reduce social distance, leading to a sense of social connection in the community. In other words, fighting the epidemic together can strengthen cooperation and shared value between individuals, thereby creating the sense of unity and seeking common ground while reserving differences.

The COVID-19 pandemic, also identified by the World Health Organization as a corona virus pandemic, will cause severe acute respiratory syndrome. The COVID-19 pandemic was first reported in Wuhan, China in December 2019 and was declared as a pandemic on January 30, 2020 by World Health Organisation. The report of the Centres for Disease Control and Prevention and published epidemiological and virological research data show that there is evidence that COVID-19 is mainly through direct contact with respiratory droplets of symptomatic or even asymptomatic patients, or through contact with space and contamination objects and surface. to reduce the contact between people in public spaces, India and many other countries worldwide implemented a lockdown that came into effect on March 15th, 2020. The main aim of

the lockdown was to fight the corona virus outbreak by applying certain regulations. Civilians or other unauthorised persons were not allowed to leave their home unless it's an emergency. Social relations refer to family members, friends, neighbours and other colleagues. The quality of social relationships is affected by positive aspects such as emotional support from other sources, as well as negative aspects such as conflict and pressure. Social relations scientists often emphasize comfortable, relaxed and easy social relationships are important in a person's life and have a great impact on health, affecting their behavioural, psychosocial, and physiological states. Therefore, the purpose of the present study is to evaluate the effects of lockdown imposed by the government in India on the social relationships of the population. Due to this, government all across the world have taken unprecedented steps to respond to the virus and contain it. Countries are implementing various health control measures to lessen the test positivity rate and avoid the collapse of their health systems. Epidemiologists predicted 7 billion infections and 40 million deaths globally for the year 2020 if no control measures are taken. Thus, the government took immediate action. Therefore, the purpose of the present study is to evaluate the effects of social distancing imposed by the curfew program in India on the social relationships of the population.

1.0.1 Significance of study

The significance of this study is to understand the consequences of COVID-19 lockdown/social distancing due to quarantine on social interaction. Social relationships refer to the existing associations among family members, friends, neighbours, co-workers, and other associates. Due to the COVID-19 pandemic, the Indian government along with many other countries implemented lockdown. Recently, the WHO announced that social disconnection is a major public health challenge. Therefore, individuals are now facing the possibility of different forms of social isolation. As a result, the goal of our research is to assess the effects of lockdown on the population's social interactions.

1.0.2 Objectives

1. To study the impact of COVID-19 lockdown in social interactions/events.
2. To study the effect of COVID-19 lockdown in the relationship among people and their perception of empathy toward others.
3. To determine the psychological impact of the lockdown on people.

1.0.3 Statistics and types of Data

Statistics is the branch of mathematics that deals with the collection, organisation, analysis, interpretation, and presentation of massive amounts of data.

Individual pieces of factual information are recorded and used for analysis as data. Primary data and secondary data are two types of data. The term "primary data" refers to information obtained by the researcher himself. Surveys, observations, experiments, questionnaires, focus groups, interviews, and other primary data sources are examples. Once it is decided what type of study will be conducted, it is required to gather information about the study, which is usually in the form of data. A poll will be run to get the data for this information. Our survey was designed to find out how the population views the quarantine period, how it affects their connections with others, and the features of their social ties and communication with various demographic groups, such as family members and co-workers.

1.0.4 Processing of data

Data processing involves translating the answers on a questionnaire into a form that can be manipulated to produce statistics. Stages of data processing include Data collection, Data preparation, Data input, Processing, Data interpretation, Data storage. Before tabulation of primary data, it should be scrutinized for completeness, consistency, accuracy and editing.

1. **Completeness:** If the answer to some important question in a schedule or questionnaire is missing it becomes necessary to contact the

informant again and complete the missing information in the report in case such an information cannot be completed the schedule or questionnaire should be discarded or revised.

2. **Consistency:** Some information given by the respondent may not be compatible in the sense that and information furnished by the individual either does not justify some other information or is contradictory to the earlier one.
3. **Accuracy:** It is of vital importance if the data is inaccurate the conclusions drawn from it have no relevance or reliability. By checking the schedules for questionnaire only a little improvement can be made. In recent times checks have been evolved to attain accuracy.
4. **Editing:** To maintain homogeneity, the information sheets are checked to see whether the unit of information or measurement is the same in all the schedules. It should also be check whether or not the same information has been supply for a particular question in all the information sheets. The ambiguity arises due to various interpretations of the same question and should be removed. Once the primary that they have undergone the above four processes is this with for further analysis.

VARIABLES DESCRIPTION

1. **Age:** The age of the person filling the form was recorded. That is to understand whether the respondent is of the age below 20 or 20 and above.
2. **Gender:** Whether the person is male or female.
3. **Place of residence:** Whether the person is living in an urban area or rural area.
4. **Interaction with your family:** Whether the respondent's interaction with family increased, decreased or had no effect during the lockdown.
5. **Interaction with your friends:** Whether the respondent's interaction with friends increased, decreased or had no effect during the lockdown.
6. **Interaction with public:** Whether the respondent's interaction with public increased, decreased or had no effect during the lockdown.
7. **Understanding the facial expressions and emotions of people with face mask:** Whether it is always difficult, sometimes difficult or not difficult.
8. **Stress:** Whether the person faced stress during lockdown or not.
9. **Self-confidence:** Whether it increased or decreased after lockdown.

Chapter 2

DATA DESCRIPTION

2.0.1 Bar Graph

A bar graph is a graph that displays all of the data in the form of rectangular bars with heights proportionate to the values they represent. The graph's bars can be displayed vertically or horizontally. Bar graphs, commonly referred to as bar charts, are a visual representation of categorised data. It's one of the methods for dealing with data. A bar graph is a visual representation of data. It's a good tool for representing data that is unrelated to one another and does not need to be in any particular format while being represented in order. The bars provide a visual representation of amounts in several categories. The bar diagrams include two lines, the horizontal and vertical lines.

Grouped bar graph is also known as clustered bar graph. It's used for representing the discrete value of two or more categorical data sets. Rectangular bars are grouped by position for levels of one categorical variable, with the secondary category level within each group shown in the same colour. It can be demonstrated both horizontally and vertically.

2.0.2 Pie Chart

A pie chart is a circular chart divided into sectors, with the size of the data represented by the area of each sector. It's also referred to as a circle graph. Because a circle has 360 degrees, each group in the pie

chart will have a proportion of 360.

2.0.3 Chi-Square Distribution

For evaluating correlations between categorical data, the Chi-Square statistic is often used. The null hypothesis of the Chi-Square test is that the categorical variables in the population have no association; they are independent. When employing a cross tabulation, the Chi-Square statistic is most typically employed to evaluate Tests of Independence (A bi-variate table is another name for it.) The distributions of two categorical variables are shown in cross tabulation. The intersections of the categories of the variables appear in the table's cells at the same time. By analysing the observed pattern of the two variables, independence determines whether there is a link between them. If the factors were actually independent of each other, the cells' responses to the pattern would be predicted. One can examine if the observed cell counts are significantly different from the expected cell counts by computing the Chi-Square statistic and comparing it to a critical value from the distribution. The Chi-Square statistic can be calculated in a number of ways, all of which are simple and intuitive:

$$\sum \frac{(f_o - f_e)^2}{f_e}$$

where f_o = the observed frequency (the observed counts in the cells) and f_e = the expected frequency if no relationship existed between the variables. The Chi-Square statistic is based on the difference between what is observed in the data and what would be predicted if there were no association between the variables, as shown in the calculation.

Chapter 3

METHODOLOGY

A cross-sectional study using an online survey was conducted between the 4th and the 13th of December 2021 in-order to study the effect of COVID-19 lockdown on social interactions. The survey was created using extensive literature reviews on the COVID-19 epidemic and social interactions. The questionnaire tool was evaluated for its appropriateness, relevancy, simplicity, and adequacy. There were 15 questions in the questionnaire, including perceptions of the quarantine period, introduction to facemasks, stress and social anxiety and how it is affecting their relationship with others, and the characteristics of social relationships and communication with various population categories, including family members, friends and work colleagues. The questionnaire was divided into three main sections. The targeted population was the rural and urban population of Kerala, with access to internet. Given the situation the use of online medium for conducting the survey was found to be more appropriate. Google form was used to conduct the survey. About 570 responses were received. The responses were collected on December 2021. However considering the current scenario and national health emergency the sampling technique was found to be appropriate. The survey questionnaire received 570 responses. The collected data was interpreted using pie charts, bar graphs and tables. Further data was analysed using Chi Square distribution. We used the software Microsoft Excel for the calculation of the p-values.

Chapter 4

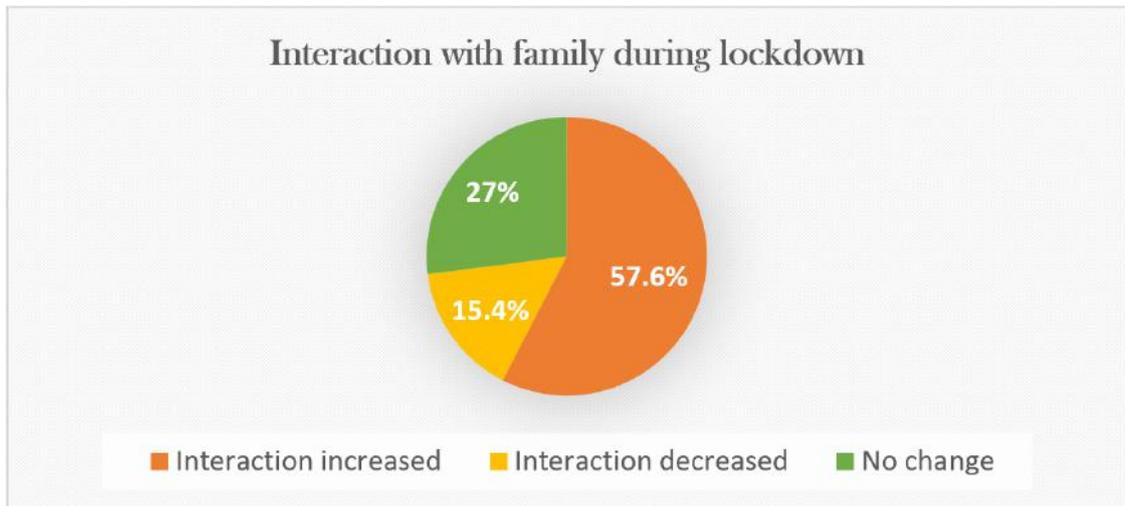
DATA ANALYSIS

Relationships between family members, friends, neighbours, co-workers, and other associates are referred to as social relationships. The government has implemented social separation as a result of the COVID-19 pandemic. Positive characteristics such as emotional support from others, as well as negative aspects, have an impact on the quality of social connections, such as a conflict or a stressful situation. Comfortable, easy going, and easy social relationships are frequently emphasised by social relationship scientists. Relationships are vital in a person's life and have a significant impact on their health, as well as their behavioural, cognitive, and emotional well-being.

4.0.1 GRAPHICAL ANALYSIS

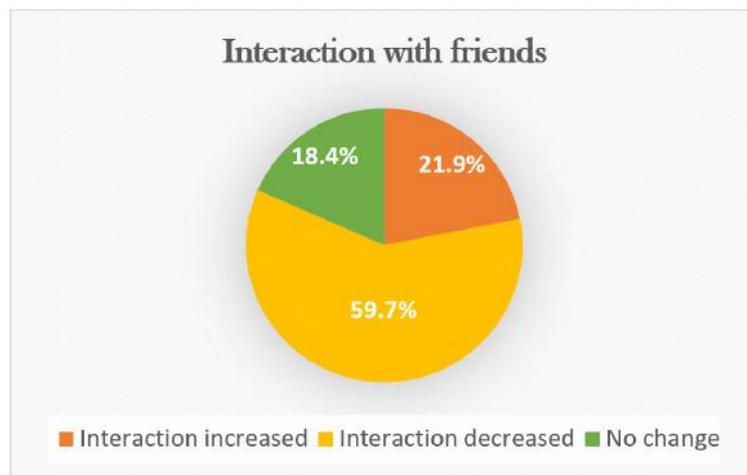
4.1.1 Interaction with family

The frequency of interaction with family during lockdown is analysed. 57.6% responded that their interaction with family increased, 15.4% responded that their interaction with family decreased and 27% found no change in their relationship with family.



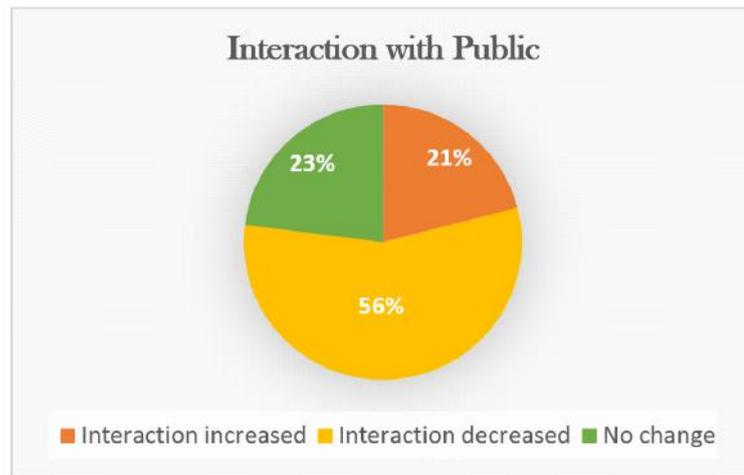
4.1.2 Interaction with friends

The frequency of interaction with friends during lockdown is analysed. 21.9% responded that their interaction with friends increased, 59.7% responded that their interaction with friends decreased and 18.4% found no change.



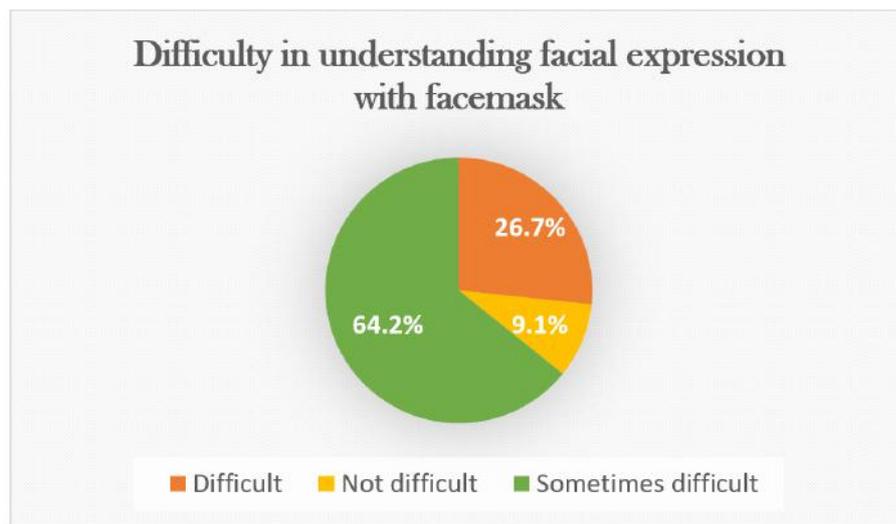
4.1.3 Interaction with Public

The frequency of interaction with public during lockdown is analysed. 21% responded that their interaction with public increased, 56% responded that their interaction with public decreased and 23% found no change.



4.1.4 Effect of mask on social interaction

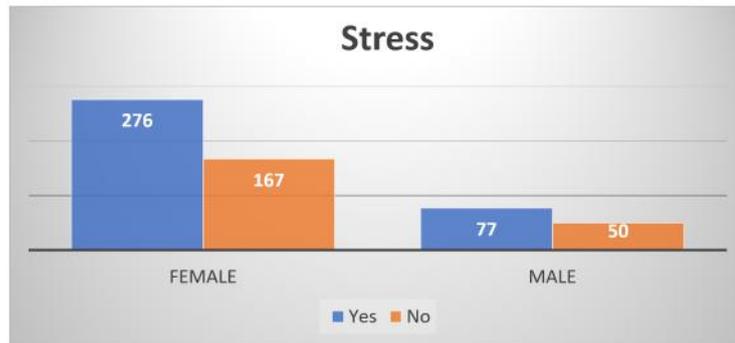
About 64.2% respondent sometimes felt it difficult to understand emotions and facial expressions of people with facemask, 26.7% people always found it difficult and 9.1% faced no difficulty.



4.1.5 Stress During Lockdown

Out of the 570 respondents, 276 females – experienced stress, 167 females – does not experience stress, 77 males- experienced stress, 50 males- does not experience stress

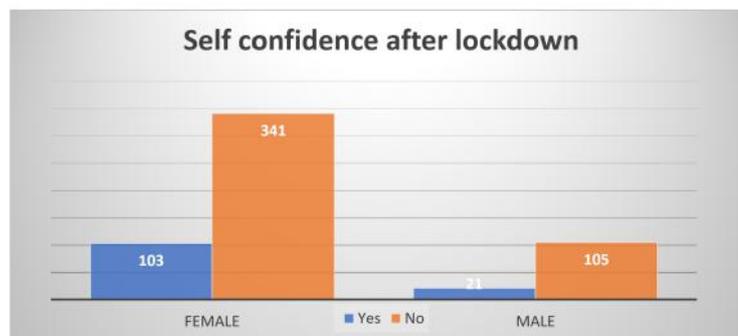
	Yes(experienced stress)	No(does not experience stress)
Female	276	167
Male	77	50



4.1.6 Self confidence after lockdown

Out of 570 respondents, 103 females and 21 males felt unconfident while interaction with public after lockdown, 341 females and 105 males felt no change in their confidence level

	Yes(felt unconfident)	No(no change in confidence level)
Female	103	341
Male	21	105



4.0.2 DATA ANALYSIS : Chi-Square Test

4.2.1 Relationship between age and interaction with family during COVID-19 lockdown

The null hypothesis is H_0 : Age and interaction with family are independent

The alternate hypothesis is H_1 : Age and interaction with family are dependent

Observed Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	129	38	69	236
20 and above 20	199	50	85	334
TOTAL	328	88	154	570

Expected Frequency in row i and column $j = \frac{(\text{Grand Total row } i)(\text{Grand Total column } j)}{\text{Total number of observations}}$

Expected Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	135.80351	36.43508772	63.7614035	236
20 and above 20	192.19649	51.56491228	90.2385965	334
TOTAL	328	88	154	570

$$\chi^2 = \sum \frac{(f_o - f_e)^2}{f_e}$$

where f_0 = observed frequency and f_e = expected frequency

Degree of freedom = $(r - 1)(c - 1)$,where r = number of rows and c = number of columns

Level of significance = 5% = 0.05

p value = 0.488972

Since $p > 0.05$, we accept H_0

Therefore, from the study, we came to the conclusion that there is no relation between age and interaction with family during COVID-19 lockdown

4.2.2 Relationship between age and interaction with friends during COVID-19 lockdown

The null hypothesis is H_0 : Age and interaction with friends are independent

The alternate hypothesis is H_1 : Age and interaction with friends are dependent

Observed Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	46	145	45	236
20 and above 20	79	195	60	334
TOTAL	125	340	105	570

Expected Frequency in row i and column $j = \frac{(\text{Grand Total row } i)(\text{Grand Total column } j)}{\text{Total number of observations}}$

Expected Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	51.754386	140.7719298	43.4736842	236
20 and above 20	73.245614	199.2280702	61.5263158	334
TOTAL	125	340	105	570

$$\chi^2 = \sum \frac{(f_o - f_e)^2}{f_e}$$

where f_0 = observed frequency and f_e = expected frequency

Degree of freedom = $(r - 1)(c - 1)$, where r = number of rows and c = number of columns

Level of significance = 5% = 0.05

p value = 0.49657

Since $p > 0.05$, we accept H_0

Therefore, from the study, we came to the conclusion that there is no relation between age and interaction with friends during COVID-19 lockdown.

4.2.3 Relationship between age and interaction with public during COVID-19 lockdown

: The null hypothesis is H_0 : Age and interaction with public are independent

The alternate hypothesis is H_1 : Age and interaction with public are dependent

Observed Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	49	129	58	236
20 and above 20	71	190	73	334
TOTAL	120	319	131	570

Expected Frequency in row i and column $j = \frac{(\text{Grand Total row } i)(\text{Grand Total column } j)}{\text{Total number of observations}}$

Expected Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	49.684211	132.077193	54.2385965	236
20 and above 20	70.315789	186.922807	76.7614035	334
TOTAL	120	319	131	570

$$\chi^2 = \sum \frac{(f_o - f_e)^2}{f_e}$$

where f_0 = observed frequency and f_e = expected frequency

Degree of freedom = $(r - 1)(c - 1)$, where r = number of rows and c = number of columns

Level of significance = 5% = 0.05

p value = 0.74692

Since $p > 0.05$, we accept H_0

Therefore, from the study, we came to the conclusion that there is no relation between age and interaction with public during COVID-19 lockdown.

4.2.4 Relationship between gender and stress during COVID-19 lockdown

The null hypothesis is H_0 : Gender and stress are independent

The alternate hypothesis is H_1 : Gender and stress are dependent

Observed Frequency

	Yes	No	TOTAL
Female	276	167	443
Male	77	50	127
TOTAL	353	217	570

Expected Frequency in row i and column $j = \frac{(\text{Grand Total row } i)(\text{Grand Total column } j)}{\text{Total number of observations}}$

Expected Frequency

	Yes	No	TOTAL
Female	274.34912	168.6508772	443
Male	78.650877	48.34912281	127
TOTAL	353	217	570

$$\chi^2 = \sum \frac{(f_o - f_e)^2}{f_e}$$

where f_0 = observed frequency and f_e = expected frequency

Degree of freedom = $(r - 1)(c - 1)$,where r = number of rows and c = number of columns

Level of significance = 5% = 0.05

p value =0.732185

Since $p > 0.05$, we accept H_0

Therefore, from the study, we came to the conclusion that there is no relation between gender and stress during COVID-19 lockdown.

4.2.5 Relationship between Age and stress during COVID-19 lockdown

The null hypothesis is H_0 : Age and stress are independent

The alternate hypothesis is H_1 : Age and stress are dependent

Observed Frequency

	Yes	No	TOTAL
Below 20	143	93	236
20 and above 20	210	124	334
TOTAL	353	217	570

Expected Frequency in row i and column $j = \frac{(\text{Grand Total row } i)(\text{Grand Total column } j)}{\text{Total number of observations}}$

Expected Frequency

	Yes	No	TOTAL
Below 20	146.15439	89.84561404	236
20 and above 20	206.84561	206.845614	334
TOTAL	353	217	570

$$\chi^2 = \sum \frac{(f_o - f_e)^2}{f_e}$$

where f_0 = observed frequency and f_e = expected frequency

Degree of freedom = $(r - 1)(c - 1)$, where r = number of rows and c = number of columns

Level of significance = $5\% = 0.05$

p value = 7.47×10^{-9}

Since $p < 0.05$, we reject H_0 and we accept H_1

Therefore, from the study, we came to the conclusion that there is relation between age and stress during COVID-19 lockdown.

Chapter 5

CONCLUSION

1. Out of the 570 respondents, about 236 were of the age below 20 and 334 of the age group equal to or above 20. Our aim was to understand if the age difference in people affected their interaction with family members during COVID-19 pandemic lockdown. About 70.76% (54.66% favourably and 16.10% unfavourably) of the respondents of age below 20 responded that the lockdown do have an effect on their interaction with family. Similarly, 74.55% (59.5% favourably and 14.97% unfavourably) respondents of age 20 and above responded the same. And the rest 29.24% of respondents of age below 20 and 25.45% of respondents above 20 felt no difference in their family interactions. So, we can conclude that age is not a factor which determine the interaction with family. From the survey, we can see that majority of the people was staying with their family during the lockdown period and this give most of them a chance to interact with their family more and built a healthy relationship with their family irrespective of their ages.
2. 191 respondents of age group below 20 have responded that lockdown had an impact on their interactions with friends favourably or unfavourably and 45 of this age group had no effect. Whereas about 274 respondents of age group 20 and above 20 have responded that COVID-19 and lockdown had an impact on their interactions with friends favourably or unfavourably and 60 of this age group had no effect. So, from the study conducted, about 80.93% of respon-

dents from the age group of below 20 have accepted that COVID-19 pandemic had an effect on their interactions with friends. And 83.035% of respondents from the age group of 20 and above 20 have accepted the same. Lockdown makes people to stay home without meeting friends face to face. During that time all age groups faced difficulties with social interactions with friends and thus we can conclude that age is not a factor of it.

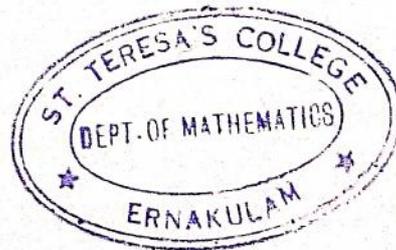
3. 191 respondents of age group below 20 have responded that lockdown had an impact on their interactions with public during COVID-19 favourably or unfavourably and 58 of this age group had no effect. About 274 responded of age group 20 and above 20 have responded that COVID-19 and lock down had an impact on their interaction with public favourably or unfavourably and 73 of this age group had no effect. So, from the study conducted, about 75.42% of respondents from the age group of below 20 have accepted that COVID-19 pandemic had an effect on their interaction with public. And 78.14% of respondents from the age group of 20 and above 20 have accepted the same. Thus we can conclude that age and the interaction with public is independent during COVID-19 pandemic lockdown makes people to stay home without meeting public face to face during that time all age groups face difficulties with social interactions with public and thus we are able to conclude that age not a factor of it.
4. 353 respondents out of total population said they faced stress during lockdown while the other 217 denied. Out of the 353, 276 were female and the others were male. And from the 217 who said no, 167 were female and 50 were male. Out of the female population 62.30% faced stress while 37.70% said they didn't. Out of the male population 60.63% faced stress and the other 39.37% denied. The lockdown had been a hard time for everyone irrespective of gender. Everyone was trying hard to make the two ends meet. So, we can say that gender plays no role in the stress factor.
5. Out of 236 people responded under the age group of 20, 143 of

them have stress and 93 of them haven't suffered stress. In the age group of above 20 years, 210 people suffered stress which is higher than the above age group and 124 out of 334 people responded haven't suffered stress. During lockdown 60.59% people under the age group of 20 suffered stress and 62.87% under the age group of above 20 suffered stress. From this study we inferred that more people from above the age group of 20 suffered stress, due to the change of work mode to online and shortage of work. As the pandemic rapidly spread across the world, a considerable degree of fear and concern was introduced among the elder population.

The purpose of our study was to understand the effects of COVID-19 lockdown on social interaction. For this a survey was conducted and several studies regarding interaction is done. We studied the relationship between age and interaction with family, age and interaction with friends and age and interaction with public during COVID-19 lockdown. Also, for understanding the psycho-physiological effects of the COVID-19 lockdown on people, we studied the relationship between age and stress and relationship between gender and stress. From the study we came to the conclusions that the relationship between age and interactions with family, relationship between age and interactions with friends, relationship between age and interactions with public, and relationship between gender and stress are all independent. Whereas the relationship between age and stress is dependent. Therefore, from the data collected and test conducted we have concluded the objectives of the study.

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Project Report

On

**THE EFFECTS OF COVID-19 LOCKDOWN
ON SOCIAL INTERACTION**

Submitted

in partial fulfilment of the requirements for the degree of
BACHELOR OF SCIENCE

in

MATHEMATICS

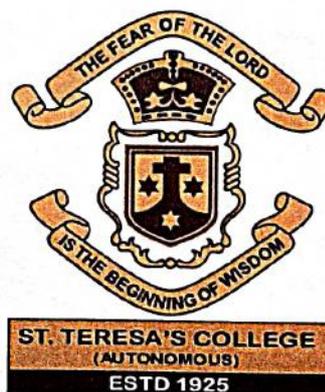
by

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(Register No. AB19BMAT056)

Under the Supervision of

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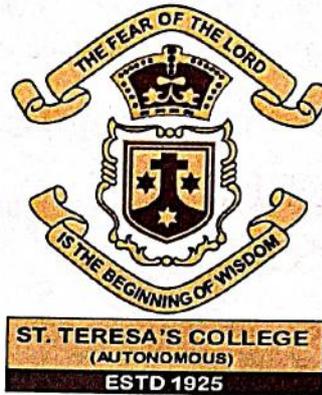


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APRIL 2022

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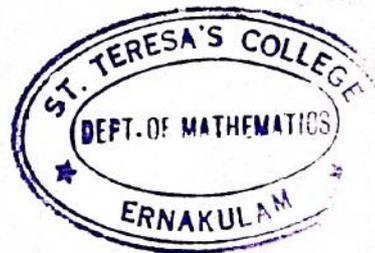
CERTIFICATE

This is to certify that the dissertation entitled, **THE EFFECTS OF COVID-19 LOCKDOWN ON SOCIAL INTERACTION** is a bonafide record of the work done by Ms. **SRUTHY K BENNI** under my guidance as partial fulfillment of the award of the degree of **Bachelor of Science in Mathematics** at St. Teresa's College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam. No part of this work has been submitted for any other degree elsewhere.

Date: 06-05-2022

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DECLARATION

I hereby declare that the work presented in this project is based on the original work done by me under the guidance of Smt. Shanty B P. Assistant Professor and Head, Department of Statistics, St. Teresa's College(Autonomous), Ernakulam and has not been included in any other project submitted previously for the award of any degree.

Ernakulam.

Date: **06-05-2022**



SRUTHY K BENNI

AB19BMAT056

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Firstly, I thank God Almighty for His grace for being able to complete this project work successfully. I express my deep sense of gratitude to our guide Smt. Shanty B P, Department of Mathematics and Statistics, St.Teresa's College, Ernakulam, for her valuable guidance and suggestions.I also thank Dr.Ursala Paul (HOD of Mathematics Department) and other teachers of the department, parents, friends, especially my group members and all those who have given me support. This project would not have been possible without the support of the people mentioned above. Thank you all.

Ernakulam.

Date: **06-05-2022**

SRUTHY K BENNI

AB19BMAT056

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Chapter 1

INTRODUCTION

Social interactions are greatly affected by the COVID-19 pandemic. In order to understand how the usage of protective equipment and social distancing affect the ability to communicate the emotions of others, thus, in order to effectively interact with others, we conducted online research on the crowd after the lockdown. The social distance with community members is adjusted in the opposite direction that is, people reduce social distance, leading to a sense of social connection in the community. In other words, fighting the epidemic together can strengthen cooperation and shared value between individuals, thereby creating the sense of unity and seeking common ground while reserving differences.

The COVID-19 pandemic, also identified by the World Health Organization as a corona virus pandemic, will cause severe acute respiratory syndrome. The COVID-19 pandemic was first reported in Wuhan, China in December 2019 and was declared as a pandemic on January 30, 2020 by World Health Organisation. The report of the Centres for Disease Control and Prevention and published epidemiological and virological research data show that there is evidence that COVID-19 is mainly through direct contact with respiratory droplets of symptomatic or even asymptomatic patients, or through contact with space and contamination objects and surface. to reduce the contact between people in public spaces, India and many other countries worldwide implemented a lockdown that came into effect on March 15th, 2020. The main aim of

the lockdown was to fight the corona virus outbreak by applying certain regulations. Civilians or other unauthorised persons were not allowed to leave their home unless it's an emergency. Social relations refer to family members, friends, neighbours and other colleagues. The quality of social relationships is affected by positive aspects such as emotional support from other sources, as well as negative aspects such as conflict and pressure. Social relations scientists often emphasize comfortable, relaxed and easy social relationships are important in a person's life and have a great impact on health, affecting their behavioural, psychosocial, and physiological states. Therefore, the purpose of the present study is to evaluate the effects of lockdown imposed by the government in India on the social relationships of the population. Due to this, government all across the world have taken unprecedented steps to respond to the virus and contain it. Countries are implementing various health control measures to lessen the test positivity rate and avoid the collapse of their health systems. Epidemiologists predicted 7 billion infections and 40 million deaths globally for the year 2020 if no control measures are taken. Thus, the government took immediate action. Therefore, the purpose of the present study is to evaluate the effects of social distancing imposed by the curfew program in India on the social relationships of the population.

1.0.1 Significance of study

The significance of this study is to understand the consequences of COVID-19 lockdown/social distancing due to quarantine on social interaction. Social relationships refer to the existing associations among family members, friends, neighbours, co-workers, and other associates. Due to the COVID-19 pandemic, the Indian government along with many other countries implemented lockdown. Recently, the WHO announced that social disconnection is a major public health challenge. Therefore, individuals are now facing the possibility of different forms of social isolation. As a result, the goal of our research is to assess the effects of lockdown on the population's social interactions.

1.0.2 Objectives

1. To study the impact of COVID-19 lockdown in social interactions/events.
2. To study the effect of COVID-19 lockdown in the relationship among people and their perception of empathy toward others.
3. To determine the psychological impact of the lockdown on people.

1.0.3 Statistics and types of Data

Statistics is the branch of mathematics that deals with the collection, organisation, analysis, interpretation, and presentation of massive amounts of data.

Individual pieces of factual information are recorded and used for analysis as data. Primary data and secondary data are two types of data. The term "primary data" refers to information obtained by the researcher himself. Surveys, observations, experiments, questionnaires, focus groups, interviews, and other primary data sources are examples. Once it is decided what type of study will be conducted, it is required to gather information about the study, which is usually in the form of data. A poll will be run to get the data for this information. Our survey was designed to find out how the population views the quarantine period, how it affects their connections with others, and the features of their social ties and communication with various demographic groups, such as family members and co-workers.

1.0.4 Processing of data

Data processing involves translating the answers on a questionnaire into a form that can be manipulated to produce statistics. Stages of data processing include Data collection, Data preparation, Data input, Processing, Data interpretation, Data storage. Before tabulation of primary data, it should be scrutinized for completeness, consistency, accuracy and editing.

1. **Completeness:** If the answer to some important question in a schedule or questionnaire is missing it becomes necessary to contact the

informant again and complete the missing information in the report in case such an information cannot be completed the schedule or questionnaire should be discarded or revised.

2. **Consistency:** Some information given by the respondent may not be compatible in the sense that and information furnished by the individual either does not justify some other information or is contradictory to the earlier one.
3. **Accuracy:** It is of vital importance if the data is inaccurate the conclusions drawn from it have no relevance or reliability. By checking the schedules for questionnaire only a little improvement can be made. In recent times checks have been evolved to attain accuracy.
4. **Editing:** To maintain homogeneity, the information sheets are checked to see whether the unit of information or measurement is the same in all the schedules. It should also be check whether or not the same information has been supply for a particular question in all the information sheets. The ambiguity arises due to various interpretations of the same question and should be removed. Once the primary that they have undergone the above four processes is this with for further analysis.

VARIABLES DESCRIPTION

1. **Age:** The age of the person filling the form was recorded. That is to understand whether the respondent is of the age below 20 or 20 and above.
2. **Gender:** Whether the person is male or female.
3. **Place of residence:** Whether the person is living in an urban area or rural area.
4. **Interaction with your family:** Whether the respondent's interaction with family increased, decreased or had no effect during the lockdown.
5. **Interaction with your friends:** Whether the respondent's interaction with friends increased, decreased or had no effect during the lockdown.
6. **Interaction with public:** Whether the respondent's interaction with public increased, decreased or had no effect during the lockdown.
7. **Understanding the facial expressions and emotions of people with face mask:** Whether it is always difficult, sometimes difficult or not difficult.
8. **Stress:** Whether the person faced stress during lockdown or not.
9. **Self-confidence:** Whether it increased or decreased after lockdown.

Chapter 2

DATA DESCRIPTION

2.0.1 Bar Graph

A bar graph is a graph that displays all of the data in the form of rectangular bars with heights proportionate to the values they represent. The graph's bars can be displayed vertically or horizontally. Bar graphs, commonly referred to as bar charts, are a visual representation of categorised data. It's one of the methods for dealing with data. A bar graph is a visual representation of data. It's a good tool for representing data that is unrelated to one another and does not need to be in any particular format while being represented in order. The bars provide a visual representation of amounts in several categories. The bar diagrams include two lines, the horizontal and vertical lines.

Grouped bar graph is also known as clustered bar graph. It's used for representing the discrete value of two or more categorical data sets. Rectangular bars are grouped by position for levels of one categorical variable, with the secondary category level within each group shown in the same colour. It can be demonstrated both horizontally and vertically.

2.0.2 Pie Chart

A pie chart is a circular chart divided into sectors, with the size of the data represented by the area of each sector. It's also referred to as a circle graph. Because a circle has 360 degrees, each group in the pie

chart will have a proportion of 360.

2.0.3 Chi-Square Distribution

For evaluating correlations between categorical data, the Chi-Square statistic is often used. The null hypothesis of the Chi-Square test is that the categorical variables in the population have no association; they are independent. When employing a cross tabulation, the Chi-Square statistic is most typically employed to evaluate Tests of Independence (A bi-variate table is another name for it.) The distributions of two categorical variables are shown in cross tabulation. The intersections of the categories of the variables appear in the table's cells at the same time. By analysing the observed pattern of the two variables, independence determines whether there is a link between them. If the factors were actually independent of each other, the cells' responses to the pattern would be predicted. One can examine if the observed cell counts are significantly different from the expected cell counts by computing the Chi-Square statistic and comparing it to a critical value from the distribution. The Chi-Square statistic can be calculated in a number of ways, all of which are simple and intuitive:

$$\sum \frac{(f_o - f_e)^2}{f_e}$$

where f_o = the observed frequency (the observed counts in the cells) and f_e = the expected frequency if no relationship existed between the variables. The Chi-Square statistic is based on the difference between what is observed in the data and what would be predicted if there were no association between the variables, as shown in the calculation.

Chapter 3

METHODOLOGY

A cross-sectional study using an online survey was conducted between the 4th and the 13th of December 2021 in-order to study the effect of COVID-19 lockdown on social interactions. The survey was created using extensive literature reviews on the COVID-19 epidemic and social interactions. The questionnaire tool was evaluated for its appropriateness, relevancy, simplicity, and adequacy. There were 15 questions in the questionnaire, including perceptions of the quarantine period, introduction to facemasks, stress and social anxiety and how it is affecting their relationship with others, and the characteristics of social relationships and communication with various population categories, including family members, friends and work colleagues. The questionnaire was divided into three main sections. The targeted population was the rural and urban population of Kerala, with access to internet. Given the situation the use of online medium for conducting the survey was found to be more appropriate. Google form was used to conduct the survey. About 570 responses were received. The responses were collected on December 2021. However considering the current scenario and national health emergency the sampling technique was found to be appropriate. The survey questionnaire received 570 responses. The collected data was interpreted using pie charts, bar graphs and tables. Further data was analysed using Chi Square distribution. We used the software Microsoft Excel for the calculation of the p-values.

Chapter 4

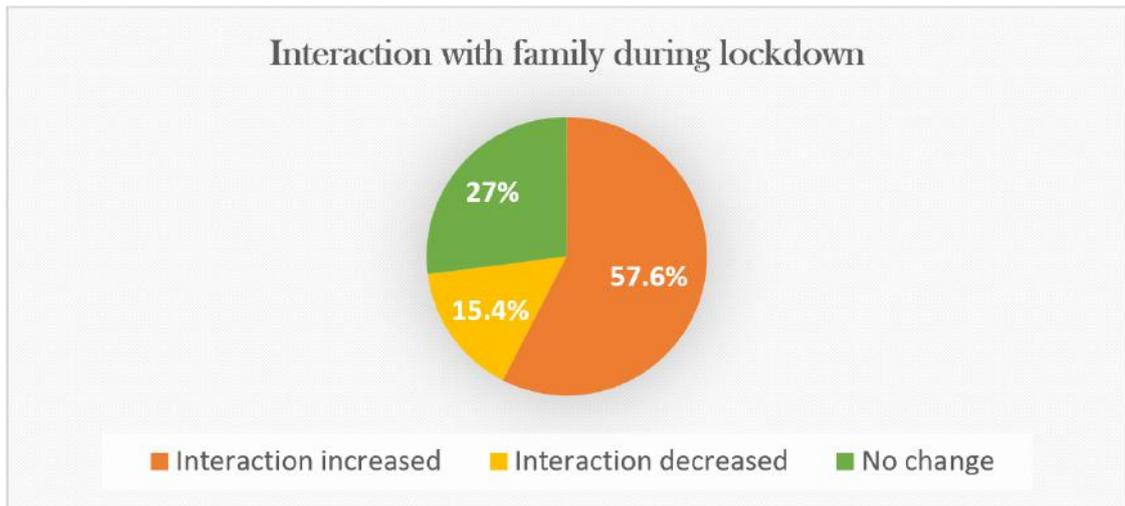
DATA ANALYSIS

Relationships between family members, friends, neighbours, co-workers, and other associates are referred to as social relationships. The government has implemented social separation as a result of the COVID-19 pandemic. Positive characteristics such as emotional support from others, as well as negative aspects, have an impact on the quality of social connections, such as a conflict or a stressful situation. Comfortable, easy going, and easy social relationships are frequently emphasised by social relationship scientists. Relationships are vital in a person's life and have a significant impact on their health, as well as their behavioural, cognitive, and emotional well-being.

4.0.1 GRAPHICAL ANALYSIS

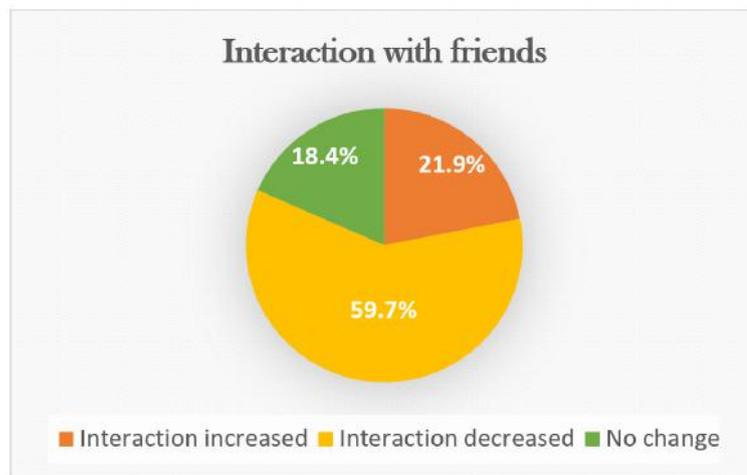
4.1.1 Interaction with family

The frequency of interaction with family during lockdown is analysed. 57.6% responded that their interaction with family increased, 15.4% responded that their interaction with family decreased and 27% found no change in their relationship with family.



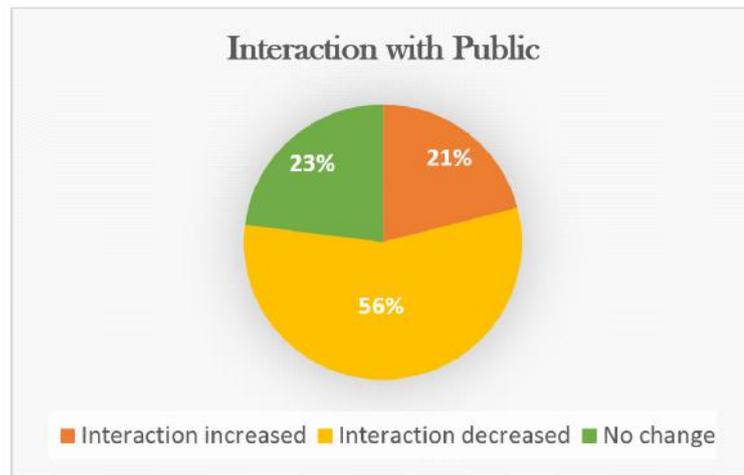
4.1.2 Interaction with friends

The frequency of interaction with friends during lockdown is analysed. 21.9% responded that their interaction with friends increased, 59.7% responded that their interaction with friends decreased and 18.4% found no change.



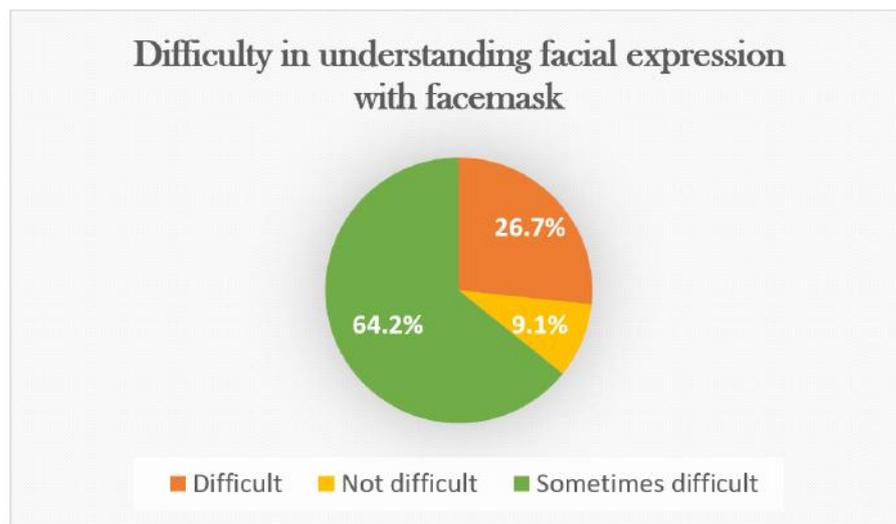
4.1.3 Interaction with Public

The frequency of interaction with public during lockdown is analysed. 21% responded that their interaction with public increased, 56% responded that their interaction with public decreased and 23% found no change.



4.1.4 Effect of mask on social interaction

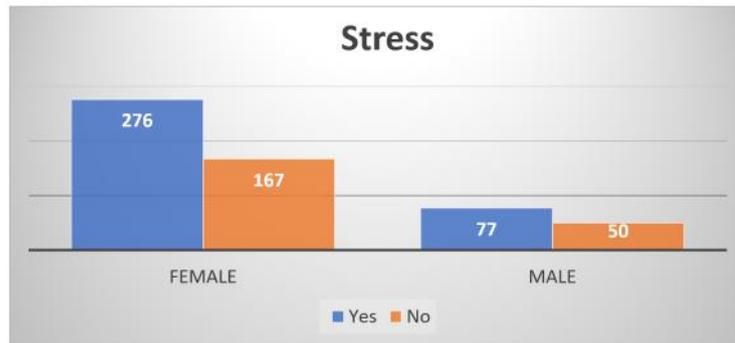
About 64.2% respondent sometimes felt it difficult to understand emotions and facial expressions of people with facemask, 26.7% people always found it difficult and 9.1% faced no difficulty.



4.1.5 Stress During Lockdown

Out of the 570 respondents, 276 females – experienced stress, 167 females – does not experience stress, 77 males- experienced stress, 50 males- does not experience stress

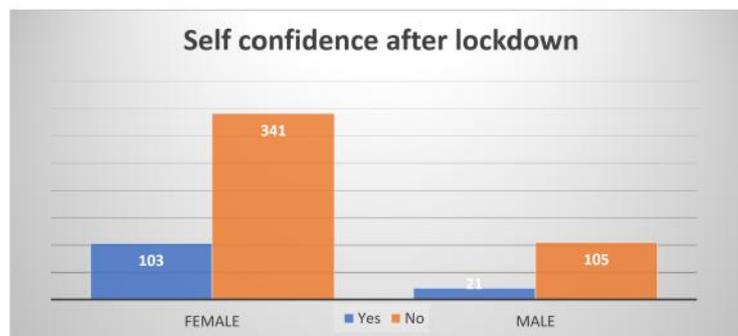
	Yes(experienced stress)	No(does not experience stress)
Female	276	167
Male	77	50



4.1.6 Self confidence after lockdown

Out of 570 respondents, 103 females and 21 males felt unconfident while interaction with public after lockdown, 341 females and 105 males felt no change in their confidence level

	Yes(felt unconfident)	No(no change in confidence level)
Female	103	341
Male	21	105



4.0.2 DATA ANALYSIS : Chi-Square Test

4.2.1 Relationship between age and interaction with family during COVID-19 lockdown

The null hypothesis is H_0 : Age and interaction with family are independent

The alternate hypothesis is H_1 : Age and interaction with family are dependent

Observed Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	129	38	69	236
20 and above 20	199	50	85	334
TOTAL	328	88	154	570

Expected Frequency in row i and column $j = \frac{(\text{Grand Total row } i)(\text{Grand Total column } j)}{\text{Total number of observations}}$

Expected Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	135.80351	36.43508772	63.7614035	236
20 and above 20	192.19649	51.56491228	90.2385965	334
TOTAL	328	88	154	570

$$\chi^2 = \sum \frac{(f_o - f_e)^2}{f_e}$$

where f_0 = observed frequency and f_e = expected frequency

Degree of freedom = $(r - 1)(c - 1)$,where r = number of rows and c = number of columns

Level of significance = 5% = 0.05

p value = 0.488972

Since $p > 0.05$, we accept H_0

Therefore, from the study, we came to the conclusion that there is no relation between age and interaction with family during COVID-19 lockdown

4.2.2 Relationship between age and interaction with friends during COVID-19 lockdown

The null hypothesis is H_0 : Age and interaction with friends are independent

The alternate hypothesis is H_1 : Age and interaction with friends are dependent

Observed Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	46	145	45	236
20 and above 20	79	195	60	334
TOTAL	125	340	105	570

Expected Frequency in row i and column $j = \frac{(\text{Grand Total row } i)(\text{Grand Total column } j)}{\text{Total number of observations}}$

Expected Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	51.754386	140.7719298	43.4736842	236
20 and above 20	73.245614	199.2280702	61.5263158	334
TOTAL	125	340	105	570

$$\chi^2 = \sum \frac{(f_o - f_e)^2}{f_e}$$

where f_0 = observed frequency and f_e = expected frequency

Degree of freedom = $(r - 1)(c - 1)$,where r = number of rows and c = number of columns

Level of significance = 5% = 0.05

p value = 0.49657

Since $p > 0.05$, we accept H_0

Therefore, from the study, we came to the conclusion that there is no relation between age and interaction with friends during COVID-19 lockdown.

4.2.3 Relationship between age and interaction with public during COVID-19 lockdown

: The null hypothesis is H_0 : Age and interaction with public are independent

The alternate hypothesis is H_1 : Age and interaction with public are dependent

Observed Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	49	129	58	236
20 and above 20	71	190	73	334
TOTAL	120	319	131	570

Expected Frequency in row i and column $j = \frac{(\text{Grand Total row } i)(\text{Grand Total column } j)}{\text{Total number of observations}}$

Expected Frequency

	Favourable	Unfavourable	No effect	TOTAL
Below 20	49.684211	132.077193	54.2385965	236
20 and above 20	70.315789	186.922807	76.7614035	334
TOTAL	120	319	131	570

$$\chi^2 = \sum \frac{(f_o - f_e)^2}{f_e}$$

where f_0 = observed frequency and f_e = expected frequency

Degree of freedom = $(r - 1)(c - 1)$, where r = number of rows and c = number of columns

Level of significance = 5% = 0.05

p value = 0.74692

Since $p > 0.05$, we accept H_0

Therefore, from the study, we came to the conclusion that there is no relation between age and interaction with public during COVID-19 lockdown.

4.2.4 Relationship between gender and stress during COVID-19 lockdown

The null hypothesis is H_0 : Gender and stress are independent

The alternate hypothesis is H_1 : Gender and stress are dependent

Observed Frequency

	Yes	No	TOTAL
Female	276	167	443
Male	77	50	127
TOTAL	353	217	570

Expected Frequency in row i and column $j = \frac{(\text{Grand Total row } i)(\text{Grand Total column } j)}{\text{Total number of observations}}$

Expected Frequency

	Yes	No	TOTAL
Female	274.34912	168.6508772	443
Male	78.650877	48.34912281	127
TOTAL	353	217	570

$$\chi^2 = \sum \frac{(f_o - f_e)^2}{f_e}$$

where f_0 = observed frequency and f_e = expected frequency

Degree of freedom = $(r - 1)(c - 1)$,where r = number of rows and c = number of columns

Level of significance = 5% = 0.05

p value =0.732185

Since $p > 0.05$, we accept H_0

Therefore, from the study, we came to the conclusion that there is no relation between gender and stress during COVID-19 lockdown.

4.2.5 Relationship between Age and stress during COVID-19 lockdown

The null hypothesis is H_0 : Age and stress are independent

The alternate hypothesis is H_1 : Age and stress are dependent

Observed Frequency

	Yes	No	TOTAL
Below 20	143	93	236
20 and above 20	210	124	334
TOTAL	353	217	570

Expected Frequency in row i and column $j = \frac{(\text{Grand Total row } i)(\text{Grand Total column } j)}{\text{Total number of observations}}$

Expected Frequency

	Yes	No	TOTAL
Below 20	146.15439	89.84561404	236
20 and above 20	206.84561	206.845614	334
TOTAL	353	217	570

$$\chi^2 = \sum \frac{(f_o - f_e)^2}{f_e}$$

where f_0 = observed frequency and f_e = expected frequency

Degree of freedom = $(r - 1)(c - 1)$, where r = number of rows and c = number of columns

Level of significance = $5\% = 0.05$

p value = 7.47×10^{-9}

Since $p < 0.05$, we reject H_0 and we accept H_1

Therefore, from the study, we came to the conclusion that there is relation between age and stress during COVID-19 lockdown.

Chapter 5

CONCLUSION

1. Out of the 570 respondents, about 236 were of the age below 20 and 334 of the age group equal to or above 20. Our aim was to understand if the age difference in people affected their interaction with family members during COVID-19 pandemic lockdown. About 70.76% (54.66% favourably and 16.10% unfavourably) of the respondents of age below 20 responded that the lockdown do have an effect on their interaction with family. Similarly, 74.55% (59.5% favourably and 14.97% unfavourably) respondents of age 20 and above responded the same. And the rest 29.24% of respondents of age below 20 and 25.45% of respondents above 20 felt no difference in their family interactions. So, we can conclude that age is not a factor which determine the interaction with family. From the survey, we can see that majority of the people was staying with their family during the lockdown period and this give most of them a chance to interact with their family more and built a healthy relationship with their family irrespective of their ages.
2. 191 respondents of age group below 20 have responded that lockdown had an impact on their interactions with friends favourably or unfavourably and 45 of this age group had no effect. Whereas about 274 respondents of age group 20 and above 20 have responded that COVID-19 and lockdown had an impact on their interactions with friends favourably or unfavourably and 60 of this age group had no effect. So, from the study conducted, about 80.93% of respon-

dents from the age group of below 20 have accepted that COVID-19 pandemic had an effect on their interactions with friends. And 83.035% of respondents from the age group of 20 and above 20 have accepted the same. Lockdown makes people to stay home without meeting friends face to face. During that time all age groups faced difficulties with social interactions with friends and thus we can conclude that age is not a factor of it.

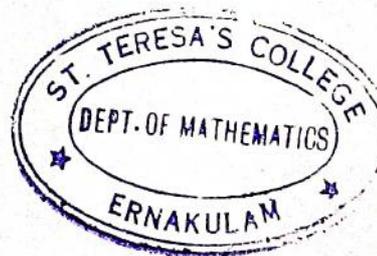
3. 191 respondents of age group below 20 have responded that lockdown had an impact on their interactions with public during COVID-19 favourably or unfavourably and 58 of this age group had no effect. About 274 responded of age group 20 and above 20 have responded that COVID-19 and lock down had an impact on their interaction with public favourably or unfavourably and 73 of this age group had no effect. So, from the study conducted, about 75.42% of respondents from the age group of below 20 have accepted that COVID-19 pandemic had an effect on their interaction with public. And 78.14% of respondents from the age group of 20 and above 20 have accepted the same. Thus we can conclude that age and the interaction with public is independent during COVID-19 pandemic lockdown makes people to stay home without meeting public face to face during that time all age groups face difficulties with social interactions with public and thus we are able to conclude that age not a factor of it.
4. 353 respondents out of total population said they faced stress during lockdown while the other 217 denied. Out of the 353, 276 were female and the others were male. And from the 217 who said no, 167 were female and 50 were male. Out of the female population 62.30% faced stress while 37.70% said they didn't. Out of the male population 60.63% faced stress and the other 39.37% denied. The lockdown had been a hard time for everyone irrespective of gender. Everyone was trying hard to make the two ends meet. So, we can say that gender plays no role in the stress factor.
5. Out of 236 people responded under the age group of 20, 143 of

them have stress and 93 of them haven't suffered stress. In the age group of above 20 years, 210 people suffered stress which is higher than the above age group and 124 out of 334 people responded haven't suffered stress. During lockdown 60.59% people under the age group of 20 suffered stress and 62.87% under the age group of above 20 suffered stress. From this study we inferred that more people from above the age group of 20 suffered stress, due to the change of work mode to online and shortage of work. As the pandemic rapidly spread across the world, a considerable degree of fear and concern was introduced among the elder population.

The purpose of our study was to understand the effects of COVID-19 lockdown on social interaction. For this a survey was conducted and several studies regarding interaction is done. We studied the relationship between age and interaction with family, age and interaction with friends and age and interaction with public during COVID-19 lockdown. Also, for understanding the psycho-physiological effects of the COVID-19 lockdown on people, we studied the relationship between age and stress and relationship between gender and stress. From the study we came to the conclusions that the relationship between age and interactions with family, relationship between age and interactions with friends, relationship between age and interactions with public, and relationship between gender and stress are all independent. Whereas the relationship between age and stress is dependent. Therefore, from the data collected and test conducted we have concluded the objectives of the study.

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**PRELIMINARY STUDIES ON
THE ANTIBACTERIAL POTENTIAL OF THE RED
SEAWEED *ACANTHOPHORA SPICIFERA* (M. Vahl) Borgesen**

**DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE AWARD OF THE DEGREE OF BACHELOR OF
SCIENCE IN
BOTANY**

By

NAME : Devika Panicker.M

Reg No: AB19BOT004



DEPARTMENT OF BOTANY

ST. TERESA'S COLLEGE (AUTONOMOUS)

ERNAKULAM

2022

CERTIFICATE

This is to certify that the dissertation entitled "Preliminary studies on the antibacterial potential of the red seaweed *Acanthophoraspicifera* (M. Vahl) Borgesen" is an authentic record of research work carried out by Miss **Devika Panicker.M**

(Reg No: AB19BOT004) under the supervision and guidance Smt. Nishitha I. K. Assistant Professor, Department of Botany and Centre for Research, St. Teresa's College (Autonomous), Emakulam, in partial fulfilment of the requirements for the award of the degree of Bachelor of Science in Botany. I further certify that no part of this work embodied in this project has been submitted for the award of any degree or diploma.

Dr. Liza Jacob
Head, Department of Botany,
St. Teresa's college (Autonomous)
Ernakulam

Smt. Nishitha I.K.
Assistant Professor,
Department of Botany,
St. Teresa's college (Autonomous)
Ernakulam

Examiners:

- 1).....  9/5/22 Anila N
- 2).....  9/5/22 Anisha S



DECLARATION

I hereby declare that the project entitled “Preliminary studies on the antibacterial potential of the red seaweed *Acanthophora spicifera* (M. Vahl) Borgesen” submitted to Mahatma Gandhi University, Kottayam, in partial fulfilment of the requirement for the Degree of Master of Science in Botany is an original project done by me under the supervision and guidance of Ms. Nishitha I.K., Department of Botany and Centre for Research, St. Teresa’s college (Autonomous), Ernakulam.

PLACE: Ernakulam

Name: **Devika Panicker.M**

DATE:4th May 2022

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Place : Ernakulam

Name: **Devika Panicker.M**

Date: 4th May 2022

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INTRODUCTION

Algae are diverse group of relatively simple, chlorophyll containing, photo-autotrophic and oxygen evolving aquatic thalloid (without differentiation into True roots, stems, leaves or leaf like organs) organisms. The word “algae” has its origin from Latin, where ‘alga’ means seaweed. The term algae was first used by Carolous Linnaeus in 1753. Most of them are photo-autotrophic but few are mixotrophic and myzotrophic (sucking through special feeding structure) study of algae is known as phycology (GK. Phykos- seaweed; logos= discourse Or study) or algology.

Algae are divided into nine main phylums, they are Phylum Rhodophycophyta, Phylum Xanthophycophyta, Phylum Chrysophycophyta, Phylum Phaeophycophyta, Phylum Bacillariophycophyta, Phylum Euglenophycophyta, Phylum Chlorophycophyta, Phylum Cryptophycophyta, and Phylum Pyrrophyphyta.

The term "seaweed" refers to a variety of marine plants and algae that can be found in the ocean, rivers, lakes, and other bodies of water. Some seaweeds, such as phytoplankton, are small and remain floating in the water column, providing the foundation for most aquatic food chains. Some are massive, such as the giant kelp that grow in dense forests. The large percentage are medium-sized, with red, green, brown, and black colours, and sometimes may wash up on beaches and shorelines. (Guiry, Michael D., 2014)

Marine algae from Indian coasts amounting to 844 species (including forma and varieties) are distributed among 217 genera. They grow in the intertidal, shallow and deep sea areas up to 180 meter depth and also in estuaries, backwaters and lagoons on solid substrates such as rocks, dead corals, pebbles, shells, mangroves and other plant materials (Anatharaman *et al.*, 2007; Sakthivel, 2007).

Mostly seen seaweeds are macro algae. They are of different types according to the colour of pigments present: brown algae (phylum Ochrophyta, class Phaeophyceae), red algae (phylum Rhodophyta, *Gelidium*), and green algae (phylum Chlorophyta, classes Chlorophyceae, Ulvophyceae etc. They differ significantly in many ultrastructural and biochemical functions, including photosynthetic pigments, storage molecules, cell wall composition, presence/absence of flagella, mitosis ultrastructure, linkage between cells, and structure of the chloroplasts, in addition to pigmentation.

Seaweed is high in vitamins, minerals, and fibre, as well as being palatable. The Japanese have a dish, 'sushi' which is nori seaweed wrapped with a mixture of fish, rice, and other ingredients for at least 1,500 years. Anti-inflammatory and anti-microbial compounds can be found in a variety of seaweeds. For thousands of years, their medicinal properties have been used; it was used to cure wounds, burns, and rashes by the ancient Romans. According to anecdotal evidence, the ancient Egyptians may have employed them to cure breast cancer. (McLachlan, J., and C. J. Bird, 1984).

In recent years, focus towards these organisms has increased due to their food and fuel production capability. In fuel industry algae biofuels have emerged as a clean, nature friendly, cost effective solution to other fuels. More recently algae have been identified and developed as renewable fuel sources, and the cultivation of algal biomass for various products is transitioning to commercial-scale systems. Large-scale cultivation of algae merges the fundamental aspects of traditional agricultural farming and aquaculture (Emily M Trentacoste *et al.*, 2014). Algae fuels are categorized into bio-ethanol, biogas, bio-hydrogen, biodiesel and bio-oil. Algae can be used in the preparation of Biodiesel, Bioethanol, Biobutanol and Hydrogen gas (Raja *et al.*, 2013)

They are considered as a potential source of bioactive substances such as proteins, lipids, and polyphenols possessing potent antibacterial, anticancer, antioxidant, antifungal, and antiviral properties (Sundaramurthy *et al.*, 2016). Seaweeds that are medicinal are rich in secondary metabolites which include alkaloids, glycosides, flavonoids, saponins, tannins, steroids, related active metabolites, which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry (Eluvakka *et al.*, 2010). Recently, their value as a source of novel bioactive substances has grown rapidly and researchers have revealed that marine algal originated compounds exhibit various biological activities (Kim and Wijesekara, 2010; Wijesekara and Kim, 2010; Wijesekara *et al.*, 2010 and Wijesekara *et al.*, 2011). The secondary metabolites of seaweeds such as Isoprenoids (terpenes, carotenoids, steroids), polyketides, phlorotannins, amino-acid derived natural products (alkaloids), and shikimates (flavonoids) have always attracted the interest of biochemists because of their diversity is comparable with those present in the leaves of higher plants (Manilal *et al.*, 2009). Seaweeds were rich in dietary fiber (>50% dry weight), particularly in the soluble form.

RHODOPHYCEAE

Rhodophyta is a phylum of macroalgae that includes the classes Phaeophyceae and Chlorophyta, which are brown and green seaweeds, respectively.

Within Archaeplastida, Rhodophyta, or red algae, is a monophyletic lineage that contains glaucophyte algae, green algae, and terrestrial plants. Bangia-like species have been found in 1.2 billion-year-old strata, indicating that Rhodophyta has a lengthy fossil history. The morphology of red algae ranges from unicellular filamentous to multicellular thalloid forms, with certain species producing economically important products like agar and carrageenan. These species can be found in a variety of marine settings, ranging from the intertidal zone to deep oceans. There are also freshwater (e.g., *Batrachospermum*) and terrestrial lineages. A triphasic life cycle with one haploid and two diploid phases, with the carposporophyte borne on female gametophytes, is one of the Rhodophyta's significant advances.

Freshwater Rhodophyta has 66 species and 27 genera in North America, although these numbers will change as molecular investigations uncover more diversity. Freshwater red algae have a limited size range than marine species, with the majority (80%) of them measuring 1-10 cm in length. Gelatinous filaments, free filaments, and pseudoparenchymatous forms are the most prevalent types.(Yoon, Hwan Su, et al., 2017)

ACANTHOPHORA

Acanthophora is a red algae that can be found in almost all tropical and subtropical oceans. Because of its changeable form, it can adapt to a wide range of environmental circumstances and hence invade a wide range of ecosystems.

Acanthophora is an erect macroalgae that may reach a height of 40cm. It has solid cylindrical branches that are 2-3mm diameter and are rarely or repeatedly branched. Short, determinate branches, irregularly shaped and spinose, with spines numerous and radially oriented, make up the major branches. The major axes have no spines. A big, oddly shaped holdfast gives rise to the plant. It has short (4 - 10cm), compact, and dense thalli in intertidal high-motion water areas. It comes in a wide range of colours, including red, purple, yellow, orange, and brown. Thalli are typically quite black in intertidal, high-motion locations, and lighter in shallow areas with low water motion and reflective sandy or silty bottoms.

In the Gulf of Mannar, Tamil Nadu's coastal region, *Acanthophora spicifera* is a common seaweed used in folkloric treatments and as a nutritional supplement. The anticancer and anti-oxidant properties of the alcoholic extract of *Acanthophora* were investigated in this study. Anti-cancer impact was measured by assessing tumour volume, tumour weight, mean survival day (MSD), and several haematological parameters after 21 days of testing and standard drug administration. The anti-oxidant state of the liver tissue was also determined. In cancerous mice treated with EAC cell lines, the ethanol extract of *Acanthophora* has a significant anti-cancer effect, reducing tumour volume and weight with mean survival day (MSD). The findings revealed that an ethanol extract of *Acanthophora* has antitumor and anti-oxidant activity, which may be due to the presence of bioactive components such as flavonoids, terpenoids, and tannins. (Lavakumar, K. F. H. Ahamed, and V. Ravichandran, 2012)

The present work was undertaken to study the antimicrobial potential of *Acanthophora spicifera*. The objectives of the study are

- Taxonomic description of the algae
- Assessment of antibacterial potential of *Acanthophora spicifera* in its dried form extracted in two different solvents; Ethanol and Chloroform.
- Comparative antibacterial activity of the algae extracted in the two different solvents against Gram positive *Staphylococcus* and Gram negative *E. coli*.
- Estimate the extractive value of the plant, in both ethanolic and chloroform solvents.

LITERATURE REVIEW

In a study conducted by Inci Tuney (2006), antibacterial activity of extracts or components from various algae has been demonstrated in vitro against both gram-positive and gram-negative bacteria. The antibacterial susceptibility test was performed using the agar disc diffusion method, with 6 mm discs impregnated with 20 µl of extracts and placed in infected agar. (Inci Tuney 2006),

Krishnapriya et al., (2013) conducted an antibacterial activity on the seaweed extracts, carried out by agar disc diffusion assay. The Muller Hinton agar (MHA) medium was used for this study using bacterial pathogens. Among the solvent extracts, methanol extract showed best results for both positive and negative strains. Chloroform extract of *G. verrucosa* gave the highest zone of inhibition measuring 21 ± 1.0 mm. Ethanol extract of *G. acerosa* also showed a zone of inhibition of 12 ± 1.0 mm. Ethanol and chloroform extracts of *G. verrucosa* gave clearly distinct zone of inhibition measuring 8 ± 1.0 and 9 ± 1.0 mm, with respect to control (25 ± 1.0 mm) against *Staphylococcus*. (Varier, KrishnapriyaMadhu, et al., 2013)

Saranraj, P. (2013) conducted a study and the methanol extract of *Gracilaria folifera* (5.0mg/ml) showed highest mean zone of inhibition (18 ± 0.4 mm) against the Gram positive cocci *Streptococcus pyogenes* followed by *Bacillus subtilis* (17 ± 0.5 mm), *Staphylococcus aureus* (17 ± 0.3 mm), *Streptococcus epidermis* (16 ± 0.6 mm) and *Bacillus cereus* (16 ± 0.2 mm). For Gram negative bacterium, the maximum zone of inhibition was recorded in methanol extract of *Gracilaria folifera* against *Klebsiella pneumoniae* (17 ± 0.5 mm) followed by *Salmonella typhi* (16 ± 0.6 mm), *Pseudomonas aeruginosa* (16 ± 0.5 mm), *Escherichia coli* (16 ± 0.3 mm). The zone of inhibition obtained from the Hexane extract of seaweed *Gracilaria folifera* against bacterial pathogens was comparatively very less when compared to the other solvent extracts. No zone of inhibition was seen in DMSO control and the positive control Ampicillin showed zone of inhibition ranging from 13 ± 0.8 mm to 20 ± 0.8 mm against the test bacterial pathogens. (Saranraj, P., 2013)

The antibacterial properties of eight crude extracts of local *Acanthophora spicifera* obtained by two distinct extraction methods were investigated by Zakaria (2010) using soxhlet extraction and solvent partitioning. By using the Disc diffusion method, these extracts were evaluated in vitro against 18 bacteria, 3 yeasts, and 6 fungal strains. The results demonstrated

that the solvent partitioning extracts of methanol and ethyl acetate had a greater spectrum of action against the tested bacterial strains. *Bacillus cereus* ATCC 10876, *Bacillus licheniformis* ATCC 12759, Menthicilin Resistance *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* ATCC 27853, *Yersinia* sp., and *Citrobacter freundii* displayed inhibitory zones against these two extracts. While methanol extracts from Soxhlet extraction and butanol from solvent partitioning had no antibacterial activity against *P. aeruginosa* ATCC 27853, the other six extracts did. (Zakaria 2010)

In a study done by Ibraheem et al. (2017), simplex extracts of *Acanthopora* showed potent inhibitory growth activities against three Gram positive bacteria [*Streptococcus agalactiae*, *pyogenes* and *Streptococcus sanguis* of inhibition ranging from [23.1±0.58 to 20.6±0.63 mm] and showed moderate activities with [*Corynebacterium diphtheriae*, *Bacillus subtilis* and *Staphylococcus aureus*] with inhibition zones ranging from [20.1±1.5 to 16.3±2.1 mm]. (Ibraheem et al.; 2017)

Also the crude extracts were found to be more active than the positive control Ampicillin, (22.3±1.5 mm), against *Streptococcus agalactiae* which showing inhibition zone. The hydro alcoholic extracts of the selected species were investigated for their antimicrobial activities using Agar well diffusion and Muller Henton against gram positive and gram negative bacteria.

In a study by Nurul Aili Zakaria et al. (2011), the antimicrobial activities of the hexane extract were evaluated using disc diffusion method against 8 Gram-negative and 10 Gram-positive bacterial strains. Out of all bacterial tested, only a Gram-positive bacterium and a Gram-negative bacterium were susceptible to the extracts. The hexane extract showed antibacterial activity against both Gram-positive bacterium and Gram-negative bacterium (*P. aeruginosa* ATCC 27853). While, chloroform and ethyl acetate extract only showed inhibitory effect on *P. aeruginosa* ATCC 27853 with inhibition zone of 9.0 mm. No inhibitory effect was showed by methanol extract on bacteria tested. (Nurul Aili Zakaria et al.; 2011)

MATERIALS AND METHODS

SPECIMEN COLLECTION

The specimen was collected by hand picking from Thikkodi beach, Calicut. The collected samples were washed immediately in seawater and then washed with fresh water and transported to the laboratory. It was again washed thoroughly to remove impurities and sand and rinsed with distilled water. The sample was identified taxonomically as *Acanthophora*. Collected sample was taxonomically evaluated using the standard literature.

SAMPLE PREPARATION

For antimicrobial studies, the cleaned samples were then shade dried, cut into small pieces and powdered in a mixer grinder. The organic solvents Chloroform and Ethanol were used for the extraction process due to its higher efficiency using Soxhlet extraction method. 20g of samples were packed in a thimble and placed in the extractor. 200ml of the solvent was added into the flask and heated. The temperature was maintained at 80°C to 85°C throughout the extraction. The soluble active constituents of the extract remained in the flask and the process was repeated until the compounds were completely extracted. The liquid extract was then cooled and concentrated by using an evaporator.

The beaker with dried extract was weighed and noted. DMSO was used to dissolve the extracts from the beaker. Later the weight of the beaker alone was noted. Hence, the actual weight of the dried extract was obtained. From the above observation, the weight of dried extract of *Acanthophora* in ethanol and chloroform was 2.44g and 0.18g respectively. From this the extractive value was calculated using the formula

Extractive value (%) = (Weight of dried extract/ Weight of plant material) X 100

PREPARATION OF EXTRACT IN VARIOUS CONCENTRATIONS

From the stock extract, concentrations of 10%, 20%, 40%, 60%(v/v) was made. The stock concentration of *Acanthophora* in ethanol and chloroform was 70mg/ml and 10mg/ml respectively. From the stock the appropriate amounts was pipetted out and made up to the required concentrations using DMSO.

ANTIBACTERIAL ACTIVITY IN ACANTHOPHORA

PREPARATION OF BACTERIAL CULTURE

In the present study, the extracts were evaluated for antimicrobial activity against *Staphylococcus* strain and *E. coli*, a Gram positive and Gram negative bacteria respectively. 3g of nutrient broth was dissolved in 100ml of distilled water in a conical flask. The broth is sterilized by autoclaving for 15 minutes. Both of the obtained bacterial stains were inoculated in the nutrient broth in laminar air flow and incubated in appropriate conditions for 24hrs.

PREPARATION OF PETRI PLATES

The selected two species of seaweeds were analysed for the antimicrobial activity for gram negative *Escherichia coli* and gram positive *Staphylococcus* by disc diffusion methods. Agar medium was prepared by dissolving 4g agar and 2.6g of nutrient broth in 100ml distilled water. The mixture is sterilized in an autoclave for 15 minutes. Just after sterilization the mixture was poured into petri plates in laminar air flow. The petri plates were allowed to solidify under aseptic conditions.

ANTIMICROBIAL TEST BY DISC DIFFUSION METHOD

Bacteria were inoculated onto the prepared agar petri plates using sterilized cotton swabs. Sterilized 6mm discs were taken from filter paper and autoclaved and are used for the method. The disc was then dipped in different concentrations of stock (10, 20, 40, 60) and placed on the agar plate using sterile forceps. Tetracycline was used as positive control and DMSO was used as negative control. This was done for both extracts of *Acanthophora* against the two strains of bacteria. The petri plates were incubated at 37°C for 24 hours and results were recorded.

OBSERVATIONS AND RESULTS

The current work was undertaken as a preliminary study of the red seaweed *Acanthophora spicifera*. The scope of the study included the estimation of extractive value in two solvents, ethanol and chloroform and the antimicrobial potential of these extracts. The antimicrobial potential activity was studied against Gram positive *Staphylococcus* and Gram-negative *E. coli*, two non-pathogenic bacteria. The results obtained are described below.

TAXONOMIC DESCRIPTION

Division: Rhodophyta
Class: Florideophyceae
Order: Ceramiales
Family: Rhodomelaceae
Genus: *Acanthophora*
Species: *spicifera*

Acanthophora spicifera (Vahl) Børgesen

Erect plants, to 40 cm tall, with solid cylindrical branches, 2 - 3 mm wide, branched either sparingly to repeatedly. Main branches have short, determinate branches, irregularly shaped and spinose, with spines numerous and radially arranged. There are no spines on main axes. The plant grows from a large, irregularly shaped holdfast. In intertidal high-motion water areas, *Acanthophora spicifera* has short (4 - 10 cm), compact and very dense thalli. In moderate or low water motion areas, the thalli are tall (10 - 25 cm), more openly branched and occur in scattered clumps.



Acanthophora spicifera(M.Vahl)Borgesen

EXTRACTIVE VALUE

Extractive values of plant materials are used to evaluate extracts of the sample, in order to get an idea about the nature of chemical constituents present in it. It can also be used to assess quality, purity and detect adulteration of the extract.

In the present study, polar and non-polar solvents were used for eluting the valuable phyto-compounds present in the sample. Extractive values of ethanol and chloroform extracts of *Acanthophora spicifera* used in the antibacterial study, are estimated in the table 1 given below;

Table 1: Extractive value of solvents administered for *Acanthophora spicifera*

Solvent	Extractive value of the sample (%)
Ethanol	12.2
Chloroform	0.9

The extractive value was greater for the ethanolic extract than for chloroform suggesting that polar solvent was more efficient in extracting the phytochemicals from the algae.

ANTIBACTERIAL ACTIVITY

The extracts of the alga exhibited only mild antibacterial activity against the two microorganisms. The activity observed can be described as being bacteriostatic showing very mild zones of inhibition. The ethanol extract of the algae showed mild antibacterial activity against *E. coli* alone. The activity is shown only at high concentration against gram negative bacteria. Chloroform extract seems to have no action on either test organisms even at higher concentrations studied. Table 2.

Table 2: Antibacterial activity of ethanolic extract of *A. spicifera* against *E. coli* and *Staphylococcus* bacteria:

Concentration(%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	No action	No action
40	No action	No action
60	Mild action	No action

Table 3: Antibacterial activity of chloroform extract of *A. spicifera* against *E. Coli* and *Staphylococcus* bacteria

Concentration(%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	No action	No action
40	No action	No action
60	No action	Mild action

The chloroform extract of *Acanthopora* has no significant effect on the growth of *E. coli*. Mild bacteriaostatic activity is observed at higher extract concentrations on *Staphylococcus*. No potential activity could be observed on the growth of *E. coli* in any of the concentrations used for the current study Table 3: Fig.

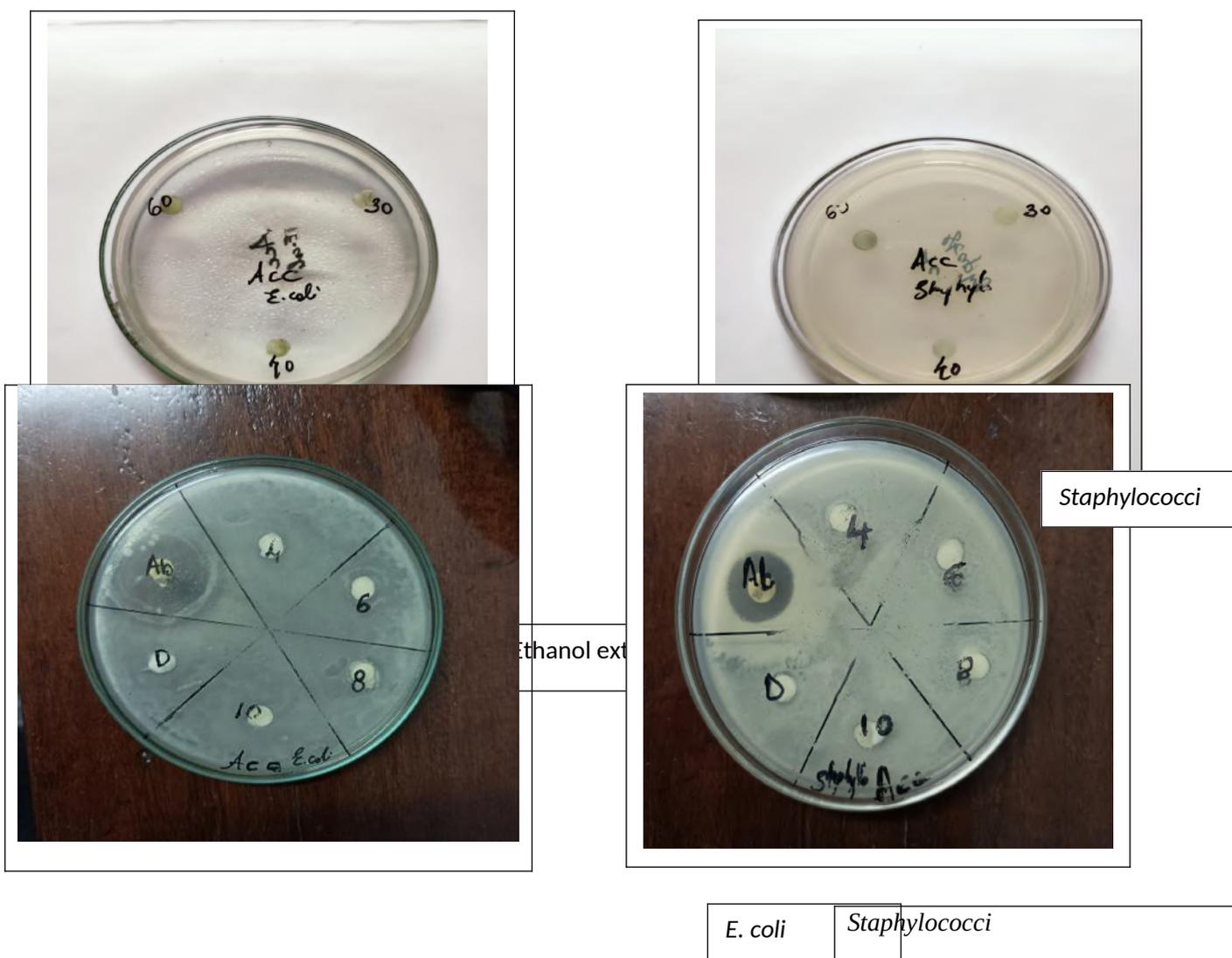


Fig 3: Antimicrobial activity of Chloroform extract of *Acanthophora spicifera*

DISCUSSION

Seaweeds are a group of marine macro algae that are now in the limelight of algal research due to their immense bioactive potentials and easy availability. Several bioactive compounds are found in high concentration in the seaweeds, because of which it exhibits various pharmacological activities. The seaweeds offer greatest wealth in terms of biomass and Rhodophytes show the largest representation among them. *Acanthophora spicifera* is a red sea with about 26 species world wide.

Natural products from marine algae have attracted the attention of biologists and chemists the as many of these compounds are used to treat diseases like cancer, acquired immune-deficiency syndrome, inflammation, pain, arthritis, as well as viral, bacterial, and fungal infections. **Sunil DS** in 2015 studied the marine red alga to analyse the phytochemical constituents. The presence of a variety of chemical constituents, such as saponins, phenols, flavonoids, alkaloids and steroids were confirmed in *Acanthophoraspicifera* by qualitative tests.

Antibacterial activity refers to the process of killing or inhibiting the disease-causing bacteria. Several plants have been traditionally used for their antibacterial activity. Like plants some algae also exhibit antibacterial properties due to the presence of terpenoids, steroids, saponins, tannins, and flavonoids. There are numerous reports regarding the inhibitory activities of macroalgae against human pathogens, fungi and yeasts. So, the use of algae as an alternative for prevention and treatment of infectious diseases has been suggested by Abirami and Kowsalya (2012). In the present study ethanolic and chloroform extract were evaluated for activity against Gram positive *Staphylococci* and Gram-negative *E. coli*. It was found that ethanolic extract had bacteriostatic activity against *E. coli* and the chloroform extract inhibited *Staphylococcus* at high concentrations.

Different solvent systems were used to extract bioactive principles from macroalgae with concomitant changes in the antibacterial activities (Thirupurasundari et al., 2008). The solvents such as acetone, benzene, butanol (Vanitha et al., 2003; Prakash et al., 2005), ethanol (Selvi et al., 2001) were used to extract antimicrobial compounds from macroalgae. The aqueous extracts prepared from seven macroalgal samples showed varying degrees of activity against tested pathogens, including the Gram positive and Gram negative bacteria. (Padmakumar (2002) is of the opinion that these differences are due to the different solubility behaviour of secondary metabolites which could be influenced by seasonal and geographical distribution of the species.

Antibacterial potential of *A. spicifera* ethanol extracts were evaluated by Meenakshi et al (2014), against six bacterial specimens. They reported high antimicrobial activity against *E. coli*. In the present study, ethanolic extract showed mild activity at higher concentration (60%) only. In their work, Meenakshi et al have also reported significant cytotoxic potential against Ehrlich Ascites carcinoma cell lines for ethanolic extract of the alga..

Several previous studies have revealed the bioactivity of active compounds isolated from *Acanthophora* sp. such as antibacterial antiviral and antioxidant activity, anti-viral, and anti-fouling. Steroids and fatty acid esters of *A. Spicifera* were reported to exhibit potent antitumor and antibacterial activity against human cancer lines and microorganisms((Laila S, 2003; Han, L et al 2006)

In their study on six sea weeds, Rajashekar et al in 2018 reported that *A. spicifera* has the least number of compounds in ethanol and chloroform extracts. This possibly explains the low antimicrobial activity of the algae in the present study also. However, they report potential inhibitory activity against *B. cereus* and *P. aeruginosa*

SUMMARY

Seaweeds are the macroalgae found in marine ecosystems where they play a multitude of roles. From being the primary producers providing nutrients and energy to other living organisms, provide shelter and home to these life forms. They also play significant roles in climate mitigation. Seaweeds have been used as traditional remedies for common ailments and have been a part of traditional cuisine in many parts of the world. Several studies have been conducted on different algae to assess their biopotentials and to exploit them in a way beneficial to man.

For this project, the red algae *Acanthophora spicifera* was selected. *Acanthophora* is an erect macroalgae, that can be found in almost all tropical and subtropical oceans. It is widely distributed in the southern and northern rocky coasts of Kerala. The alga was studied to identify its extractive values in different solvents and to assess its antibacterial potential. The dried algae was extracted in the solvents using the Soxhlet apparatus. The extractive values of the algae in ethanol and chloroform, two solvents with very different polarities was estimated and found that the polar solvent, ethanol had

greater extractive value of 12.2% than the non-polar solvent chloroform with only 0.9% extractive value.

Propanol (polar) and Benzene, Chloroform and Hexane (non polar) were evaluated. Only the extracts showing antibacterial activity (Ethanol, Chloroform, Isoamyl alcohol, Methanol and Propanol) were considered for further study.

The antioxidant potential was studied by the disc diffusion method. The test organisms used were the Gram positive *Staphylococcus* and Gram negative *E. Coli*. The analysis of antimicrobial activity of *Acanthophora spicifera* displayed little to no microbial activity in both ethanolic and chloroform extracts for gram positive (*Staphylococcus*) and gram negative (*E. coli*) bacteria taken. A mild reaction suggesting bacteriostatic activity was observed in ethanolic extract at higher concentration for gram negative bacteria while the chloroform extract showed mild reaction for gram positive bacteria at higher concentration. This can be used as an indicator for further studies of antimicrobial activity of the species.

Acanthophora possess antioxidant, antitumor and antibacterial activity, which may be due to the presence of bioactive components such as phenolics, terpenoids, and tannins. The chemical constituent of *Acanthophora* is rich in non halogenated steroid. Various other extracts of the algae have highlighted the nutritional and anticancer properties which supports its widespread usage in folklore medicine. Thus the proper identification and analysis of the seaweed can be used for producing natural alternatives to the synthetic medicines available in today's market.

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**MORPHOLOGICAL AND ANATOMICAL EVALUATION OF
PTERIDOPHYTES FROM THE BOTANICAL GARDEN OF
ST. TERESA'S COLLEGE OF ERNAKULAM DISTRICT**

**A DISSERTATION SUBMITTED TO
MAHATMA GANDHI UNIVERSITY, KOTTAYAM
IN PARTIAL FULFILLMENT OF REQUIREMENTS FOR
AWARD OF THE DEGREE OF
“BACHELOR OF SCIENCE IN BOTANY”**

**BY
JIJI JOSEPH
REG NO: AB19BOT009**



**DEPARTMENT OF BOTANY
ST. TERESA'S COLLEGE (AUTONOMOUS)
ERNAKULAM**

MAY - 2022

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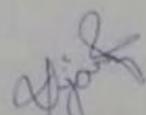
**DEPARTMENT OF BOTANY
ST. TERESA'S COLLEGE (AUTONOMOUS)
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CERTIFICATE

This is to certify that this dissertation entitled "MORPHOLOGICAL AND ANATOMICAL EVALUATION OF PTERIDOPHYTES FROM THE BOTANICAL GARDEN OF ST. TERESA'S COLLEGE OF ERNAKULAM DISTRICT" is an authentic record of research work carried out by Miss. Jiji Joseph (AB19BOT009) under the supervision and guidance of Dr. Alphonsa Vijaya Joseph of St. Teresa's College (Autonomous), Ernakulam. I further certify that no part of the work embodied in the project has been submitted for the award of any other degree or diploma.

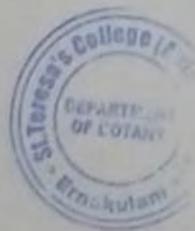


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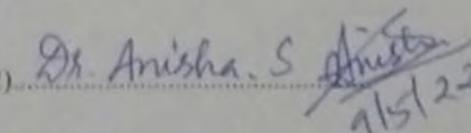


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Examiners



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Dr. Anila V

2)  7/5/22
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Place: Ernakulam

Date: 09-05-2022

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**MORPHOLOGICAL AND ANATOMICAL EVALUATION OF
PTERIDOPHYTES FROM THE BOTANICAL GARDEN OF
ST. TERESA'S COLLEGE OF ERNAKULAM DISTRICT**

INTRODUCTION

An ecosystem can be categorized into its abiotic constituents, including minerals, climate, soil, water, sunlight, and all other nonliving elements, and its biotic constituents, consisting of all its living members. Linking these constituents together are two major forces: the flow of energy through the ecosystem and the cycling of nutrients within the ecosystem. Ecosystems vary in size: some are small enough to be contained within single water droplets while others are large enough to encompass entire landscapes and regions. The fundamental source of energy in almost all ecosystems is radiant energy from the Sun. The energy of sunlight is used by the ecosystem's autotrophic, or self-sustaining, organisms (that is, those that can make their own food). Consisting largely of green vegetation, these organisms are capable of photosynthesis—i.e., they can use the energy of sunlight to convert carbon dioxide and water into simple, energy-rich carbohydrates. The autotrophs use the energy stored within the simple carbohydrates to produce the more complex organic compounds, such as proteins, lipids, and starches, that maintain the organisms' life processes. The autotrophic segment of the ecosystem is commonly referred to as the producer level (Spellman et al, 2001).

Plants are predominantly photosynthetic eukaryotes of the kingdom Plantae. Plants are a large and varied group of organisms. There are close to 300,000 species of catalogued plants.¹ Of these, about 260,000 are plants that produce seeds. Mosses, ferns, conifers, and flowering plants are all members of the plant kingdom. The plant kingdom contains mostly photosynthetic organisms; a few parasitic forms have lost the ability to photosynthesize. The process of photosynthesis uses chlorophyll, which is located in organelles called chloroplasts. Plants possess cell walls containing cellulose. Most plants reproduce sexually, but they also have diverse methods of asexual reproduction. Plants exhibit indeterminate growth, meaning they do not have a final body form, but continue to grow body mass until they die.

Pteridophytes are the first terrestrial vascular plants. Carolus Linnaeus classified them under the group cryptogamae. Pteridophytes occupy a transitional position between bryophytes and spermatophytes. They do not produce flowers and seeds, hence they are also called Cryptogams. More than 12,000 species of pteridophytes are found on Earth. The word 'Pteridophytes' comes from Greek word Pteron meaning "feather" and python meaning "plants".

Pteridophytes mainly thrive on moist and shady places and some of them grow in sandy soil. The main plant body is sporophyte which differentiated into roots, stem and leaves. The stem is underground rhizome. Some pteridophytes have some leaves called microphylls (*Lycopodium*) and some have large leaves called megaphylls (*Pteris*). Leaves may also have spores on the underside. They are called Sporophylls. Pteridophytes are flowerless and seedless hence they reproduce through spores. Sometimes sporophylls form compact structures called Strobili. A well developed vascular system with xylem and phloem is present (Soni, N. K. 2010).

Like other plants, pteridophytes constitute a good source of food to animals. Sporocarps of *Marsilea*, a water fern, yield starch that is cooked and eaten by certain tribal. By their growth, pteridophytes bind the soil even along hill slopes. The soil is protected from erosion. *Equisetum* stems have been used in scouring (cleaning of utensils) and polishing of metals. *Equisetum* species are, therefore, also called scouring rushes. *Azolla* (a water fern) has a symbiotic association with nitrogen fixing cyanobacterium *Anabaena azollae*. It is inoculated to paddy fields to function as biofertilizer. An anthelmintic drug is obtained from rhizomes of *Dryopteris*. Ferns are grown as ornamental plants for their delicate and graceful leaves (Eisenberg et al, 2009)

Selaginella is a pteridophyte. It is also called spikemoss or club moss. It is the largest and the only living genus of the family Selaginellaceae. It contains more than 800 species distributed all around the world with the highest diversity found in the tropical regions. They are seedless vascular plants. They are mostly found in shady areas, some species are also present in seasonal dry or xerophytic conditions. They are found on tree trunks, rocks, forest floors, etc. The main plant body is a sporophyte. It is a vascular plant and is differentiated into root, stem and leaves (Leitch et al, 2013).

Lygodium is a climbing fern native to large parts of tropical and subtropical Africa, Asia and Oceania. This climbing fern causes significant ecological impacts, forming thick mats which climb over and smother undergrowth, shrubs and even tall trees and modify fire regimes so that ground fires are carried into the forest canopy.

Equisetum is also called horse tail, scouring rush, fifteen species of rush like conspicuously jointed perennial herbs, the only living genus of plants in the order Equisetales and the class Equisetopsida. Horsetails grow in moist, rich soils in all parts of the world except Australasia. Some species produce two kinds of shoots: those with conelike clusters (strobili) of spore capsules

and those lacking such structures. Some are evergreen; others send up new shoots annually from underground rootstalks. Their hollow, jointed, ridged stems contain silicate and other minerals. The leaves are reduced to sheaths that clasp and encircle the shoots. Stems that bear terminal spore cones are often flesh-coloured and are present (Kachhiyapatel, R. N. 2022).

Angiopteris is a genus of huge evergreen ferns from the family Marattiaceae, found throughout the paleotropics from Madagascar to the South Pacific islands. This fern consists of a short, fleshy stem that bears many leaves at the tip. Leaves are deciduous. The sporangia develop on the underside along the sides of the pinnules.

The genus *Microsorium* is derived from the Greek word for small and the spore-containing structure known as sorus. It is epiphytic, rhizomatous fern, able to grow up to 60 - 80 cm tall and 20 - 30 cm wide. Leaves are lanceolate shape with pointed tips. Stem has an underground horizontal stem known as a rhizome. Spores occur on the frond underside in small, dark brown spots. This cultivar is grown for its ornamental foliage. It is suitable for container plantings and may be placed indoors or outdoors.

Nephrolepis means "kidney-scale" and refers to the shape of the indusia of the sori. Is a sub-erect fern, with slightly drooping fronds, able to grow up to 60 - 90 cm tall and 0.5 - 1 m wide. The plant body is sporophyte differentiated in to rhizomes, roots and leaves. Leaves are tufted, long, narrow and simply with fish tail. Sori round and arranged in 2 rows near the margins of the pinnae.

Pteris is a genus of about 300 species of ferns in the subfamily Pteridoideae of the family Pteridaceae. They are native to tropical and subtropical regions of the world. The frond margin is reflexed over the marginal sori. The sporophytic plant body is differentiated in to roots, stem and leaves. The underground stem is branched and perennial, rhizomatous. Leaves arise from the upper portion of the rhizome. Leaves are unipinnately compound arranged in acropetal manner. Rhizome is differentiated in to node and internode (Kamau, 2012).

The genus *Marsilea* comprises about 58 living species. The species of *Marsilea* are aquatic or amphibious plants the grow either completely sub-merged in water, or partly or wholly out of water with their roots embedded in the muddy soil (Preston and Croft, 2001). The plant body is sporophyte and is differentiated in to stem ,roots and leaves. Stem consists of an elongated stolon-

like rhizome with distinct nodes and internodes. Rhizome grows either on the surface of the soil or slightly embedded in the muddy soil. The stem is freely branched. Leaves are long-petioles and compound, developing at the nodes. They are arranged alternately in two rows along the upper side of the rhizome. Roots arise from the underside of the stem at the nodes. The roots are unbranched, adventitious type and arise in one or two clusters (Larson,1993).

Adiantum is commonly known as 'Maiden hair fern' or 'Walking fern'. They are distributed in the tropical and temperate regions of the world. The sporophyte is differentiated into rhizome, roots and leaves. The rhizome is a perennial, subterranean dichotomously branched structure. It is covered with persistent leaf bases and hairy outgrowths called ramenta. The roots are adventitious and arise from the rhizome. The leaves are also called fronds and are pinnately compound, the young leaves are circinate. The petiole is long, black and shiny. The venation is free and dichotomous in all the species. The leaves bear marginal sori which are covered by a false indusium (Britton & Brown,1896).

The flora of this Ernakulam district is tropical. The heavy rainfall combined with moderate temperature and fertile soil support a luxuriant vegetation. Many of the common plants are found in the coastal area which forms the low land region. Coconut is extensively cultivated here. Mangalavanam Bird Sanctuary is located at the centre of Kochi. It covers 2.74 acres supports many species of mangroves and is a nesting ground for a variety of migratory birds. The Mangalavanam is called the "green lung of Kochi", considering its role in controlling the city's air pollution. Thattekad Bird Sanctuary lies on the northern bank of the Periyar River and covers about 25 km² (10 sq mi). It was founded by ornithologist Salim Ali. The sanctuary is 80 km (50 mi) from Kochi. Birds found here include falcons, jungle fowl, water hens, and hornbills. The flora of this area consists mainly of plantations of teak, rosewood, and mahogany (Vannucci, 2002).

St. Teresa's College, Ernakulam have lot of vegetation in their Botanical Garden. Plants in the garden help students for their academic purpose.

So, My study is concerned with Morphological and Anatomical evaluation of Pteridophytes from the botanical garden of St. Teresa's College of Ernakulam district.

OBJECTIVES OF THE STUDY

- Survey of Pteridophytes in the Botanical Garden of St. Teresa's College, Ernakulam.
- Collection of fresh samples of Pteridophytes from the study area.
- Morphological evaluation of collected Pteridophytes.
- Anatomical evaluation of collected Pteridophytes.

REVIEW OF LITERATURE

Satti N L Malati and Geddada Mohan Narasimha investigated the ‘Pteridophytes in India’ in the year 2020. Studies on Pteridophytes in India were reported since British India by Beddome 1. The Fern studies gained momentum after the establishment of the Indian Fern Society in 1983. Varieties of Pteridophytes are present in Himalayan mountain range, Eastern Ghats and Western Ghats of India and are in use by tribal people as food, medicine and ornamental plants since ancient periods. The distribution and diversity of Pteridophytes has been studied along east coast in parts of Tamil Nadu, Andhra Pradesh and Odisha. This review focussed on the effects of elevation, humidity, temperature and environment on Pteridophytes distribution. The present paper provides comprehensive review of studies on Pteridophytes carried out by researchers on east coast of India and presents the species of Pteridophytes that are becoming extinct. The effects of climatic conditions, rainfall, land use pattern and environmental effects on pteridophytes distribution and sustainability are highlighted (Malati and Rao, 2020).

K P Rajesh and Vijisha Palakkal pointed that “ Pteridophyte Flora of Aralam Wildlife Sanctuary, Kerala” in 2016. A total of 69 species of pteridophytes including 63 Ferns and six Lycophytes were recorded from the Aralam Wildlife Sanctuary in the Western Ghats of Kerala. It harbours some of the rare and endemic species, including the members of Hymenophyllaceae, the most sensitive fern family. The preliminary analysis on the pteridophytes reveals the rich pteridophytes diversity of the Aralam Wildlife Sanctuary. It also indicates the need of detailed survey on the pteridophyte flora (Vijisha and Rajesh, 2016).

. Alexandru M F Tomescu in 2017 studied on ‘Developmental programmes in the evolution of *Equisetum* reproductive morphology’ .The origin of the *Equisetum* strobilus has long been debated and the fossil record has played an important role in these discussions. The paradigm underlying these debates has been the perspective of the shoot as node–internode alternation, with sporangiophores attached at nodes. However, fossils historically excluded from these discussions (e.g. *Cruciaetheca* and *Peltotheca*) exhibit reproductive morphologies that suggest attachment of sporangiophores along internodes, challenging traditional views. This has rekindled discussions around the evolution of the *Equisetum* strobilus, but lack of mechanistic explanations has led discussions to a stalemate. This model has implications – testable by studies of the fossil record, phylogeny and development – for directionality in the evolution of reproductive morphology

(*Cruciaetheca–Peltotheca–Equisetum*) and for the homology of the *Equisetum* stobilus. Furthermore, this model implies that sporangiophore development is independent of node–internode identity, suggesting that the sporangiophore represents the expression of an ancestral euphyllphyte developmental module that pre-dates the evolution of leaves (Tomescu et al, 2017).

In the year 2002 Harald Schneider and Kathleen Pryer enumerate that ‘Structure and function of spores in the aquatic heterosporous fern, family Marsileaceae’. Spores of the aquatic heterosporous fern family Marsileaceae differ markedly from spores of Salviniaceae, the only other family of heterosporous ferns and sister group to Marsileaceae, and from spores of all homosporous ferns. The marsileaceous outer spore wall (perine) is modified above the aperture into a structure, the acrolamella, and the perine and acrolamella are further modified into a remarkable gelatinous layer that envelops the spore. Observations with light and scanning electron microscopy indicate that the three living marsileaceous fern genera (*Marsilea*, *Pilularia*, and *Regnellidium*) each have distinctive spores, particularly with regard to the perine and acrolamella. Several spore characters support a division of *Marsilea* into two groups. Spore character evolution is discussed in the context of developmental and possible functional aspects. The gelatinous perine layer acts as a flexible, floating organ that envelops the spores only for a short time and appears to be an adaptation of marsileaceous ferns to amphibious habitats. The gelatinous nature of the perine layer is likely the result of acidic polysaccharide components in the spore wall that have hydrogel (swelling and shrinking) properties. Megaspores floating at the water/air interface form a concave meniscus, at the center of which is the gelatinous acrolamella that encloses a “sperm lake.” This meniscus creates a vortex-like effect that serves as a trap for free-swimming sperm cells, propelling them into the sperm lake (Schneider and Pryer, 2002).

A study on ‘Morphology, Anatomy and Ontogeny of *Adiantum capillus veneris* : An experimental system to study the development of ferns in 2013 by Xia Li, Yu Han Fang, Ji Yang, Shou Nong Bai and GUANG yuan Rao. *Adiantum capillus-veneris* L., commonly regarded as a good experimental plant for the study of fern development, is investigated, and its life cycle under laboratory conditions is described. In our study, the life cycle of *A. capillus-veneris* was completed in 100 days. Features of spore germination, antheridium and archegonium ontogeny and development, and embryogenesis are investigated. Exosporic gametophytes of *A. capillus-veneris* came from spores, and ended with the cordate prothallia. Antheridia and archegonia were

generally situated toward the posterior end and the notch of the prothallia, respectively. Sporophyte was also studied with emphasis on leaf morphology and the vascular system. There were vascular bundles of dictyostele without secondary vascular tissues. Sporogenesis was scrutinized from sporangial initial of a single surface cell to the annulus shedding spores explosively. Our findings provide insight into the life cycle of the leptosporangiate ferns under experimental conditions, and make it possible for *A. capillus-veneris* to be an underlying model plant for the study of evolutionary and developmental biology (Li et al, 2013).

MATERIALS AND METHOD

- **Study area:**

The Botanical Garden of St. Teresa's College has been selected as the area for study.

- **Survey of plant samples from the study area:**

Cryptogams were selected as the plant samples for the study. A detailed survey has been conducted for the collection of plant samples. 9 species of cryptogams were found in the study area.

- **Collection of plant samples:**

The fresh plant samples such as *Selaginella*, *Equisetum*, *Angiopteris*, *Microsorium*, *Lygodium*, *Nephrolepis*, *Pteris*, *Adiantum* and *Marsilea* were collected for the study from the study area.

- **Morphological evaluation of collected plant samples:**

Fresh plant samples were collected from the study area and morphological characters were analyzed and recorded.

- **Anatomical evaluation of collected plant samples:**

For examining anatomical characteristics, a thin cross-section of the stem or petiole is taken by using a clean and sharp blade. Then the cross-section was mounted with safranin stain and glycerine on a clean glass slide and observed under a compound microscope. The anatomical features were recorded and photographs were taken.

**OBSERVATIONS
AND
RESULTS**

1. *SELAGINELLA*

Selaginella is a fascinating plant that spreads and acts like a moss. It is also called Spike moss or club moss. It is the largest and only living genus of the family Selaginellaceae. They were found in a shady area.



PLATE 1: HABITAT OF *SELAGINELLA*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Lycopodiopsida
Order : Selaginellales
Family : Selaginellaceae
Genus : Selaginella

- **MORPHOLOGICAL EVALUATION OF *SELAGINELLA***

SPOROPHYTE: The sporophyte is an evergreen, delicate herb. Plants are found to be erect. The plant body consists of Root, Stem, Leaves, Ligules and Rhizophores

ROOT: The root of the young sporophyte is the primary root while others are adventitious. Aerial roots have developed caps, and cutinized epidermal cells enter the soil. Their origin is endogenous. They originate either from the tips of rhizophores or directly from the stem or from the swollen base of the hypocotyl.

STEM: The stem is profusely branched, delicate and evergreen. The branching is of monopodial type.

LEAVES: Leaves are small, simple and lanceolate with a pointed apex. Each leaf is provided with a single unbranched midrib. Leaves near the apical portion of the branch, bear sporangia (micro-or mega) and are called sporophylls (micro-or mega) respectively. The sporophylls are usually aggregated into a condensed structure which is known as a strobilus. Small leaves are present on the dorsal side of the stem and bigger ones on the ventral side of the stem. Microphylls are present, Anisophyllous and Isophyllous based on the unequal and equal size of leaves.

LIGULES: The Ligules are found on the adaxial side of the leaf and are a small membranous out-growth present at the base of the leaf in a pit-like structure known as a ligule pit. The structure of the ligule consists of two parts, glossopodium and the body of the ligule

RHIZOPHORES: It is a colourless, leafless, unbranched and cylindrical structure. A tuft of adventitious roots gets developed when it touches the soil.

- **ANATOMICAL EVALUATION OF *SELAGINELLA* STEM**

A Transverse - section of the *Selaginella* stem is circular in outline. It includes Epidermis, Cortex and Stele

EPIDERMIS: It is the outermost covering layer comprising of a single cell in thickness. The epidermal cells are covered by a thick coating of cuticle. Hairs and stomata are absent.

CORTEX: it is seen inner to the epidermis. The cortex is differentiated into the inner and outer cortex. it is made up of parenchymatous cells. The parenchymatous cortex is made up of angular cells without intercellular spaces

STELE: The central portion of the stem is occupied by a well-developed stele. The stele is of protostelic type i.e., the xylem is present in the center and surrounded by phloem on all sides. Phloem, in turn, is surrounded by a single-layered pericycle. Pith is absent. The stele is surrounded by a single - layered pericycle made of parenchymatous cells.



PLATE 2: T.S OF *SELAGINELLA* STEM

2. *LYGODIUM*

It is an evergreen fern with climbing fronds. They are epiphytic and perennial in nature. It grows thick into mats which smother the undergrowth and up over shrubby trees. It is found growing in moist and shady places which are rich in humus and other organic matters,



PLATE 3: HABITAT OF *LYGODIUM*

- **TAXONOMIC POSITION**

Kingdom : Plantae

Class : Polypodiopsida
Order : Schizaeles
Family : Lygodiaceae
Genus : Lygodium

- **MORPHOLOGICAL EVALUATION OF *LYGODIUM***

SPOROPHYTE: The plant body consists of leaves, stem and creeping rhizome

STEM: Dichotomously branched, Indeterminate rachis growth

LEAVES: Pinnately compound leaves

- **ANATOMICAL EVALUATION OF *LYGODIUM* STEM**

A transverse section (T.S.) of the stem of Lygodium is circular in outline.

EPIDERMIS: Single-layered and broader Parenchymatous cells

CORTEX: The cortex is differentiated into outer and inner sclerenchymatous cells and middle parenchymatous cells. The whole of the cortex is made up of parenchymatous cells with small or large intercellular spaces and the sclerenchymatous cells, without intercellular spaces.

STELE: Protostelic type. Stele comprises primary xylem and primary phloem. pith is absent and the stele is situated in the centre.

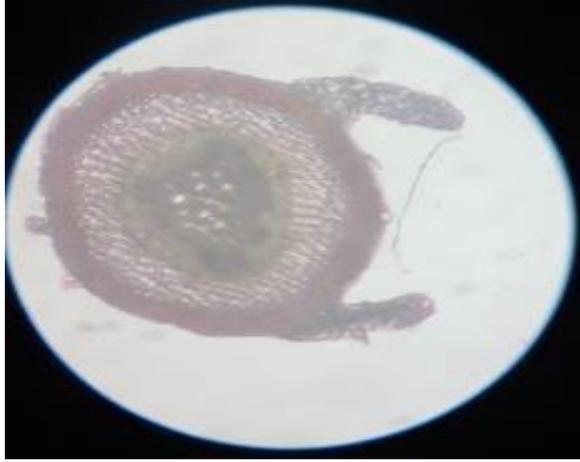


PLATE 4: T.S OF *LYGODIUM* STEM

1. *EQUISETUM*

Equisetum is a herbaceous plant. It is also known as field horsetail or common horsetail.

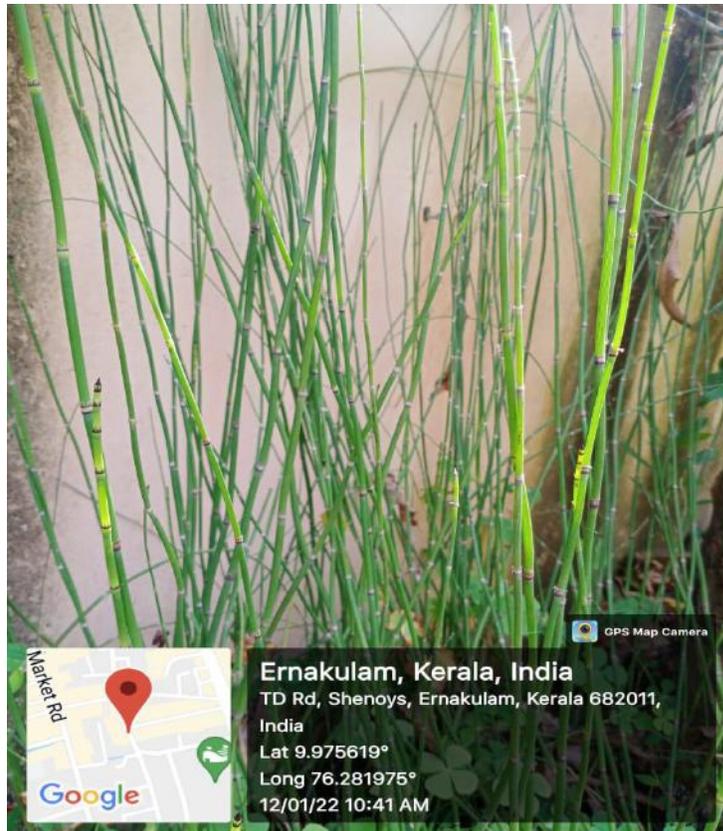


PLATE 5: HABITAT OF *EQUISETUM*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Equisetales
Family : Equisetaceae
Genus : Equisetum

- **MORPHOLOGICAL EVALUATION OF *EQUISETUM***

SPOROPHYTE: The plant body is sporophyte and erect. The sporophyte is differentiated into root, stem and leaves.

STEM: Stem consists of underground rhizome and upright green branches. Jointed stem with nodes and internodes with longitudinal ridges and furrows.

LEAVES: Silica deposits in stem make it rough. Leaves nodes with small, sessile microphyllous scale leaves in whorls. Fertile branches bear strobili after some vegetative growth.

ROOTS: Adventitious roots arise from nodes of rhizome. Roots are photosynthetic in nature.

SPORES: Equisetum is homosporous and Eusporangiate. Strobili are borne terminally and singly on aerial fertile branches. Strobilus consists of a central axis on which stalked sporangiophores with sporangium are arranged in whorls.

- **ANATOMICAL EVALUATION OF *EQUISETUM* STEM**

A transverse - section of the *Equisetum* stem is wavy in outline because of the presence of ridges and grooves. It includes Epidermis, Cortex and Stele

EPIDERMIS: The epidermis is single-layered with stomata and heavily coated with silica deposits.

CORTEX: Outer cortex is sclerenchymatous and chlorenchymatous. Inner cortex is made up of large parenchymatous cells with vallecular canals. Vallecular canals are large air-filled, intercellular spaces below the furrows in the inner cortex. It shows a hydrophytic character. Endodermis and pericycle single layered.

STELE: Xylem is V-shaped. Protoxylem is endarch lying opposite to carinal cavity. Two strands of metaxylem are present. Phloem is present between two strands of metaxylem and made up of phloem parenchyma and sieve tubes. Pith is present in the form of pith cavity, located in the centre of the aerial shoot. A water-containing cavity is present in each vascular bundle known as a carinal canal. The Carinal canal is a water-filled region present in the vascular bundle.

PITH: Pith is large central cavity filled with water.

XEROPHYTIC CHARACTERS:

1. Presence of ridges and furrows.
2. Presence of sunken stomata.
3. Presence of well-developed sclerenchymatous hypodermis.
4. Presence of reduced and scaly leaves.
5. Presence of well-developed vascular cylinder.

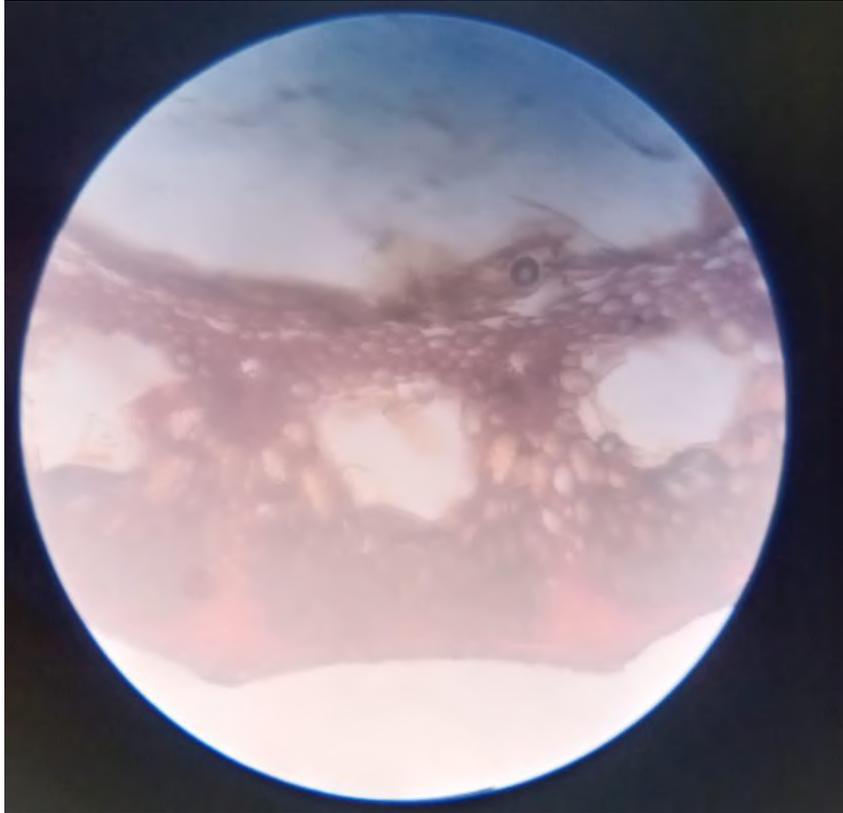


PLATE 6: T.S OF *EQUISETUM* STEM

2. ANGIOPTERIS

Angiopteris is an evergreen fern plant. They are unique among ferns in having explosively dispersed spores.



PLATE 7: HABITAT OF ANGIOPTERIS

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Marattiales

Family : Marattiaceae

Genus : *Angiopteris*

- **MORPHOLOGICAL EVALUATION OF *ANGIOPTERIS***

SPOROPHYTE: The sporophytic plant body consists of an upright, tuberous, conical, fleshy rhizomatous stem.

STEM: The stem is thick and inhabits the plant resembles a tree fern. The stem is often called a caudex or trunk which may be a foot or two in height and almost the same girth.

RHIZOME: The upper surface of the rhizome bears a crown of graceful, stately leaves.

LEAVES: Leaves are deciduous. The leaves are 5-6 metres long in a luxuriously growing plant, with a petiole as thick as a man's arm. The leaves are typically bi-pinnately compound. The venation is of the open dichotomous type Pinnae are glabrous (smooth). a pair of thick fleshy stipules at the base of the leaf is present. When the leaves fall off, these persist with the leaf bases and form a protective armour around the stem. The base of the petiole and the stipules together appear like a horse's hoof. The pinnae are long, dorsoventrally flattened and have a long drawn tip. The margin is serrate. The sori occupy a near-terminal position on the dichotomously branched veins.

ROOTS: Roots are produced from the undersurface of the rhizome at the base of each leaf. Root hairs are peculiar in being multicellular. At the margins of the pinnae on the abaxial surface are borne, the sori. Roots are perennial, thick and have a mycorrhizal association.

- **ANATOMICAL EVALUATION OF *ANGIOPTERIS* PETIOLE**

Transverse - section of petiole shows a circular outline. It includes the epidermis, cortex and stele.

EPIDERMIS: single-layered epidermis which consists of thin-walled cells.

CORTEX: The bulk of petiole is composed of ground tissue and is differentiated into three zones. The outermost zones consist of 3-4 layers of cells which are made up of thin-walled parenchymatous cells. The middle zone consists of 3-4 layers of cells made up of thick-walled sclerenchymatous cells, being comparatively smaller in size than the cells of the outer and inner zone. Some of the cells of the middle and inner zone contain tannin. The endodermis is followed by a pericycle containing thin-walled cells, which are 1-3 layers in thickness. Xylem lies in the centre of the vascular strand. It is plate-like with several protoxylem points in exarch conditions. Xylem is surrounded by phloem. Phloem consists of sieve cells and parenchyma and xylem have simple tracheids of various sizes. Metaxylem tracheids have scalariform and pitted thickening while protoxylem tracheids have annular and spiral thickening.

STARCH GRAINS: The inner zone consists of large and thin-walled polygonal cells filled with starch grains. Starch grains are usually large and spherical or oval in shape. The concentrations of these grains are more towards the base of the petiole and gradually decrease towards the apex.



PLATE 8: C.S OF ANGIOPTERIS PETIOLE

3. *MICROSORUM*

Microsorium is an epiphyte and perennial in nature. They grow from rhizomes, rather than roots.



PLATE 9: HABITAT OF *MICROSORUM*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Polypodiaceae

Genus : Microsorium

- **MORPHOLOGICAL EVALUATION OF *MICROSORUM***

SPOROPHYTE: The plant body consists of hard stem and long petiole.

LEAVES: Lanceolate shape, pointed tip, the winged base of leaf, midrib is raised and highly prominent.

RHIZOME: The underground horizontal stem is known as the rhizome

SPORES: occur on the frond underside in small and dark brown spots

- **ANATOMICAL EVALUATION OF *MICROSORUM* PETIOLE**

EPIDERMIS: single-layered and parenchymatous

CORTEX: made up of parenchymatous cells

STELE: vascular bundles are present. Endodermis is present



PLATE 10: T.S OF *MICROSORUM* PETIOLE

4. *NEPHROLEPIS*

It is an epiphytic fern. Feather-like fronds in shades of green make these ferns valuable.



PLATE 11: HABITAT OF *NEPHROLEPIS*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Nephrolepidaceae

Genus : Nephrolepis

- **MORPHOLOGICAL EVALUATION OF *NEPHROLEPIS***

SPOROPHYTE: The plant body is sporophytic differentiated into rhizomes, roots and leaves.

RHIZOME: Rhizome is short, slender and wide creeping. It bears a close tuft of leaves and long, slender lateral branches called runners. Runners bear adventitious roots and in acropetal succession.

LEAVES: The leaves are tufted, long, narrow and simply with fish tail. Margins are present.

- **ANATOMICAL EVALUATION OF *NEPHROLEPIS* STEM**

The transverse section of the petiole has an adaxial groove.

EPIDERMIS: It is composed of small thick walled cells

CORTEX: Epidermis is followed by 3-4 layers of sclerenchymatous cortex and compact parenchyma. Conducting strands are embedded in the parenchymatous cortex and are arranged in a horse - shoe like shape.

STELE: Protoxylem lies towards outside. Phloem completely surrounds xylem which in turn is surrounded by a layer of pericycle and endodermis.

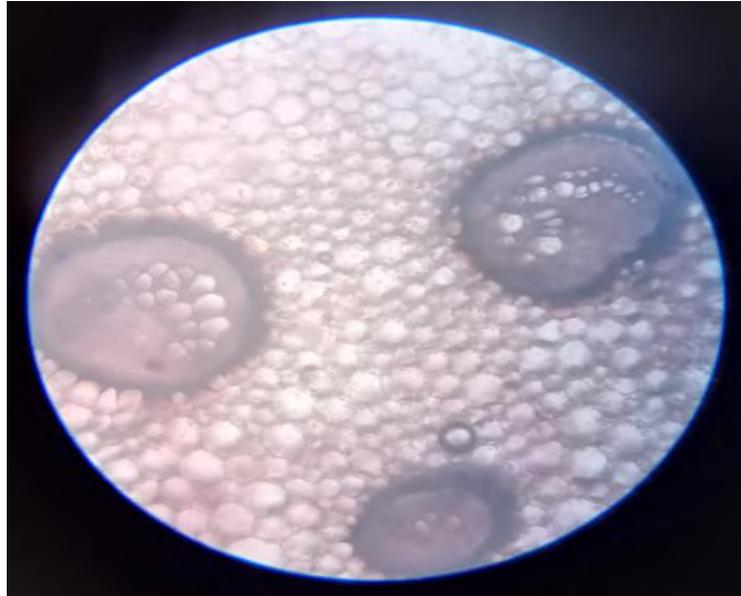


PLATE 12: T.S OF *NEPHROLEPIS* STEM

5. *PTERIS*

They inhabit shady and moist areas.

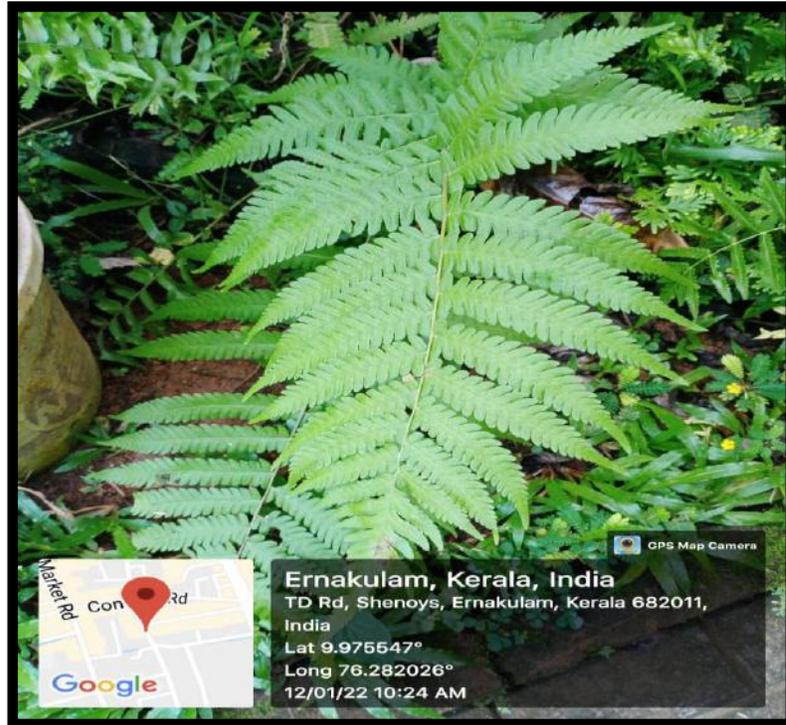


PLATE 13: HABITAT OF *PTERIS*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Pteridaceae

Genus : Pteris

- **MORPHOLOGICAL EVALUATION OF *PTERIS***

SPOROPHYTE: The sporophytic plant body is differentiated into Root, Rhizomatous stem and Leaves

ROOT: The roots are small and branched.

RHIZOME: The rhizome is differentiated into nodes and internodes and its entire surface is covered with scales. The growing point of rhizome is covered with ramenta.

LEAVES: The leaves are borne on the upper surface of the rhizome. The young leaves are spirally coiled and show circinate vernation. The leaves are multipinnately compound with long rachis. The pinnae are small near the base as well as towards the apex, while they are large towards the middle.

- **ANATOMICAL EVALUATION OF *PTERIS* PETIOLE:**

EPIDERMIS: single-layered and covered with cuticle. Ramenta arise from the epidermis

CORTEX: Differentiated into outer sclerenchymatous and inner parenchymatous zone

STELE: Xylem has two adaxial hooks; xylem is surrounded by phloem. Pericycle and endodermis is present

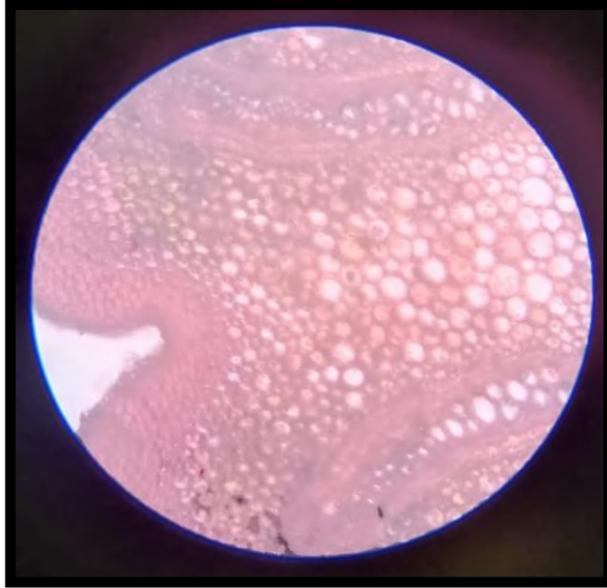


PLATE 14: T.S OF *PTERIS* PETIOLE

6. *MARSILEA*

Marsilea is herbaceous plant. It is also known as a water clover plant and four-leaf clover plant. It does not resemble common ferns. They either present above water or submerged

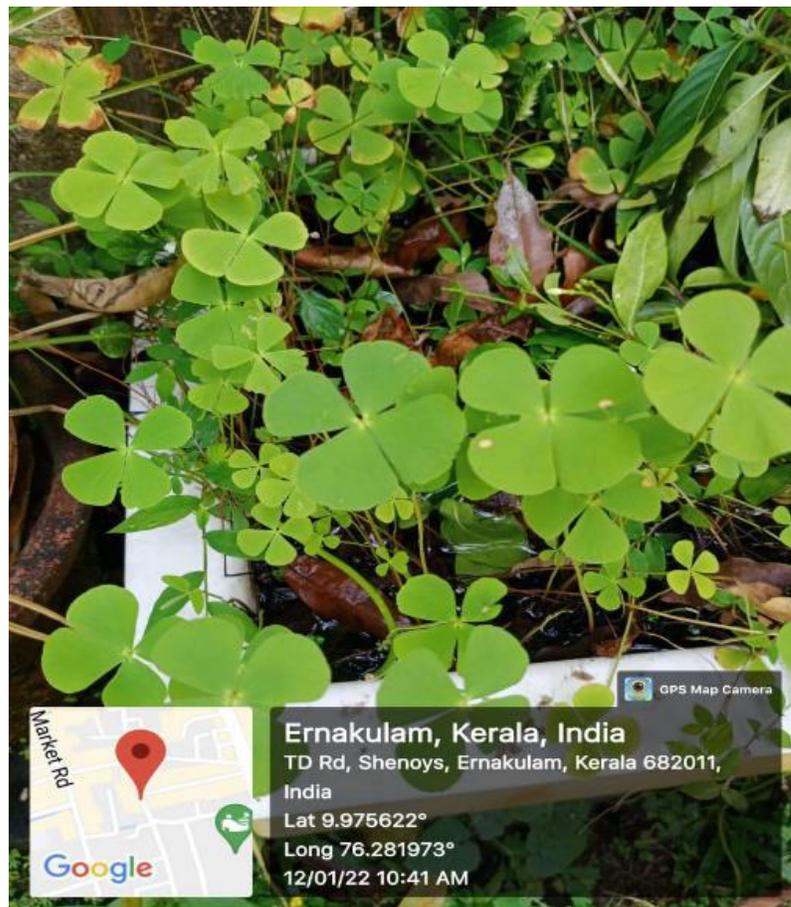


PLATE 15: HABITAT OF *MARSILEA*

- **TAXONOMIC POSITION**

Kingdom : Palantae

Class : Polypodiopsida

Order : Salviniiales

Family : Marsileaceae

Genus : Marsilea

- **MORPHOLOGICAL EVALUATION OF *MARSILEA***

SPOROPHYTE: The plant body is differentiated into rhizomes, leaves and roots.

RHIZOME: It is slender, dichotomously branched with distinct nodes and internodes and is capable of indefinite growth in all directions.

LEAVES: They are borne alternately on the upper side of the rhizome at nodes, in two rows. They show circinate vernation. In submerged plants the petiole is long and the lamina floats over the surface of the water but in muddy or marshy plants the petiole of the leaf is short and rigid with short lamina spreading in the air. The lamina consists of 4 leaflets/pinnae which are present at the apex of the petiole. Near the base of the petiole, the stalked bean-shaped sporocarps are borne.

ROOTS: The roots are adventitious, arising from the underside of the node of the rhizome, either singly or in groups.

- **ANATOMICAL EVALUATION OF *MARSILEA* PETIOLE**

EPIDERMIS: The outermost layer is the epidermis which consists of rectangular cells. Below the epidermis is the hypodermis followed by the cortex.

CORTEX: The cortex is differentiated into the outer and inner cortex. The outer cortex consists of aerenchyma having many air cavities or air chambers separated from each other with the help of

one-celled thick trabeculae or septa. The inner cortex is parenchymatous and contains starch and tannin-filled cells.

STELE; The stele is triangular in shape, the present shows the protostelic structure. The stele is bounded by a layer of endodermis and an unilayered pericycle. Xylem is V-shaped and the arms of 'V' contain a metaxylem in the center and protoxylem towards the ends. The xylem remains surrounded by the phloem in the centre and

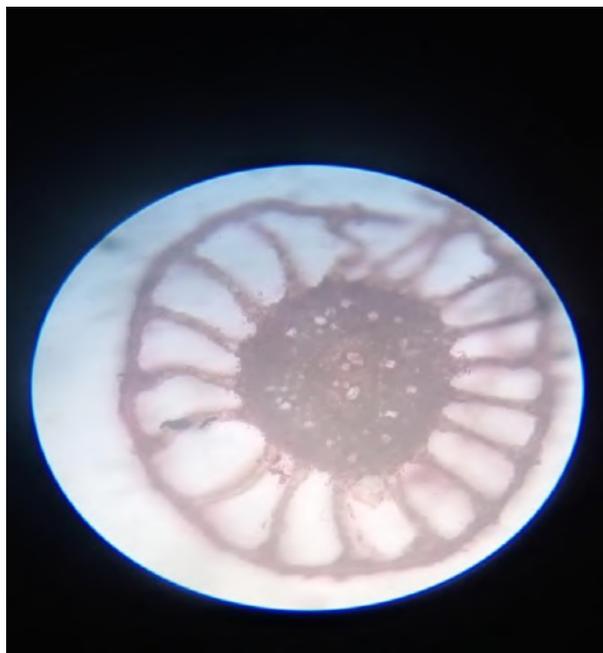


PLATE 16: T S OF *MARSILEA* PETIOLE

7. *ADIANTUM*

Adiantum is also known as maidenhair fern or walking fern. They are small, perennial and evergreen plant.

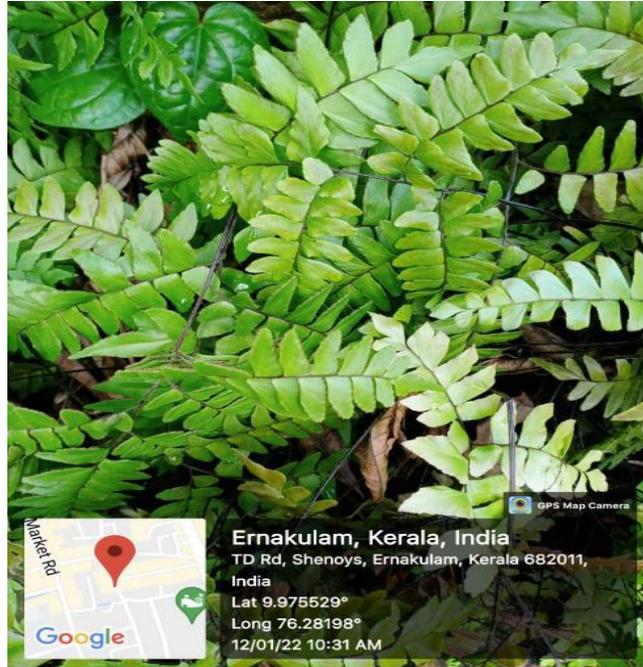


PLATE 17: HABITAT OF *ADIANTUM*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Nephrolepidaceae

Genus : Nephrolepis

- **MORPHOLOGICAL EVALUATION OF *ADIANTUM***

SPOROPHYTE: The plant is divided into stem, root and leaves.

RHIZOME: Rhizome grows horizontally near the soil surface. Scales, called palea covered the surface of rhizome.

LEAVES: Leaves of *Adiantum* are called fronds. These leaves are large, about 4-6 inches in length and are bipinnately compound. Leaflets of the first order are called pinnae and leaflets of second order are called pinnules. Main axis of leaf on which leaflets are produced is rachis. The rachis is black in color and shiny. The leaves are produced in acropetalous succession on the creeping rhizome. They show circinate venation. The pinnae are stalked and have a dichotomous venation. There is no distinction between fertile and sterile leaves. The whole leaf may be sporangiferous or only certain pinnae may bear sporangia. The soral organisation is very evident. Sori are borne on the ventral surface of the pinnae.

- **ANATOMICAL EVALUATION OF *ADIANTUM* PETIOLE**

EPIDERMIS: The petiole in T.S. shows a single-layered epidermis with a thick cuticle. The epidermis is followed by a sclerenchymatous hypodermis which provides mechanical support.

CORTEX: Consists of parenchymatous cells. The central region possesses a single large horseshoe-shaped stele. Xylem forms central core surrounded by phloem

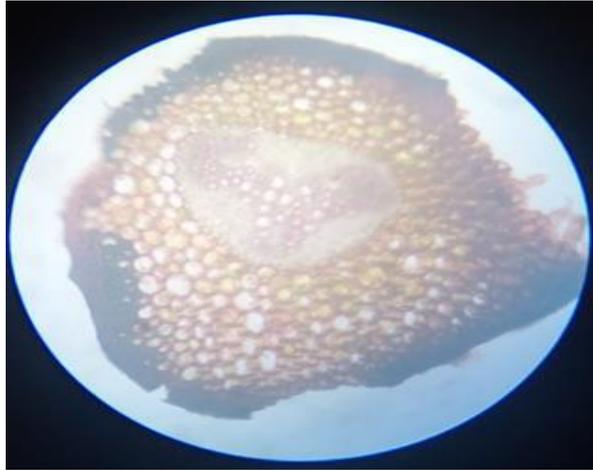


PLATE 18: T S OF *ADIANTUM* PETIOLE

DISCUSSION

Ferns and their allies are one of the Pteridophyta's oldest major divisions, with approximately 12 000 species scattered over 250 distinct genera (Baskaran *et al.*, 2018). Sushruta (about 100 AD) and Charaka (around 100 AD) of the biomedical and Ayurvedic schools of medicine, respectively, proposed the use of various ferns in the Samhita literature. Pteridophytes are also employed by physicians in the Unani medical system. Several ferns are suggested by native doctors in the traditional Chinese medical system (Kimura and Noro, 1965). Several researchers have recently conducted ethnobotanical and advanced pharmacological investigations on ferns and their companions. Most ferns and fern allies provided several health advantages to ancient civilizations that utilized them for food, tea, and medications. Modern techniques have merged interdisciplinary technology, as well as particular chemical components collected and identified, allowing the production of extremely pure medications from plant parts.

Plants that produce high quality and quantity of polysaccharides, steroids, terpenoids, flavonoids, alkaloids, and antibiotics are ideal for developing medications for a variety of ailments/diseases, including cancer therapies. Modern studies on the functional activities of pteridophytes for human health, such as the discovery of particular chemicals and their use in medications, have broadened the scope of pteridophytes, transforming these plants into a godsend for pharmaceutical businesses and associated industries. Plants that produce high levels of polysaccharides, steroids, terpenoids, flavonoids, alkaloids, and antibiotics are useful for producing drugs for a number of ailments/diseases, including cancer therapy. Modern research on the functional activities of pteridophytes for human health, such as the identification of specific compounds and their application in drugs, has widened the scope of pteridophytes, making these plants a boon to pharmaceutical companies and related industries. Earlier pteridophytes' pharmacological activity suggests an alternate therapy for treating human illnesses. Pteridophytes (ferns and fern allies) are an old lineage that humans have been discovering and exploiting for over 2000 years due to their beneficial characteristics as the earliest vascular plants. Previous research has demonstrated that the lycophyte *Selaginella* sp. has a wide range of pharmacological activity, including antioxidant, anti-inflammatory, anti-cancer, antidiabetic, antiviral, antibacterial, and anti-Alzheimer capabilities. Among all the pteridophytes studied, taxa from the Pteridaceae, Polypodiaceae, and

Adiantaceae showed the most therapeutic efficacy. According to our findings, several pteridophytes have characteristics that might be employed in alternative medicine to treat a variety of human ailments.

Ferns are common denominators of abundant and diverse biodiversity in practically every corner of the world. The comparison of evolutionary adaptations and natural innovations reveals the genetic underpinning for organism development. It is highlighted that good field stations with big greenhouses at the periphery of protected forests should work as 'Fernariums/ Mossariums/ and/or Lichenariums' to conserve and nurture rare, endangered, and medicinally outstanding species present in such areas/forests. Gene networks (DNA stretches) that preserve comparable wiring schematics (some or many similar DNA sequences) throughout related, distantly related, or completely different animals reveal how regulatory sections of the genome have developed. Without a doubt, comparative genomics can assist us in deciphering the evolvability of gene networks and conservation modes.

The potential use of Pteridophytes as Ecological Indicators is becoming more widely recognized. The use of pteridophytes as EIs is becoming more common. This demonstrates the group's significant potential as EIs, which is further backed by several studies using similar approaches, resulting in a huge number of species, genera, and families being proposed as EIs.

The ferns have also been shown to be having an important role in the bioremediation of wastewater. (Dudani *et al.*, 2001) found the Chinese Bracken fern namely *Pteris vittata* L. to be a hyperaccumulator of the toxic metal Arsenic. Besides producing large biomass, they also found this fern to be efficient in Ar accumulation with concentrations as high as 2.3% in the aerial portions of the fern. Later on, many researchers provided reports of the hyperaccumulation properties of *Pteris* as well as many other ferns also. (Zhang, 2002) suggested that *P. vittata* could be an excellent model to study arsenic uptake, translocation, speciation, distribution and detoxification in plants and for phytoremediation of arsenic-contaminated soil and water.

Besides having all these wonderful properties, the pteridophytes are also greatly valued as ornamentals. Prior to the discovery of these benefits obtained from this group of plants, ferns were used to enhance the beauty of the landscape and are continued to be used so till now. Ferns

like *Adiantum sp.*, *Selaginella sp.*, *Lygodium sp.*, *Pteris sp.*, etc. are also grown in the gardens or in the pots.

The pteridophytes are moisture and shade-loving plants and are dependent upon the microclimatic conditions of the region for their successful survival in that region. Any kind of disturbance in these microclimatic conditions can hinder the growth and evolutionary processes occurring naturally in these plants thereby, leading to declining in their populations. Thus, factors like climate change, increasing urbanization, industrialization, encroachment of forest lands, unplanned developmental activities, and overexploitation of natural resources, pose a major threat to the survival of these groups of plants. Due to the unplanned felling of trees in the forests the members of epiphytic pteridophytes belonging to the families Polypodiaceae, Davalliaceae, Aspleniaceae, Vittariaceae, have been reduced day by day (Nambiar and Shimna, 2022). Large-scale collection of ferns from the forests by the visitors and local people for ornamental purpose, medicinal purposes and during excursions also increases the pressure on these plants.

Biodiversity conservation is the need of time and hence, it has become imperative to develop *in situ* and *ex situ* conservation methods for conservation of the diminishing biodiversity. The *in-situ* conservation is very beneficial as it allows the evolution of the species to continue within the area of natural occurrence. Hence, the steps for conserving the ferns *in situ* should be focused upon. The *ex-situ* conservation includes the development of botanical gardens or conservatories, germplasm banks, DNA banks, seed banks and involve the use of techniques such as tissue culture, cryopreservation; incorporation of disease, pest and stress tolerance traits through genetic transformation and ecological restoration of rare plant species and their populations (Pritchard *et al.*, 2010). The conservation of flowering plants has been achieved to a good extent by developing conservatories and botanical gardens which also help in creating awareness among the local people. Developing a fern conservatory or fern garden is not preferred much and hence, such steps should be considered and implemented for conserving the rare and endangered species. The tissue culture is a very useful technique for the mass multiplication of the plant species in a short time and hence, research focusing on developing a protocol for *in vitro* regeneration of ferns and fern-allies should be encouraged. Parts of areas rich in abundant pteridophyte diversity can be declared as pteridophyte biosphere reserves or small gene sanctuaries can be established to save the epiphytic pteridophytes.

CONCLUSION

The present study demonstrates the relevance of Pteridophytes in nature. Pteridophytes are an ancient lineage of plants, composed of ferns and fern allies, which are spread across the globe. There is also a long record of humans using pteridophytes to their benefit, which includes the broad categories of medicine, ornamentation, food, phytoremediation, and agriculture.

Pteridophytes have qualities that might be employed in alternative medicine to treat a variety of human ailments. Biopharmaceutical approaches can be used to preserve and even improve bioactive molecules for the development of anti-disease medications. Despite the fact that several studies have revealed that ferns have medicinal potential. Ferns have also been found to play an essential part in wastewater bioremediation.

There is a dearth of studies demonstrating their real usefulness and the criteria utilized to choose the Ecological indicators. The genetic foundations for organism development are revealed through evolutionary interactions and natural innovations. For the sake of the future, research into the development of pteridophytes should be supported. According to the current study, pteridophytes should be kept and safeguarded in the future. Pteridophytes are highly prized foliage ornamentals.

Documentation on the economic importance of pteridophytes is needed to reveal the importance of this plant group to the public and the indigenous knowledge about them. It is also important that field botanists should avoid the ruthless collection of rare species and make sure that they leave the bulk of plants to continue to grow and reproduce in the world. Providing proper awareness about the conservation of pteridophytes among the local people is needed. Further studies on pteridophytes can bring many more species that are of economic importance to light.

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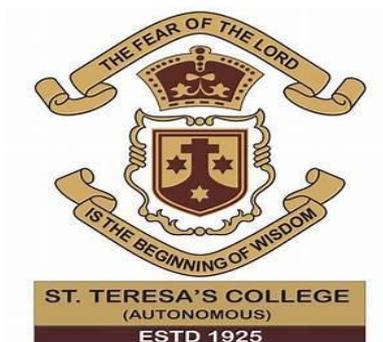
**STUDIES ON THE ANTIBACTERIAL POTENTIAL OF
GRACILARIA CORTICATA (J. Agardh) J. Agardh IN ETHANOL AND
CHLOROFORM EXTRACTS**

**DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE AWARD OF THE DEGREE OF BACHELOR OF SCIENCE
IN
BOTANY**

By

KRISHNENDU N M

AB19BOT011

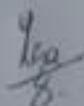


**DEPARTMENT OF BOTANY
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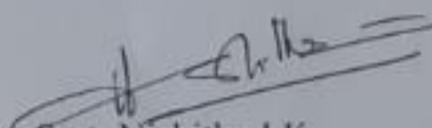
2022

CERTIFICATE

This is to certify that the dissertation entitled "Studies on the antibacterial potential of *Gracilaria corticata* (J. Agardh) J. Agardh in ethanol and chloroform extracts" is an authentic record of research work carried out by Miss **Krishnendu N M** (Reg No:ABI9BOT011) under the supervision and guidance Smt. Nishitha I. K. Assistant Professor, Department of Botany and Centre for Research, St. Teresa's College (Autonomous), Emakulam, in partial fulfilment of the requirements for the award of the degree of Bachelor of Science in Botany. I further certify that no part of this work embodied in this project has been submitted for the award of any degree or diploma.

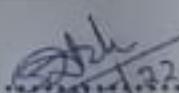
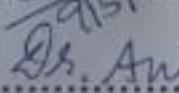


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DECLARATION

I hereby declare that the project entitled “Studies on the antibacterial potential of *Gracilaria corticata* (J. Agardh) J. Agardh in ethanol and chloroform extracts” submitted to Mahatma Gandhi University, Kottayam, in partial fulfilment of the requirement for the Degree of Master of Science in Botany is an original project done by under the supervision and guidance of Ms. Nishitha I.K Department of Botany and Centre for Research, St. Teresa’s college (Autonomous), Ernakulam.

PLACE: ERNAKULAM

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INTRODUCTION

Algae are diverse group of relatively simple, chlorophyll containing, photo-autotrophic and oxygen evolving aquatic thalloid (without differentiation into True roots, stems, leaves or leaf like organs) organisms. The word “algae” has its origin from Latin, where ‘alga’ means seaweed. The term algae was first used by Carolous Linnaeus in 1753. Most of them are photo-autotrophic but few are mixotrophic and myzotrophic (sucking through special feeding structure) study of algae is known as phycology (GK. Phykos- seaweed; logos= discourse Or study) or algology.

Algae are divided into nine main phylums, they are Phylum Rhodophycophyta, Phylum Xanthophycophyta, Phylum Chrysophycophyta, Phylum Phaeophycophyta, Phylum Bacillariophycophyta, Phylum Euglenophycophyta, Phylum Chlorophycophyta, Phylum Cryptophycophyta, and Phylum Pyrrophyphyta.

(<https://www.slideshare.net/BIYYANISUMAN/algae-suman-81289656>)

The term "seaweed" refers to a variety of marine plants and algae that can be found in the ocean, rivers, lakes, and other bodies of water. Some seaweeds, such as phytoplankton, are small and remain floating in the water column, providing the foundation for most aquatic food chains. Some are massive, such as the giant kelp that grow in dense forests. The large percentage are medium-sized, with red, green, brown, and black colours, and sometimes may wash up on beaches and shorelines. (Guiry, Michael D., 2014)

Marine algae from Indian coasts amounting to 844 species (including forma and varieties) are distributed among 217 genera. They grow in the intertidal, shallow and deep sea areas up to 180 meter depth and also in estuaries, backwaters and lagoons on solid substrates such as rocks, dead corals, pebbles, shells, mangroves and other plant materials (Anatharaman *et al.*, 2007; Sakthivel, 2007).

Mostly seen seaweeds are macro algae. They are of different types according to the colour of pigments present: brown algae (phylum Ochrophyta, class Phaeophyceae), red algae (phylum Rhodophyta, *Gelidium*), and green algae (phylum Chlorophyta, classes Chlorophyceae, Ulvophyceae etc. They differ significantly in many ultrastructural and biochemical functions, including photosynthetic pigments, storage molecules, cell wall composition, presence/absence of flagella, mitosis ultrastructure, linkage between cells, and structure of the chloroplasts, in addition to pigmentation.

Seaweed is high in vitamins, minerals, and fibre, as well as being palatable. The Japanese have a dish, 'sushi' which is nori seaweed wrapped with a mixture of fish, rice, and other ingredients for at least 1,500 years. Anti-inflammatory and anti-microbial compounds can be found in a variety of seaweeds. For thousands of years, their medicinal properties have been used; it was used to cure wounds, burns, and rashes by the ancient Romans. According to anecdotal evidence, the ancient Egyptians may have employed them to cure breast cancer. (McLachlan, J., and C. J. Bird, 1984).

In recent years, focus towards these organisms has increased due to their food and fuel production capability. In fuel industry algae biofuels have emerged as a clean, nature friendly, cost effective solution to other fuels. More recently algae have been identified and developed as renewable fuel sources, and the cultivation of algal biomass for various products is transitioning to commercial-scale systems. Large-scale cultivation of algae merges the fundamental aspects of traditional agricultural farming and aquaculture (Emily M Trentacoste *et al.*, 2014). Algae fuels are categorized into bio-ethanol, biogas, bio-hydrogen, biodiesel and bio-oil. Algae can be used in the preparation of Biodiesel, Bioethanol, Biobutanol and Hydrogen gas (Raja *et al.*, 2013)

They are considered as a potential source of bioactive substances such as proteins, lipids, and polyphenols possessing potent antibacterial, anticancer, antioxidant, antifungal, and antiviral properties (Sundaramurthy *et al.*, 2016). Seaweeds that are medicinal are rich in secondary metabolites which include alkaloids, glycosides, flavonoids, saponins, tannins, steroids, related active metabolites, which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry (Eluvakkal *et al.*, 2010). Recently, their value as a source of novel bioactive substances has grown rapidly and researchers have revealed that marine algal originated compounds exhibit various biological activities (Kim and Wijesekara, 2010; Wijesekara and Kim, 2010; Wijesekara *et al.*, 2010 and Wijesekara *et al.*, 2011). The secondary metabolites of seaweeds such as Isoprenoids (terpenes, carotenoids, steroids), polyketides, phlorotannins, amino-acid derived natural products (alkaloids), and shikimates (flavonoids) have always attracted the interest of biochemists because of their diversity is comparable with those present in the leaves of higher plants (Manilal *et al.*, 2009). Seaweeds were rich in dietary fibre (>50% dry weight), particularly in the soluble form.

RHODOPHYCEAE

Rhodophyta is a phylum of macroalgae that includes the classes Phaeophyceae and Chlorophyta, which are brown and green seaweeds, respectively.

Within Archaeplastida, Rhodophyta, or red algae, is a monophyletic lineage that contains glaucophyte algae, green algae, and terrestrial plants. Bangia-like species have been found in 1.2 billion-year-old strata, indicating that Rhodophyta has a lengthy fossil history. The morphology of red algae ranges from unicellular filamentous to multicellular thalloid forms, with certain species producing economically important products like agar and carrageenan. These species can be found in a variety of marine settings, ranging from the intertidal zone to deep oceans. There are also freshwater (e.g., *Batrachospermum*) and terrestrial lineages. A triphasic life cycle with one haploid and two diploid phases, with the carposporophyte borne on female gametophytes, is one of the Rhodophyta's significant advances.

Freshwater Rhodophyta has 66 species and 27 genera in North America, although these numbers will change as molecular investigations uncover more diversity. Freshwater red algae have a limited size range than marine species, with the majority (80%) of them measuring 1-10 cm in length. Gelatinous filaments, free filaments, and pseudoparenchymatous forms are the most prevalent types. (Yoon, Hwan Su, et al., 2017)

GRACILLARIA

In terms of the number of species, the genus *Gracilaria* is one of the largest genera of red algae. It's also a wide spread genus, with species found in all oceans except the Arctic. Nearly 28 species of *Gracilaria* have been reported from the Indian coast (Sahoo *et al.*, 2001). Because of its size and extensive range, it's suitable for biogeographic investigation. The greatest number of *Gracilaria* species can be found in tropical waters. Large beds of *Gracilaria* usually grow in the eulittoral zone, or just below it in the beginning of the sublittoral, on sandy or muddy sediments that are protected from waves. Sometimes it can be found free-floating in tidal lakes of salt or brackish water. It can adapt to large variations in growing conditions such as freshwater dilution, increase in fertilizer concentration from runoff, and raised temperatures. Large biomasses can grow when there is little competition from other species, and vegetative propagation may be a normal method of reproduction (McLachlan, J.,

et al., 1984). In tropical and subtropical oceans, these are frequently red, green, or greenish brown.

Gracilaria are found as branched thalli, terete to flattened, branching sub-dichotomous to irregular. It has holdfast a disc or crust giving rise to one to many erect axes. The thalli are red, olive, green to purple, Spermatangia are seen in pits or shallow depressions. Sporophytes with tetrasporangia are scattered in the outer cortex, cruciately divide (R Iyer, et al., 2004)

Because they have phycocolloids, the major source of agar- (1, 4)-3, 6-anhydro-1-galactose and -(1,3)-d-galactose with low esterification in the cell wall, *Gracilaria* species are essential for industrial and biotechnological uses. Agar and other polysaccharides are found in *G. confervoides*, *G. dura*, *G. chilensi*, and *G. secundata* among the carbohydrates.

The present work was undertaken to study the antimicrobial potential of *Gracilaria corticata*. The objectives of the study are

- Taxonomic description of the algae
- Assessment of antibacterial potential of the common seaweed *Gracilaria corticata* in its dried form extracted in two different solvents; Ethanol and Chloroform.
- Evaluate the difference in antibacterial potential shown by the alga in the two different solvents against Gram positive *Staphylococcus* and Gram negative *E. coli*.
- Estimate the extractive value of the plant, in both ethanolic and chloroform solvents.

LITERATURE REVIEW

In a study conducted by Inci Tuney (2006), antibacterial activity of extracts or components from various algae has been demonstrated in vitro against both gram-positive and gram-negative bacteria. The antibacterial susceptibility test was performed using the agar disc diffusion method, with 6 mm discs impregnated with 20 µl of extracts and placed in infected agar. *Gracilaria* chloroform extract was tested for antibacterial properties against *Staphylococcus aureus* bacterial strains. *Gracilaria* extract showed action in *S. aureus* extract. Ethanol extracts from *G. domigensis* and *G. sjostedii* showed antibacterial activity against *E. coli* and *S. aureus*. (TÜney, İnci, et al., 2006)

Krishnapriya et al., (2013) conducted an antibacterial activity on the seaweed extracts, carried out by agar disc diffusion assay. The Muller Hinton agar (MHA) medium was used for this study using bacterial pathogens. Among the solvent extracts, methanol extract showed best results for both positive and negative strains. Chloroform extract of *G. verrucosa* gave the highest zone of inhibition measuring 21±1.0 mm. Ethanol extract of *G. acerosa* also showed a zone of inhibition of 12±1.0 mm. Ethanol and chloroform extracts of *G. verrucosa* gave clearly distinct zone of inhibition measuring 8±1.0 and 9±1.0 mm, with respect to control (25±1.0 mm) against *Staphylococcus*. (Varier, Krishnapriya Madhu, et al., 2013)

Saranraj, P. (2013) conducted a study and the methanol extract of *Gracilaria folifera* (5.0mg/ml) showed highest mean zone of inhibition (18±0.4mm) against the Gram positive cocci *Streptococcus pyogenes* followed by *Bacillus subtilis* (17±0.5mm), *Staphylococcus aureus* (17±0.3mm), *Streptococcus epidermis* (16±0.6mm) and *Bacillus cereus* (16±0.2mm). For Gram negative bacterium, the maximum zone of inhibition was recorded in methanol extract of *Gracilaria folifera* against *Klebsiella pneumoniae* (17±0.5mm) followed by *Salmonella typhi* (16±0.6mm), *Pseudomonas aeruginosa* (16±0.5mm), *Escherichia coli* (16±0.3mm). The zone of inhibition obtained from the Hexane extract of seaweed *Gracilaria folifera* against bacterial pathogens was comparatively very less when compared to the other solvent extracts. No zone of inhibition was seen in DMSO control and the positive control Ampicillin showed zone of inhibition ranging from 13±0.8 mm to 20±0.8mm against the test bacterial pathogens. (Saranraj, P., 2013)

The antibacterial properties of eight crude extracts of local *Acanthophora spicifera* obtained by two distinct extraction methods were investigated by Zakaria (2010) using soxhlet extraction and solvent partitioning. By using the Disc diffusion method, these extracts were

evaluated in vitro against 18 bacteria, 3 yeasts, and 6 fungal strains. The results demonstrated that the solvent partitioning extracts of methanol and ethyl acetate had a greater spectrum of action against the tested bacterial strains. *Bacillus cereus* ATCC 10876, *Bacillus licheniformis* ATCC 12759, Menthicilin Resistance *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* ATCC 27853, *Yersinia* sp., and *Citrobacter freundii* displayed inhibitory zones against these two extracts. While methanol extracts from Soxhlet extraction and butanol from solvent partitioning had no antibacterial activity against *P. aeruginosa* ATCC 27853, the other six extracts did. (Zakaria, et al., 2010)

In a study done by Ibraheem et al.; 2017; simplex extracts of *Acanthopora* showed potent inhibitory growth activities against three Gram positive bacteria [*Streptococcus agalactiae*, *pyogenes* and *Streptococcus sanguis* of inhibition ranging from [23.1±0.58 to 20.6±0.63 mm] and showed moderate activities with [*Corynebacterium diphtheriae*, *Bacillus subtilis* and *Staphylococcus aureus*] with inhibition zones ranging from [20.1±1.5 to 16.3±2.1 mm].

Also the crude extracts were found to be more active than the positive control Ampicillin, (22.3±1.5 mm), against *Streptococcus agalactiae* which showing inhibition zone. The hydro alcoholic extracts of the selected species were investigated for their antimicrobial activities using Agar well diffusion and Muller Henton against gram positive and gram negative bacteria. (Ibraheem, Ibraheem BM, et al., 2017)

In a study by Nurul Aili Zakaria et al.; (2011), the antimicrobial activities of the hexane extract were evaluated using disc diffusion method against 8 Gram-negative and 10 Gram-positive bacterial strains. Out of all bacterial tested, only a Gram-positive bacterium and a Gram-negative bacterium were susceptible to the extracts. The hexane extract showed antibacterial activity against both Gram-positive bacterium and Gram-negative bacterium (*P. aeruginosa* ATCC 27853). While, chloroform and ethyl acetate extract only showed inhibitory effect on *P. aeruginosa* ATCC 27853 with inhibition zone of 9.0 mm. No inhibitory effect was showed by methanol extract on bacteria tested. (Zakaria, Nurul Aili, et al., 2011)

MATERIALS AND METHODS

SPECIMEN COLLECTION

The specimen was collected by hand picking from Thikkodi beach, Calicut. The collected samples were washed immediately in seawater and then washed with fresh water and transported to the laboratory. It was again washed thoroughly to remove impurities and sand and rinsed with distilled water. The sample was identified taxonomically as *Gracilaria corticata*. Collected sample was taxonomically evaluated using the standard literature.

SAMPLE PREPARATION

For antimicrobial studies, the cleaned samples were then shade dried, cut into small pieces and powdered in a mixer grinder. The organic solvents Chloroform and Ethanol were used for the extraction process due to its higher efficiency using Soxhlet extraction method. 20g of samples were packed in a thimble and placed in the extractor. 200ml of the solvent was added into the flask and heated. The temperature was maintained at 80°C to 85°C throughout the extraction. The soluble active constituents of the extract remained in the flask and the process was repeated until the compounds were completely extracted. The liquid extract was then cooled and concentrated by using an evaporator.

The beaker with dried extract was weighed and noted. DMSO was used to dissolve the extracts from the beaker. Later the weight of the beaker alone was noted. Hence, the actual weight of the dried extract was obtained. Similarly, the weight of dried extract of *Gracilaria*, in ethanol and chloroform was 0.46g and 10mg respectively. From this the extractive value was calculated using the formula

Extractive value (%) = (Weight of dried extract/ Weight of plant material) X 100

PREPARATION OF EXTRACT IN VARIOUS CONCENTRATIONS

From the stock extract, concentrations of 10%, 20%, 40%, 60% (v/v) was made. The stock concentration of *Gracilaria* in ethanol and chloroform was 10mg/ml and 10mg/ml respectively. From the stock the appropriate amounts was pipetted out and made up to the required concentrations using DMSO.

ANTIBACTERIAL ACTIVITY IN GRACILARIA

PREPARATION OF BACTERIAL CULTURE

In the present study, the extracts were evaluated for antimicrobial activity against *Staphylococcus* strain and *E. coli*, a Gram positive and a Gram negative bacteria respectively. 3g of nutrient broth was dissolved in 100ml of distilled water in a conical flask. The broth is sterilized by autoclaving for 15 minutes. Both of the obtained bacterial stains were inoculated in the nutrient broth in laminar air flow and incubated in appropriate conditions for 24hrs.

PREPARATION OF PETRI PLATES

The selected two species of seaweeds were analysed for the antimicrobial activity for gram negative *Escherichia coli* and gram positive *Staphylococcus* by disc diffusion methods. Agar medium was prepared by dissolving 4g agar and 2.6g of nutrient broth in 100ml distilled water. The mixture is sterilized in an autoclave for 15 minutes. Just after sterilization the mixture was poured into petri plates in laminar air flow. The petri plates were allowed to solidify under aseptic conditions.

ANTIMICROBIAL TEST BY DISC DIFFUSION METHOD

Bacteria were inoculated onto the prepared agar petri plates using sterilized cotton swabs. Sterilized 6mm discs were taken from filter paper and autoclaved and is used for the method. The disc was then dipped in different concentrations of stock (10, 20, 40, 60) and placed on the agar plate using sterile forceps. Tetracycline was used as positive control and DMSO was used as negative control. This was done for both extracts of *Gracilaria* against the two strains of bacteria. The petri plates were incubated at 37°C for 24 hours and results were recorded.

OBSERVATION AND RESULTS

The current study aimed at the taxonomic description of the red seaweed *Gracilaria corticata* and the estimation of its extractive value and antimicrobial potential in two solvents, ethanol and chloroform. The antimicrobial potential activity was studied against Gram positive *Staphylococcus* and Gram negative *E. coli*, two non-pathogenic bacteria. The results obtained are described below.

TAXONOMIC DESCRIPTION

Kingdom: Plantae

Phylum: Rhodophyta

Class: Florideophyceae

Order: Gracilariales

Family: Gracilariaceae

Genus: *Gracilaria*

Species: *corticata*

Gracilaria corticata (J. Agardh) J. Agardh

Thallus erect, up to 14cm in length, arising singly from a discoid holdfast. Stipe very short, terete, up to 5mm long, often inconspicuous. Branching frequently, becoming denser in upper parts of the plant; mostly dichotomous, and producing a bushy appearance. Axes compressed, almost cartilaginous; constricted at the base in basal branches. Blades linear, up to 15cm long, up to 4mm wide; apices generally obtuse, acute in finer branches. Blade surface and margins smooth. Fresh specimens purple to green and firm but pliable (Iyer et al, 2004).



Gracilaria corticata (J. Agardh) J. Agardh

EXTRACTIVE VALUE

Extractive values of plant materials are used to evaluate extracts of the sample, in order to get an idea about the nature of chemical constituents present in it. It can also be used to assess quality, purity and detect adulteration of the extract.

In the present study, polar and non-polar solvents were used for eluting the valuable phyto-compounds present in the sample. Extractive values of ethanol and chloroform extracts of *Gracilaria corticata* used in the antibacterial study, are estimated in the table 1 given below;

Table 1: Extractive value of solvents administered for *Gracilaria corticata*

Solvent	Extractive value of the sample (%)
Ethanol	2.3
Chloroform	0.5

The extractive value was greater for the ethanolic extract than for chloroform suggesting that polar solvent was more efficient in extracting the phytochemicals from the algae.

ANTIBACTERIAL ACTIVITY

The extracts of the algae exhibited moderate to mild antibacterial activity against the two microorganisms. The activity observed can be described as being bacteriostatic showing very

mild zones of inhibition. The ethanol extract of the algae showed mild antibacterial activity against both the test organisms. *Gracilaria* shows mild action against gram negative bacteria at all concentrations of ethanol extract used in the current study against both test organisms.

Table 2; Fig. 2

Table 2: Antibacterial activity of ethanolic extract of *Gracilaria corticata* against *E. coli* and *Staphylococcus* bacteria:

Concentration (%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	Mild action	Mild action
40	Mild action	Mild action
60	Mild action	Mild action

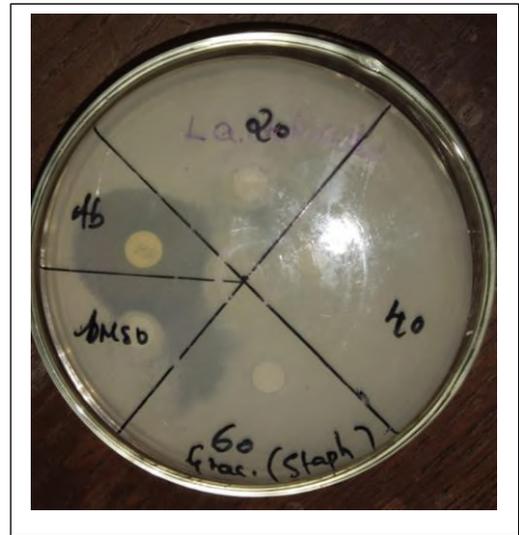
Table 3: Antibacterial activity of chloroform extract of *Gracilaria corticata* against *E. coli* and *Staphylococcus* bacteria

Concentration (%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	No action	No action
40	No action	No action
60	No action	Mild action

The chloroform extract of *Gracilaria* has no significant effect on the bacterial growth. Mild bacteriaostatic activity is observed at higher extract concentrations on *Staphylococcus*. No potential activity could be observed on the growth of *E. coli* in any of the concentrations used for the current study Table 3: Fig. 3

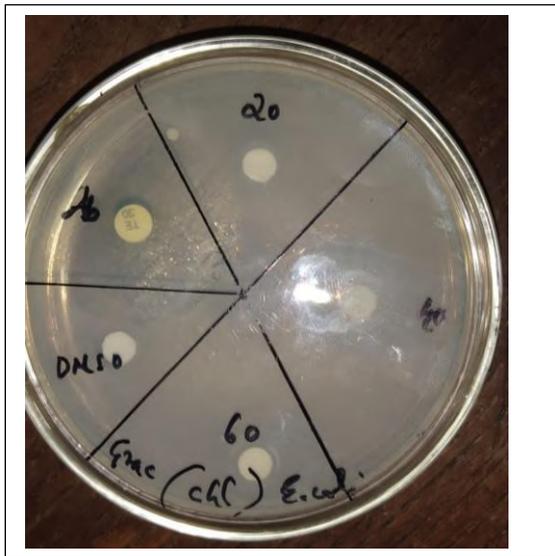


E. coli

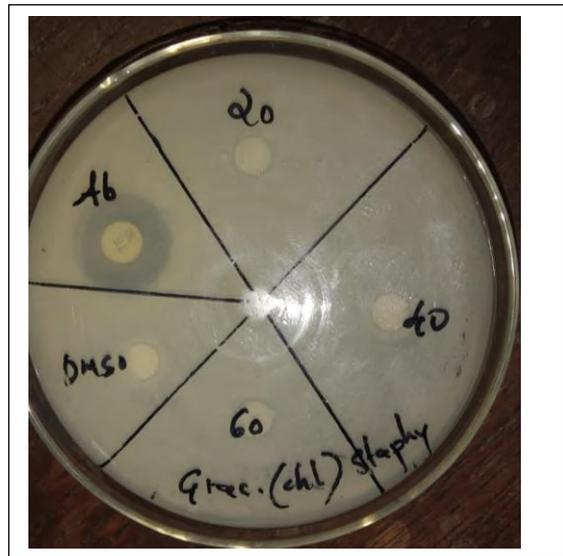


Staphylococci

Fig 2: Antimicrobial activity of Ethanol extract of Gracilaria



E. coli



Staphylococci

Fig 3: Antimicrobial activity of Chloroform extract of Gracilaria

DISCUSSION

Algae have attracted great importance in the recent years due to the large number and amounts of bioactive components in them. More than 600 trace elements are found in high concentration in the seaweeds compared to the terrestrial plants, because of which it has various pharmacological activities. The seaweeds offer greatest wealth in terms of biomass and Rhodophytes show the largest representation among them. The red sea weed *Gracilaria* is amongst the largest group with over 150 species world wide and nearly 28 species in India (Sahoo *et al.*, 2001).

Gracilaria has been identified as a rich source of various bioactive compounds as assessed by the studies on its different species such as *G. corticata*, *G. dentata*, *G. edulis*, *G. opuntia*, *G. pygmaea* and *G. verrucosa* carried out by various researchers. In a study carried out by Balakrishnan *et al.* in 2013, phytochemical screening of *G. corticata* was done using different solvents like methanol, ethanol, petroleum ether and acetone revealing the presence of most of the bioactive components of which alkaloids, phenols, quinones and steroids are most abundant. In a similar investigation led by Gnanaprakasam *et al.*, (2017), the hexane, chloroform, ethyl acetate, acetone and methanol extracts of *G. corticata* were used to analyse the phytochemicals, and results revealed that the terpenoids, tannins and phenolic compounds were present in the all the extracts and alkaloids were present only in the chloroform and ethyl acetate extracts.

Antibacterial activity refers to the process of killing or inhibiting the disease-causing bacteria. Several plants have been traditionally used for their antibacterial activity. Like plants some algae also exhibit antibacterial properties due to the presence of terpenoids, steroids, saponins, tannins, and flavonoids. There are numerous reports regarding the inhibitory activities of macroalgae against human pathogens, fungi and yeasts. So, the use of algae as an alternative for prevention and treatment of infectious diseases has been suggested by Abirami and Kowsalya (2012). In the present study ethanolic and chloroform extract were evaluated for activity against Gram positive *Staphylococci* and Gram-negative *E. coli*. It was found that ethanolic extract had bacteriostatic activity against both the bacteria at all concentrations treated and the effect was dose dependent. Sanaraj P., 2013, in his study on *G. edulis* also reported maximum activity against Gram positive bacteria in ethanol extracts. Rashida *et al.* (2019) also report ethanol extract of *Gracilaria* to have higher antibacterial activity than other solvents.

In the preliminary assay conducted to evaluate antibacterial activity of *Gracilaria* species against human pathogens by Susanth (2012), ten different organic solvents were considered. This study also reported that the extracts of ethanol and chloroform were the most potent of all.

Johnsi et al (2011), studied the antibacterial activity of aqueous extract of four seaweeds against ten pathogenic bacteria. This study reports the aqueous extract of *Gracilaria corticata* as having the highest potency against the pathogen *Proteus mirabilis*. In the current study however extracts in both solvents show bacteriostatic activity against *E. coli* and *Staphylococci*. Neither extracts are bactericidal and show a mild inhibitory zone of 7 - 8 mm.

Different solvent systems were used to extract bioactive principles from macroalgae with concomitant changes in the antibacterial activities (Thirupurasundari et al., 2008). The solvents such as acetone, benzene, butanol (Vanitha et al., 2003; Prakash et al., 2005), ethanol (Selvi et al., 2001) were used to extract antimicrobial compounds from macroalgae. The aqueous extracts prepared from seven macroalgal samples showed varying degrees of activity against tested pathogens, including the Gram positive and Gram negative bacteria. (Johnsi et al, 2011). Padmakumar (2002) is of the opinion that these differences are due to the different solubility behaviour of secondary metabolites which could be influenced by seasonal and geographical distribution of the species.

SUMMARY

Algae are an important constituent of the aquatic ecosystems and can be seen in water bodies like oceans, seas, lakes, estuaries and soon. They can be of different types and in different colours. Mostly seen seaweeds are macroalgae. They can be used as food and are a storehouse of bioactive components like vitamins, phenolics, terpenoids and other secondary metabolites. They also possess antibacterial, antioxidant, antifungal properties. Red algae is used for the extraction of agar (*Gracilaria*). It also shows few antibacterial properties.

The present study was done to estimate the difference in extractive yield, and the antimicrobial potential of the dried form of, *Gracilaria corticata* in polar and non-polar solvents, ethanol and chloroform respectively. The whole plant body was taken for the study. The cleaned, dried and powdered sample was extracted using the sohxlet apparatus. Extractive values of plant materials are often used to evaluate extracts of the sample, in order to get an idea about the nature of chemical constituents present in it. It can also be used to assess quality, purity and detect adulteration of the extract. *G. corticata* showed a better elution for polar solvent than non-polar solvent. The extractive yield obtained was more for ethanolic extract (2.3%) as compared to chloroform extract (0.5%).

In the present project, antibacterial potential of *Gracilaria corticata* was tested against two non pathogenic bacteria, the Gram negative *E. coli* and the Gram negative *Staphylococcus* by the disc diffusion method. It is concluded that the organic solvent extraction by ethanol and chloroform was suitable to verify the antimicrobial properties of *Gracilaria corticata* and they were supported by many investigations.

The current investigation showed that *Gracilaria corticata* has antimicrobial potential. The ethanol extract has better antimicrobial activity when compared to chloroform extracts. Ethanol extract was bacteriostatic for both gram negative (*E. coli*) and gram positive (*Staphylococcus*) bacteria at all concentrations studied. Whereas the extract in chloroform showed no significant activity except in the higher concentration (60%) and only against the Gram positive *Staphylococcus*.

The present study justifies the claimed uses of *Gracilaria corticata* in the traditional system of medicine to treat various infectious diseases caused by the microbes. These results suggest the possibility of using marine algae extracts in therapy as natural alternatives to antibiotics currently in the market, and clearly show that seaweeds are a valuable source of biologically active compounds.

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**“CHARACTERIZATION OF EPICUTICULAR WAX
FROM *IXORA COCCINEA* AND *IXORA CHINENSIS*
AND ITS APPLICATION FOR DEVELOPMENT OF
HYDROPHOBIC PAPERS”**

Dissertation submitted in partial fulfillment of the requirements for the award of the degree of

“BACHELOR OF SCIENCE IN BOTANY”

By

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DEPARTMENT OF BOTANY

ST. TERESA'S COLLEGE (AUTONOMOUS) ERNAKULAM

2022

CERTIFICATE

This is to certify that the dissertation entitled "Characterization of epicuticular wax from *Ixora coccinea* and *Ixora chinensis* And its application for development of Hydrophobic paper" is an authentic record of work carried out by Tincy B F under my Supervision and guidance in partial fulfilment of the requirement of B.Sc. degree affiliated to Mahatma Gandhi University, Kottayam. I further certify that no part of the work embodied in This dissertation work has been submitted for the award of any other degree or diploma.



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Place: Ernakulam

Date: 09/05/2022



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INTRODUCTION

On these times plastic bags are commonly used by many because of their unique properties like durability, water resistant, light weight, good transparency and wear resistance etc. Plastic may cause environmental effects such as animal choking, pollution, blockage of channels, rivers and streams. The major impact of plastic to the environment is it may take so many years to decompose. In addition, toxic substances are released into the soil when they perish under sunlight and, if plastics are burned, they release a toxic substance into the air causing ambient pollution. The presence of plastics in the marine environment poses several challenges that hinder economic development. Production of plastics increased exponentially from 2.3 million tons in 1950 to 448 million tons by 2015. To avoid the use of plastic biodegradable plastic are made and then paper bags are invented and it is scientifically proved that it won't cause any damage to environment.

Paper bags and cloth bags can be used to avoid these problems of plastics, but they do not resist to water and more expensive than using plastic bags so that their usage became limited. However paper is very resource –heavy to produce: manufacturing a paper bag takes about four times as much energy as it takes to produce plastic bags, plus the chemicals and fertilizers used in producing paper bags create additional harm to the environment. In an attempt we have to find suitable organic material which might be able to solve the above –mentioned problems, answers were sought in the nature.

Plants have the ability to resist water by the presence of the wax present on it. The quantity of wax depends upon the plant characters. Some plants have high amount of wax in it, and so their hydrophobicity will be more. *Ixora coccinea* and *Ixora chinensis* have this wax on the surface of the leaves.



Ixora coccinea belongs to the family Rubiaceae. It is a dense, multi-branched evergreen shrub with 4-6 ft in height but capable of reaching up to 12 ft high, common flowering shrub native to southern India, Bangladesh and Sri Lanka. Although there are 500 species in the genus *Ixora*, only a handful are commonly cultivated. Phytochemical studies indicate that the plant contains the phytochemicals lupeol, ursolic acid, oleanolic acid, sitosterol, rutin, leucocyanadin, anthocyanins, proanthocyanidins and glycosides. The flowers, leaves, roots, and the stem are used to treat various ailments in the Indian traditional system of medicine, the Ayurveda, and in various folk medicines, in traditional Indian medicine the fusion of juice leaves and the fruit of *Ixora coccinea* is used for care for dysentery, ulcers and gonorrhoea.

Ixora chinensis is a multi-stemmed, erect, evergreen shrub growing up to 2 meters tall, but more commonly less than 1 meter. The plant is harvested for local medicinal use. It is widely cultivated as an ornamental, valued especially for its long-lasting flower. The glossy, leathery, oblong leaves are about 4 in (10 cm) long, with entire margins, and are carried in opposite pairs or whorled on the stems. Small tubular, scarlet flowers in dense rounded clusters 2-5 in (5.1-12.7 cm) across are produced almost all year long.

Hydrophobic paper is very important because nowadays, most of the people used plastic bags as their food containers and packaging. Researches revealed that using plastics especially for food is dangerous because of the chemicals present in it. Hydrophobic papers can be a substitute for plastics because they are made from renewable sources, can be recycled and are biodegradable.

Ixora coccinea and *Ixora chinensis* were selected to this investigation. Epicuticular waxes are generally the waterproofing components found in an amorphous layer on the outer surface of the leaves. They are essential for plants as barrier protection against environmental stress. The waxy covering on plant leaves is called the "cuticle". It is composed of cutin, a wax-like material

produced by the plant that is chemically a hydroxy fatty acid. The purpose of this covering is to help the plant retain water.

The present study was done for characterization of epicuticular wax from *Ixora coccinea* and *Ixora chinensis* and its application for developing a hydrophobic paper.

OBJECTIVES

- To isolate and analyze the epicuticular wax from *Ixora coccinea* and *Ixora chinensis* using suitable solvents.
- To quantify the wax obtained from the leaves.
- To apply the epicuticular wax obtained for development of hydrophobic paper .

REVIEW OF LITERATURE

Epicuticular wax layers have a considerable influence on the wettability of plant surfaces in contrast to the corresponding intracuticular waxes, which control their permeability to water. The degree of surface wetness is functionally important to plants in several ways. Several properties of the plant cuticles are mainly based on the waxes. Intracuticular waxes mainly function as water transpiration barrier (Riederer and Schreiber, 1995), whereas epicuticular waxes strongly influence the wettability, self-cleaning behavior and the light reflection at the cuticle interface (Bargel et al., 2006a).

In a study conducted by Maria Jecemie A. Acebuche aimed to produce hydrophobic paper from the wax of *Colocasia esculenta* (taro leaf) and chitin from crab shells. Based on the findings of that study, the researcher concludes that the prepared hydrophobic paper is better than photographic paper in terms of solubility, liquid dropping, pH and moisture content. Those results proves that the combination of wax from taro leaves and chitin from crab shells can produce an acid free hydrophobic paper with low moisture content that can withstand penetration of most solvents. This process can make a good hydrophobic paper that will last longer than ordinary paper.

M Gobalakrishnan (2014) done a study of superhydrophobicity In that review, the leafs, *Alocasia macrorrhizos*, *Alchemilla mollis*, *Nelumbo Nucifera*, *Colocasia esculenta*, *Rosa Rubiginosa*, *Salvina Molesta*, which possess the superhydrophobic properties are explained. and he concluded that Super hydrophobic structure is a structure which has the contact angle more than 150 degrees. Super hydrophobic is also called as ultra-hydrophobic. The microstructures present in the plant leaves has this properties. The plants *Alocasia macrorrhizos*,

Alchemilla mollis, *Nelumbo Nucifera*, *Colocasia esculenta*, *Rosa Rubiginosa*, *Salvina Molesta* has excellent super hydrophobic structure. The microstructured wax present in these leaves, exhibits this property. These microstructures can be extracted and used to other object to improve the hydrophobicity of the object.

Akash Kalita (2018) studied about the hydrophobicity of the plant *Colocasia esculenta* which possesses a highly hydrophobic layer of bio-wax on its leaves. The bio-wax was extracted by from the leaves by organic solvent extraction method which in this case is chloroform. During the course of study quantitative analysis shows that the amount of bio-wax present per gram of leaf is equal to 0.116 gram approximately. The extracted bio-wax shows hydrophobic property even when it is exposed to high temperatures which is around 95-100 degree Celsius. These characteristics make the bio-wax of *Colocasia esculenta* a suitable substance for coating papers to make them hydrophobic. The hydrophobic papers thus created may be then used to make biodegradable hydrophobic paper bags. It is observed that the bio-wax extracted by the process of organic solvent extraction possesses a greenish pigment which might be unsuitable for future industrial use. The pigment was successfully removed by treating the leaves with steam (at 100°C for 30 minutes) prior to the process of extraction. The bio-wax extracted after steam treatment exhibited white color rather than green which proved that the green pigment was successfully eliminated by the process of steam treatment. It is due to degradation of chlorophyll at high temperature provided by steam. The white colored wax was subjected to a temperature of 60°C for 15 minutes to remove any water molecule that may have got trapped into the wax due to steam.

MATERIALS AND METHODS

MATERIALS

Leaves of *Ixora coccinea* , and leaves *Ixora chinensis* , solvents such as Chloroform and Benzene, Weighing machine, Beakers, measuring cylinder, Test tube, Petri plate, Glass rod, Whatman filter paper.

METHODS

Collection of plant sample

Isolation of wax from surface of leaves

Wax confirmatory test

Quantitative analysis of bio-wax

Test for hydrophobicity

Heat sensitivity test

1)COLLECTION OF PLANT SAMPLE

The leaves of *Ixora coccinea* and *Ixora chinensis* were collected from the nearby local area of Ernakulam.

2)ISOLATION OF WAX FROM THE SURFACE OF LEAVES

Fresh leaves of *Ixora coccinea* and *Ixora chinensis* were collected have to cut into small fragments. From that fragments 2 and 3 grams of each plant are weighed using a weighing machine. Then to the 2g of the each plants, 10 ml of solvents such as chloroform and benzene

was added in respective beakers. Like this to the 3g of the fragments 15 ml of solvents (chloroform, benzene) are added in the beaker. Then the leaf fragments were completely immersed into the solvents using a glass rod for 3 minutes. After 3 minutes these two solvents (chloroform, benzene) are moved to different petriplates and allowed them to evaporate. A white layer of wax was appeared on the surface of the petriplates.

3) WAX CONFIRMATORY TEST

The wax was extracted from *Ixora coccinea* and *Ixora chinensis* by solvent extraction method. The solvent (chloroform, benzene) is allowed to evaporate. Then the wax obtained was dissolved in ethanol and transferred into a test tube. To this few drops of distilled water is added to the solution and shaken well. A milky white appearance is noticed, and so the presence of wax is confirmed.

4) QUANTITATIVE ANALYSIS OF BIO-WAX

Fresh leaves of *Ixora coccinea* and *Ixora chinensis* were collected from the nearby local area are cut it into small fragments. From this 2 and 3 grams of each plant were weighed using a weighing machine. 2g of the fragments of each plants are immersed in 10ml of solvents (chloroform, benzene) and 3g of each plants are immersed in 15 ml of the solvents respectively. After 3 minutes the solvent is transferred into a petri plate. Then the fragments of leaves are allowed to dry. And the weight of the leaves again measured. By subtracting the weight of the fresh leaves before the solvent treatment to the weight of fresh leaves after the solvent extraction the amount of the wax can be calculated.

5) TEST FOR HYDROPHOBICITY

Bio-wax is isolated by solvent extraction method, using the solvents chloroform and benzene is then transferred into a petriplate and the solvent is allowed to cool. The wax appeared in white color on the surface of the petri plate is again mixed with 2ml of respective solvents. A rectangular piece of whatman filter paper is dipped into the solvent which contain the wax. After the filter paper is dried, water is added drop by drop to the paper. The water resisting capacity of the wax on each solvent can be evaluated by observing the time until the paper shows hydrophobicity.

6) HEAT SENSITIVITY TEST

Wax was extracted by the solvent extraction method using the solvents chloroform and benzene in different petriplates and the solvent are allowed to evaporate at room temperature. The petriplate is heated for about 70 degree Celsius for 2 minutes after the wax is completely vapourised. To the heated petriplate 2ml of each solvents are added. The hydrophobic property of the wax is noted.



OBSERVATION AND RESULT

1) ISOLATION OF WAX FROM THE SURFACE OF LEAVES

The isolation of wax from the surface of the leaves of *Ixora coccinea* and *Ixora chinensis* are done by solvent extraction method. Chloroform and benzene are the solvents used here. Wax appeared as white cloudy layer on the surface of the petriplates. Wax obtained using the solvent benzene is lesser than that of the wax obtained from the solvent chloroform. So from the study it is well understood that the solvent chloroform is more suitable for the extraction of wax from the leaves of *Ixora coccinea* and *Ixora chinensis*.

2) WAX CONFIRMATORY TEST

The wax obtained from the solvent extraction method was dissolved in ethanol and transferred into test tubes and few drops of distilled water is added and shaken well. Milky white appearance is noticed and so the presence of wax is confirmed. *Ixora coccinea*(plate:7,9) and *Ixora chinensis* (plate:8,10) in chloroform gives more milky white color than in benzene

3) QUANTITATIVE ANALYSIS OF BIO-WAX

In quantitative analysis the amount of wax obtained was by subtracting the weight of the fresh leaves before the solvent treatment to the weight of fresh leaves after the solvent extraction.

In *Ixora coccinea* the initial weight of the leaf fragments with 2g in case of chloroform the final weight is 1.83 so the wax obtained is 0.17 and in 3g of leaves the final weight is 2.78g and so the wax obtained is 0.22. In the case of benzene for 2g of the leaves the final weight is 1.87 and the wax obtained is 0.13 and in 3g of leaf fragments the final weight is 2.86g and the wax obtained is 0.14g.(Table-1.1)

TABLE-1.1. Quantitative analysis of bio-wax of *Ixora coccinea*

Name of the plant	Name of the solvent	Initial weight (gm)	Final weight (gm)	Amount of wax obtained (gm)
<i>Ixora coccinea</i>	Chloroform	2	1.83	0.17
		3	2.78	0.22
	<u>Benzene</u>	2	1.87	0.13
		3	2.86	0.14

Similarly in the case of In *Ixora chinensis* the initial weight of the leaf fragments with 2g in case of chloroform the final weight is 1.89g so the wax obtained is 0.11 and in 3g of leaves the final weight is 2.88g and so the wax obtained is 0.12. In the case of benzene for 2g of the leaves the final weight is 1.92g and the wax obtained is 0.08 and in 3g of leaf fragments the final weight is 2.94g and the wax obtained is 0.06g.(Table-1.2)

TABLE-1.2 Quantitative analysis of bio-wax obtained from *Ixora chinensis*

Name of the plant	Name of the solvent	Initial weight (gm)	Final weight (gm)	Amount of wax obtained (gm)
<i>Ixora chinensis</i>	Chloroform	2	1.89	0.11
		3	2.88	0.12
	Benzene	2	1.92	0.08
		3	2.94	0.06

4) TEST FOR HYDROPHOBICITY

The hydrophobicity of *Ixora coccinea* and *Ixora chinensis* was tested by dropping water on the filter paper which is dipped in the solvent contained wax. And the water retaining capacity was noted by the time till the paper shows hydrophobicity. In this test it is clear that *Ixora coccinea* wax coated filter paper using chloroform the paper shows hydrophobicity for about 50 minutes (Plate;11) the solvent benzene shows hydrophobicity for about 3 minutes (plate;12)and in case of *Ixora chinensis* wax coated using chloroform shows hydrophobicity for about 1 minutes (plate;13)and in the solvent benzene shows hydrophobicity for about 1 minutes (plate;14).

TABLE -2 Test for hydrophobicity

Name of the plant	<i>Ixora coccinea</i>	<i>Ixora chinensis</i>
Time of hydrophobicity in chloroform	50 minutes	3 minutes
Time of hydrophobicity in benzene	1 minute	1 minute

5) HEAT SENSITIVITY TEST

In this heat sensitivity test the wax obtained from the solvent extraction method is subjected to heat at 70 degree Celsius and to this 2ml of solvent is added and the water retaining capacity is noted. Wax obtained from the solvent chloroform is showing hydrophobicity for about 3 minutes (plate;15) in *Ixora coccinea* and 1 minutes(plate;17) in *Ixora chinensis* wax obtained from the solvent benzene not retained its hydrophobic property.(plate;16 and 18).

PLATE-1



Leaf of *Ixora coccinea*

Plate-2



Leaf of *Ixora chinensis*

ISOLATION WAX FROM THE SURFACE OF LEAVES

Ixora coccinea



Chloroform-PLATE-3



Benzene -PLATE-4

Ixora chinensis



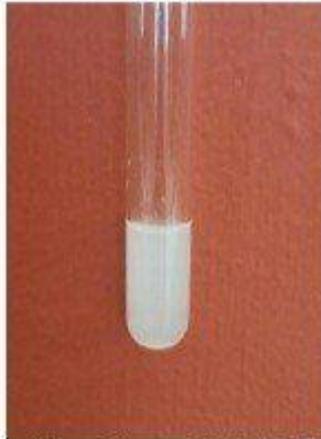
Chloroform-PLATE-5

Benzene-PLATE-6



WAX CONFIRMATORY TEST

Ixora coccinea



Chloroform -PLATE -7



Benzene-PLATE-8



Ixora chinensis

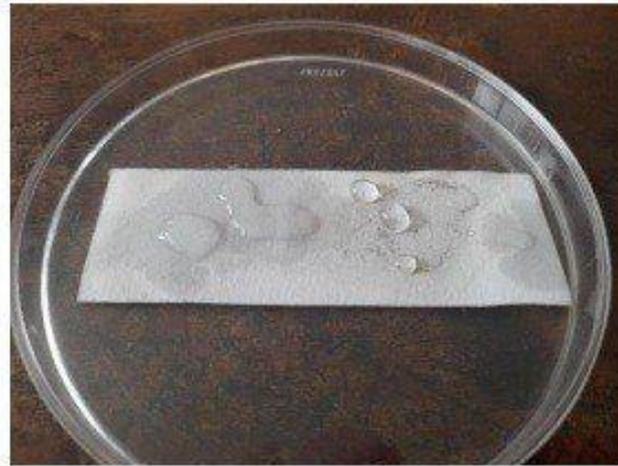


Chloroform-PLATE-9

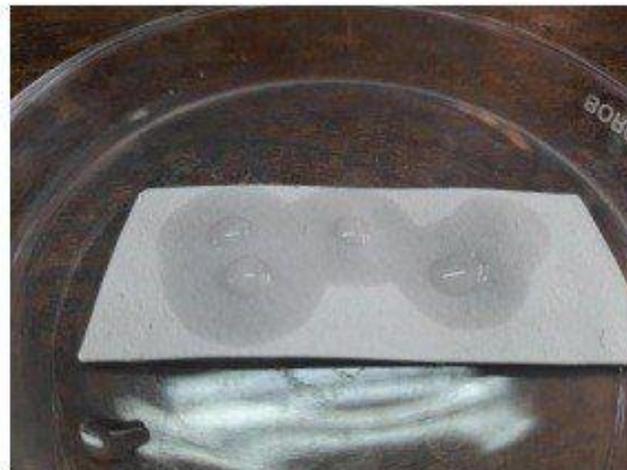
Benzene-PLATE-10

TEST FOR HYDROPHOBICITY

Ixora coccinea



Chloroform-PLATE-11



Benzene-PLATE-12

Ixora chinensis



Chloroform-PLATE-13



Benzene-PLATE-14

HEAT SENSITIVITY TEST

Ixora coccinea

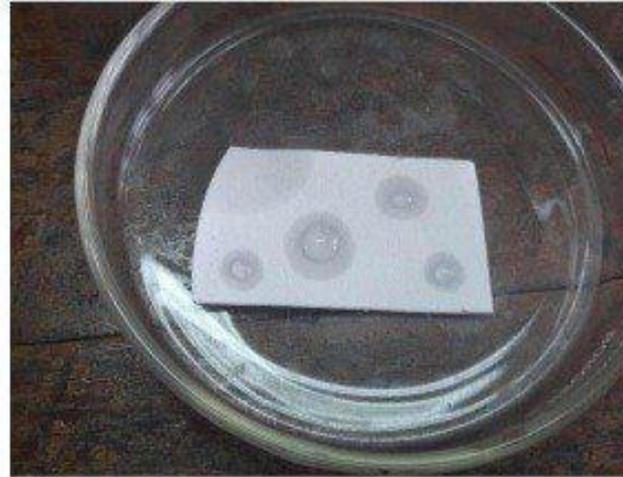


Chloroform-PLATE-15

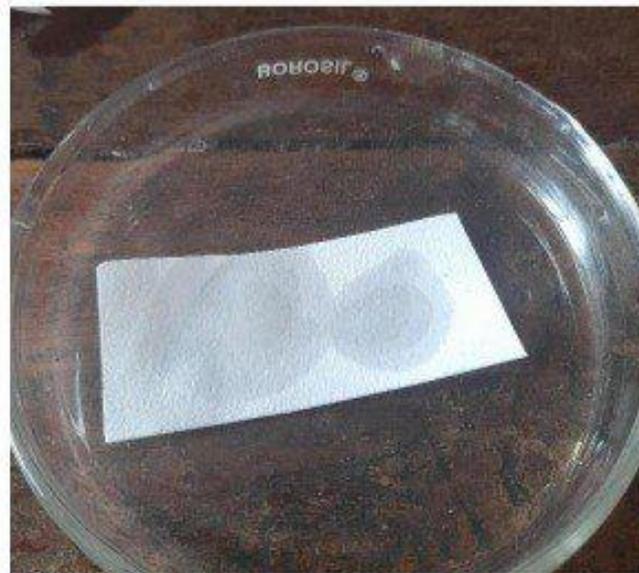


Benzene-PLATE-16

Ixora chinensis



Chloroform-PLATE-17



Benzene-PLATE-18



DISCUSSION

Epicuticular wax is a coating of wax covering the outer surface of the plant cuticle in land plants. It may form a whitish film or bloom on leaves, fruits and other plant organs. Most of the epicuticular coating consists of hard wax composed of oleanolic acid dimers attached to the edges of plates consisting of C24–C26 alcohols, notably n-hexacosanol (Casado and Heredia, 1999). Maximum hydrophobicity occurs with n-alkanes, where the terminal methyl groups are arranged in the tightest possible packing. The situation is less clear with the molecular composition of the surfaces of solid cyclic wax components but they tend to be more wettable than their aliphatic counterparts.

The present study deals with the development of hydrophobic paper using epicuticular wax obtained from the leaves of *Ixora coccinea* and *Ixora chinensis*. Both the plants have epicuticular waxy surface in their leaves. The wax is extracted from solvent extraction method. The solvents used for extraction method are chloroform and benzene.

In this study the wax is extracted using the solvents chloroform and benzene among these chloroform is the best solvent for wax extraction. Because from *Ixora coccinea* and *Ixora chinensis* more amount of wax is obtained by using the solvent chloroform. The wax obtained is confirmed by dissolving it in ethanol and few ml of distilled water, as a result the solution turns milky white and indicates that the presence of wax is confirmed.

On this study the amount wax obtained is measured by quantitative analysis, and so in *Ixora coccinea* and *Ixora chinensis* more wax is obtained from the solvent chloroform.

During this study the wax is coated in whatman's filter paper and water drops are dripped to it to check the hydrophobicity of paper. The hydrophobicity is observed by which time till the paper shows hydrophobic property. And the wax extracted using chloroform shows more hydrophobicity in *Ixora coccinea*.

In a study conducted by Akash.k and Nayan.T the amount of wax present per 1 gm of *colacasia esculenta* leaves is approximately 0.116g. And the extracted bio-wax shows hydrophobic properties even when it exposed to higher temperature which is around 95-100°C.

The hydrophobic paper made from the wax obtained from the leaves of *colacasia esculenta* is found to be better than photographic paper in terms of solubility, liquid dropping, pH and moisture content. And also it is found that the combination of wax from taro leaves and Chitin from crab shells can produce an acid free hydrophobic paper with low moisture content.

During the study heat sensitivity test is done by heating the wax with a petriplate for 70°C and Whatman's filter paper is dipped in it, then water drops are added drop by drop to the paper to check the hydrophobicity. Wax obtained from chloroform retained hydrophobic property after heating also.

SUMMARY AND CONCLUSIONS

Developing hydrophobic papers are very important because nowadays most of the people are using plastic bags as their food containers and for packaging food items. Researchers revealed that using plastic for covering food is very dangerous because of the chemical substances present in it. So it is important to prohibit plastic bag completely because it is becoming threat to humans.

In the present study isolation of wax by solvent extraction method using the solvents chloroform and benzene, wax confirmatory test, hydrophobicity and heat sensitivity tests are carried out. From this we can conclude that wax is obtained in a minimal quantity in *Ixora coccinea* and *Ixora chinensis* by using the solvent chloroform. It can be used for making hydrophobic papers. *Ixora coccinea* and *Ixora chinensis* are easily available in the environment and so the production of hydrophobic papers using the wax obtained from these plants are possible.

The quantitative analysis of wax concluded that more wax is obtained from the solvent extraction method using the solvent chloroform and so chloroform is the best solvent for extraction of wax. The quantity of wax is taken by subtracting the final weight of leaves after isolation of wax and the initial weight of leaves before isolation of wax.

Hydrophobicity test shows wax obtained with the solvent chloroform shows more hydrophobicity in both the plants *Ixora coccinea* and *Ixora chinensis*. So the solvent chloroform is more suitable for isolation of wax from the surface of leaves by solvent extraction method.

In heat sensitivity test shows that the wax obtained from *Ixora coccinea* and *Ixora chinensis* retained their hydrophobicity in the solvent chloroform and in benzene it doesn't retained hydrophobic property.

From this study we can conclude that since the plants *Ixora coccinea* and *Ixora chinensis* are abundantly available, and its bio-wax possess hydrophobic properties, the bio-wax can be used as a surface coating for the biodegradable papers which is to replace the use of plastic bags, which will really helpful to the human as well as to the environment. It will reduce the amount of pollution.

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**PRELIMINARY STUDIES ON
THE ANTIBACTERIAL POTENTIAL OF THE RED
SEAWEED *ACANTHOPHORASPICIFERA* (M. Vahl) Borgesen**

**DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE AWARD OF THE DEGREE OF BACHELOR OF
SCIENCE IN
BOTANY**

By

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**DEPARTMENT OF BOTANY
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ERNAKULAM**

2022

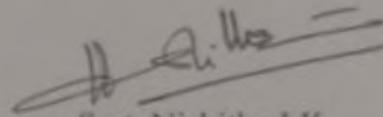
CERTIFICATE

This is to certify that the dissertation entitled "Preliminary studies on the antibacterial potential of the red seaweed *Acanthopora spicifera* (M. Vahl) Borgesen" is an authentic record of research work carried out by Miss **Tinu Petson**

(Reg No: AB19BOT018) under the supervision and guidance Smt. Nishitha I. K. Assistant Professor, Department of Botany and Centre for Research, St. Teresa's College (Autonomous), Emakulam, in partial fulfilment of the requirements for the award of the degree of Bachelor of Science in Botany. I further certify that no part of this work embodied in this project has been submitted for the award of any degree or diploma.

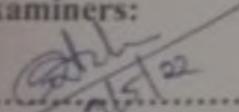
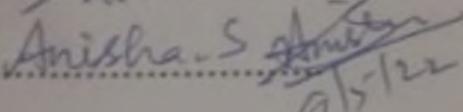


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DECLARATION

I hereby declare that the project entitled "Preliminary studies on the antibacterial potential of the red seaweed *Acanthoporphoraspicifera* (M. Vahl) Borgesen" submitted to Mahatma Gandhi University, Kottayam, in partial fulfilment of the requirement for the Degree of Master of Science in Botany is an original project done by me under the supervision and guidance of Ms. Nishitha I.K., Department of Botany and Centre for Research, St. Teresa's college (Autonomous), Ernakulam.

PLACE: Ernakulam

DATE: 4th May 2022



Name: **Tinu Petson**

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Place : Ernakulam

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Date: 4th May 2022

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INTRODUCTION

Algae are diverse group of relatively simple, chlorophyll containing, photo-autotrophic and oxygen evolving aquatic thalloid (without differentiation into True roots, stems, leaves or leaf like organs) organisms. The word "algae" has its origin from Latin, where 'alga' means seaweed. The term algae was first used by Carolous Linnaeus in 1753. Most of them are photo-autotrophic but few are mixotrophic and myzotrophic (sucking through special feeding structure) study of algae is known as phycology (GK. Phykos- seaweed; logos= discourse Or study) or algology.

Algae are divided into nine main phylums, they are Phylum Rhodophycophyta, Phylum Xanthophycophyta, Phylum Chrysophycophyta, Phylum Phaeophycophyta, Phylum Bacillariophycophyta, Phylum Euglenophycophyta, Phylum Chlorophycophyta, Phylum Cryptophycophyta, and Phylum Pyrrophyta.

The term "seaweed" refers to a variety of marine plants and algae that can be found in the ocean, rivers, lakes, and other bodies of water. Some seaweeds, such as phytoplankton, are small and remain floating in the water column, providing the foundation for most aquatic food chains. Some are massive, such as the giant kelp that grow in dense forests. The large percentage are medium-sized, with red, green, brown, and black colours, and sometimes may wash up on beaches and shorelines. (Guiry, Michael D., 2014)

Marine algae from Indian coasts amounting to 844 species (including forma and varieties) are distributed among 217 genera. They grow in the intertidal, shallow and deep sea areas up to 180 meter depth and also in estuaries, backwaters and lagoons on solid substrates such as rocks, dead corals, pebbles, shells, mangroves and other plant materials (Anatharaman *et al.*, 2007; Sakthivel, 2007).

Mostly seen seaweeds are macro algae. They are of different types according to the colour of pigments present: brown algae (phylum Ochrophyta, class Phaeophyceae), red algae (phylum Rhodophyta, *Gelidium*), and green algae (phylum Chlorophyta, classes Chlorophyceae, Ulvophyceae etc. They differ significantly in many ultrastructural and biochemical functions, including photosynthetic pigments, storage molecules, cell wall composition, presence/absence of flagella, mitosis ultrastructure, linkage between cells, and structure of the chloroplasts, in addition to pigmentation.

Seaweed is high in vitamins, minerals, and fibre, as well as being palatable. The Japanese have a dish, 'sushi' which is nori seaweed wrapped with a mixture of fish, rice, and other ingredients for at least 1,500 years. Anti-inflammatory and anti-microbial compounds can be found in a variety of seaweeds. For thousands of years, their medicinal properties have been used; it was used to cure wounds, burns, and rashes by the ancient Romans. According to anecdotal evidence, the ancient Egyptians may have employed them to cure breast cancer. (McLachlan, J., and C. J. Bird, 1984).

In recent years, focus towards these organisms has increased due to their food and fuel production capability. In fuel industry algae biofuels have emerged as a clean, nature friendly, cost effective solution to other fuels. More recently algae have been identified and developed as renewable fuel sources, and the cultivation of algal biomass for various products is transitioning to commercial-scale systems. Large-scale cultivation of algae merges the fundamental aspects of traditional agricultural farming and aquaculture (Emily M Trentacoste *et al.*, 2014). Algae fuels are categorized into bio-ethanol, biogas, bio-hydrogen, biodiesel and bio-oil. Algae can be used in the preparation of Biodiesel, Bioethanol, Biobutanol and Hydrogen gas (Raja *et al.*, 2013)

They are considered as a potential source of bioactive substances such as proteins, lipids, and polyphenols possessing potent antibacterial, anticancer, antioxidant, antifungal, and antiviral properties (Sundaramurthy *et al.*, 2016). Seaweeds that are medicinal are rich in secondary metabolites which include alkaloids, glycosides, flavonoids, saponins, tannins, steroids, related active metabolites, which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry (Eluvakka *et al.*, 2010). Recently, their value as a source of novel bioactive substances has grown rapidly and researchers have revealed that marine algal originated compounds exhibit various biological activities (Kim and Wijesekara, 2010; Wijesekara and Kim, 2010; Wijesekara *et al.*, 2010 and Wijesekara *et al.*, 2011). The secondary metabolites of seaweeds such as Isoprenoids (terpenes, carotenoids, steroids), polyketides, phlorotannins, amino-acid derived natural products (alkaloids), and shikimates (flavonoids) have always attracted the interest of biochemists because of their diversity is comparable with those present in the leaves of higher plants (Manilal *et al.*, 2009). Seaweeds were rich in dietary fiber (>50% dry weight), particularly in the soluble form.

RHODOPHYCEAE

Rhodophyta is a phylum of macroalgae that includes the classes Phaeophyceae and Chlorophyta, which are brown and green seaweeds, respectively.

Within Archaeplastida, Rhodophyta, or red algae, is a monophyletic lineage that contains glaucophyte algae, green algae, and terrestrial plants. Bangia-like species have been found in 1.2 billion-year-old strata, indicating that Rhodophyta has a lengthy fossil history. The morphology of red algae ranges from unicellular filamentous to multicellular thalloid forms, with certain species producing economically important products like agar and carrageenan. These species can be found in a variety of marine settings, ranging from the intertidal zone to deep oceans. There are also freshwater (e.g., *Batrachospermum*) and terrestrial lineages. A triphasic life cycle with one haploid and two diploid phases, with the carposporophyte borne on female gametophytes, is one of the Rhodophyta's significant advances.

Freshwater Rhodophyta has 66 species and 27 genera in North America, although these numbers will change as molecular investigations uncover more diversity. Freshwater red algae have a limited size range than marine species, with the majority (80%) of them measuring 1-10 cm in length. Gelatinous filaments, free filaments, and pseudoparenchymatous forms are the most prevalent types. (Yoon, Hwan Su, et al., 2017)

ACANTHOPHORA

Acanthophora is a red algae that can be found in almost all tropical and subtropical oceans. Because of its changeable form, it can adapt to a wide range of environmental circumstances and hence invade a wide range of ecosystems.

Acanthophora is an erect macroalgae that may reach a height of 40cm. It has solid cylindrical branches that are 2-3mm diameter and are rarely or repeatedly branched. Short, determinate branches, irregularly shaped and spinose, with spines numerous and radially oriented, make up the major branches. The major axes have no spines. A big, oddly shaped holdfast gives rise to the plant. It has short (4 - 10cm), compact, and dense thalli in intertidal high-motion water areas. It comes in a wide range of colours, including red, purple, yellow, orange, and brown. Thalli are typically quite black in intertidal, high-motion locations, and lighter in shallow areas with low water motion and reflective sandy or silty bottoms.

In the Gulf of Mannar, Tamil Nadu's coastal region, *Acanthoporphoraspicifera* is a common seaweed used in folkloric treatments and as a nutritional supplement. The anticancer and anti-oxidant properties of the alcoholic extract of *Acanthoporphora* were investigated in this study. Anti-cancer impact was measured by assessing tumour volume, tumour weight, mean survival day (MSD), and several haematological parameters after 21 days of testing and standard drug administration. The anti-oxidant state of the liver tissue was also determined. In cancerous mice treated with EAC cell lines, the ethanol extract of *Acanthoporphora* has a significant anti-cancer effect, reducing tumour volume and weight with mean survival day (MSD). The findings revealed that an ethanol extract of *Acanthoporphora* has antitumor and anti-oxidant activity, which may be due to the presence of bioactive components such as flavonoids, terpenoids, and tannins. (Lavakumar, K. F. H. Ahamed, and V. Ravichandran, 2012)

The present work was undertaken to study the antimicrobial potential of *Acanthoporphoraspicifera*. The objectives of the study are

- Taxonomic description of the algae
- Assessment of antibacterial potential of *Acanthoporphoraspicifera* in its dried form extracted in two different solvents; Ethanol and Chloroform.
- Comparative antibacterial activity of the alga extracted in the two different solvents against Gram positive *Staphylococcus* and Gram negative *E. coli*.
- Estimate the extractive value of the plant, in both ethanolic and chloroform solvents.

LITERATURE REVIEW

In a study conducted by Inci Tuney (2006), antibacterial activity of extracts or components from various algae has been demonstrated in vitro against both gram-positive and gram-negative bacteria. The antibacterial susceptibility test was performed using the agar disc diffusion method, with 6 mm discs impregnated with 20 μ l of extracts and placed in infected agar. (Inci Tuney 2006),

Krishnapriya et al., (2013) conducted an antibacterial activity on the seaweed extracts, carried out by agar disc diffusion assay. The Muller Hinton agar (MHA) medium was used for this study using bacterial pathogens. Among the solvent extracts, methanol extract showed best results for both positive and negative strains. Chloroform extract of *G. verrucosa* gave the highest zone of inhibition measuring 21 ± 1.0 mm. Ethanol extract of *G. acerosa* also showed a zone of inhibition of 12 ± 1.0 mm. Ethanol and chloroform extracts of *G. verrucosa* gave clearly distinct zone of inhibition measuring 8 ± 1.0 and 9 ± 1.0 mm, with respect to control (25 ± 1.0 mm) against *Staphylococcus*. (Varier, KrishnapriyaMadhu, et al., 2013)

Saranraj, P. (2013) conducted a study and the methanol extract of *Gracilariabifolifera* (5.0mg/ml) showed highest mean zone of inhibition (18 ± 0.4 mm) against the Gram positive cocci *Streptococcus pyogenes* followed by *Bacillus subtilis* (17 ± 0.5 mm), *Staphylococcus aureus* (17 ± 0.3 mm), *Streptococcus epidermis* (16 ± 0.6 mm) and *Bacillus cereus* (16 ± 0.2 mm). For Gram negative bacterium, the maximum zone of inhibition was recorded in methanol extract of *Gracilariabifolifera* against *Klebsiella pneumoniae* (17 ± 0.5 mm) followed by *Salmonella typhi* (16 ± 0.6 mm), *Pseudomonas aeruginosa* (16 ± 0.5 mm), *Escherichia coli* (16 ± 0.3 mm). The zone of inhibition obtained from the Hexane extract of seaweed *Gracilariabifolifera* against bacterial pathogens was comparatively very less when compared to the other solvent extracts. No zone of inhibition was seen in DMSO control and the positive control Ampicillin showed zone of inhibition ranging from 13 ± 0.8 mm to 20 ± 0.8 mm against the test bacterial pathogens. (Saranraj, P., 2013)

The antibacterial properties of eight crude extracts of local *Acanthoporaspicifera* obtained by two distinct extraction methods were investigated by Zakaria (2010) using Soxhlet extraction and solvent partitioning. By using the Disc diffusion method, these extracts were evaluated in vitro against 18 bacteria, 3 yeasts, and 6 fungal strains. The results demonstrated

that the solvent partitioning extracts of methanol and ethyl acetate had a greater spectrum of action against the tested bacterial strains. *Bacillus cereus* ATCC 10876, *Bacillus licheniformis* ATCC 12759, Menthicilin Resistance *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* ATCC 27853, *Yersinia* sp., and *Citrobacter freundii* displayed inhibitory zones against these two extracts. While methanol extracts from Soxhlet extraction and butanol from solvent partitioning had no antibacterial activity against *P. aeruginosa* ATCC 27853, the other six extracts did. (Zakaria 2010)

In a study done by Ibraheem et al. (2017), simplex extracts of *Acanthophora* showed potent inhibitory growth activities against three Gram positive bacteria [*Streptococcus agalactiae*, *pyogenes* and *Streptococcus sanguis* of inhibition ranging from [23.1±0.58 to 20.6±0.63 mm] and showed moderate activities with [*Corynebacterium diphtheriae*, *Bacillus subtilis* and *Staphylococcus aureus*] with inhibition zones ranging from [20.1±1.5 to 16.3±2.1 mm]. (Ibraheem et al.; 2017)

Also the crude extracts were found to be more active than the positive control Ampicillin, (22.3±1.5 mm), against *Streptococcus agalactiae* which showing inhibition zone. The hydro alcoholic extracts of the selected species were investigated for their antimicrobial activities using Agar well diffusion and Muller Henton against gram positive and gram negative bacteria.

In a study by Nurul Aili Zakaria et al. (2011), the antimicrobial activities of the hexane extract were evaluated using disc diffusion method against 8 Gram-negative and 10 Gram-positive bacterial strains. Out of all bacterial tested, only a Gram-positive bacterium and a Gram-negative bacterium were susceptible to the extracts. The hexane extract showed antibacterial activity against both Gram-positive bacterium and Gram-negative bacterium (*P. aeruginosa* ATCC 27853). While, chloroform and ethyl acetate extract only showed inhibitory effect on *P. aeruginosa* ATCC 27853 with inhibition zone of 9.0 mm. No inhibitory effect was showed by methanol extract on bacteria tested. (Nurul Aili Zakaria et al.; 2011)

MATERIALS AND METHODS

SPECIMEN COLLECTION

The specimen was collected by hand picking from Thikkodi beach, Calicut. The collected samples were washed immediately in seawater and then washed with fresh water and transported to the laboratory. It was again washed thoroughly to remove impurities and sand and rinsed with distilled water. The sample was identified taxonomically as *Acanthophora*. Collected sample was taxonomically evaluated using the standard literature.

SAMPLE PREPARATION

For antimicrobial studies, the cleaned samples were then shade dried, cut into small pieces and powdered in a mixer grinder. The organic solvents Chloroform and Ethanol were used for the extraction process due to its higher efficiency using Soxhlet extraction method. 20g of samples were packed in a thimble and placed in the extractor. 200ml of the solvent was added into the flask and heated. The temperature was maintained at 80°C to 85°C throughout the extraction. The soluble active constituents of the extract remained in the flask and the process was repeated until the compounds were completely extracted. The liquid extract was then cooled and concentrated by using an evaporator.

The beaker with dried extract was weighed and noted. DMSO was used to dissolve the extracts from the beaker. Later the weight of the beaker alone was noted. Hence, the actual weight of the dried extract was obtained. From the above observation, the weight of dried extract of *Acanthophora* in ethanol and chloroform was 2.44g and 0.18g respectively. From this the extractive value was calculated using the formula

Extractive value (%) = (Weight of dried extract/ Weight of plant material) X 100

PREPARATION OF EXTRACT IN VARIOUS CONCENTRATIONS

From the stock extract, concentrations of 10%, 20%, 40%, 60%(v/v) was made. The stock concentration of *Acanthophora* in ethanol and chloroform was 70mg/ml and 10mg/ml respectively. From the stock the appropriate amounts was pipetted out and made up to the required concentrations using DMSO.

ANTIBACTERIAL ACTIVITY IN ACANTHOPHORA

PREPARATION OF BACTERIAL CULTURE

In the present study, the extracts were evaluated for antimicrobial activity against *Staphylococcus* strain and *E. coli*, a Gram positive and a Gram negative bacteria respectively. 3g of nutrient broth was dissolved in 100ml of distilled water in a conical flask. The broth is sterilized by autoclaving for 15 minutes. Both of the obtained bacterial stains were inoculated in the nutrient broth in laminar air flow and incubated in appropriate conditions for 24hrs.

PREPARATION OF PETRI PLATES

The selected two species of seaweeds were analysed for the antimicrobial activity for gram negative *Escherichia coli* and gram positive *Staphylococcus* by disc diffusion methods. Agar medium was prepared by dissolving 4g agar and 2.6g of nutrient broth in 100ml distilled water. The mixture is sterilized in an autoclave for 15 minutes. Just after sterilization the mixture was poured into petri plates in laminar air flow. The petri plates were allowed to solidify under aseptic conditions.

ANTIMICROBIAL TEST BY DISC DIFFUSION METHOD

Bacteria were inoculated onto the prepared agar petri plates using sterilized cotton swabs. Sterilized 6mm discs were taken from filter paper and autoclaved and is used for the method. The disc was then dipped in different concentrations of stock (10, 20, 40, 60) and placed on the agar plate using sterile forceps. Tetracycline was used as positive control and DMSO was used as negative control. This was done for both extracts of *Acanthophora* against the two strains of bacteria. The petri plates were incubated at 37°C for 24 hours and results were recorded.

OBSERVATIONS AND RESULTS

The current work was undertaken as a preliminary study of the red seaweed *Acanthophora spicifera*. The scope of the study included the estimation of extractive value in two solvents, ethanol and chloroform and the antimicrobial potential of these extracts. The antimicrobial potential activity was studied against Gram positive *Staphylococcus* and Gram-negative *E. coli*, two non-pathogenic bacteria. The results obtained are described below.

TAXONOMIC DESCRIPTION

- Division: Rhodophyta
Class: Florideophyceae
Order: Ceramiales
Family: Rhodomelaceae
Genus: *Acanthophora*
Species: *spicifera*

Acanthophora spicifera (Vahl) Børgesen

Erect plants, to 40 cm tall, with solid cylindrical branches, 2 - 3 mm wide, branched either sparingly to repeatedly. Main branches have short, determinate branches, irregularly shaped and spinose, with spines numerous and radially arranged. There are no spines on main axes. The plant grows from a large, irregularly shaped holdfast. In intertidal high-motion water areas, *Acanthophora spicifera* has short (4 - 10 cm), compact and very dense thalli. In moderate or low water motion areas, the thalli are tall (10 - 25 cm), more openly branched and occur in scattered clumps.



Acanthophoras pecifera(M.Vahl)Borgesen

EXTRACTIVE VALUE

Extractive values of plant materials are used to evaluate extracts of the sample, in order to get an idea about the nature of chemical constituents present in it. It can also be used to assess quality, purity and detect adulteration of the extract.

In the present study, polar and non-polar solvents were used for eluting the valuable phyto-compounds present in the sample. Extractive values of ethanol and chloroform extracts of *Acanthophora spicifera* used in the antibacterial study, are estimated in the table 1 given below;

Table 1: Extractive value of solvents administered for *Acanthophora spicifera*.

Solvent	Extractive value of the sample (%)
Ethanol	12.2
Chloroform	0.9

The extractive value was greater for the ethanolic extract than for chloroform suggesting that polar solvent was more efficient in extracting the phytochemicals from the algae.

ANTIBACTERIAL ACTIVITY

The extracts of the algae exhibited only mild antibacterial activity against the two microorganisms. The activity observed can be described as being bacteriostatic showing very mild zones of inhibition. The ethanol extract of the algae showed mild antibacterial activity against *E. coli* alone. The activity is shown only at high concentration against gram negative bacteria.

Table 2: Antibacterial activity of ethanolic extract of *A. spicifera* against *E. coli* and *Staphylococcus* bacteria:

Concentration(%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	No action	No action
40	No action	No action
60	Mild action	No action

Table 3: Antibacterial activity of chloroform extract of *A. spicifera* against *E. coli* and *Staphylococcus* bacteria

Concentration(%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	No action	No action
40	No action	No action
60	No action	Mild action

The chloroform extract of *Acanthophora* has no significant effect on the growth of *E. coli*. Mild bacteriaostatic activity is observed at higher extract concentrations on *Staphylococcus*. No potential activity could be observed on the growth of *E. coli* in any of the concentrations used for the current study Table 3: Fig.



E. coli

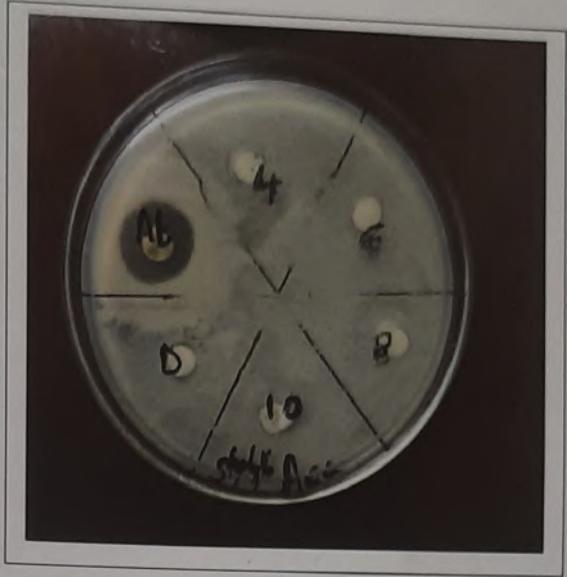


Staphylococci

Fig 2: Antimicrobial activity of Ethanol extract of *Acanthophora spicifera*



E. coli



Staphylococci

Fig 3: Antimicrobial activity of Chloroform extract of *Acanthoporaspicifera*

DISCUSSION

Seaweeds are a group of marine macro algae that are now in the limelight of algal research due to their immense bioactive potentials and easy availability. Several bioactive compounds are found in high concentration in the seaweeds, because of which it exhibits various pharmacological activities. The seaweeds offer greatest wealth in terms of biomass and Rhodophytes show the largest representation among them. *Acanthophoraspicifera* is a red sea with about 26 species world wide.

Natural products from marine algae have attracted the attention of biologists and chemists the asmany of these compounds are used to treat diseases like cancer, acquired immune-deficiency syndrome, inflammation, pain, arthritis, as well as viral, bacterial, and fungal infections. Sunil DS in 2015 studied the marine red alga to analyse the phytochemical constituents. The presence of a variety of chemical constituents, such as saponins, phenols, flavonoids, alkaloids and steroids were confirmed in *Acanthophoraspicifera* by qualitative tests.

Antibacterial activity refers to the process of killing or inhibiting the disease-causing bacteria. Several plants have been traditionally used for their antibacterial activity. Like plants some algae also exhibit antibacterial properties due to the presence of terpenoids, steroids, saponins, tannins, and flavonoids. There are numerous reports regarding the inhibitory activities of macroalgae against human pathogens, fungi and yeasts. So, the use of algae as an alternative for prevention and treatment of infectious diseases has been suggested by Abirami and Kowsalya (2012). In the present study ethanolic and chloroform extract were evaluated for activity against Gram positive *Staphylococci* and Gram-negative *E. coli*. It was found that ethanolic extract had bacteriostatic activity against *E. coli* and the chloroform extract inhibited *Staphylococcus* at high concentrations.

Different solvent systems were used to extract bioactive principles from macroalgae with concomitant changes in the antibacterial activities (Thirupurasundari et al., 2008). The solvents such as acetone, benzene, butanol (Vanitha et al., 2003; Prakash et al., 2005), ethanol (Selvi et al., 2001) were used to extract antimicrobial compounds from macroalgae. The aqueous extracts prepared from seven macroalgal samples showed varying degrees of activity against tested pathogens, including the Gram positive and Gram negative bacteria. (Padmakumar (2002) is of the opinion that these differences are due to the different solubility behaviour of secondary metabolites which could be influenced by seasonal and geographical distribution of the species.

Antibacterial potential of *A. spicifera* ethanol extracts were evaluated by Meenakshi et al (2014), against six bacterial specimens. They reported high antimicrobial activity against *E. coli*. In the present study, ethanolic extract showed mild activity at higher concentration (60%) only. In their work, Meenakshi et al have also reported significant cytotoxic potential against Ehrlich Ascites carcinoma cell lines for ethanolic extract of the alga..

Several previous studies have revealed the bioactivity of active compounds isolated from *Acanthophora* sp. such as antibacterial antiviral and antioxidant activity, anti-viral, and anti-fouling. Steroids and fatty acid esters of *A. spicifera* were reported to exhibit potent antitumor and antibacterial activity against human cancer lines and microorganisms ((Laila S, 2003; Han, L et al 2006)

In their study on six sea weeds, Rajashekar et al in 2018 reported that *A. spicifera* has the least number of compounds in ethanol and chloroform extracts. This possibly explains the low antimicrobial activity of the algae in the present study also. However, they report potential inhibitory activity against *B. cereus* and *P. aeruginosa*

SUMMARY

Seaweeds are the macroalgae found in marine ecosystems where they play a multitude of roles. From being the primary producers providing nutrients and energy to other living organisms, provide shelter and home to these life forms. They also play significant roles in climate mitigation. Seaweeds have been used as traditional remedies for common ailments and have been a part of traditional cuisine in many parts of the world. Several studies have been conducted on different algae to assess their biopotentials and to exploit them in a way beneficial to man.

For this project, the red algae *Acanthophora spicifera* was selected. *Acanthophora* is an erect macroalgae, that can be found in almost all tropical and subtropical oceans. It is widely distributed in the southern and northern rocky coasts of Kerala. The alga was studied to identify its extractive values in different solvents and to assess its antibacterial potential. The dried algae was extracted in the solvents using the Soxhlet apparatus. The extractive values of the algae in ethanol and chloroform, two solvents with very different polarities was estimated and found that the polar solvent, ethanol had greater extractive value of 12.2% than the non-polar solvent chloroform with only 0.9% extractive value.

The antioxidant potential was studied by the disc diffusion method. The test organisms used were the Gram positive *Staphylococcus* and Gram negative *E. coli*. The analysis of antimicrobial activity of *Acanthophora spicifera* displayed little to no microbial activity in both ethanolic and chloroform extracts for gram positive (*Staphylococcus*) and gram negative (*E. coli*) bacteria taken. A mild reaction suggesting bacteriostatic activity was observed in ethanolic extract at higher concentration for gram negative bacteria while the chloroform extract showed mild reaction for gram positive bacteria at higher concentration. This can be used as an indicator for further studies of antimicrobial activity of the species.

Acanthophora possess antioxidant, antitumor and antibacterial activity, which may be due to the presence of bioactive components such as phenolics, terpenoids, and tannins. The chemical constituent of *Acanthophora* is rich in non halogenated steroid. Various other extracts of the algae have highlighted the nutritional and anticancer properties which supports its widespread usage in folklore medicine. Thus the proper identification and analysis of the seaweed can be used for producing natural alternatives to the synthetic medicines available in today's market.

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**CHARACTERIZATION OF EPICUTICULAR
WAX FROM *Rhizophora mucronata* Lam. and
Avicennia officinalis L.**

**AND ITS APPLICATION FOR DEVELOPMENT
OF HYDROPHOBIC PAPER**

Dissertation submitted in partial fulfillment of the requirements for the award of the
degree of

BACHELOR OF SCIENCE IN BOTANY

By

JAYALAKSHMI. S. B

Reg. No. : AB19BOT024

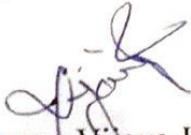


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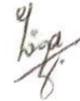
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CERTIFICATE

This is to certify that the dissertation entitled "**Characterization of epicuticular wax from Rhizophora mucronata Lam. and Avicennia officinalis L. and its application for development of hydrophobic paper**" is an authentic record of work carried out by Jayalakshmi S. B under my supervision and guidance in partial fulfillment of the requirement of B.Sc. degree affiliated to Mahatma Gandhi University, Kottayam. I further certify that no part of the work embodied in this dissertation work has been submitted for the award of any other degree or diploma.



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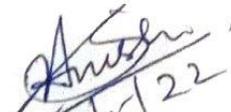


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Place: Ernakulam.

Date: 9/5/2022



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INTRODUCTION

Plastic is found more useful in day today life due to its high durability, non- corrosive nature, light weight, electrical and thermal insulation, temperature resistance, low permeability, high strength to weight ratio, water resistance etc. Plastic bags have become a global concern because of the fact that they cause serious threat to environment because of its non-biodegradable nature. Around 5-6 billion plastic bags are used across the globe. Attempts were made to create alternatives like biodegradable plastic bags. Plastic and polythene as hydrophobic material become a great concern due to their non-biodegradable nature. Therefore to avoid these problems, use of paper bags are encouraged because it has been proved that paper bags do not harm environment.

Paper bags are not water resistant due to which it's use become limited. Paper bags are coated with synthetic compounds like lacquer to have advantage over plain paper than their degradation might cause water and soil pollution. So, attempts were made to find suitable organic material which might be able to solve the above mentioned problem.

Paper is an inherent hydrophilic material because of hydroxyl groups contained in cellulose. The hydrophilic nature of cellulose pose obvious limitations in the use of paper when hydrophobicity is highly demanded.

In general term wax is used for variety of natural or artificial commercial products that contain fatty materials of various kinds. Well known examples are bees wax, Paraffin wax and carnauba wax from the wax palm. Epicuticular wax are sometimes visible as a bluish covering on leaves of cabbage, fruit plums etc.

Aerial surface of plants contain hydrophobic wax which is water proof (Bio- wax) and provide protection against environmental stresses. In some plants their bio- wax is highly hydrophobic.

The leaves of plants are available throughout the year. The hydrophobic surface have attracted considerable attraction in the last two decades because of their various potential applications relevant to water repellent and self-cleaning.

Rhizophora mucronata Lam. belongs to Rhizophoraceae family (Mangrove). It is commonly known as loop- root mangrove or red mangrove, distributed in the tropics. They thrive on coastline in brackish water and salt marshes. It is a small medium sized evergreen tree growing to a height of about 20 to 30 meters on water banks. Leaf arrangement is opposite and simple with dark green color. Its leaves are very thick with cuticle. The tallest trees are closest to the water and shorter trees are further inland. The tree has a large number of areal stilt roots from the trunk. It seems to be more tolerant of inundation than other mangrove species and often forms an evergreen fringe to mangrove areas. It is used to help prevent coastal erosion and restoration of mangrove habitats. Timber is used for fire wood and in the construction of buildings.

Avicennia officinalis L. belongs to Acanthaceae family known as Indian Mangrove. It possess pneumatophores. Grows just above the high tide in coastal lagoons and brackish water estuaries. . Leaves are elliptical, leathery, green and shiny with rounded apex. The young tree forms a columnar tree up to 15m and grow up to 30. The bark is smooth. It prefers clay soil and usually found inland.

The present study was done for the characterization of epicuticular wax from *Rhizophora mucronata* Lam. And *Avicennia officinalis* L. and its application for the development of hydrophobic paper.

OBJECTIVES

- To isolate and analyse the epicuticular wax from *Rhizophora mucronata* Lam. and *Avicennia officinalis* L. using suitable solvents.
- To quantify the wax obtained and characterize it for hydrophobicity and heat sensitivity.
- To apply the epicuticular wax for the development of hydrophobic paper.

REVIEW OF LITERATURE

Epicuticular wax layers have influence on the wettability of plants surface and also control their permeability to water. Plant waxes are generally the water proofing components found in an amorphous layer on the outer surface of the plants. They are essential for the plants as barrier protection against environmental stresses. Surface wetness is functionally important to plants. Plant waxes are hydrophobic substance, this property is important to the orientation of their molecules in solid state. Epicuticular wax decrease surface wetting and moisture loss. Other functions of epicuticular wax include reflection of Ultra Violet light, formation of an ultra hydrophobic and self-cleaning surface and acting as anti-climb surface.

In a study conducted by Yadav *et al.* (2014), epicuticular wax has been extracted from the leaves of *Calotropis procera* R.Br. using various solvent (ethanol, methanol, benzene, acetone). The highest hydrophobicity (29.5nt7%) was found to be in paper coated with epicuticular wax with benzene from the adaxial surface of *Calotropis procera* R.Br.

A study conducted by Mukherjee *et al.* (2019) focuses on the development of a super hydrophobic layer containing chemically extracted bio wax from lotus and taro leaves on silicon substrate. Contact angle measurement is performed to determine the hydrophobicity of the surface by measuring the respective contact angle at the interphase if liquid and bio wax coated surface

Nayan *et al.* (2018) conducted a study on the hydrophobicity of *Colocassia esculenta* leaf. The surface of taro leaves is covered with a layer of highly hydrophobic layer of bio wax. The main objective of the study was to isolate the bio wax layer of the leaves using organic solvent extraction method using chloroform and coated in a surface of a hydrophobic paper. Also the bio wax extracted is subjected to various tests like heat test, hydrophobicity test, antimicrobial test, quantitative analysis to check it's viability for industrial uses. The quantitative analysis showed that 1gm of the leaf sample contain 0.116g of wax. It was observed that the paper coated with wax attained hydrophobic property. Heat sensitivity test showed that bio wax retains hydrophobicity even at high temperatures. Bio wax also possess anti – bacterial property.

Nazri *et al.* (2014) conducted a study on the hydrophobic properties as well as the presence of 1- octacosanol of the taro wax extracted from the taro leaf using various analytical techniques. Bio- wax extraction was achieved by immersing taro leaves in 500 ml Chloroform at 50°C for 30 seconds and the step was repeated with sample using chloroform. The solvent was evaporated using evaporator and raw bio wax solution was extracted. Plant based taro wax can be a source of sustainable and renewable hydrophobic material used in HVAC application system.

The epicuticular wax derived from *Calotropis procera* R.Br is explored as an ecofriendly and safe hydrophobic material. Leaf with smallest area was found to be most suitable for extraction of wax. To evaluate the hydrophobic potential of wax is developing hydrophobic paper water regains and contact angle has been measured.

Alvarez *et. al.*, (2019). Developed hydrophobic paper containing wax of taro leaves and chitin from Crab shell. The physical properties (color, odour, texture, tensile strength, thickness, density, solubility, liquid dropping) and chemical properties (ash content, PH, moisture content) of the formulated hydrophobic paper were compared to the properties of commercial photographic paper were compared to the properties of commercial photo graphic paper. Fourier transformed Infrared (FTIR) was also used to determine functional groups present in the paper. The prepared hydrophobic paper is dirty white in color, has a pleasant odour, rough texture. Physical properties of the hydrophobic paper were found better than photographic paper except for its dirty white color due to the pigment of leaves that was present in wax from Taro leaves and its rough texture.

MATERIALS AND METHODS

MATERIALS

Leaves of *Rhizophora mucronata* Lam., Leaves of *Avicennia officinalis* L., Solvents such as Chloroform and Benzene, weighing balance, beaker, petri plate, ethanol, distilled water, Whatman filter paper, test tube.

METHODS

- 1) Collection of plant sample
- 2) Isolation of wax from leaf surface
- 3) Wax confirmatory test
- 4) Quantitative analysis of Bio- wax
- 5) Test for hydrophobicity
- 6) Heat sensitivity test

1. COLLECTION OF PLANT SAMPLE

The leavaes of the *Rhizophora mucronata* Lam. and *Avicennia officinalis* L. were collected from local coastal areas near Alappuzha.

2. ISOLATION OF WAX FROM SURFACE OF LEAVES

Fresh leaves of *Rhizophora mucronata* Lam. and *Avicennia officinalis* L. were collected. Leaves of each plant were cut into fragments and from that fragments 2g and 3g of each plant leaves were weighed out.

Then 10 ml and 15 ml of solvents such as Chloroform and Benzene was taken in beaker. For the two plants the leaf fragments were immersed in these two solvents for 3 minutes. A glass rod was used to immerse the leaves completely. After 3 minutes these solvents were transferred into different petri plate and allowed to evaporate. A white cloudy wax layer is appeared on the surface of petri plate in different quantities. The petri plate is labelled respectively.

3. WAX CONFIRMATORY TEST

Wax was extracted from leaves of *Rhizophora mucronata* Lam. and *Avicennia officinalis* L. by solvent extraction method. The solvent was evaporated. The wax was then dissolved in Ethanol and transferred in to a test tube. The wax confirmatory test was characterized by the presence of milky white appearance. So when few ml of distilled water is added to the solution and shaken well the solution turned milky white and confirmed the presence of wax.

4. QUANTITATIVE ANALYSIS OF BIO-WAX

Fresh leaves of *Rhizophora mucronata* Lam. and *Avicennia officinalis* L. collected and cut into fragments. 2g and 3g of these two plants were measured then these fragments were dipped in 10 ml and 15 ml of solvents such as Chloroform and Benzene respectively. The solvent was discarded and the remaining solvent was allowed to air dry. The weight if the leaf fragments was then measured again. By subtracting the weight of the leaves after the solvent treatment from the weight of the fresh leaves, the amount of wax can be calculated.

5. TEST FOR HYDROPHOBICITY

Bio wax was isolated from the leaves by solvent extraction method. The solvent such as Chloroform and Benzene containing wax was poured into a petri dish and the solvent was allowed to evaporate. After the solvent had evaporated the wax coating on the petri plate was again dissolved in 2ml of solvents to get higher concentration of wax. Whatman filter paper cut into rectangular piece was dipped in the solvent and test for hydrophobicity was done by dropping water on it using a dropper and was compared to filter paper without wax coating. The water resistance was evaluated by observing the time till the wax coated filter paper shows hydrophobicity.

6. HEAT SENSITIVITY TEST

Wax was extracted in different petri plates using solvents such as Chloroform and Benzene. The solvent was allowed to evaporate. Different petri plates was subjected to heat for 2 minutes. The retention of hydrophobic property of wax were observed.

OBSERVATIONS AND RESULTS

1. ISOLATION OF WAX FROM LEAF SURFACE

Isolation of wax from leaves is caused by solvent extraction method. The solvents such as Chloroform and Benzene is used. Cloudy white layer of wax is obtained by the solvent Chloroform on *Rhizophora mucronata* Lam. (plate: 3) and *Avicennia officinalis* L. (plate: 5) and less amount of wax layer in case of Benzene in both plants compared to Chloroform. So from the study it is well understood that the solvent Chloroform will give more wax from the leaves of *Rhizophora mucronata* Lam. and *Avicennia officinalis* L. than Benzene. (Plate: 4&6) *Rhizophora mucronata* Lam. in Chloroform has comparatively more wax than *Avicennia officinalis* L. in Chloroform. So, chloroform is the best solvent used for extraction of wax from the leaves of *Rhizophora mucronata* Lam. and *Avicennia officinalis* L.

2. WAX CONFIRMATORY TEST

The wax obtained by the solvent extraction method was dissolved in Ethanol and few ml of distilled water in a test tube and shaken well. The solution leaving a milky white color which confirms the presence of wax. So in this case *Rhizophora mucronata* Lam. gives more white solution than *Avicennia officinalis* L. (Plate: 7 & 8)

3. QUANTITATIVE ANALYSIS OF BIO WAX

The amount of wax was calculated by subtracting the weight of the leaves after solvent treatment from the weight of the fresh leaves. In *Rhizophora mucronata* Lam. the initial weight in all two solvents is 3g. In Chloroform the final weight is 2.5g so, the amount of wax obtained is 0.5

In Benzene the final weight is 2.27g and the wax obtained is 0.27 so, in case of *Rhizophora officinalis* L. it is clear that Chloroform will give more amount of wax than Benzene. (Table 1.1)

Table 1.1 Quantitative analysis of Bio-wax in *Rhizophora mucronata* Lam.

Name of plant	Name of solvent	Initial weight (g)	Final weight (g)	Amount of wax obtained (g)
<i>Rhizophora mucronata</i> Lam.	Chloroform	3	2.50	0.5
	Benzene	3	2.73	0.2

Similarly in *Avicennia officinalis* L. the initial weight of all the solvents is 3g. In case of Chloroform the final weight is 2.74g. So the amount of wax obtained is 0.26

In Benzene, final weight is 2.87g so the amount of wax obtained is 0.13

So we can infer that in *Avicennia officinalis* L. more wax is obtained in Chloroform than Benzene. (Table: 1.2)

Table1.2 Quantitative analysis of bio wax in *Avicennia officinalis* L.

Name of plant	Name of solvent	Initial weight (g)	Final weight (g)	Amount of wax obtained (g)
<i>Avicennia officinalis</i> L.	Chloroform	3	2.47	0.26
	Benzene	3	2.87	0.13

4. TEST FOR HYDROPHOBICITY

Hydrophobicity test was carried out by dropping water on wax coated Whatman filter paper and was compared to a filter paper without wax coating and water resistance was evaluated by observing the time till the paper shows hydrophobicity. So from this test it is clear that the *Rhizophora mucronata* Lam. wax coated filter paper with solvent Chloroform shows hydrophobicity for about 3 hour 13 minutes.(Plate: 9) The solvent Benzene shows hydrophobicity for about 1.5 minutes.(plate: 10). In case of *Avicennia officinalis* L. the solvent Chloroform shows hydrophobicity for about 3 minutes (plate: 11) and in solvent Benzene it shows hydrophobicity for only 1 minute (plate: 12). (Table: 2)

Table 2 Test for hydrophobicity

Name of solvent	<i>Rhizophora mucronata</i> Lam.	<i>Avicennia officinalis</i> L.
Chloroform	3hour 13minutes	3minutes
Benzene	1.5minute	1 minute

5. HEAT SENSITIVITY TEST

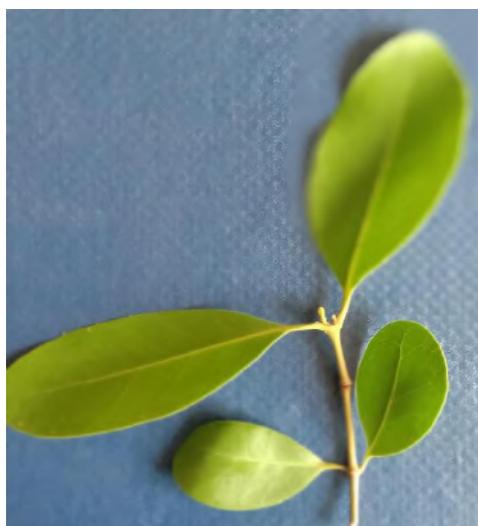
In heat sensitivity test the obtained wax was subjected to heat for 2 minutes. The retention of hydrophobicity and integrity of wax was observed. From this test, *Rhizophora mucronata* Lam. in solvent Chloroform is showing hydrophobicity even after subjected to heat for 2 minutes .In solvent Benzene hydrophobicity is not retained (plate: 13&14)

Similarly in case of *Avicennia officinalis* L. only in solvent Chloroform shows hydrophobicity even after subjected to heat for 2 minutes. In Solvent benzene the hydrophobicity is not retained (plate: 15&16).

PLATE 1
Leaf of *Rhizophora mucronata* Lam.



PLATE 2
Leaf of *Avicennia officinalis* L.

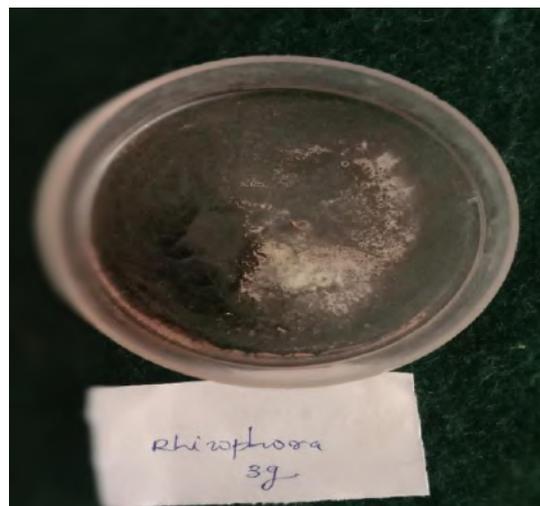


ISOLATION OF WAX FROM LEAF SURFACE

Rhizophora mucronata Lam.

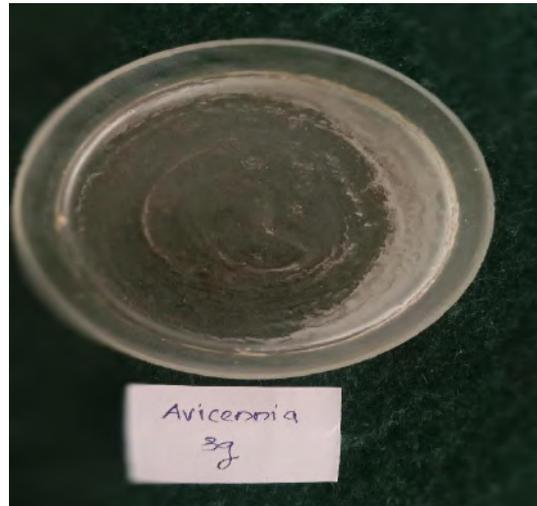


Chloroform PLATE 3



Benzene PLATE 4

Avicennia officinalis L.



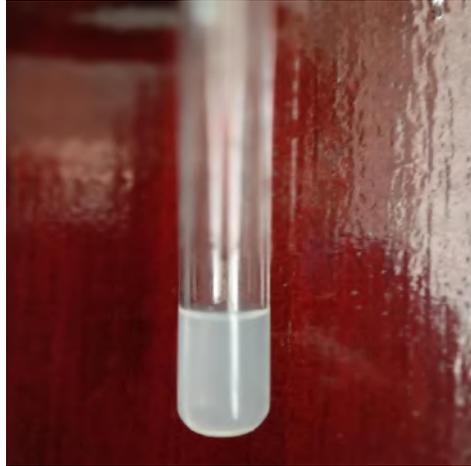
Chloroform PLATE 5



Benzene PLATE 6

WAX CONFIRMATORY TEST

PLATE 7



Rhizophora mucronata Lam. (Chloroform)

PLATE 8



Avicennia officinalis L. (Benzene)

TEST FOR HYDROPHOBICITY

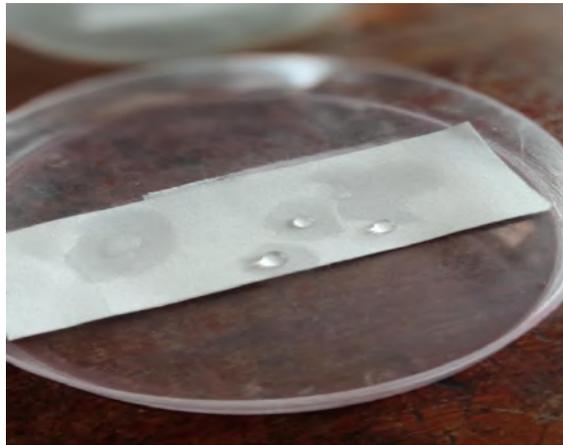
Rhizophora mucronata Lam.

Chloroform PLATE 9

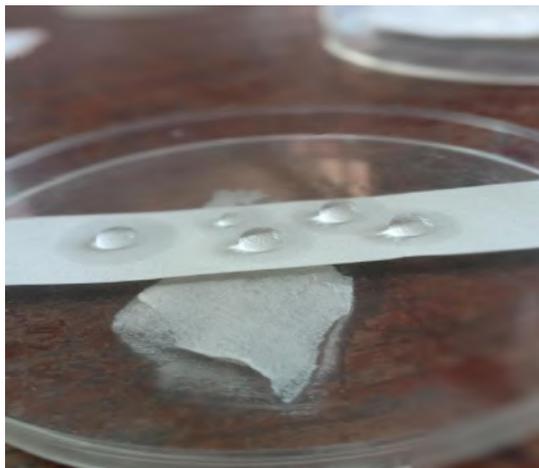


Benzene PLATE 10

Avicennia officinalis L.



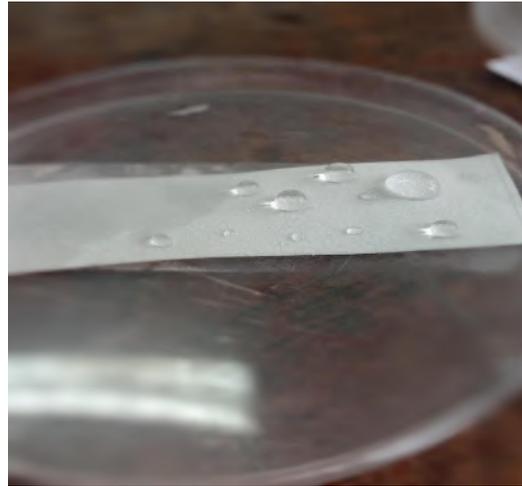
Chloroform PLATE 11



Benzene PLATE 12

HEAT SENSITIVITY TEST

Rhizophora mucronata Lam.



Chloroform PLATE 13



Benzene PLATE 14

Avicennia officinalis L.



Chloroform PLATE 15



Benzene PLATE 16

DISCUSSION

Plant epicuticular wax are mixture of aliphatic compounds. The wax present on the surface on the other surface of plants acts as a protective barrier to environmental stresses. The epicuticular wax from leaves provide the hydrophobic coating on the paper. The hydrophobic nature of paper is due to presence of hydroxyl group.

The present investigation deals with characterization of epicuticular wax from *Rhizophora mucronata* Lam. and *Avicennia officinalis* L. in these two plants *Rhizophora mucronata* Lam. possess a highly hydrophobic layer of bio wax on leaves compared to *Avicennia officinalis* L. These plant bio wax was extracted from these plants leaves by organic solvent extraction method. The solvents such as Chloroform and Benzene was used.

In the quantitative analysis of bio wax it is observed that the amount of wax that is obtained from the leaves of *Rhizophora mucronata* Lam. is in large amount when extracted with solvent Chloroform. The leaves of *Avicennia officinalis* L. also showed a slight hydrophobicity solvent Chloroform. Chloroform is more suitable for wax extraction than Benzene.

The extracted bio wax from both the leaves of *Rhizophora mucronata* Lam. and *Avicennia officinalis* L. shows hydrophobicity. The water resistance was evaluated by observing the time till the paper coated with wax shows hydrophobicity. In the study it is evident that the *Rhizophora mucronata* Lam. wax coated filter paper in solvent Chloroform and Benzene shows hydrophobicity for about 3 hour 13 minutes and 1.5 minutes respectively. On the other hand *Avicennia officinalis* L. Coated filter paper in Chloroform and Benzene shows hydrophobicity for about 3 minutes and 1 minute respectively. In both plants chloroform shows more hydrophobicity compared to Benzene.

The epicuticular wax from both plants were subjected to heat for about 2 minutes and the hydrophobicity of *Rhizophora mucronata* Lam. wax was retained even after treating with heat. This character shows that the bio wax from *Rhizophora mucronata* Lam. Is a suitable substance for coating paper to make them hydrophobic. The above study reveals *Rhizophora mucronata* Lam. Is more suitable for extraction of bio wax than *Avicennia officinalis* L. when treated with Chloroform.

In a study conducted by Yadav *et al.*(2014) the highest hydrophobicity was found to be in paper disc coated with epicuticular wax extracted with Benzene from adaxial surface of *Calotropis procera* R. Br. The hydrophobicity is retained even though it is exposed to high temperatures about 70° c.

The quantitative analysis of *Colocassia esculenta* by Nayan *et al.* (2018) show that 1 gram of sample contained 0.116 gram of wax. It was observed that the paper coated with bio wax attained hydrophobicity which was similar to *Colocassia* leaf.

Only a few researches have been done in this field, still it is restricted to laboratory and not applied in real life applications. There are issues related to its cost and durability. Although the use of other non-biodegradable chemicals are dangerous to humans as well as cause harm to environment and need to find an alternative eco-friendly method

SUMMARY AND CONCLUSION

Plastic is found more useful because of its high durability, light weight, water resistance and several other properties. Researchers have found that the use of plastic is dangerous because of the chemicals present in it. Hydrophobic paper was found as an alternative. They are made from renewable resources, can be recycled and are biodegradable.

The present study was on characterization of epicuticular wax from *Rhizophora mucronata* Lam. and *Avicennia officinalis* L. The studies on these plants revealed that the bio wax leaves of *Rhizophora mucronata* Lam. Have the potent ability to make hydrophobic paper compared to *Avicennia officinalis* L.

From the present study isolation of wax from leaf surface, wax confirmatory test, quantitative analysis of bio wax, test for hydrophobicity and heat sensitivity test were carried out.

Quantitative analysis of bio wax reveals that more amount of wax obtained when we use the solvent Chloroform in both plants than solvent Benzene. Benzene gives comparatively low bio wax than Chloroform in both plants.

Test for hydrophobicity reveals that more time for hydrophobicity has occurred in the case of *Rhizophora mucronata* Lam. wax extracted with Chloroform than *Avicennia officinalis* L. in Chloroform. The solvent Benzene in both plants shows comparatively low hydrophobicity than Chloroform.

Heat sensitivity test reveals that when the solvent Chloroform is used in both the plants, *Rhizophora mucronata* Lam. retains their hydrophobic property even though they were subjected to heat treatment and *Avicennia officinalis* L.

Shows only a slight hydrophobicity after heat treatment. When heat sensitivity test is done on plant wax on Solvent Benzene *Rhizophora mucronata* Lam. Shows a very low hydrophobicity and *Avicennia officinalis* L. does not show hydrophobic property after heat treatment.

From the study it can be concluded that the leaves of *Rhizophora mucronata* Lam. Possess high hydrophobic property than *Avicennia officinalis* L. Therefore the bio wax from *Rhizophora mucronata* Lam. could be used as surface coating for papers which could be used for making them biodegradable, hydrophobic paper bags which can be used instead of plastic bags and normal paper bags, thus reduce a greater extent of environmental degradation

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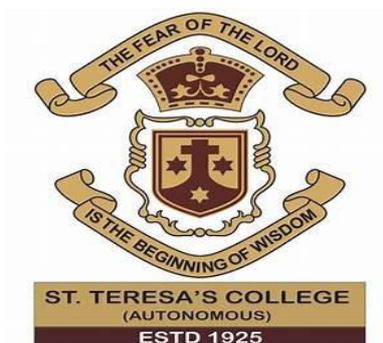
**STUDIES ON THE ANTIBACTERIAL POTENTIAL OF
GRACILARIA CORTICATA (J. Agardh) J. Agardh IN ETHANOL AND
CHLOROFORM EXTRACTS**

**DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE AWARD OF THE DEGREE OF BACHELOR OF SCIENCE
IN
BOTANY**

By

NAME LAKSHMI P S

Reg No: AB19BOT026



**DEPARTMENT OF BOTANY
ST. TERESA'S COLLEGE (AUTONOMOUS)
ERNAKULAM**

2022

CERTIFICATE

This is to certify that the dissertation entitled "Studies on the antibacterial potential of *Gracilaria corticata* (J. Agardh) J. Agardh in ethanol and chloroform extracts" is an authentic record of research work carried out by Miss Lakshmi P.S., AB19BOT026 under the supervision and guidance Smt. Nishitha I. K. Assistant Professor, Department of Botany and Centre for Research, St. Teresa's College (Autonomous), Emakulam, in partial fulfilment of the requirements for the award of the degree of Bachelor of Science in Botany. I further certify that no part of this work embodied in this project has been submitted for the award of any degree or diploma.



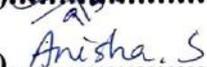
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9/5/22

DECLARATION

I hereby declare that the project entitled “Studies on the antibacterial potential of *Gracilaria corticata* (J. Agardh) J. Agardh in ethanol and chloroform extracts” submitted to Mahatma Gandhi University, Kottayam, in partial fulfilment of the requirement for the Degree of Master of Science in Botany is an original project done by under the supervision and guidance of Ms. Nishitha I.K Department of Botany and Centre for Research, St. Teresa’s college (Autonomous), Ernakulam.

PLACE: ERNAKULAM

NAME : LAKSHMI P S

DATE:

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Above all, I thank God Almighty for his Blessing which Enlightened me throughout the course of the dissertation.

Place: Ernakulam

Name: LAKSHMI P S

Date:

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INTRODUCTION

Algae are diverse group of relatively simple, chlorophyll containing, photo-autotrophic and oxygen evolving aquatic thalloid (without differentiation into True roots, stems, leaves or leaf like organs) organisms. The word “algae” has its origin from Latin, where ‘alga’ means seaweed. The term algae was first used by Carolous Linnaeus in 1753. Most of them are photo-autotrophic but few are mixotrophic and myzotrophic (sucking through special feeding structure) study of algae is known as phycology (GK. Phykos= seaweed; logos= discourse Or study) or algology.

Algae are divided into nine main phylums, they are Phylum Rhodophycophyta, Phylum Xanthophycophyta, Phylum Chrysophycophyta, Phylum Phaeophycophyta, Phylum Bacillariophycophyta, Phylum Euglenophycophyta, Phylum Chlorophycophyta, Phylum Cryptophycophyta, and Phylum Pyrrophyphyta.

(<https://www.slideshare.net/BIYYANISUMAN/algae-suman-81289656>)

The term "seaweed" refers to a variety of marine plants and algae that can be found in the ocean, rivers, lakes, and other bodies of water. Some seaweeds, such as phytoplankton, are small and remain floating in the water column, providing the foundation for most aquatic food chains. Some are massive, such as the giant kelp that grow in dense forests. The large percentage are medium-sized, with red, green, brown, and black colours, and sometimes may wash up on beaches and shorelines. (Guiry, Michael D., 2014)

Marine algae from Indian coasts amounting to 844 species (including forma and varieties) are distributed among 217 genera. They grow in the intertidal, shallow and deep sea areas up to 180 meter depth and also in estuaries, backwaters and lagoons on solid substrates such as rocks, dead corals, pebbles, shells, mangroves and other plant materials (Anatharaman *et al.*, 2007; Sakthivel, 2007).

Mostly seen seaweeds are macro algae. They are of different types according to the colour of pigments present: brown algae (phylum Ochrophyta, class Phaeophyceae), red algae (phylum Rhodophyta, *Gelidium*), and green algae (phylum Chlorophyta, classes Chlorophyceae, Ulvophyceae etc. They differ significantly in many ultrastructural and biochemical functions, including photosynthetic pigments, storage molecules, cell wall composition, presence/absence of flagella, mitosis ultrastructure, linkage between cells, and structure of the chloroplasts, in addition to pigmentation.

Seaweed is high in vitamins, minerals, and fibre, as well as being palatable. The Japanese have a dish, 'sushi' which is nori seaweed wrapped with a mixture of fish, rice, and other ingredients for at least 1,500 years. Anti-inflammatory and anti-microbial compounds can be found in a variety of seaweeds. For thousands of years, their medicinal properties have been used; it was used to cure wounds, burns, and rashes by the ancient Romans. According to anecdotal evidence, the ancient Egyptians may have employed them to cure breast cancer. (McLachlan, J., and C. J. Bird, 1984).

In recent years, focus towards these organisms has increased due to their food and fuel production capability. In fuel industry algae biofuels have emerged as a clean, nature friendly, cost effective solution to other fuels. More recently algae have been identified and developed as renewable fuel sources, and the cultivation of algal biomass for various products is transitioning to commercial-scale systems. Large-scale cultivation of algae merges the fundamental aspects of traditional agricultural farming and aquaculture (Emily M Trentacoste *et al.*, 2014). Algae fuels are categorized into bio-ethanol, biogas, bio-hydrogen, biodiesel and bio-oil. Algae can be used in the preparation of Biodiesel, Bioethanol, Biobutanol and Hydrogen gas (Raja *et al.*, 2013)

They are considered as a potential source of bioactive substances such as proteins, lipids, and polyphenols possessing potent antibacterial, anticancer, antioxidant, antifungal, and antiviral properties (Sundaramurthy *et al.*, 2016). Seaweeds that are medicinal are rich in secondary metabolites which include alkaloids, glycosides, flavonoids, saponins, tannins, steroids, related active metabolites, which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry (Eluvakkal *et al.*, 2010). Recently, their value as a source of novel bioactive substances has grown rapidly and researchers have revealed that marine algal originated compounds exhibit various biological activities (Kim and Wijesekara, 2010; Wijesekara and Kim, 2010; Wijesekara *et al.*, 2010 and Wijesekara *et al.*, 2011). The secondary metabolites of seaweeds such as Isoprenoids (terpenes, carotenoids, steroids), polyketides, phlorotannins, amino-acid derived natural products (alkaloids), and shikimates (flavonoids) have always attracted the interest of biochemists because of their diversity is comparable with those present in the leaves of higher plants (Manilal *et al.*, 2009). Seaweeds were rich in dietary fibre (>50% dry weight), particularly in the soluble form.

RHODOPHYCEAE

Rhodophyta is a phylum of macroalgae that includes the classes Phaeophyceae and Chlorophyta, which are brown and green seaweeds, respectively.

Within Archaeplastida, Rhodophyta, or red algae, is a monophyletic lineage that contains glaucophyte algae, green algae, and terrestrial plants. Bangia-like species have been found in 1.2 billion-year-old strata, indicating that Rhodophyta has a lengthy fossil history. The morphology of red algae ranges from unicellular filamentous to multicellular thalloid forms, with certain species producing economically important products like agar and carrageenan. These species can be found in a variety of marine settings, ranging from the intertidal zone to deep oceans. There are also freshwater (e.g., *Batrachospermum*) and terrestrial lineages. A triphasic life cycle with one haploid and two diploid phases, with the carposporophyte borne on female gametophytes, is one of the Rhodophyta's significant advances.

Freshwater Rhodophyta has 66 species and 27 genera in North America, although these numbers will change as molecular investigations uncover more diversity. Freshwater red algae have a limited size range than marine species, with the majority (80%) of them measuring 1-10 cm in length. Gelatinous filaments, free filaments, and pseudoparenchymatous forms are the most prevalent types. (Yoon, Hwan Su, et al., 2017)

GRACILLARIA

In terms of the number of species, the genus *Gracilaria* is one of the largest genera of red algae. It's also a wide spread genus, with species found in all oceans except the Arctic. Nearly 28 species of *Gracilaria* have been reported from the Indian coast (Sahoo *et al.*, 2001). Because of its size and extensive range, it's suitable for biogeographic investigation. The greatest number of *Gracilaria* species can be found in tropical waters. Large beds of *Gracilaria* usually grow in the eulittoral zone, or just below it in the beginning of the sublittoral, on sandy or muddy sediments that are protected from waves. Sometimes it can be found free-floating in tidal lakes of salt or brackish water. It can adapt to large variations in growing conditions such as freshwater dilution, increase in fertilizer concentration from runoff, and raised temperatures. Large biomasses can grow when there is little competition from other species, and vegetative propagation may be a normal method of reproduction

(McLachlan, J., et al., 1984). In tropical and subtropical oceans, these are frequently red, green, or greenish brown.

Gracilaria are found as branched thalli, terete to flattened, branching sub-dichotomous to irregular. It has holdfast a disc or crust giving rise to one to many erect axes. The thalli are red, olive, green to purple, Spermatangia are seen in pits or shallow depressions. Sporophytes with tetrasporangia are scattered in the outer cortex, cruciately divide (R Iyer, et al., 2004)

Because they have phycocolloids, the major source of agar- (1, 4)-3, 6-anhydro-l-galactose and -(1,3)-d-galactose with low esterification in the cell wall, *Gracilaria* species are essential for industrial and biotechnological uses. Agar and other polysaccharides are found in *G. confervoides*, *G. dura*, *G. chilensi*, and *G. secundata* among the carbohydrates.

The present work was undertaken to study the antimicrobial potential of *Gracilaria corticata*. The objectives of the study are

- Taxonomic description of the algae
- Assessment of antibacterial potential of the common seaweed *Gracilaria corticata* in its dried form extracted in two different solvents; Ethanol and Chloroform.
- Evaluate the difference in antibacterial potential shown by the alga in the two different solvents against Gram positive *Staphylococcus* and Gram negative *E. coli*.
- Estimate the extractive value of the plant, in both ethanolic and chloroform solvents.

LITERATURE REVIEW

In a study conducted by Inci Tuney (2006), antibacterial activity of extracts or components from various algae has been demonstrated in vitro against both gram-positive and gram-negative bacteria. The antibacterial susceptibility test was performed using the agar disc diffusion method, with 6 mm discs impregnated with 20 µl of extracts and placed in infected agar. *Gracilaria* chloroform extract was tested for antibacterial properties against *Staphylococcus aureus* bacterial strains. *Gracilaria* extract showed action in *S. aureus* extract. Ethanol extracts from *G. domigensis* and *G. sjoestedii* showed antibacterial activity against *E. coli* and *S. aureus*. (TÜney, İnci, et al., 2006)

Krishnapriya et al., (2013) conducted an antibacterial activity on the seaweed extracts, carried out by agar disc diffusion assay. The Muller Hinton agar (MHA) medium was used for this study using bacterial pathogens. Among the solvent extracts, methanol extract showed best results for both positive and negative strains. Chloroform extract of *G. verrucosa* gave the highest zone of inhibition measuring 21±1.0 mm. Ethanol extract of *G. acerosa* also showed a zone of inhibition of 12±1.0 mm. Ethanol and chloroform extracts of *G. verrucosa* gave clearly distinct zone of inhibition measuring 8±1.0 and 9±1.0 mm, with respect to control (25±1.0 mm) against *Staphylococcus*. (Varier, Krishnapriya Madhu, et al., 2013)

Saranraj, P. (2013) conducted a study and the methanol extract of *Gracilaria folifera* (5.0mg/ml) showed highest mean zone of inhibition (18±0.4mm) against the Gram positive cocci *Streptococcus pyogenes* followed by *Bacillus subtilis* (17±0.5mm), *Staphylococcus aureus* (17±0.3mm), *Streptococcus epidermis* (16±0.6mm) and *Bacillus cereus* (16±0.2mm). For Gram negative bacterium, the maximum zone of inhibition was recorded in methanol extract of *Gracilaria folifera* against *Klebsiella pneumoniae* (17±0.5mm) followed by *Salmonella typhi* (16±0.6mm), *Pseudomonas aeruginosa* (16±0.5mm), *Escherichia coli* (16±0.3mm). The zone of inhibition obtained from the Hexane extract of seaweed *Gracilaria folifera* against bacterial pathogens was comparatively very less when compared to the other solvent extracts. No zone of inhibition was seen in DMSO control and the positive control Ampicillin showed zone of inhibition ranging from 13±0.8 mm to 20±0.8mm against the test bacterial pathogens. (Saranraj, P., 2013)

The antibacterial properties of eight crude extracts of local *Acanthophora spicifera* obtained by two distinct extraction methods were investigated by Zakaria (2010) using soxhlet extraction and solvent partitioning. By using the Disc diffusion method, these extracts were

evaluated in vitro against 18 bacteria, 3 yeasts, and 6 fungal strains. The results demonstrated that the solvent partitioning extracts of methanol and ethyl acetate had a greater spectrum of action against the tested bacterial strains. *Bacillus cereus* ATCC 10876, *Bacillus licheniformis* ATCC 12759, Methicillin Resistance *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* ATCC 27853, *Yersinia* sp., and *Citrobacter freundii* displayed inhibitory zones against these two extracts. While methanol extracts from Soxhlet extraction and butanol from solvent partitioning had no antibacterial activity against *P. aeruginosa* ATCC 27853, the other six extracts did. (Zakaria, et al., 2010)

In a study done by Ibraheem et al.; 2017; simplex extracts of *Acanthopora* showed potent inhibitory growth activities against three Gram positive bacteria [*Streptococcus agalactiae*, *pyogenes* and *Streptococcus sanguis* of inhibition ranging from [23.1±0.58 to 20.6±0.63 mm] and showed moderate activities with [*Corynebacterium diphtheriae*, *Bacillus subtilis* and *Staphylococcus aureus*] with inhibition zones ranging from [20.1±1.5 to 16.3±2.1 mm].

Also the crude extracts were found to be more active than the positive control Ampicillin, (22.3±1.5 mm), against *Streptococcus agalactiae* which showing inhibition zone. The hydro alcoholic extracts of the selected species were investigated for their antimicrobial activities using Agar well diffusion and Muller Henton against gram positive and gram negative bacteria. (Ibraheem, Ibraheem BM, et al., 2017)

In a study by Nurul Aili Zakaria et al.; (2011), the antimicrobial activities of the hexane extract were evaluated using disc diffusion method against 8 Gram-negative and 10 Gram-positive bacterial strains. Out of all bacterial tested, only a Gram-positive bacterium and a Gram-negative bacterium were susceptible to the extracts. The hexane extract showed antibacterial activity against both Gram-positive bacterium and Gram-negative bacterium (*P. aeruginosa* ATCC 27853). While, chloroform and ethyl acetate extract only showed inhibitory effect on *P. aeruginosa* ATCC 27853 with inhibition zone of 9.0 mm. No inhibitory effect was showed by methanol extract on bacteria tested. (Zakaria, Nurul Aili, et al., 2011)

MATERIALS AND METHODS

SPECIMEN COLLECTION

The specimen was collected by hand picking from Thikkodi beach, Calicut. The collected samples were washed immediately in seawater and then washed with fresh water and transported to the laboratory. It was again washed thoroughly to remove impurities and sand and rinsed with distilled water. The sample was identified taxonomically as *Gracilaria corticata*. Collected sample was taxonomically evaluated using the standard literature.

SAMPLE PREPARATION

For antimicrobial studies, the cleaned samples were then shade dried, cut into small pieces and powdered in a mixer grinder. The organic solvents Chloroform and Ethanol were used for the extraction process due to its higher efficiency using Soxhlet extraction method. 20g of samples were packed in a thimble and placed in the extractor. 200ml of the solvent was added into the flask and heated. The temperature was maintained at 80°C to 85°C throughout the extraction. The soluble active constituents of the extract remained in the flask and the process was repeated until the compounds were completely extracted. The liquid extract was then cooled and concentrated by using an evaporator.

The beaker with dried extract was weighed and noted. DMSO was used to dissolve the extracts from the beaker. Later the weight of the beaker alone was noted. Hence, the actual weight of the dried extract was obtained. Similarly, the weight of dried extract of *Gracilaria*, in ethanol and chloroform was 0.46g and 10mg respectively. From this the extractive value was calculated using the formula

Extractive value (%) = (Weight of dried extract/ Weight of plant material) X 100

PREPARATION OF EXTRACT IN VARIOUS CONCENTRATIONS

From the stock extract, concentrations of 10%, 20%, 40%, 60% (v/v) was made. The stock concentration of *Gracilaria* in ethanol and chloroform was 10mg/ml and 10mg/ml respectively. From the stock the appropriate amounts was pipetted out and made up to the required concentrations using DMSO.

ANTIBACTERIAL ACTIVITY IN GRACILARIA

PREPARATION OF BACTERIAL CULTURE

In the present study, the extracts were evaluated for antimicrobial activity against *Staphylococcus* strain and *E. coli*, a Gram positive and a Gram negative bacteria respectively. 3g of nutrient broth was dissolved in 100ml of distilled water in a conical flask. The broth is sterilized by autoclaving for 15 minutes. Both of the obtained bacterial stains were inoculated in the nutrient broth in laminar air flow and incubated in appropriate conditions for 24hrs.

PREPARATION OF PETRI PLATES

The selected two species of seaweeds were analysed for the antimicrobial activity for gram negative *Escherichia coli* and gram positive *Staphylococcus* by disc diffusion methods. Agar medium was prepared by dissolving 4g agar and 2.6g of nutrient broth in 100ml distilled water. The mixture is sterilized in an autoclave for 15 minutes. Just after sterilization the mixture was poured into petri plates in laminar air flow. The petri plates were allowed to solidify under aseptic conditions.

ANTIMICROBIAL TEST BY DISC DIFFUSION METHOD

Bacteria were inoculated onto the prepared agar petri plates using sterilized cotton swabs. Sterilized 6mm discs were taken from filter paper and autoclaved and is used for the method. The disc was then dipped in different concentrations of stock (10, 20, 40, 60) and placed on the agar plate using sterile forceps. Tetracycline was used as positive control and DMSO was used as negative control. This was done for both extracts of *Gracilaria* against the two strains of bacteria. The petri plates were incubated at 37°C for 24 hours and results were recorded.

OBSERVATION AND RESULTS

The current study aimed at the taxonomic description of the red seaweed *Gracilaria corticata* and the estimation of its extractive value and antimicrobial potential in two solvents, ethanol and chloroform. The antimicrobial potential activity was studied against Gram positive *Staphylococcus* and Gram negative *E. coli*, two non-pathogenic bacteria. The results obtained are described below.

TAXONOMIC DESCRIPTION

Kingdom: Plantae

Phylum: Rhodophyta

Class: Florideophyceae

Order: Gracilariales

Family: Gracilariaceae

Genus: *Gracilaria*

Species: *corticata*

Gracilaria corticata (J. Agardh) J. Agardh

Thallus erect, up to 14cm in length, arising singly from a discoid holdfast. Stipe very short, terete, up to 5mm long, often inconspicuous. Branching frequently, becoming denser in upper parts of the plant; mostly dichotomous, and producing a bushy appearance. Axes compressed, almost cartilaginous; constricted at the base in basal branches. Blades linear, up to 15cm long, up to 4mm wide; apices generally obtuse, acute in finer branches. Blade surface and margins smooth. Fresh specimens purple to green and firm but pliable (Iyer et al, 2004).



Gracilaria corticata (J. Agardh) J. Agardh

EXTRACTIVE VALUE

Extractive values of plant materials are used to evaluate extracts of the sample, in order to get an idea about the nature of chemical constituents present in it. It can also be used to assess quality, purity and detect adulteration of the extract.

In the present study, polar and non-polar solvents were used for eluting the valuable phyto-compounds present in the sample. Extractive values of ethanol and chloroform extracts of *Gracilaria corticata* used in the antibacterial study, are estimated in the table 1 given below;

Table 1: Extractive value of solvents administered for *Gracilaria corticata*

Solvent	Extractive value of the sample (%)
Ethanol	2.3
Chloroform	0.5

The extractive value was greater for the ethanolic extract than for chloroform suggesting that polar solvent was more efficient in extracting the phytochemicals from the algae.

ANTIBACTERIAL ACTIVITY

The extracts of the algae exhibited moderate to mild antibacterial activity against the two microorganisms. The activity observed can be described as being bacteriostatic showing very

mild zones of inhibition. The ethanol extract of the algae showed mild antibacterial activity against both the test organisms. *Gracilaria* shows mild action against gram negative bacteria at all concentrations of ethanol extract used in the current study against both test organisms.

Table 2; Fig. 2

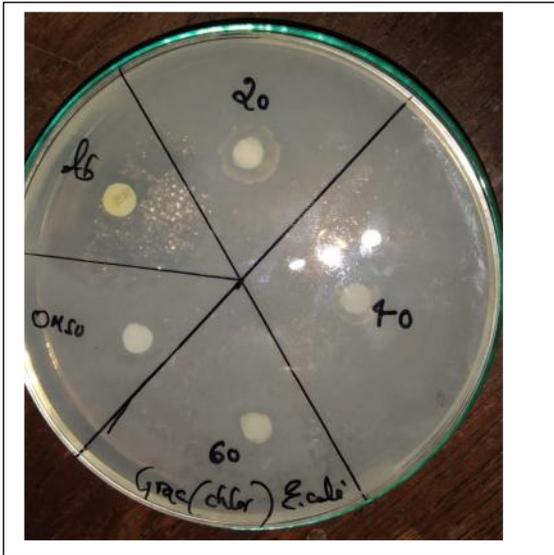
Table 2: Antibacterial activity of ethanolic extract of *Gracilaria corticata* against *E. coli* and *Staphylococcus* bacteria:

Concentration (%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	Mild action	Mild action
40	Mild action	Mild action
60	Mild action	Mild action

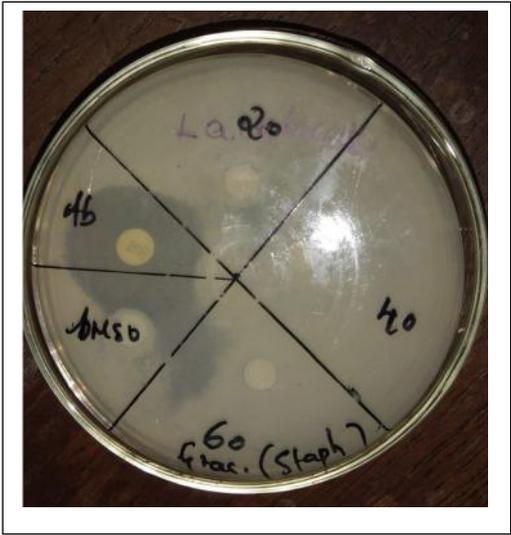
Table 3: Antibacterial activity of chloroform extract of *Gracilaria corticata* against *E. coli* and *Staphylococcus* bacteria

Concentration (%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	No action	No action
40	No action	No action
60	No action	Mild action

The chloroform extract of *Gracilaria* has no significant effect on the bacterial growth. Mild bacteriaostatic activity is observed at higher extract concentrations on *Staphylococcus*. No potential activity could be observed on the growth of *E. coli* in any of the concentrations used for the current study Table 3: Fig. 3



E. coli



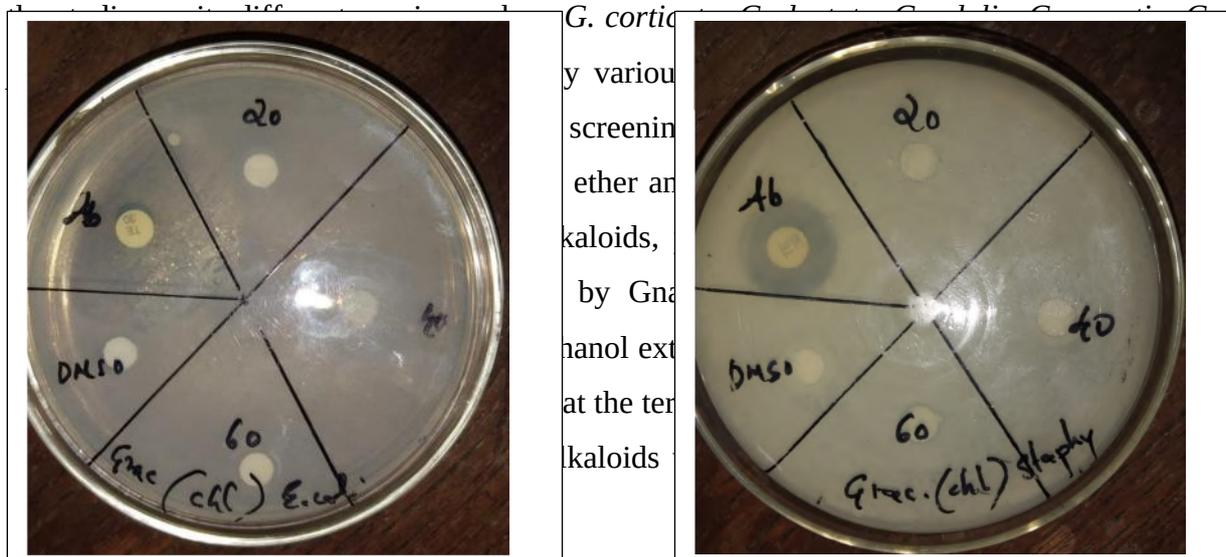
Gram (staph)

Fig 2: Antimicrobial activity of Ethanol extract of Gram (chlor) E. coli and Gram (staph) Staphylococcus aureus

DISCUSSION

Algae have attracted great importance in the recent years due to the large number and amounts of bioactive components in them. More than 600 trace elements are found in high concentration in the seaweeds compared to the terrestrial plants, because of which it has various pharmacological activities. The seaweeds offer greatest wealth in terms of biomass and Rhodophytes show the largest representation among them. The red sea weed *Gracilaria* is amongst the largest group with over 150 species world wide and nearly 28 species in India (Sahoo *et al.*, 2001).

Gracilaria has been identified as a rich source of various bioactive compounds as assessed by



Antibacterial activity refers to the process of killing or inhibiting the disease-causing bacteria. Several plants have been traditionally used for their antibacterial properties. Some algae also exhibit antibacterial properties due to the presence of terpenoids, steroids, saponins,

Fig 3: Antimicrobial activity of Chloroform extract of *Gracilaria corticata* against human pathogens, fungi and yeasts. So, the use of algae as an alternative for prevention and treatment of infectious diseases has been suggested by Abirami and Kowsalya (2012). In the present study ethanolic and chloroform extract were evaluated for activity against Gram positive *Staphylococci* and Gram-negative *E. coli*. It was found that ethanolic extract had bacteriostatic activity against both the bacteria at all concentrations treated and the effect was dose dependent. Sanaraj P., 2013, in his study on *G. edulis* also reported maximum activity against Gram positive bacteria in ethanol extracts. Rashida et al

(2019) also report ethanol extract of *Gracilaria* to have higher antibacterial activity than other solvents.

the preliminary assay ten different organic solvents like Acetone, Butanol, Ethanol, Ethyl acetate, Isoamyl alcohol, Methanol and Propanol (polar) and Benzene, Chloroform and Hexane (non polar) were evaluated. Only the extracts showing antibacterial activity (Ethanol, Chloroform, Isoamyl alcohol, Methanol and Propanol) were considered for further study. In the preliminary assay ten different organic solvents like Acetone, Butanol, Ethanol, Ethyl acetate, Isoamyl alcohol, Methanol and Propanol (polar) and Benzene, Chloroform and Hexane (nonpolar) were evaluated. Only the extracts showing antibacterial activity (Ethanol, Chloroform, Isoamyl alcohol, Methanol and Propanol) were considered for further study. The antibacterial activity of five *Gracilaria* species was determined in both gram positive and gram negative bacteria. In the preliminary assay ten different organic solvents like Acetone, Butanol, Ethanol, Ethyl acetate, Isoamyl alcohol, Methanol and Propanol (polar) and Benzene, Chloroform and Hexane (non polar) were evaluated. Only the extracts showing antibacterial activity (Ethanol, Chloroform, Isoamyl alcohol, Methanol and Propanol) were considered for further study.

In the preliminary assay conducted to evaluate antibacterial activity of *Gracilaria* species against human pathogens by Susanth (2012), ten different organic solvents were considered. This study also reported that the extracts of ethanol and chloroform were the most potent of all.

Johnsi et al (2011), studied the antibacterial activity of aqueous extract of four seaweeds against ten pathogenic bacteria. This study reports the aqueous extract of *Gracilaria corticata* as having the highest potency against the pathogen *Proteus mirabilis*. In the current study however extracts in both solvents show bacteriostatic activity against *E. coli* and *Staphylococci*. Neither extracts are bactericidal and show a mild inhibitory zone of 7 - 8 mm.

Different solvent systems were used to extract bioactive principles from macroalgae with concomitant changes in the antibacterial activities (Thirupurasundari et al., 2008). The solvents such as acetone, benzene, butanol (Vanitha et al., 2003; Prakash et al., 2005), ethanol (Selvi et al., 2001) were used to extract antimicrobial compounds from macroalgae. The aqueous extracts prepared from seven macroalgal samples showed varying degrees of

activity against tested pathogens, including the Gram positive and Gram negative bacteria. (Johnsi et al, 2011). Padmakumar (2002) is of the opinion that these differences are due to the different solubility behaviour of secondary metabolites which could be influenced by seasonal and geographical distribution of the species.

SUMMARY

Algae are an important constituent of the aquatic ecosystems and can be seen in water bodies like oceans, seas, lakes, estuaries and soon. They can be of different types and in different colours. Mostly seen seaweeds are macroalgae. They can be used as food and are a storehouse of bioactive components like vitamins, phenolics, terpenoids and other secondary metabolites. They also possess antibacterial, antioxidant, antifungal properties. Red algae is used for the extraction of agar (*Gracilaria*). It also shows few antibacterial properties.

The present study was done to estimate the difference in extractive yield, and the antimicrobial potential of the dried form of, *Gracilaria corticata* in polar and non-polar solvents, ethanol and chloroform respectively. The whole plant body was taken for the study. The cleaned, dried and powdered sample was extracted using the sohxlet apparatus. Extractive values of plant materials are often used to evaluate extracts of the sample, in order to get an idea about the nature of chemical constituents present in it. It can also be used to assess quality, purity and detect adulteration of the extract. *G. corticata* showed a better elution for polar solvent than non-polar solvent. The extractive yield obtained was more for ethanolic extract (2.3%) as compared to chloroform extract (0.5%).

In the present project, antibacterial potential of *Gracilaria corticata* was tested against two non pathogenic bacteria, the Gram negative *E. coli* and the Gram negative *Staphylococcus* by the disc diffusion method. It is concluded that the organic solvent extraction by ethanol and chloroform was suitable to verify the antimicrobial properties of *Gracilaria corticata* and they were supported by many investigations.

The current investigation showed that *Gracilaria corticata* has antimicrobial potential. The ethanol extract has better antimicrobial activity when compared to chloroform extracts. Ethanol extract was bacteriostatic for both gram negative (*E. coli*) and gram positive (*Staphylococcus*) bacteria at all concentrations studied. Whereas the extract in chloroform showed no significant activity except in the higher concentration (60%) and only against the Gram positive *Staphylococcus*.

The present study justifies the claimed uses of *Gracilaria corticata* in the traditional system of medicine to treat various infectious diseases caused by the microbes. These results suggest the possibility of using marine algae extracts in therapy as natural alternatives to antibiotics currently in the market, and clearly show that seaweeds are a valuable source of biologically active compounds.

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**MORPHOLOGICAL AND ANATOMICAL EVALUATION OF
PTERIDOPHYTES FROM THE BOTANICAL GARDEN OF ST.
TERESA'S COLLEGE OF ERNAKULAM DISTRICT**

**A DISSERTATION SUBMITTED TO
MAHATMA GANDHI UNIVERSITY, KOTTAYAM
IN PARTIAL FULFILLMENT OF REQUIREMENTS FOR
AWARD OF THE DEGREE OF
"BACHELOR OF SCIENCE IN BOTANY"**

BY

SHWETHA U

REG NO: AB19BOT031



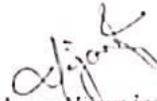
**DEPARTMENT OF BOTANY
ST. TERESA'S COLLEGE (AUTONOMOUS)
ERNAKULAM
MAY - 2022**

CERTIFICATE

This is to certify that this dissertation entitled "MORPHOLOGICAL AND ANATOMICAL EVALUATION OF PTERIDOPHYTES FROM THE BOTANICAL GARDEN OF ST. TERESA'S COLLEGE OF ERNAKULAM DISTRICT" is an authentic record of research work carried out by Miss. Shwetha U AB19BO1031 under the supervision and guidance of Dr. Alphonsa Vijaya Joseph of St. Teresa's College (Autonomous), Ernakulam. I further certify that no part of the work embodied in the project has been submitted for the award of any other degree or diploma.



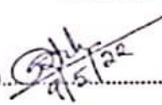
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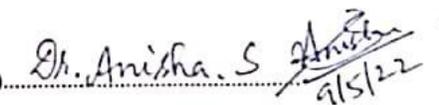


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Place: Ernakulam

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Shwetha U

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**MORPHOLOGICAL AND ANATOMICAL EVALUATION OF
PTERIDOPHYTES FROM THE BOTANICAL GARDEN OF
ST. TERESA'S COLLEGE OF ERNAKULAM DISTRICT**

INTRODUCTION

An ecosystem is a geographic area where plants, animals, and other organisms, as well as weather and landscape, work together to form a bubble of life. Ecosystems contain biotic or living, parts, as well as abiotic factors, or nonliving parts. Biotic factors include plants, animals, and other organisms (Balasubramanian, 2021). Four types of ecosystem Forest Ecosystems, Grassland Ecosystems, Tundra Ecosystems, Desert Ecosystem (Rustad *et al.*, 2001). An ecosystem consists of all the organisms and the physical environment with which they interact. These biotic and abiotic components are linked together through nutrient cycles and energy flows. Energy enters the system through photosynthesis and is incorporated into plant tissue (Odenbaugh, 2010).

R.H. Whittaker gave the Five Kingdom classification for living organisms. He categorized living organisms based on multiple characteristics such as cellular structure, mode of nutrition, body organization, reproduction, phylogenetic relationship, etc. These five kingdoms were Monera, Protista, Fungi, Plantae and Animalia (Whittaker, 1969). Kingdom Plantae includes all the plants. They are eukaryotic, multicellular and autotrophic organisms. The plant cell contains a rigid cell wall. Plants have chloroplast and chlorophyll pigment, which is required for photosynthesis (Hoek *et al.*, 1995). The plant kingdom has been classified into five subgroups they are Thallophyta, Bryophyta, Pteridophyta, Gymnosperms, Angiosperms (Sharma and Pawar, 2020).

A pteridophyte is a vascular plant (with xylem and phloem) that disperses spores. Because pteridophytes produce neither flowers nor seeds, they are sometimes referred to as "cryptogams", meaning that their means of reproduction is hidden. Ferns, horsetails (often treated as ferns), and lycophytes (clubmosses, spikemosses and quillworts) are all pteridophytes. However, they do not form a monophyletic group because ferns (and horsetails) are more closely related to seed plants than to lycophytes. "Pteridophyta" is thus no longer a widely accepted taxon, but the term pteridophyte remains in common parlance, as do

pteridology and pteridologist as a science and its practitioner, respectively. Ferns and lycophytes share a life cycle and are often collectively treated or studied, for example by the International Association of Pteridologists and the Pteridophyte Phylogeny Group (Nepal, 2018).

Pteridophytes commonly known as Vascular Cryptogams, are the seedless vascular plants that evolved after bryophytes. Besides being a lower plant, pteridophytes are economically very important. Dry fronds of many ferns are used as a cattle feed. Pteridophytes are also used as a medicine (Dudani *et al.*, 2011). The decoction of foliage of *Lycopodium* is used in homeopathy to treat diarrhoea, bladder irritability, eczema, rheumatism, constipation and inflammation of liver. The Flavonoids and saponins present in *Equisetum* have diuretic affect. The fern, *Dryopteris* yield an anti-helminthic drug. Sporocarps of Marsilea are rich source of starch and eaten for their nutritive value as food. *Osmunda cinnamomea* is used externally for rheumatism and internally for joint pain. The chemically active principal 'Marsiline' isolated from *Marsilea* is found to be very effective against sedative and anti-convulsant principal (Al-Achi ,2020).

Selaginella species are creeping or ascendant plants with simple, scale-like leaves (microphylls) on branching stems from which roots also arise. The stems are aerial, horizontally creeping on the substratum (as in *Selaginella kraussiana*), sub-erect (*Selaginella trachyphylla*) or erect (as in *Selaginella erythropus*). The Selaginellaceae are mostly distributed in tropical and warm regions, worldwide. Economic importance includes cultivated ornamentals and local medicinal plants. See Jermy (1990) for general information and Korall and Kenrick (2002, 2004) for phylogenetic analyses of the family (Setyawan, 2011). *Selaginella* is the sole genus of vascular plants in the family Selaginellaceae, the spikemosses or lesser clubmosses.

Lygodium is a genus of about 40 species of ferns, native to tropical regions across the world, with a few temperate species in eastern Asia and eastern North America. It is the sole genus in the family Lygodiaceae in the Pteridophyte Phylogeny Group classification of 2016. (Pemberton, 1998). *Lygodium* is widely used in treating various ailments like jaundice, dysmenorrhoea, wound healing and eczema. It is the rich source of alkaloids, flavonoids, saponins and cumarin. *Lygodium japonicum* is recorded as having medicinal value in its native range. CABI (2017) gives the following details. In China, it is used as a diuretic (Puri, 1970)

and to treat colds, inflammation, kidney stones and renal ailments (Eisenberg *et al.*, 2009), (Yadav *et al.*, 2012).

Equisetum is the only living genus in Equisetaceae, a family of ferns, which reproduce by spores rather than seeds. *Equisetum* is a "living fossil", the only living genus of the entire subclass Equisetidae, which for over 100 million years was much more diverse and dominated the understory of late Paleozoic forests (Christenhusz *et al.*, 2017). Horsetail (*Equisetum arvense*) is an herbal remedy that dates back to ancient Roman and Greek times. It was used traditionally to stop bleeding, heal ulcers and wounds, and treat tuberculosis and kidney problems. The name *Equisetum* is derived from the Latin roots equus, meaning "horse," and seta, meaning "bristle" (Parkash and Dhungana, 2011).

Angiopteris is a genus of huge evergreen ferns from the family Marattiaceae, found throughout the paleotropics from Madagascar to the South Pacific islands (Heim, 2015). Species of smaller stature with elongate synangia and creeping rhizomes are sometimes segregated into the genus, and a once-pinnate monotypic segregate genus has been called *Macroglossum*, but molecular data supports inclusion of these taxa within a broad concept of *Angiopteris*. Many traditional medicinal uses are known: a decoction of the rhizome has been used to arrest the discharge of blood after a miscarriage and rhizome boiled with green beans to treat beriberi. In Siberut (Indonesia) a decoction of the leaves of *A. evecta* and *Diplazium esculentum* (Retz.) (De Winter and Amoroso, 2003).

Microsorium is a genus of ferns in the family Polypodiaceae, subfamily Microsoroideae, according to the Pteridophyte Phylogeny Group classification of 2016. The species are tropical. Like most ferns, they grow from rhizomes, rather than roots. The genus name is often misspelled "*Microsorium*" or "*Microsoreum*" (Wei *et al.*, 2017). *Microsorium scolopendria* is an important ornamental and medicinal fern. The fronds arise from black-scaly stems (rhizomes) that creep along the soil surface or grow on other plants as epiphyte. The plant paste provides a significant protection against phytophagous insects (Mehrnejad, 2001).

Nephrolepis is Sword Fern. Family: Nephrolepidaceae (Formerly: Davalliaceae)
Nephrolepis is a genus of about 30 species of ferns in the family Davalliaceae (included in

Lomariopsidaceae in some classifications). *Nephrolepis* species are frost tender but are easily grown in mild areas in a position in full shade or partshade with moist humus-rich soil and steady high humidity. They are widely cultivated as indoor plants. In cooler climates, if they are grown in heated indoor situations, ensure they receive plenty of bright filtered light and ample water. They can be propagated by division or from spores.

Pteris is a genus of about 300 species of ferns in the subfamily Pteridoideae of the family Pteridaceae. They are native to tropical and subtropical regions of the world. Many of them have linear frond segments, and some have sub-palmate division (Ara, 2015). Some of these ferns are popular in cultivation as houseplants. These smaller species are often called "table ferns". *Pteris vittata* (commonly known as brake fern) was discovered to have the ability to "hyperaccumulate" (absorb large amounts of) arsenic from soil. The fern was growing at a central Florida site contaminated with large amounts of copper arsenate in the soil. Dr. Lena Q. Ma of the University of Florida later discovered that it had hyperaccumulated considerable amounts of arsenic from the soil. The discovery may lead to the use of *Pteris vittata* as a potential bioremediation plant (Chen *et al.*, 2006).

Marsilea is herbaceous plant. It is also known as a water clover plant and four leaf clover plant. It does not resemble common ferns. They either present above water or submerged. *Marsilea* is a genus of approximately 65 species of aquatic ferns of the family Marsileaceae. The name honours Italian naturalist Luigi Ferdinando Marsili. These small plants are of unusual appearance and do not resemble common ferns. Distribution of the Marsileaceae is subcosmopolitan. Economic importance of family members includes use of *Marsilea* species as food (sporocarps or leaves) and cultivated ornamentals (especially *Marsilea* spp. & *Regnellidium diphyllum*) (Sharma, P).

Adiantum the maidenhair fern, is a genus of about 250 species of ferns in the subfamily Vittarioideae of the family Pteridaceae, though some researchers place it in its own family, Adiantaceae. The genus name comes from Greek, meaning "not wetting", referring to the fronds' ability to shed water without becoming wet. In Europe, the use of *Adiantum* as a drug has been well known since antiquity. It is used to treat various illnesses of the respiratory tract and is taken in the form of tea (infusion) or syrup (extract) (Rastogi *et al.*, 2018).

Ernakulam district is in the state of Kerala, which is known as God's own country. The flora of the Ernakulam district is tropical in nature. The heavy rain fall combined with moderate temperature and fertile soil supports luxuriant vegetation. Many of the common plants are found in the coastal area which forms the low land region. The Mangalavanam bird sanctuary in Ernakulam district is the "green lung of Kochi", considering its role in controlling the city's air pollution. St Teresa's College is a prestigious autonomous institution situated in the centre of Ernakulam district. The campus botanical garden possesses a vast range of flora. So, the study is concerned with Morphological and Anatomical evaluation of pteridophytes from the Botanical garden of St. Teresa's College of Ernakulam district.

OBJECTIVES OF THE STUDY

- Survey of Pteridophytes in the Botanical Garden of St. Teresa's College, Ernakulam.
- Collection of fresh samples of Pteridophytes from the study area.
- Morphological evaluation of collected Pteridophytes.
- Anatomical evaluation of collected Pteridophytes.

REVIEW OF LITERATURE

Pteridophytes (ferns and lycophytes) are free-sporing vascular plants that have a life cycle with alternating, free-living gametophyte and sporophyte phases that are independent at maturity. The body of the sporophyte is well differentiated into roots, stem and leaves. The root system is always adventitious.

The earliest record on the ferns and fern allies of Western Ghats is perhaps the one by Van Rheedee (1703) in his classical work *Hortus Malabaricus* in which 20 ferns and fern allies were illustrated and described. The first survey of South Indian Ferns was made by Col. R.H. Beddome (1873) who gave a brief description and illustration of these plants, After Beddome's work, emphasis was given to intensive exploration of other areas.

The most authentic and foremost attempt to record the endemic Pteridophytes of India was made by Chandra (1982) and he reported 96 endemic species to India. Later, Chandra and Kaur (1984) added 41 species to the previous list. Dhir and Saiki (1984) listed 58 species of ferns as endemic to the Himalaya, whereas Dixit (1984) listed 214 species of Pteridophytes endemic to India. Further information on the distribution of endemic Pteridophytes was provided by Bir (1987 and 1989). After that a phytogeographic analysis of the endemic species was done by Dixit and Bal Krishna (1990). Manickam and Irudayaraj (1992) in the Pteridophytic Flora of the Western Ghats enumerated 16 endemics from South India, whereas 17 endemic species were reported from South India by Nayar and Geevarghese (1988, 1993).

The another more significant and valuable contribution has been made recently by Fraser-Jenkins (2008). He has removed many of the earlier endemic species of Pteridophytes as pseudo-endemics which arose mainly due to mistaken synonyms or the lack of understanding of the range of species. He has listed only 47 species of Indian Pteridophytes as endemics. It is observed that there is a vast difference among the observations of Pteridologist regarding the endemic status of the Indian Pteridophytes. One of the reasons

for this is that the taxonomy and nomenclature of Pteridophytes is much more confusing than those of any other group of plants.

According to Moran (2008), there are 13,600 species of ferns globally, and of this, approximately 1200 species with 70 families and 192 genera are seen in India (Dixit, 1984;2000;Sukumaran *et al.*, 2009;Dudani *et al.*, 2011; Patil *et al.*,2013;Kavitha *et al.*, 2017; Patil *et al.*, 2016). In the year 1883, Beddome published the most authentic work on Indian pteridophytes known as Beddome's "Handbook of the ferns of British India, Ceylon, and the Malay Peninsula".

The upper elevations (2300-4200m) of a national park located on the eastern slopes of the northern Peruvian Andes were surveyed for their pteridophyte flora. A total of 174 species in 43 genera were found. The most diverse ecological zone was the montane rain forest zone located from 3100m upwards to timberline, which contained 109 pteridophyte species. This was high species richness compared to adjacent ecological zones: tropical alpine, 65 species; montane wet forest, 61 species. We suggest that reasons for this were a high and constant humidity, the abundance of speciose genera such as *Elaphoglossum*, and the sharing of species with both of the adjacent zones. (Youn, 1990).

The Bauer and other scientists have discovered the bacterial strains of *Agrobacterium tumefaciens*, *Escherichia coli*, *Salmonella arizonae*, *Salmonella typhi* and *Staphylococcus aureus* were procured from the Institute of Microbial Technology (IMTECH), Chandigarh and the aqueous and alcoholic leaves extract of twelve important pteridophytic plants were prepared and tested for their antimicrobial activity against the bacteria selected by Disc diffusion method as suggested. It has been observed that, nearly all the leaves extracts have shown inhibitory effect against the bacterial strains selected and some of the extracts were more competent than the selected antibiotic. Our findings provide the novel insights with regards to antimicrobial agents and these could be further enhanced through *in vivo* studies and isolation and characterization of active constituents for human health (Bauer *et al.*,1966).

MATERIALS AND METHODS

- **Study area:**

The Botanical Garden of St. Teresa's College has been selected as the area for study.

- **Survey of plant samples from the study area:**

Cryptogams were selected as the plant samples for the study. A detailed survey has been conducted for the collection of plant samples. 9 species of cryptogams were found in the study area.

- **Collection of plant samples:**

The fresh plant samples such as Selaginella, Equisetum, Angiopteris, Microsorium, Lygodium, Nephrolepis, Pteris, Adiantum and Marsilea were collected for the study from the study area.

- **Morphological evaluation of collected plant samples:**

Fresh plant samples were collected from the study area and morphological characters were analyzed and recorded.

- **Anatomical evaluation of collected plant samples:**

For examining anatomical characteristics, a thin cross-section of the stem or petiole is taken by using a clean and sharp blade. Then the cross-section was mounted with safranin stain and glycerine on a clean glass slide and observed under a compound microscope. The anatomical features were recorded and photographs were taken

**OBSERVATIONS
AND
RESULTS**

1. *SELAGINELLA*

Selaginella is a fascinating plant that spreads and acts like a moss. It is also called Spike moss or club moss. It is the largest and only living genus of the family Selaginellaceae. They were found in a shady area.



PLATE 1: HABITAT OF *SELAGINELLA*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Lycopodiopsida

Order : Selaginellales

Family : Selaginellaceae

Genus : *Selaginella*

- **MORPHOLOGICAL EVALUATION OF *SELAGINELLA***

SPOROPHYTE: The sporophyte is an evergreen, delicate herb. Plants are found to be erect. The plant body consists of Root, Stem, Leaves, Ligules and Rhizophores

ROOT: The root of the young sporophyte is the primary root while others are adventitious. Aerial roots have developed caps, and cutinized epidermal cells enter the soil. Their origin is endogenous. They originate either from the tips of rhizophores or directly from the stem or from the swollen base of the hypocotyl.

STEM: The stem is profusely branched, delicate and evergreen. The branching is of monopodial type.

LEAVES: Leaves are small, simple and lanceolate with a pointed apex. Each leaf is provided with a single unbranched midrib. Leaves near the apical portion of the branch, bear sporangia (micro-or mega) and are called sporophylls (micro-or mega) respectively. The sporophylls are usually aggregated into a condensed structure which is known as a strobilus. Small leaves are present on the dorsal side of the stem and bigger ones on the ventral side of the stem. Microphylls are present, Anisophyllous and Isophyllous based on the unequal and equal size of leaves.

LIGULES: The Ligules are found on the adaxial side of the leaf and are a small membranous out-growth present at the base of the leaf in a pit-like structure known as a ligule pit. The structure of the ligule consists of two parts, glossopodium and the body of the ligule

RHIZOPHORES: It is a colourless, leafless, unbranched and cylindrical structure. A tuft of adventitious roots gets developed when it touches the soil.

- **ANATOMICAL EVALUATION OF *SELAGINELLA* STEM**

A Transverse - section of the Selaginella stem is circular in outline. It includes Epidermis, Cortex and Stele

EPIDERMIS: It is the outermost covering layer comprising of a single cell in thickness. The epidermal cells are covered by a thick coating of cuticle. Hairs and stomata are absent.

CORTEX: it is seen inner to the epidermis. The cortex is differentiated into the inner and outer cortex. it is made up of parenchymatous cells. The parenchymatous cortex is made up of angular cells without intercellular spaces

STELE: The central portion of the stem is occupied by a well-developed stele. The stele is of protostelic type i.e., the xylem is present in the center and surrounded by phloem on all sides. Phloem, in turn, is surrounded by a single-layered pericycle. Pith is absent. The stele is surrounded by a single - layered pericycle made of parenchymatous cells.



PLATE 2: T.S OF *SELAGINELLA* STEM

2. *LYGODIUM*

It is an evergreen fern with climbing fronds. They are epiphytic and perennial in nature. It grows thick into mats which smother the undergrowth and up over shrubby trees. It is found growing in moist and shady places which are rich in humus and other organic matters,



PLATE 3: HABITAT OF *LYGODIUM*

● **TAXONOMYIC POSITION**

Kingdom : Plantae

Class : Polypodiopsida

Order : Schizaeles

Family : Lygodiaceae

Genus : Lygodium

- **MORPHOLOGICAL EVALUATION OF *LYGODIUM***

SPOROPHYTE: The plant body consists of leaves, stem and creeping rhizome

STEM: Dichotomously branched, Indeterminate rachis growth

LEAVES: Pinnately compound leaves

- **ANATOMICAL EVALUATION OF *LYGODIUM* STEM**

A transverse section (T.S.) of the stem of *Lygodium* is circular in outline.

EPIDERMIS: Single-layered and broader Parenchymatous cells

CORTEX: The cortex is differentiated into outer and inner sclerenchymatous cells and middle parenchymatous cells. The whole of the cortex is made up of parenchymatous cells with small or large intercellular spaces and the sclerenchymatous cells, without intercellular spaces.

STELE: Protostelic type. Stele comprises primary xylem and primary phloem. pith is absent and the stele is situated in the centre.

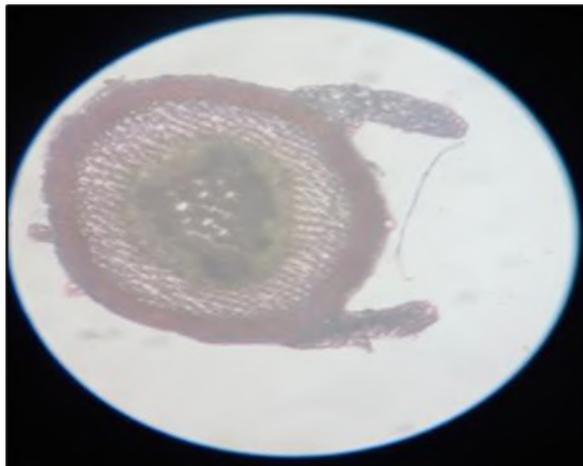


PLATE 4: T.S OF *LYGODIUM* STEM

3. *EQUISETUM*

Equisetum is a herbaceous plant. It is also known as field horsetail or common horsetail.



PLATE 5: HABITAT OF *EQUISETUM*

● **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Equisetales

Family : Equisetaceae

Genus : *Equisetum*

- **MORPHOLOGICAL EVALUATION OF *EQUISETUM***

SPOROPHYTE: The plant body is sporophyte and erect. The sporophyte is differentiated into root, stem and leaves.

STEM: Stem consists of underground rhizome and upright green branches. Jointed stem with nodes and internodes with longitudinal ridges and furrows.

LEAVES: Silica deposits in stem make it rough. Leaves nodes with small, sessile microphyllous scale leaves in whorls. Fertile branches bear strobili after some vegetative growth.

ROOTS: Adventitious roots arise from nodes of rhizome. Roots are photosynthetic in nature.

SPORES: Equisetum is homosporous and Eusporangiate. Strobili are borne terminally and singly on aerial fertile branches. Strobilus consists of a central axis on which stalked sporangiophores with sporangium are arranged in whorls.

- **ANATOMICAL EVALUATION OF *EQUISETUM* STEM**

A transverse - section of the *Equisetum* stem is wavy in outline because of the presence of ridges and grooves. It includes Epidermis, Cortex and Stele

EPIDERMIS: The epidermis is single-layered with stomata and heavily coated with silica deposits.

CORTEX: Outer cortex is sclerenchymatous and chlorenchymatous. Inner cortex is made up of large parenchymatous cells with vallecular canals. Vallecular canals are large air-filled, intercellular spaces below the furrows in the inner cortex. It shows a hydrophytic character. Endodermis and pericycle single layered.

STELE: Xylem is V-shaped. Protoxylem is endarch lying opposite to carinal cavity. Two strands of metaxylem are present. Phloem is present between two strands of metaxylem and made up of phloem parenchyma and sieve tubes. Pith is present in the form of pith cavity, located in the centre of the aerial shoot. A water-containing cavity is present in each vascular bundle known as a carinal canal. The Carinal canal is a water-filled region present in the vascular bundle.

PITH: Pith is large central cavity filled with water.

XEROPHYTIC CHARACTERS:

1. Presence of ridges and furrows.
2. Presence of sunken stomata.
3. Presence of well-developed sclerenchymatous hypodermis.
4. Presence of reduced and scaly leaves.
5. Presence of well-developed vascular cylinder.

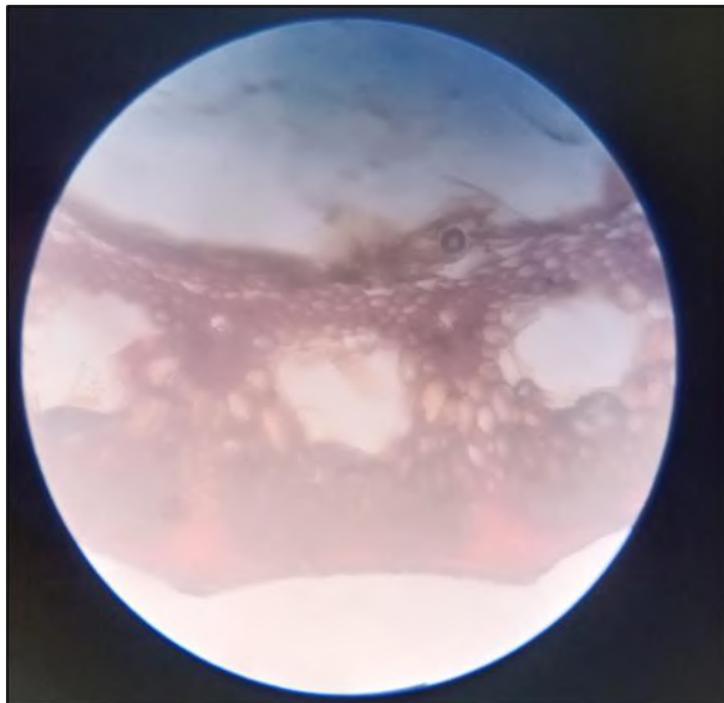


PLATE 6: T.S OF *EQUISETUM* STEM

4. *ANGIOPTERIS*

Angiopteris is an evergreen fern plant. They are unique among ferns in having explosively dispersed spores.



PLATE 7: HABITAT OF *ANGIOPTERIS*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Marattiales

Family : Marattiaceae

Genus : *Angiopteris*

- **MORPHOLOGICAL EVALUATION OF *ANGIOPTERIS***

SPOROPHYTE: The sporophytic plant body consists of an upright, tuberous, conical, fleshy rhizomatous stem.

STEM: The stem is thick and inhabits the plant resembles a tree fern. The stem is often called a caudex or trunk which may be a foot or two in height and almost the same girth.

RHIZOME: The upper surface of the rhizome bears a crown of graceful, stately leaves.

LEAVES: Leaves are deciduous. The leaves are 5-6 metres long in a luxuriously growing plant, with a petiole as thick as a man's arm. The leaves are typically bi-pinnately compound. The venation is of the open dichotomous type. Pinnae are glabrous (smooth). A pair of thick fleshy stipules at the base of the leaf is present. When the leaves fall off, these persist with the leaf bases and form a protective armour around the stem. The base of the petiole and the stipules together appear like a horse's hoof. The pinnae are long, dorsoventrally flattened and have a long drawn tip. The margin is serrate. The sori occupy a near-terminal position on the dichotomously branched veins.

ROOTS: Roots are produced from the undersurface of the rhizome at the base of each leaf. Root hairs are peculiar in being multicellular. At the margins of the pinnae on the abaxial surface are borne, the sori. Roots are perennial, thick and have a mycorrhizal association.

- **ANATOMICAL EVALUATION OF *ANGIOPTERIS* PETIOLE**

Angiopteris is an evergreen fern plant. They are unique among ferns in having explosively dispersed spores

EPIDERMIS: single-layered epidermis which consists of thin-walled cells.

CORTEX: The bulk of petiole is composed of ground tissue and is differentiated into three zones. The outermost zones consist of 3-4 layers of cells which are made up of thin-walled parenchymatous cells. The middle zone consists of 3-4 layers of cells made up of thick-walled sclerenchymatous cells, being comparatively smaller in size than the cells of the outer and inner

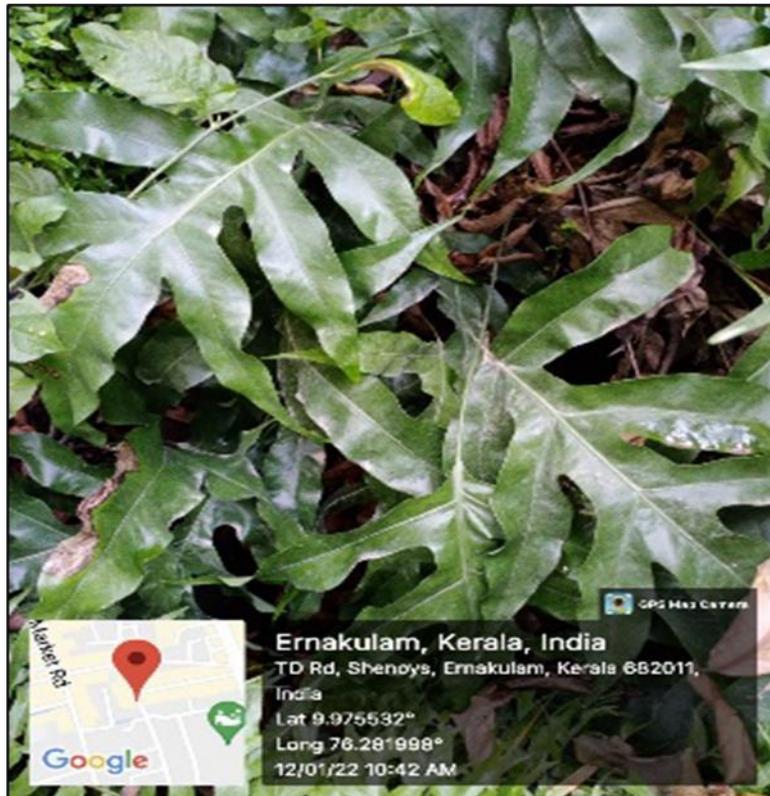
zone. Some of the cells of the middle and inner zone contain tannin. The endodermis is followed by a pericycle containing thin-walled cells, which are 1-3 layers in thickness. Xylem lies in the centre of the vascular strand. It is plate-like with several protoxylem points in exarch conditions. Xylem is surrounded by phloem. Phloem consists of sieve cells and parenchyma and xylem have simple tracheids of various sizes. Metaxylem tracheids have scalariform and pitted thickening while protoxylem tracheids have annular and spiral thickening.

STARCH GRAINS: The inner zone consists of large and thin-walled polygonal cells filled with starch grains. Starch grains are usually large and spherical or oval in shape. The concentrations of these grains are more towards the base of the petiole and gradually decrease towards the apex.



PLATE 8: C.S OF ANGIOPTERIS PETIOLE

5. *MICROSORUM*



Microsorium is an epiphyte and perennial in nature. They grow from rhizomes, rather than roots.

PLATE 9: HABITAT OF *MICROSORUM*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Polypodiaceae

Genus : Microsorium

- **MORPHOLOGICAL EVALUATION OF *MICROSORUM***

SPOROPHYTE: The plant body consists of hard stem and long petiole.

LEAVES: Lanceolate shape, pointed tip, the winged base of leaf, midrib is raised and highly prominent.

RHIZOME: The underground horizontal stem is known as the rhizome

SPORES: occur on the frond underside in small and dark brown spots

- **ANATOMICAL EVALUATION OF *MICROSORUM* PETIOLE**

EPIDERMIS: single-layered and parenchymatous

CORTEX: made up of parenchymatous cells

STELE: vascular bundles are present. Endodermis is present

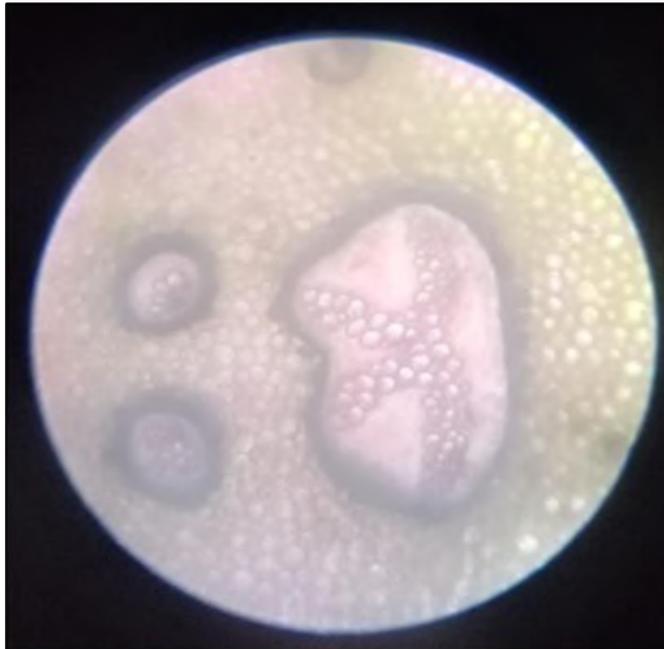


PLATE 10: T.S OF *MICROSORUM* PETIOLE

6. *NEPHROLEPIS*

It is an epiphytic fern. Feather-like fronds in shades of green make these ferns valuable.

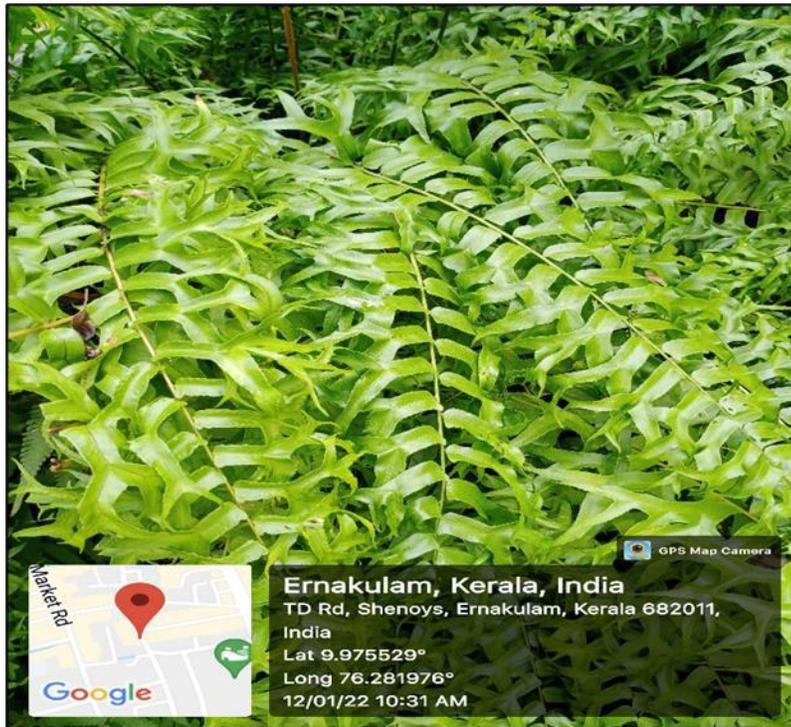


PLATE 11: HABITAT OF *NEPHROLEPIS*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Nephrolepidaceae

Genus : Nephrolepis

- **MORPHOLOGICAL EVALUATION OF *NEPHROLEPIS***

SPOROPHYTE: The plant body is sporophytic differentiated into rhizomes, roots and leaves.

RHIZOME: Rhizome is short, slender and wide creeping. It bears a close tuft of leaves and long, slender lateral branches called runners. Runners bear adventitious roots and in acropetal succession.

LEAVES: The leaves are tufted, long, narrow and simply with fish tail. Margins are present.

- **ANATOMICAL EVALUATION OF *NEPHROLEPIS* STEM**

The transverse section of the petiole has an adaxial groove.

EPIDERMIS: It is composed of small thick walled cells.

CORTEX: Epidermis is followed by 3-4 layers of sclerenchymatous cortex and compact parenchyma. Conducting strands are embedded in the parenchymatous cortex and are arranged in a horse - shoe like shape.

STELE: Protoxylem lies towards outside. Phloem completely surrounds xylem which in turn is surrounded by a layer of pericycle and endodermis.



PLATE 12: T.S OF *NEPHROLEPIS* STEM

7. *PTERIS*

They inhabit shady and moist areas.

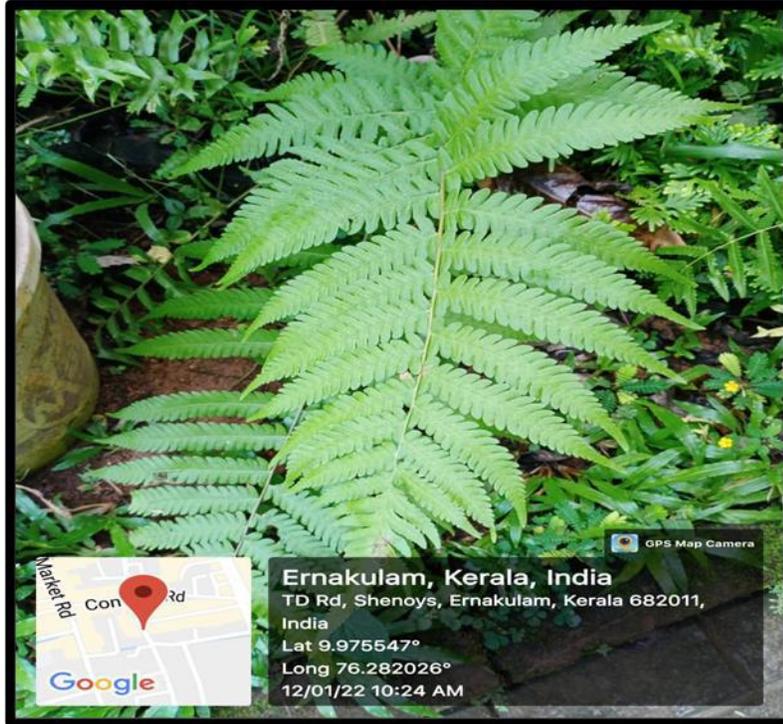


PLATE 13: HABITAT OF *PTERIS*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Pteridaceae

Genus : *Pteris*

- **MORPHOLOGICAL EVALUATION OF *PTERIS***

SPOROPHYTE: The sporophytic plant body is differentiated into Root, Rhizomatous stem and Leaves

ROOT: The roots are small and branched.

RHIZOME: The rhizome is differentiated into nodes and inter-nodes and its entire surface is covered with scales. The growing point of rhizome is covered with ramanta.

LEAVES: The leaves are borne on the upper surface of the rhizome. The young leaves are spirally coiled and show circinate vernation. The leaves are multipinnately compound with long rachis. The pinnae are small near the base as well as towards the apex, while they are large towards the middle.

- **ANATOMICAL EVALUATION OF *PTERIS* PETIOLE:**

EPIDERMIS: single-layered and covered with cuticle. Ramanta arise from the epidermis

CORTEX: Differentiated into outer sclerenchymatous and inner parenchymatous zone

STELE: Xylem has two adaxial hooks; xylem is surrounded by phloem. Pericycle and endodermis is present



PLATE 14: T.S OF *PTERIS* PETIOLE

8. *MARSILEA*

Marsilea is herbaceous plant. It is also known as a water clover plant and four-leaf clover plant. It does not resemble common ferns. They either present above water or submerged

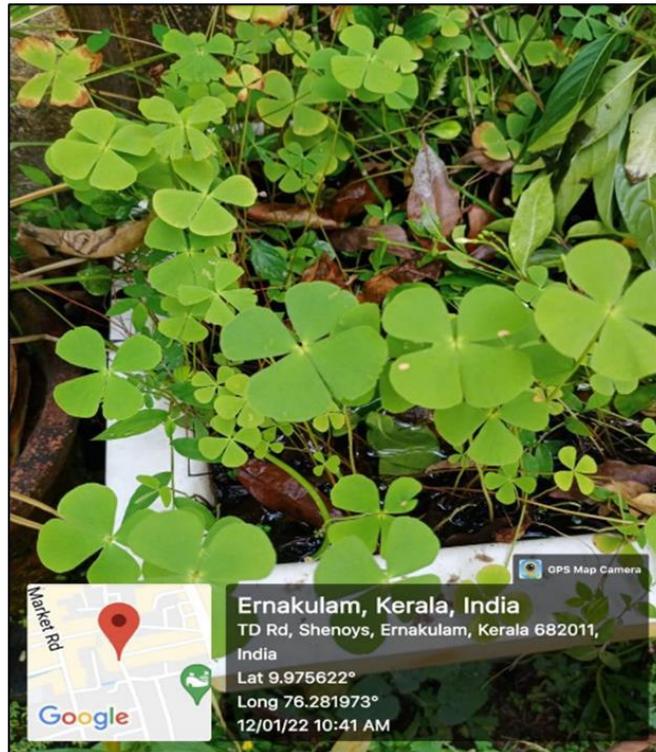


PLATE 15: HABITAT OF *MARSILEA*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Salviniiales

Family : Marsileaceae

Genus : *Marsilea*

- **MORPHOLOGICAL EVALUATION OF *MARSILEA***

SPOROPHYTE: The plant body is differentiated into rhizomes, leaves and roots.

RHIZOME: It is slender, dichotomously branched with distinct nodes and internodes and is capable of indefinite growth in all directions.

LEAVES: They are borne alternately on the upper side of the rhizome at nodes, in two rows. They show circinate vernation. In submerged plants the petiole is long and the lamina floats over the surface of the water but in muddy or marshy plants the petiole of the leaf is short and rigid with short lamina spreading in the air. The lamina consists of 4 leaflets/pinnae which are present at the apex of the petiole. Near the base of the petiole, the stalked bean-shaped sporocarps are borne.

ROOTS: The roots are adventitious, arising from the underside of the node of the rhizome, either singly or in groups.

- **ANATOMICAL EVALUATION OF *MARSILEA* PETIOLE**

EPIDERMIS: The outermost layer is the epidermis which consists of rectangular cells. Below the epidermis is the hypodermis followed by the cortex.

CORTEX: The cortex is differentiated into the outer and inner cortex. The outer cortex consists of aerenchyma having many air cavities or air chambers separated from each other with the help

of one-celled thick trabeculae or septa. The inner cortex is parenchymatous and contains starch and tannin-filled cells.

STELE; The stele is triangular in shape, the present shows the protostelic structure. The stele is bounded by a layer of endodermis and an unilayered pericycle. Xylem is V-shaped and the arms of 'V' contain a metaxylem in the center and protoxylem towards the ends. The xylem remains surrounded by the phloem in the center.

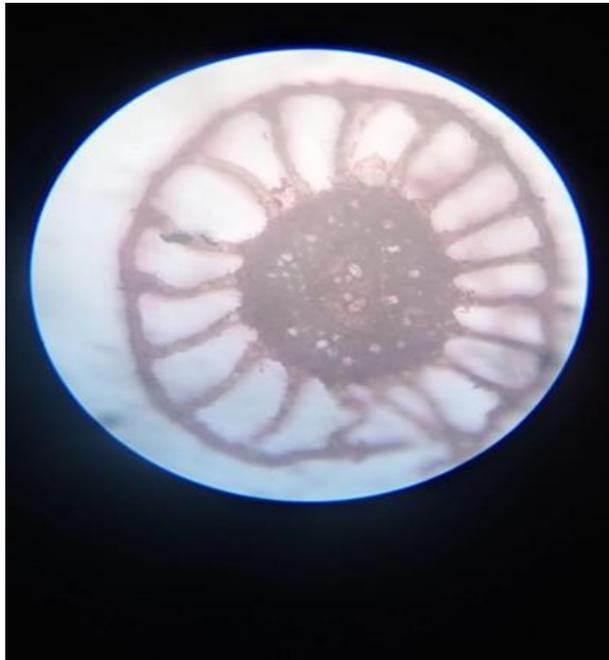


PLATE 16: ANATOMY OF *MARSILEA* PETIOLE

9. *ADIANTUM*

Adiantum is also known as maidenhair fern or walking fern. They are small, perennial and evergreen plant.

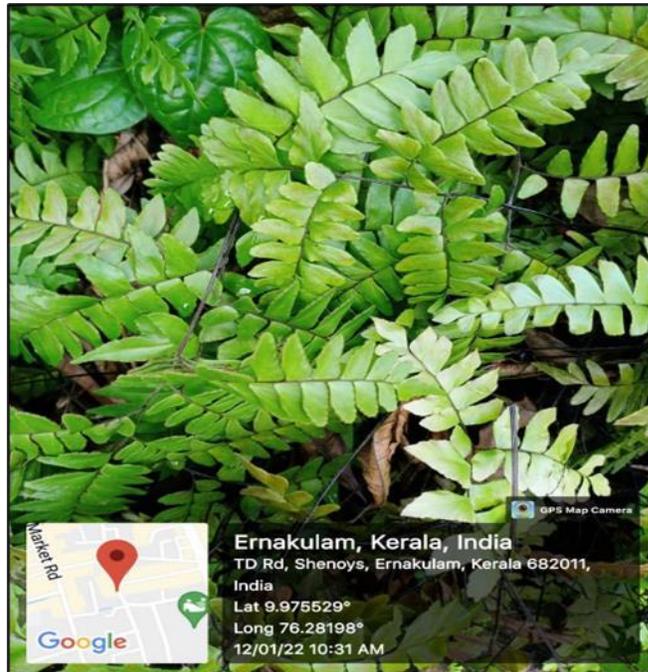


PLATE 17: HABITAT OF *ADIANTUM*

- **TAXONOMIC POSITION**

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Nephrolepidaceae

Genus : *Nephrolepis*

- **MORPHOLOGICAL EVALUATION OF *ADIANTUM***

SPOROOPHYTE: The plant is divided into stem, root and leaves.

RHIZOME: Rhizome grows horizontally near the soil surface. Scales, called palea covered the surface of rhizome.

LEAVES: Leaves of *Adiantum* are called fronds. These leaves are large, about 4-6 inches in length and are bipinnately compound. Leaflets of the first order are called pinnae and leaflets of second order are called pinnules. Main axis of leaf on which leaflets are produced is rachis. The rachis is black in color and shiny. The leaves are produced in acropetalous succession on the creeping rhizome. They show circinate venation. The pinnae are stalked and have a dichotomous venation. There is no distinction between fertile and sterile leaves. The whole leaf may be sporangiferous or only certain pinnae may bear sporangia. The soral organisation is very evident. Sori are borne on the ventral surface of the pinnae.

- **ANATOMICAL EVALUATION OF *ADIANTUM* PETIOLE**

EPIDERMIS: The petiole in T.S. shows a single-layered epidermis with a thick cuticle. The epidermis is followed by a sclerenchymatous hypodermis which provides mechanical support.

CORTEX: Consists of parenchymatous cells. The central region possesses a single large horseshoe-shaped stele. Xylem forms central core surrounded by phloem.

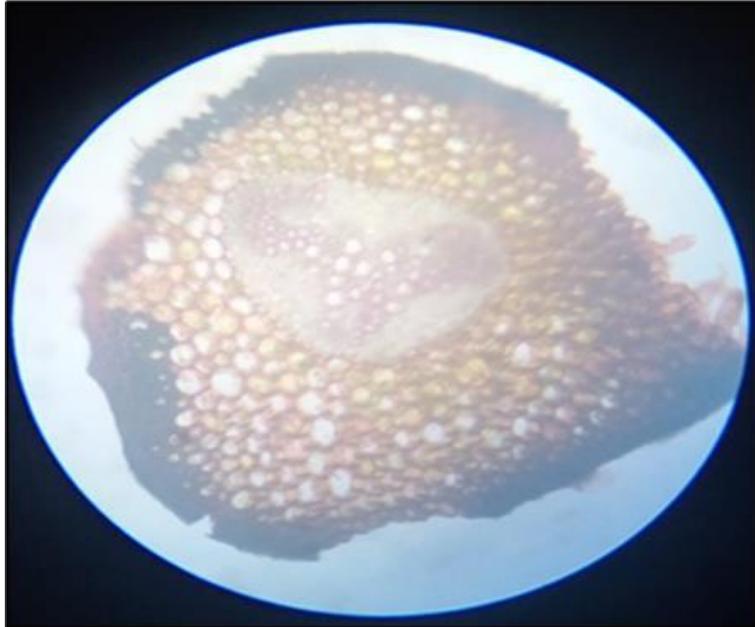


PLATE 18: ANATOMICAL EVALUATION OF *ADIANTUM* PETIOLE

DISCUSSION

Ferns and their allies are one of the Pteridophyta's oldest major divisions, with approximately 12 000 species scattered over 250 distinct genera (Baskaran *et al.*,2018). Sushruta (about 100 AD) and Charka (around 100 AD) of the biomedical and Ayurvedic schools of medicine, respectively, proposed the use of various ferns in the Samhita literature. Pteridophytes are also employed by physicians in the Unani medical system. Several ferns are suggested by native doctors in the traditional Chinese medical system. Several researchers have recently conducted ethnobotanical and advanced pharmacological investigations on ferns and their companions. Most ferns and fern allies provided several health advantages to ancient civilizations that utilized them for food, tea, and medications. Modern techniques have merged interdisciplinary technology, as well as particular chemical components collected and identified, allowing the production of extremely particle medications from plant parts.

Plants that produce high quality and quantity of polysaccharides, steroids, terpenoids, flavonoids, alkaloids, and antibiotics are ideal for developing medications for a variety of ailments/diseases, including cancer therapies. Modern studies on the functional activities of pteridophytes for human health, such as the discovery of particular chemicals and their use in medications, have broadened the scope of pteridophytes, transforming these plants into a godsend for pharmaceutical businesses and associated industries. Plants that produce high levels of polysaccharides, steroids, terpenoids, flavonoids, alkaloids, and antibiotics are useful for producing drugs for a number of ailments/diseases, including cancer therapy. Modern research on the functional activities of pteridophytes for human health, such as the identification of specific compounds and their application in drugs, has widened the scope of pteridophytes, making these plants a boon to pharmaceutical companies and related industries. Earlier pteridophytes' pharmacological activity suggests an alternate therapy for treating human illnesses. Pteridophytes (ferns and fern allies) are an old lineage that humans have been discovering and exploiting for over 2000 years due to their beneficial characteristics as the earliest vascular plants. Previous research has demonstrated that the lycophyte *Selaginella* sp. has a wide range of pharmacological activity, including antioxidant, anti-inflammatory, anti-

cancer, antidiabetic, antiviral, antibacterial, and anti-Alzheimer capabilities. Among all the pteridophytes studied, taxa from the Pteridaceae, Polypodiaceae, and Adiantaceae showed the most therapeutic efficacy. According to our findings, several pteridophytes have characteristics that might be employed in alternative medicine to treat a variety of human ailments.

Ferns are common denominators of abundant and diverse biodiversity in practically every corner of the world. The comparison of evolutionary adaptations and natural innovations reveals the genetic underpinning for organism development. It is highlighted that good field stations with big greenhouses at the periphery of protected forests should work as 'Fernariums/ Mossariums/ and/or Lichenariums' to conserve and nurture rare, endangered, and medicinally outstanding species present in such areas/forests. Gene networks (DNA stretches) that preserve comparable wiring schematics (some or many similar DNA sequences) throughout related, distantly related, or completely different animals reveal how regulatory sections of the genome have developed. Without a doubt, comparative genomics can assist us in deciphering the evolvability of gene networks and conservation modes.

The potential use of Pteridophytes as Ecological Indicators is becoming more widely recognized. The use of pteridophytes as EIs is becoming more common. This demonstrates the group's significant potential as EIs, which is further backed by several studies using similar approaches, resulting in a huge number of species, genera, and families being proposed as EIs.

The ferns have also been shown to be having an important role in the bioremediation of wastewater. (Dudani *et al.*, 2001) found the Chinese Bracken fern namely *Pteris vittata* L. to be a hyperaccumulator of the toxic metal Arsenic. Besides producing large biomass, they also found this fern to be efficient in Ar accumulation with concentrations as high as 2.3% in the aerial portions of the fern. Later on, many researchers provided reports of the hyperaccumulation properties of *Pteris* as well as many other ferns also (Zhang, 2002) suggested that *P. vittata* could be an excellent model to study arsenic uptake, translocation, speciation,

distribution and detoxification in plants and for phytoremediation of arsenic-contaminated soil and water.

Besides having all these wonderful properties, the pteridophytes are also greatly valued as ornamentals. Prior to the discovery of these benefits obtained from this group of plants, ferns were used to enhance the beauty of the landscape and are continued to be used so till now. Ferns like *Adiantum* sp., *Selaginella* sp., *Lygodium* sp., *Pteris* sp., etc. are also grown in the gardens or in the pots.

The pteridophytes are moisture and shade-loving plants and are dependent upon the microclimatic conditions of the region for their successful survival in that region. Any kind of disturbance in these microclimatic conditions can hinder the growth and evolutionary processes occurring naturally in these plants thereby, leading to declining in their populations. Thus, factors like climate change, increasing urbanization, industrialization, encroachment of forest lands, unplanned developmental activities, and overexploitation of natural resources, pose a major threat to the survival of these groups of plants. Due to the unplanned felling of trees in the forests the members of epiphytic pteridophytes belonging to the families Polypodiaceae, Davalliaceae, Aspleniaceae, Vittariaceae, have been reduced day by day (Nambiar and Shimna, 2022). Large-scale collection of ferns from the forests by the visitors and local people for ornamental purpose, medicinal purposes and during excursions also increases the pressure on these plants.

Biodiversity conservation is the need of time and hence, it has become imperative to develop in situ and ex situ conservation methods for conservation of the diminishing biodiversity. The in-situ conservation is very beneficial as it allows the evolution of the species to continue within the area of natural occurrence. Hence, the steps for conserving the ferns in situ should be focused upon. The ex-situ conservation includes the development of botanical gardens or conservatories, germplasm banks, DNA banks, seed banks and involve the use of techniques such as tissue culture, cryopreservation; incorporation of disease, pest and stress tolerance traits through genetic transformation and ecological restoration of rare plant species and their populations (Pritchard *et al.*, 2012). The conservation of flowering plants has been achieved to a good extent by developing conservatories and botanical gardens which also help

in creating awareness among the local people. Developing a fern conservatory or fern garden is not preferred much and hence, such steps should be considered and implemented for conserving the rare and endangered species. The tissue culture is a very useful technique for the mass multiplication of the plant species in a short time and hence, research focusing on developing a protocol for in vitro regeneration of ferns and fern-allies should be encouraged. Parts of areas rich in abundant pteridophyte diversity can be declared as pteridophyte biosphere reserves or small gene sanctuaries can be established to save the epiphytic pteridophytes.

CONCLUSION

The present study demonstrates the relevance of Pteridophytes in nature. Pteridophytes are an ancient lineage of plants, composed of ferns and fern allies, which are spread across the globe. There is also a long record of humans using pteridophytes to their benefit, which includes the broad categories of medicine, ornamentation, food, phytoremediation, and agriculture. Biopharmaceutical approaches can be used to preserve and even improve bioactive molecules for the development of anti-disease medications. Several studies have revealed that ferns have medicinal potential and an essential role in wastewater bioremediation.

There is a dearth of studies demonstrating their real usefulness and the criteria utilized to choose the Ecological indicators. The genetic foundations for organism development are revealed through evolutionary interactions and natural innovations. For the sake of the future, research into the development of pteridophytes should be supported. According to the current study, pteridophytes should be kept and safeguarded in the future. Pteridophytes are highly prized foliage ornamentals.

Documentation on the economic importance of pteridophytes is needed to reveal the importance of this plant group to the public and the indigenous knowledge about them. It is also important that field botanists should avoid the ruthless collection of rare species and make sure that they leave the bulk of plants to continue to grow and reproduce in the world. Providing proper awareness about the conservation of pteridophytes among the local people is needed. Further studies on pteridophytes can bring many more species that are of economic importance to light.

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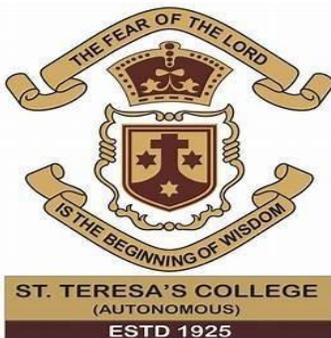
**PRELIMINARY STUDIES ON
THE ANTIBACTERIAL POTENTIAL OF THE RED
SEAWEED *ACANTHOPHORA SPICIFERA* (M. Vahl) Borgesen**

**DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE AWARD OF THE DEGREE OF BACHELOR OF
SCIENCE IN
BOTANY**

By

NAME : A V Sona Samardh

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DEPARTMENT OF BOTANY

ST. TERESA'S COLLEGE (AUTONOMOUS)

ERNAKULAM

2022

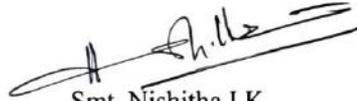
CERTIFICATE

This is to certify that the dissertation entitled "Preliminary studies on the antibacterial potential of the red seaweed *Acanthophoraspicifera* (M. Vahl) Borgesen" is an authentic record of research work carried out by Miss **A V Sona Samadh**

(Reg No: AB19BOT33) under the supervision and guidance Smt. Nishitha I. K. Assistant Professor, Department of Botany and Centre for Research, St. Teresa's College (Autonomous), Emakulam, in partial fulfilment of the requirements for the award of the degree of Bachelor of Science in Botany. I further certify that no part of this work embodied in this project has been submitted for the award of any degree or diploma.

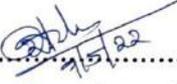
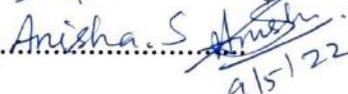


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- 2)  Anisha S
9/5/22



DECLARATION

I hereby declare that the project entitled “Preliminary studies on the antibacterial potential of the red seaweed *Acanthophora spicifera* (M. Vahl) Borgesen” submitted to Mahatma Gandhi University, Kottayam, in partial fulfilment of the requirement for the Degree of Master of Science in Botany is an original project done by me under the supervision and guidance of Ms. Nishitha I.K., Department of Botany and Centre for Research, St. Teresa’s college (Autonomous), Ernakulam.

PLACE: Ernakulam
DATE: 4th May 2022

NAME: A V Sona Samardh

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Place : Ernakulam

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Date: 4th May 2022

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INTRODUCTION

Algae are diverse group of relatively simple, chlorophyll containing, photo-autotrophic and oxygen evolving aquatic thalloid (without differentiation into True roots, stems, leaves or leaf like organs) organisms. The word “algae” has its origin from Latin, where ‘alga’ means seaweed. The term algae was first used by Carolous Linnaeus in 1753. Most of them are photo-autotrophic but few are mixotrophic and myzotrophic (sucking through special feeding structure) study of algae is known as phycology (GK. Phykos- seaweed; logos= discourse Or study) or algology.

Algae are divided into nine main phylums, they are Phylum Rhodophycophyta, Phylum Xanthophycophyta, Phylum Chrysophycophyta, Phylum Phaeophycophyta, Phylum Bacillariophycophyta, Phylum Euglenophycophyta, Phylum Chlorophycophyta, Phylum Cryptophycophyta, and Phylum Pyrrophytocyta.

The term "seaweed" refers to a variety of marine plants and algae that can be found in the ocean, rivers, lakes, and other bodies of water. Some seaweeds, such as phytoplankton, are small and remain floating in the water column, providing the foundation for most aquatic food chains. Some are massive, such as the giant kelp that grow in dense forests. The large percentage are medium-sized, with red, green, brown, and black colours, and sometimes may wash up on beaches and shorelines. (Guiry, Michael D., 2014)

Marine algae from Indian coasts amounting to 844 species (including forma and varieties) are distributed among 217 genera. They grow in the intertidal, shallow and deep sea areas up to 180 meter depth and also in estuaries, backwaters and lagoons on solid substrates such as rocks, dead corals, pebbles, shells, mangroves and other plant materials (Anatharaman *et al.*, 2007; Sakthivel, 2007).

Mostly seen seaweeds are macro algae. They are of different types according to the colour of pigments present: brown algae (phylum Ochrophyta, class Phaeophyceae), red algae (phylum Rhodophyta, *Gelidium*), and green algae (phylum Chlorophyta, classes Chlorophyceae, Ulvophyceae etc. They differ significantly in many ultrastructural and biochemical functions, including photosynthetic pigments, storage molecules, cell wall composition, presence/absence of flagella, mitosis ultrastructure, linkage between cells, and structure of the chloroplasts, in addition to pigmentation.

Seaweed is high in vitamins, minerals, and fibre, as well as being palatable. The Japanese have a dish, 'sushi' which is nori seaweed wrapped with a mixture of fish, rice, and other ingredients for at least 1,500 years. Anti-inflammatory and anti-microbial compounds can be found in a variety of seaweeds. For thousands of years, their medicinal properties have been used; it was used to cure wounds, burns, and rashes by the ancient Romans. According to anecdotal evidence, the ancient Egyptians may have employed them to cure breast cancer. (McLachlan, J., and C. J. Bird, 1984).

In recent years, focus towards these organisms has increased due to their food and fuel production capability. In fuel industry algae biofuels have emerged as a clean, nature friendly, cost effective solution to other fuels. More recently algae have been identified and developed as renewable fuel sources, and the cultivation of algal biomass for various products is transitioning to commercial-scale systems. Large-scale cultivation of algae merges the fundamental aspects of traditional agricultural farming and aquaculture (Emily M Trentacoste *et al.*, 2014). Algae fuels are categorized into bio-ethanol, biogas, bio-hydrogen, biodiesel and bio-oil. Algae can be used in the preparation of Biodiesel, Bioethanol, Biobutanol and Hydrogen gas (Raja *et al.*, 2013)

They are considered as a potential source of bioactive substances such as proteins, lipids, and polyphenols possessing potent antibacterial, anticancer, antioxidant, antifungal, and antiviral properties(Sundaramurthy *et al.*, 2016). Seaweeds that are medicinal are rich in secondary metabolites which include alkaloids, glycosides, flavonoids, saponins, tannins, steroids, related active metabolites, which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry(Eluvakkalet *et al.*, 2010). Recently, their value as a source of novel bioactive substances has grown rapidly and researchers have revealed that marine algal originated compounds exhibit various biological activities (Kim and Wijesekara, 2010; Wijesekara and Kim, 2010; Wijesekara *et al.*, 2010 and Wijesekara *et al.*, 2011). The secondary metabolites of seaweeds such as Isoprenoids (terpenes, carotenoids, steroids), polyketides, phlorotannins, amino-acid derived natural products (alkaloids), and shikimates (flavonoids) have always attracted the interest of biochemists because of their diversity is comparable with those present in the leaves of higher plants (Manilal *et al.*, 2009). Seaweeds were rich in dietary fiber (>50% dry weight), particularly in the soluble form.

RHODOPHYCEAE

Rhodophyta is a phylum of macroalgae that includes the classes Phaeophyceae and Chlorophyta, which are brown and green seaweeds, respectively.

Within Archaeplastida, Rhodophyta, or red algae, is a monophyletic lineage that contains glaucophyte algae, green algae, and terrestrial plants. Bangia-like species have been found in 1.2 billion-year-old strata, indicating that Rhodophyta has a lengthy fossil history. The morphology of red algae ranges from unicellular filamentous to multicellular thalloid forms, with certain species producing economically important products like agar and carrageenan. These species can be found in a variety of marine settings, ranging from the intertidal zone to deep oceans. There are also freshwater (e.g., *Batrachospermum*) and terrestrial lineages. A triphasic life cycle with one haploid and two diploid phases, with the carposporophyte borne on female gametophytes, is one of the Rhodophyta's significant advances.

Freshwater Rhodophyta has 66 species and 27 genera in North America, although these numbers will change as molecular investigations uncover more diversity. Freshwater red algae have a limited size range than marine species, with the majority (80%) of them measuring 1-10 cm in length. Gelatinous filaments, free filaments, and pseudoparenchymatous forms are the most prevalent types. (Yoon, Hwan Su, et al., 2017)

ACANTHOPHORA

Acanthophora is a red algae that can be found in almost all tropical and subtropical oceans. Because of its changeable form, it can adapt to a wide range of environmental circumstances and hence invade a wide range of ecosystems.

Acanthophora is an erect macroalgae that may reach a height of 40cm. It has solid cylindrical branches that are 2-3mm diameter and are rarely or repeatedly branched. Short, determinate branches, irregularly shaped and spinose, with spines numerous and radially oriented, make up the major branches. The major axes have no spines. A big, oddly shaped holdfast gives rise to the plant. It has short (4 - 10cm), compact, and dense thalli in intertidal high-motion water areas. It comes in a wide range of colours, including red, purple, yellow, orange, and brown. Thalli are typically quite black in intertidal, high-motion locations, and lighter in shallow areas with low water motion and reflective sandy or silty bottoms.

In the Gulf of Mannar, Tamil Nadu's coastal region, *Acanthophora spicifera* is a common seaweed used in folkloric treatments and as a nutritional supplement. The anticancer and anti-oxidant properties of the alcoholic extract of *Acanthophora* were investigated in this study. Anti-cancer impact was measured by assessing tumour volume, tumour weight, mean survival day (MSD), and several haematological parameters after 21 days of testing and standard drug administration. The anti-oxidant state of the liver tissue was also determined. In cancerous mice treated with EAC cell lines, the ethanol extract of *Acanthophora* has a significant anti-cancer effect, reducing tumour volume and weight with mean survival day (MSD). The findings revealed that an ethanol extract of *Acanthophora* has antitumor and anti-oxidant activity, which may be due to the presence of bioactive components such as flavonoids, terpenoids, and tannins. (Lavakumar, K. F. H. Ahamed, and V. Ravichandran, 2012)

The present work was undertaken to study the antimicrobial potential of *Acanthophora spicifera*. The objectives of the study are

- Taxonomic description of the algae
- Assessment of antibacterial potential of *Acanthophora spicifera* in its dried form extracted in two different solvents; Ethanol and Chloroform.
- Comparative antibacterial activity of the algae extracted in the two different solvents against Gram positive *Staphylococcus* and Gram negative *E. coli*.
- Estimate the extractive value of the plant, in both ethanolic and chloroform solvents.

LITERATURE REVIEW

In a study conducted by Inci Tuney (2006), antibacterial activity of extracts or components from various algae has been demonstrated in vitro against both gram-positive and gram-negative bacteria. The antibacterial susceptibility test was performed using the agar disc diffusion method, with 6 mm discs impregnated with 20 µl of extracts and placed in infected agar. (Inci Tuney 2006),

Krishnapriya et al., (2013) conducted an antibacterial activity on the seaweed extracts, carried out by agar disc diffusion assay. The Muller Hinton agar (MHA) medium was used for this study using bacterial pathogens. Among the solvent extracts, methanol extract showed best results for both positive and negative strains. Chloroform extract of *G. verrucosa* gave the highest zone of inhibition measuring 21±1.0 mm. Ethanol extract of *G. acerosa* also showed a zone of inhibition of 12±1.0 mm. Ethanol and chloroform extracts of *G. verrucosa* gave clearly distinct zone of inhibition measuring 8±1.0 and 9±1.0 mm, with respect to control (25±1.0 mm) against *Staphylococcus*. (Varier, KrishnapriyaMadhu, et al., 2013)

Saranraj, P. (2013) conducted a study and the methanol extract of *Gracilaria folifera* (5.0mg/ml) showed highest mean zone of inhibition (18±0.4mm) against the Gram positive cocci *Streptococcus pyogenes* followed by *Bacillus subtilis* (17±0.5mm), *Staphylococcus aureus* (17±0.3mm), *Streptococcus epidermis* (16±0.6mm) and *Bacillus cereus* (16±0.2mm). For Gram negative bacterium, the maximum zone of inhibition was recorded in methanol extract of *Gracilaria folifera* against *Klebsiella pneumoniae* (17±0.5mm) followed by *Salmonella typhi* (16±0.6mm), *Pseudomonas aeruginosa* (16±0.5mm), *Escherichia coli* (16±0.3mm). The zone of inhibition obtained from the Hexane extract of seaweed *Gracilaria folifera* against bacterial pathogens was comparatively very less when compared to the other solvent extracts. No zone of inhibition was seen in DMSO control and the positive control Ampicillin showed zone of inhibition ranging from 13±0.8 mm to 20±0.8mm against the test bacterial pathogens. (Saranraj, P., 2013)

The antibacterial properties of eight crude extracts of local *Acanthophora spicifera* obtained by two distinct extraction methods were investigated by Zakaria (2010) using Soxhlet extraction and solvent partitioning. By using the Disc diffusion method, these extracts were evaluated in vitro against 18 bacteria, 3 yeasts, and 6 fungal strains. The results demonstrated

that the solvent partitioning extracts of methanol and ethyl acetate had a greater spectrum of action against the tested bacterial strains. *Bacillus cereus* ATCC 10876, *Bacillus licheniformis* ATCC 12759, Menthicilin Resistance *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* ATCC 27853, *Yersinia* sp., and *Citrobacter freundii* displayed inhibitory zones against these two extracts. While methanol extracts from Soxhlet extraction and butanol from solvent partitioning had no antibacterial activity against *P. aeruginosa* ATCC 27853, the other six extracts did. (Zakaria 2010)

In a study done by Ibraheem et al. (2017), simplex extracts of *Acanthopora* showed potent inhibitory growth activities against three Gram positive bacteria [*Streptococcus agalactiae*, *pyogenes* and *Streptococcus sanguis* of inhibition ranging from [23.1±0.58 to 20.6±0.63 mm] and showed moderate activities with [*Corynebacterium diphtheriae*, *Bacillus subtilis* and *Staphylococcus aureus*] with inhibition zones ranging from [20.1±1.5 to 16.3±2.1 mm]. (Ibraheem et al.; 2017)

Also the crude extracts were found to be more active than the positive control Ampicillin, (22.3±1.5 mm), against *Streptococcus agalactiae* which showing inhibition zone. The hydro alcoholic extracts of the selected species were investigated for their antimicrobial activities using Agar well diffusion and Muller Henton against gram positive and gram negative bacteria.

In a study by Nurul Aili Zakaria et al. (2011), the antimicrobial activities of the hexane extract were evaluated using disc diffusion method against 8 Gram-negative and 10 Gram-positive bacterial strains. Out of all bacterial tested, only a Gram-positive bacterium and a Gram-negative bacterium were susceptible to the extracts. The hexane extract showed antibacterial activity against both Gram-positive bacterium and Gram-negative bacterium (*P. aeruginosa* ATCC 27853). While, chloroform and ethyl acetate extract only showed inhibitory effect on *P. aeruginosa* ATCC 27853 with inhibition zone of 9.0 mm. No inhibitory effect was showed by methanol extract on bacteria tested. (Nurul Aili Zakaria et al.; 2011)

MATERIALS AND METHODS

SPECIMEN COLLECTION

The specimen was collected by hand picking from Thikkodi beach, Calicut. The collected samples were washed immediately in seawater and then washed with fresh water and transported to the laboratory. It was again washed thoroughly to remove impurities and sand and rinsed with distilled water. The sample was identified taxonomically as *Acanthophora*. Collected sample was taxonomically evaluated using the standard literature.

SAMPLE PREPARATION

For antimicrobial studies, the cleaned samples were then shade dried, cut into small pieces and powdered in a mixer grinder. The organic solvents Chloroform and Ethanol were used for the extraction process due to its higher efficiency using Soxhlet extraction method. 20g of samples were packed in a thimble and placed in the extractor. 200ml of the solvent was added into the flask and heated. The temperature was maintained at 80°C to 85°C throughout the extraction. The soluble active constituents of the extract remained in the flask and the process was repeated until the compounds were completely extracted. The liquid extract was then cooled and concentrated by using an evaporator.

The beaker with dried extract was weighed and noted. DMSO was used to dissolve the extracts from the beaker. Later the weight of the beaker alone was noted. Hence, the actual weight of the dried extract was obtained. From the above observation, the weight of dried extract of *Acanthophora* in ethanol and chloroform was 2.44g and 0.18g respectively. From this the extractive value was calculated using the formula

Extractive value (%) = (Weight of dried extract/ Weight of plant material) X 100

PREPARATION OF EXTRACT IN VARIOUS CONCENTRATIONS

From the stock extract, concentrations of 10%, 20%, 40%, 60% (v/v) was made. The stock concentration of *Acanthophora* in ethanol and chloroform was 70mg/ml and 10mg/ml respectively. From the stock the appropriate amounts was pipetted out and made up to the required concentrations using DMSO.

ANTIBACTERIAL ACTIVITY IN ACANTHOPHORA

PREPARATION OF BACTERIAL CULTURE

In the present study, the extracts were evaluated for antimicrobial activity against *Staphylococcus* strain and *E. coli*, a Gram positive and Gram negative bacteria respectively. 3g of nutrient broth was dissolved in 100ml of distilled water in a conical flask. The broth is sterilized by autoclaving for 15 minutes. Both of the obtained bacterial stains were inoculated in the nutrient broth in laminar air flow and incubated in appropriate conditions for 24hrs.

PREPARATION OF PETRI PLATES

The selected two species of seaweeds were analysed for the antimicrobial activity for gram negative *Escherichia coli* and gram positive *Staphylococcus* by disc diffusion methods. Agar medium was prepared by dissolving 4g agar and 2.6g of nutrient broth in 100ml distilled water. The mixture is sterilized in an autoclave for 15 minutes. Just after sterilization the mixture was poured into petri plates in laminar air flow. The petri plates were allowed to solidify under aseptic conditions.

ANTIMICROBIAL TEST BY DISC DIFFUSION METHOD

Bacteria were inoculated onto the prepared agar petri plates using sterilized cotton swabs. Sterilized 6mm discs were taken from filter paper and autoclaved and are used for the method. The disc was then dipped in different concentrations of stock (10, 20, 40, 60) and placed on the agar plate using sterile forceps. Tetracycline was used as positive control and DMSO was used as negative control. This was done for both extracts of *Acanthophora* against the two strains of bacteria. The petri plates were incubated at 37°C for 24 hours and results were recorded.

OBSERVATIONS AND RESULTS

The current work was undertaken as a preliminary study of the red seaweed *Acanthophora spicifera*. The scope of the study included the estimation of extractive value in two solvents, ethanol and chloroform and the antimicrobial potential of these extracts. The antimicrobial potential activity was studied against Gram positive *Staphylococcus* and Gram-negative *E. coli*, two non-pathogenic bacteria. The results obtained are described below.

TAXONOMIC DESCRIPTION

Division: Rhodophyta
Class: Florideophyceae
Order: Ceramiales
Family: Rhodomelaceae
Genus: *Acanthophora*
Species: *spicifera*

Acanthophora spicifera (Vahl) Børgesen

Erect plants, to 40 cm tall, with solid cylindrical branches, 2 - 3 mm wide, branched either sparingly to repeatedly. Main branches have short, determinate branches, irregularly shaped and spinose, with spines numerous and radially arranged. There are no spines on main axes. The plant grows from a large, irregularly shaped holdfast. In intertidal high-motion water areas, *Acanthophora spicifera* has short (4 - 10 cm), compact and very dense thalli. In moderate or low water motion areas, the thalli are tall (10 - 25 cm), more openly branched and occur in scattered clumps.



Acanthophora spicifera(M.Vahl)Borgesen

EXTRACTIVE VALUE

Extractive values of plant materials are used to evaluate extracts of the sample, in order to get an idea about the nature of chemical constituents present in it. It can also be used to assess quality, purity and detect adulteration of the extract.

In the present study, polar and non-polar solvents were used for eluting the valuable phyto-compounds present in the sample. Extractive values of ethanol and chloroform extracts of *Acanthophora spicifera* used in the antibacterial study, are estimated in the table 1 given below;

Table 1: Extractive value of solvents administered for *Acanthophora spicifera*

Solvent	Extractive value of the sample (%)
Ethanol	12.2
Chloroform	0.9

The extractive value was greater for the ethanolic extract than for chloroform suggesting that polar solvent was more efficient in extracting the phytochemicals from the algae.

ANTIBACTERIAL ACTIVITY

The extracts of the alga exhibited only mild antibacterial activity against the two microorganisms. The activity observed can be described as being bacteriostatic showing very mild zones of inhibition. The ethanol extract of the algae showed mild antibacterial activity against *E. coli* alone. The activity is shown only at high concentration against gram negative bacteria. Chloroform extract seems to have no action on either test organisms even at higher concentrations studied. Table 2.

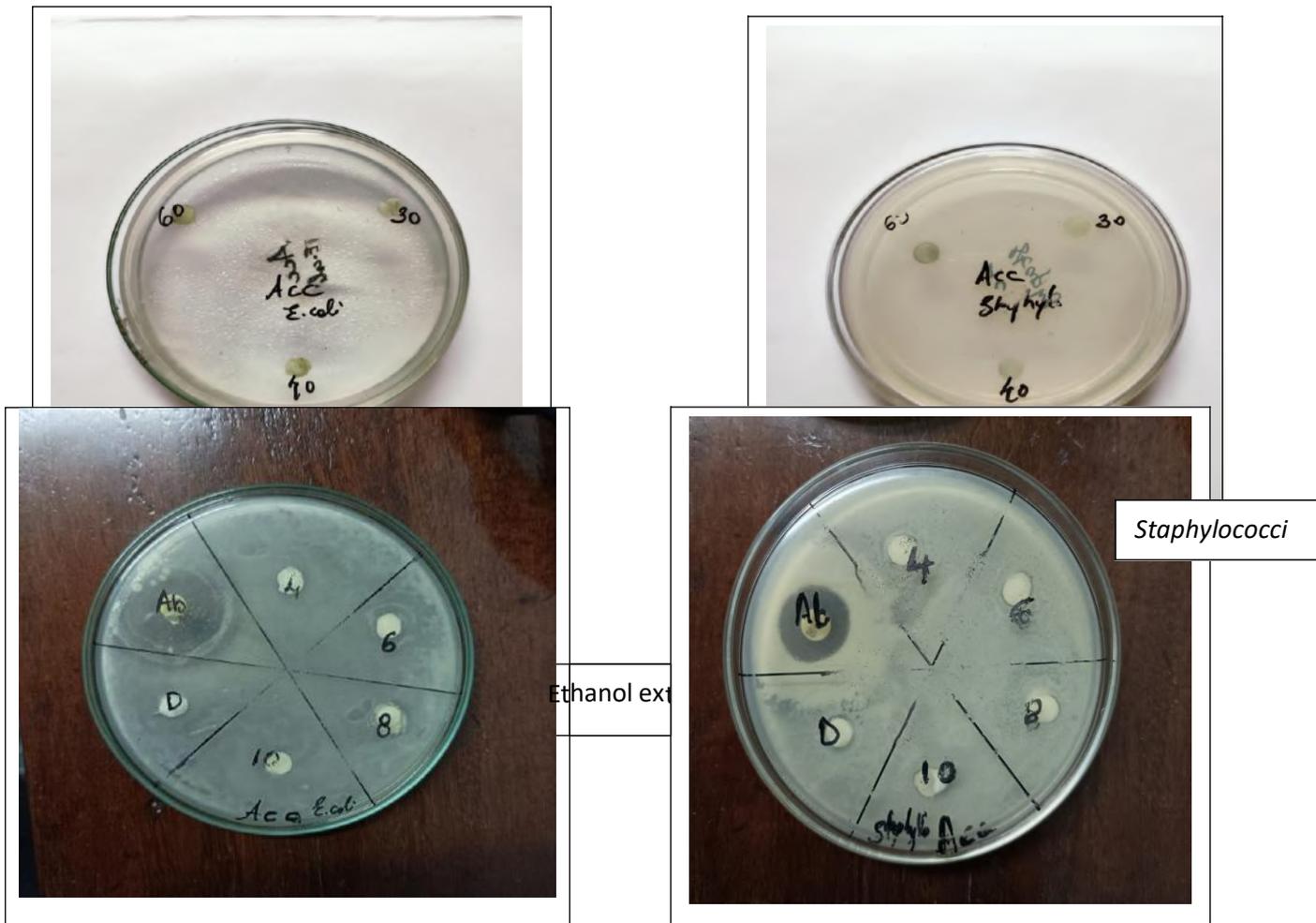
Table 2: Antibacterial activity of ethanolic extract of *A. spicifera* against *E. coli* and *Staphylococcus* bacteria:

Concentration(%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	No action	No action
40	No action	No action
60	Mild action	No action

Table 3: Antibacterial activity of chloroform extract of *A. spicifera* against *E. Coli* and *Staphylococcus* bacteria

Concentration(%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	No action	No action
40	No action	No action
60	No action	Mild action

The chloroform extract of *Acanthophora* has no significant effect on the growth of *E. coli*. Mild bacteriaostatic activity is observed at higher extract concentrations on *Staphylococcus*. No potential activity could be observed on the growth of *E. coli* in any of the concentrations used for the current study Table 3: Fig.



<i>E. coli</i>	<i>Staphylococci</i>
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Fig 3: Antimicrobial activity of Chloroform extract of *Acanthophora spicifera*

DISCUSSION

Seaweeds are a group of marine macro algae that are now in the limelight of algal research due to their immense bioactive potentials and easy availability. Several bioactive compounds are found in high concentration in the seaweeds, because of which it exhibits various pharmacological activities. The seaweeds offer greatest wealth in terms of biomass and Rhodophytes show the largest representation among them. *Acanthophora spicifera* is a red sea with about 26 species world wide.

Natural products from marine algae have attracted the attention of biologists and chemists the as many of these compounds are used to treat diseases like cancer, acquired immune-deficiency syndrome, inflammation, pain, arthritis, as well as viral, bacterial, and fungal infections. **Sunil DS** in 2015 studied the marine red alga to analyse the phytochemical constituents. The presence of a variety of chemical constituents, such as saponins, phenols, flavonoids, alkaloids and steroids were confirmed in *Acanthophoraspicifera* by qualitative tests.

Antibacterial activity refers to the process of killing or inhibiting the disease-causing bacteria. Several plants have been traditionally used for their antibacterial activity. Like plants some algae also exhibit antibacterial properties due to the presence of terpenoids, steroids, saponins, tannins, and flavonoids. There are numerous reports regarding the inhibitory activities of macroalgae against human pathogens, fungi and yeasts. So, the use of algae as an alternative for prevention and treatment of infectious diseases has been suggested by Abirami and Kowsalya (2012). In the present study ethanolic and chloroform extract were evaluated for activity against Gram positive *Staphylococci* and Gram-negative *E. coli*. It was found that ethanolic extract had bacteriostatic activity against *E. coli* and the chloroform extract inhibited *Staphylococcus* at high concentrations.

Different solvent systems were used to extract bioactive principles from macroalgae with concomitant changes in the antibacterial activities (Thirupurasundari et al., 2008). The solvents such as acetone, benzene, butanol (Vanitha et al., 2003; Prakash et al., 2005), ethanol (Selvi et al., 2001) were used to extract antimicrobial compounds from macroalgae. The aqueous extracts prepared from seven macroalgal samples showed varying degrees of activity against tested pathogens, including the Gram positive and Gram negative bacteria. (Padmakumar (2002) is of the opinion that these differences are due to the different solubility behaviour of secondary metabolites which could be influenced by seasonal and geographical distribution of the species.

Antibacterial potential of *A. spicifera* ethanol extracts were evaluated by Meenakshi et al (2014), against six bacterial specimens. They reported high antimicrobial activity against *E. coli*. In the present study, ethanolic extract showed mild activity at higher concentration (60%) only. In their work, Meenakshi et al have also reported significant cytotoxic potential against Ehrlich Ascites carcinoma cell lines for ethanolic extract of the alga..

Several previous studies have revealed the bioactivity of active compounds isolated from *Acanthophora* sp. such as antibacterial antiviral and antioxidant activity, anti-viral, and anti-fouling. Steroids and fatty acid esters of *A. Spicifera* were reported to exhibit potent antitumor and antibacterial activity against human cancer lines and microorganisms((Laila S, 2003; Han, L et al 2006)

In their study on six sea weeds, Rajashekar et al in 2018 reported that *A. spicifera* has the least number of compounds in ethanol and chloroform extracts. This possibly explains the low antimicrobial activity of the algae in the present study also. However, they report potential inhibitory activity against *B. cereus* and *P. aeruginosa*

SUMMARY

Seaweeds are the macroalgae found in marine ecosystems where they play a multitude of roles. From being the primary producers providing nutrients and energy to other living organisms, provide shelter and home to these life forms. They also play significant roles in climate mitigation. Seaweeds have been used as traditional remedies for common ailments and have been a part of traditional cuisine in many parts of the world. Several studies have been conducted on different algae to assess their biopotentials and to exploit them in a way beneficial to man.

For this project, the red algae *Acanthophora spicifera* was selected. *Acanthophora* is an erect macroalgae, that can be found in almost all tropical and subtropical oceans. It is widely distributed in the southern and northern rocky coasts of Kerala. The alga was studied to identify its extractive values in different solvents and to assess its antibacterial potential. The dried algae was extracted in the solvents using the Soxhlet apparatus. The extractive values of the algae in ethanol and chloroform, two solvents with very different polarities was estimated and found that the polar solvent, ethanol had

greater extractive value of 12.2% than the non-polar solvent chloroform with only 0.9% extractive value.

Propanol (polar) and Benzene, Chloroform and Hexane (non polar) were evaluated. Only the extracts showing antibacterial activity (Ethanol, Chloroform, Isoamyl alcohol, Methanol and Propanol) were considered for further study.

The antioxidant potential was studied by the disc diffusion method. The test organisms used were the Gram positive *Staphylococcus* and Gram negative *E. Coli*. The analysis of antimicrobial activity of *Acanthophora spicifera* displayed little to no microbial activity in both ethanolic and chloroform extracts for gram positive (*Staphylococcus*) and gram negative (*E. coli*) bacteria taken. A mild reaction suggesting bacteriostatic activity was observed in ethanolic extract at higher concentration for gram negative bacteria while the chloroform extract showed mild reaction for gram positive bacteria at higher concentration. This can be used as an indicator for further studies of antimicrobial activity of the species.

Acanthophora possess antioxidant, antitumor and antibacterial activity, which may be due to the presence of bioactive components such as phenolics, terpenoids, and tannins. The chemical constituent of *Acanthophora* is rich in non halogenated steroid. Various other extracts of the algae have highlighted the nutritional and anticancer properties which supports its widespread usage in folklore medicine. Thus the proper identification and analysis of the seaweed can be used for producing natural alternatives to the synthetic medicines available in today's market.

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**MORPHOLOGICAL AND ANATOMICAL EVALUATION OF
PTERIDOPHYTES FROM THE BOTANICAL GARDEN OF
ST. TERESA'S COLLEGE OF ERNAKULAM DISTRICT**

**A DISSERTATION SUBMITTED TO
MAHATMA GANDHI UNIVERSITY, KOTTAYAM
IN PARTIAL FULFILLMENT OF REQUIREMENTS FOR
AWARD OF THE DEGREE OF
"BACHELOR OF SCIENCE IN BOTANY"**

BY

LIBIYA GILBERT

REG NO: AB19BOT039



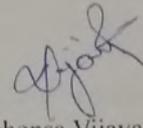
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May 2022**

CERTIFICATE

This is to certify that this dissertation entitled "MORPHOLOGICAL AND ANATOMICAL EVALUATION OF PTERIDOPHYTES FROM THE BOTANICAL GARDEN OF ST. TERESA'S COLLEGE OF ERNAKULAM DISTRICT" is an authentic record of research work carried out by Miss. Libiya Gilbert (AB19BOT039) under the supervision and guidance of Dr. Alphonsa Vijaya Joseph of St. Teresa's College (Autonomous), Ernakulam. I further certify that no part of the work embodied in the project has been submitted for the award of any other degree or diploma.



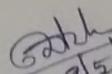
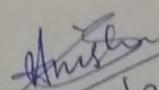
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Place: Ernakulam

Date: 09-05-2022

Libiya Gilbert

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**MORPHOLOGICAL AND ANATOMICAL EVALUATION OF
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INTRODUCTION

An ecosystem is a geographic area where plants, animals, and other organisms, as well as weather and landscape, work together to form a bubble of life. An ecosystem (or ecological system) consists of all the organisms and the physical environment with which they interact (Chapin and Stuart, 2011). These biotic and abiotic components are linked together through nutrient cycles and energy flows. Energy enters the system through photosynthesis and is incorporated into plant tissue. By feeding on plants and on one another, animals play an important role in the movement of matter and energy through the system. They also influence the quantity of plant and microbial biomass present. By breaking down dead organic matter, decomposers release carbon back to the atmosphere and facilitate nutrient cycling by converting nutrients stored in dead biomass back to a form that can be readily used by plants and microbes.

Plants are the incredibly important Kingdom of organisms. Kingdom Plantae includes all the plants. They are eukaryotic, multicellular and autotrophic organisms. Whittaker, in 1963, divided living organisms into five kingdoms and Kingdom Plantae. the Five Kingdom Grouping, categorizes all the living organisms into five territories – Protista, Monera, Fungi, Plantae, and Animalia. They are multicellular organisms with the amazing ability to make their own food from carbon dioxide in the atmosphere. They provide the foundation of any food webs and animal life would not exist if plants were not around. Man from time immortal has been dependent on the plant world for innumerable needs. This dependency urged even the prehistoric man to identify the plants and also to classify them into different groups such as food plants, poisonous plants, and medicinal plants etc. Which are important for him. This was the beginning of plant taxonomy which induced the identification, nomenclature and classification of plants. From the simple method of recognition of economically useful plants by the earliest man, today it has become a highly complex and all-embracing biological science. Plants are vital for many for groups of living being having spread nearly all around the world, plants have many various forms and structures as a result of living conditions, genetic structure and phylogenetic characteristics.

Pteridophytes are an important group of epiphyte plants, of which 29% are epiphyte Pteridophyte means (pteron- feather, phyton- plant) the name was originally given to those

groups of plants which have well developed pinnate or frond like leaves. Pteridophytes is a vascular plant that disperses spores. Because, pteridophytes produce neither flower nor seeds, they are sometimes referred to as “Cryptogams”, meaning that their means of reproduction is hidden ferns, horsetails and lycophytes are all pteridophytes. Therefore, these plants are also known as vascular cryptogams or snakes of plant kingdom. They are represented by about 400 living and fossil genera and some 10,500 species. Palaeobotanical studies reveal that these plants were dominant on the earth during the Devonian period and they were originated about 400 million years ago in the Silurian period of the Palaeozoic era. Earliest known Pteridophyte is Cooksonia.

Majority of the living Pteridophytes are terrestrial and prefer to grow in cool, moist and shady places e.g., ferns. Some members are aquatic (eg., *Marsilea*, *Azolla*), xerophytic (eg., *Selaginella*). Majority of the living Pteridophytes are terrestrial and prefer to grow in cool, moist and shady places e.g., ferns. Some members are aquatic (e.g., *Marsilea*, *Azolla*), xerophytic (e.g., *Selaginella rupestris*, *Equisetum*) or epiphytic (e.g., *Lycopodium squarrosum*). Pteridophytes are herbaceous but a few are perennial and tree like (e.g., *Angiopteris*). Smallest Pteridophyte is *Azolla* (an aquatic fern) and largest is *Cyathea* (tree fern). Plant body is sporophytic that can be differentiated in to root, stem and leaves. Roots are adventitious in nature with monopodial or dichotomous branching. Internally usually they are diarch. Stem is usually branched. Branching is monopodial or dichotomous. Branches do not arise in the axil of the leaves. In many pteridophytes stem is represented by rhizome. Leaves may be small, thin, scaly and sessile (eg: *selginella*) or large and pinnately compound (eg: *dryopteris*, *adiantum*). Cambium is absent; hence, they do not show secondary growth.

Reproduction take place by means of spores which produced inside sporangia. Water is essential for fertilization (zooidogamous). Therefore, pteridophytes are also known as Amphibians of plant kingdom. Fertilization results in the well-developed formation of zygote or oospore, which ultimately develops in to well developed sporophyte. The fertilization egg divides transversely or vertically. Another cross wall from a quadrant stage producing stem, leaf, foot and root. Plants shows heteromorphic alternations of generation. The main plant body is sporophytic and forms a dominant phase in the life cycle

Pteridophytes are little economic value. Many species of Pteridophyte are grown as ornamental plants (eg: *selginella*). Plants is a ball like structure under dry condition and an availability of water. It become green and flat on soil. Other species like *Lycopodium*, *osmanda*, *polypodium*, *pteridium* etc are also grown in gardens. Many species of Pteridophyte are used in soil conservation (eg: *Lycopodium*, *Selginella* etc). They also used as a medicine. Extract of *Lycopodium* plants are used as kidney stimulant. *Lycopodium clavatum* is used in skin diseases. *Equisitum arvense* is used as diuretic (promoting urine discharge). Rhizome and frond bases of *dryopteris filix - max* are used as taenifuge. Several species of *Lycopodium* (eg: *L.obscurum*) are used in Christmas wreaths and other decorations. It is commonly called Christmas green. *Equisitum* deposit large amount of silica in their cell wall. So formerly, it was used in cleaning and polishing the metal pots. Therefore, the plants has been given the name 'scouring rushes'. *Equisitum arvense* in biological indicators (for the presence of gold in soil). Young shoot of *dryopteris filix-max* are used as vegetables. Starchy paste of sporocarps of *marsilea drummandii* is used in making cakes and is called ' nardoo'. Axola is groun in the rice fields to maintain its fertility because it has the symbiotic association with cyanobacteria (blue green algae) *anabaena*, *nostoc*.

Selaginella - It is a sole genus of vascular plants in the family *selaginellaceae*, occurs mostly in the tropical regions of the world, with in the handful of species to be found in the artic - alpine zones of both hemispheres. Under dry conditions, some species of *selaginella* can survive dehydration in this state, they may also rollup into brown balls and be uprooted, but can rehydrated under moist condition became green again and resume growth. This phenomenon is known as *poikilohydry*.

Lygodium - *Lygodium* (climbing fern) is a genus of about 40 species of ferns, native to tropical regions across the world, with a few temperate species in eastern Asia and eastern North America. It is the sole genus in the family *Lygodiaceae* in the Pteridophyte Phylogeny Group classification of 2016 (PPG I). *Lygodium* are unusual in that the rachis, or midrib, of the frond is thin, flexible, and long, the frond unrolling with indeterminate growth and the rachis twining around supports, so that each frond forms a distinct vine. *Lygodium* species, known as *nito*, are used as a source of fibers in the Philippines. The fibers are used as material for weaving, most notably of traditional *salakot* headgear.

Equisitum – *Equisetum* (horsetail, snake grass, puzzlegrass) is the only living genus in *Equisetaceae*, a family of ferns, which reproduce by spores rather than seeds (Dunmire et al,

1995). *Equisetum* leaves are greatly reduced and usually non-photosynthetic. They contain a single, non-branching vascular trace, which is the defining feature of microphylls. However, it has recently been recognised that horsetail microphylls are probably not ancestral as in lycophytes (clubmosses and relatives), but rather derived adaptations, evolved by reduction of megaphylls (Rutishauser, 1999).

Angiopteris - It is a genus of huge evergreen ferns that are found throughout the paleotropics from Madagascar to south Pacific Island. This is a large, attractive, hardy, ornamental ferns, for shady sites. *Angiopteris* is unique among ferns in having explosively dispersed spores. Thought to be caused by the cavitation of an airspace between spores layers. Some leaves are edible. For medical uses, the leaves are pounded to relieve coughs, the roots are used to stop bleeding after miscarriage. The rhizomes are used medicinally in Thailand. (Murdock and Andrew, 2008)

Microsorium - *Microsorium* is a genus of ferns in the family Polypodiaceae, subfamily Microsoroideae, according to the Pteridophyte Phylogeny Group classification of 2016 (PPG I). The species are tropical. Like most ferns, they grow from rhizomes, rather than roots. The genus name is often misspelled "Microsorium" or "Microsoreum". It includes some species that are lithophytic rheophytes.

Nephrolepis - *Nephrolepis* is a genus of about 30 species of ferns. It is the only genus in the family Nephrolepidaceae, placed in the suborder Aspleniineae (eupolypods I) of the order Polypodiales in the Pteridophyte Phylogeny Group classification of 2016 (PPG I). They are semi-evergreen ferns from tropical and subtropical regions around the world. Some are terrestrial and some are epiphytic. Dense mounds of feather-like fronds in shades of green and sometimes chartreuse make these ferns valuable foliage plants, both indoors and out. Many cultivars are sterile, or do not come true from spores. For those that are fertile, sow spores at 70°F when ripe. Separate rooted runners in late winter or early spring.

Pteris - *pteris* is a genus of about 300 species ferns. They are native tropical and subtropical regions of the world. Many of them have linear frond segments and some have sub-palmate division. Although it grows readily in the wild it is sometimes cultivated. It is grown in gardens for its attractive appearance, or used for pollution control schemes. Some of these ferns are popular in cultivation as houseplants. These smaller species are often called table ferns.

Marsilea -*Marsilea* is a genus of approximately 65 species of aquatic ferns of the family Marsileaceae. The name honours Italian naturalist Luigi Ferdinando Marsili (1656–1730). These small plants are of unusual appearance and do not resemble common ferns. Common names include water clover and four-leaf clover because of the long-stalked leaves have four clover-like lobes and are either present above water or submerged. The sporocarps of some Australian species are very drought-resistant, surviving up to 100 years in dry conditions. On wetting, the gelatinous interior of the sporocarp swells, splitting it and releasing a worm-like mass that carries sori, eventually leading to germination of spores and fertilization.

Adiantum - *Adiantum* the maidenhair fern, is a genus of about 250 species of ferns in the subfamily Vittarioideae of the family Pteridaceae, though some researchers place it in its own family, Adiantaceae. The genus name comes from Greek, meaning "unwetted", referring to the fronds' ability to shed water without becoming wet (Christenhusz *et al.*, 2011). They are distinctive in appearance, with dark, often black stipes and rachises, and bright green, often delicately cut leaf tissue. The sori are borne submarginally, and are covered by reflexed flaps of leaf tissue which resemble indusia. Dimorphism between sterile and fertile fronds is generally subtle.

Ernakulam district is in the state of Kerala, which is known as God's own country. The flora of the Ernakulam district is tropical in nature. The heavy rainfall combined with moderate temperature and fertile soil support luxuriant vegetation. Many of the common plants are found in the coastal area which forms the low land region. The Mangalavanam bird sanctuary in Ernakulam district is the "green lung of Kochi", considering its role in controlling city's air pollution. St. Teresa's College is a prestigious autonomous institution situated in the centre of Ernakulam district. The campus botanical garden possesses a vast range of flora

So, the study is concerned with the morphological and anatomical evaluation of Pteridophytes from the botanical garden of St. Teresa's College Ernakulam district.

OBJECTIVES OF THE STUDY

- Survey of Pteridophytes in the Botanical Garden of St. Teresa's College, Ernakulam.
- Collection of fresh samples of Pteridophytes from the study area.
- Morphological evaluation of collected Pteridophytes.
- Anatomical evaluation of collected Pteridophytes.

REVIEW OF LITERATURE

Selaginella, *Lygodium*, *Equisitum*, *Angiopteris*, *Microsorium*, *Marsilea*, *Pteris*, *Adiantum* these are great genus, that come under pteridophytes division that has attracted researchers from various field of botanical science and laymen a like. Since long, plant explores, morphologist, anatomist, taxonomist etc. They contributed these works for our understanding.

(William and Terry, 1976) gave a sparse description and Comparative studies of the anatomy and morphology of *S. apoda* (L.) Spring and *S. ludoviciana* A. Br. were undertaken as a basis for understanding the anatomy and taxonomy of the two taxa. *Selaginella ludoviciana* has an erect habit, while *S. apoda* remains prostrate. Both species branch pseudomonopodially. The microphylls of *S. ludoviciana* have a hyaline border, whereas a distinctive differentiated margin is lacking in the leaves of *S. apoda*. Differences in leaf morphology, stomatal distribution and leaf anatomy occur in the taxa. In both species, adventitious roots arise at the dichotomies of the stem and exhibit a monarch form and exarch xylem maturation. The stelar pattern of the stem is protostelic, with the stele suspended by trabeculae in a lacuna. Xylem maturation is exarch in the stem. A basic nodal pattern of a single, unbranched trace associated with no leaf gap occurs in both species. The taxonomic recognition of a single species versus two species is discussed.

(Robert John Harvey-Gibson ,1902) give a brief description about Contributions towards a knowledge of the anatomy of the genus *Selaginella*. Hofmeister, it is true, makes a brief reference to the subject, but confines his remarks to certain general points with regard to the origin and branching of the roots. He speaks of adventitious roots arising at the forkings of the stem in such species as 5. *Denticulata*, *Helvetica*, *Martensii*, and either generally throughout its length or on the basal region of the stem only. The root, Hofmeister says, arises in the axil of the ventral leaf situated at the forking of the shoot axis, and from the outer side of the cross-band which unites the vascular systems of the two branches (eg. *denticulata*). The roots branch freely, the first forking

being in the plane of the leaf in whose axil the root arises, and the second branching being at right angles to the first. Hofmeister also draws attention to the swelling.

(Uma and Ganeshaiah,1997) gave a sparse description about the wide spectrum of surface structural and anatomical details of the Chinese brake fern (*Pteris vittata*) using scanning electron microscopy (SEM). SEM revealed that the epidermal cells of the pinnae were elongated with raised periclinal and sinuous anticlinal walls. The pinnae were hypostomatous with randomly scattered anomocytic stomatal complexes positioned at the same level as the epidermis. Stomates were large and elliptical (27.4 μm \times 10.2 μm). Cross sections from the central regions of the rachis and the stipe revealed V-and U-shaped vascular bundles, respectively. In each vascular bundle, the xylem strands were sea-horse shaped (hippocampus). In contrast, the pinnae possessed a triangular vascular bundle with uniform mesophyll organization comprising of homogenous lobed parenchyma cells. The indumentum consisted of trichomes and scales, which formed various types of vestiture. Trichomes were borne only on the pinnae and scales on the rachis and stipe. The roots developed a dense network of long root hairs averaging 244 μm long, and the xylem consisted of tracheids with scalariform pitting. Sori were submarginal; continuous along both margins of the pinna and were covered with a false indusium. The sporangia were oblong with a short thick stalk and the annulus was positioned vertically resulting in transverse dehiscence of the sporangium. The paraphyses were uniseriate, unbranched, septate and found to be intermixed with the sporangia. The exine of the globose spores was adorned with thick reticulum in which the areoles contained round tubercles. This study describes surface features in detail, which is essential to studies examining the issue of whether morphological characteristics are related to arsenic hyperaccumulation in *P. vittata*.

MATERIALS AND METHODS

Study area:

The Botanical Garden of St. Teresa's College has been selected as the area for study.

Survey of plant samples from the study area:

Cryptogams were selected as the plant samples for the study. A detailed survey has been conducted for the collection of plant samples. 9 species of cryptogams were found in the study area.

Collection of plant samples:

The fresh plant samples such as Selaginella, Equisetum, Angiopteris, Microsorium, Lygodium, Nephrolepis, Pteris, Adiantum and Marsilea were collected for the study from the study area.

Morphological evaluation of collected plant samples:

Fresh plant samples were collected from the study area and morphological characters were analyzed and recorded.

Anatomical evaluation of collected plant samples:

For examining anatomical characteristics, a thin cross-section of the stem or is taken by using a clean and sharp blade. Then the cross-section was mounted with safranin stain and glycerine on a clean glass slide and observed under a compound microscope. The anatomical features were recorded and photographs were taken.

OBSERVATION AND RESULTS

1. *SELAGINELLA*

Selaginella is a fascinating plant that spreads and acts like a moss. It is also called Spike moss or club moss. It is the largest and only living genus of the family Selaginellaceae. They were found in a shady area.



PLATE 1: HABITAT OF *SELAGINELLA*

TAXONOMIC POSITION

Kingdom: Plantae

Class : Lycopodiopsida

Order : Selaginellales

Family : Selaginellaceae

Genus : Selaginella

MORPHOLOGICAL EVALUATION OF SELAGINELLA

SPOROPHYTE

- The sporophyte is an evergreen, delicate herb.
- Plants are found to be erect. The plant body consists of Root, Stem, Leaves, Ligules and Rhizophores

ROOT

- The root of the young sporophyte is the primary root while others are adventitious.
- Aerial roots have developed caps, and cutinized epidermal cells enter the soil.
- Their origin is endogenous. They originate either from the tips of rhizophores or directly from the stem or from the swollen base of the hypocotyl.

STEM

- The stem is profusely branched, delicate and evergreen.
- The branching is of monopodial type.

LEAVES

- Leaves are small, simple and lanceolate with a pointed apex.
- Each leaf is provided with a single unbranched midrib.
- Leaves near the apical portion of the branch, bear sporangia (micro-or mega) and are called sporophylls (micro-or mega) respectively.
- The sporophylls are usually aggregated into a condensed structure which is known as a strobilus.
- Small leaves are present on the dorsal side of the stem and bigger ones on the ventral side of the stem.
- Microphylls are present, Anisophyllous and Isophyllous based on the unequal and equal size of leaves.

LIGULES

- The Ligules are found on the adaxial side of the leaf and are a small membranous out-growth present at the base of the leaf in a pit-like structure known as a ligule pit.

- The structure of the ligule consists of two parts, glossopodium and the body of the ligule

RHIZOPHORES

- It is a colourless, leafless, unbranched and cylindrical structure.
- A tuft of adventitious roots gets developed when it touches the soil.

ANATOMICAL EVALUATION OF *SELAGINELLA* STEM

A Transverse section (T.S.) of the stem of *Selaginella* is somewhat circular in outline and shows the following structures:

- 1.Epidermis
- 2.Cortex
- 3.Stele

EPIDERMIS

- It is the outermost covering layer comprising of a single cell in thickness.
- The epidermal cells are covered by a thick coating of cuticle.
- Hairs and stomata are absent.

CORTEX

- It is seen inner to the epidermis.
- The cortex is differentiated into the inner and outer cortex.
- It is made up of parenchymatous cells.
- The parenchymatous cortex is made up of angular cells without intercellular spaces

STELE

- The central portion of the stem is occupied by a well-developed stele.
- The stele is of protostelic type i.e., the xylem is present in the center and surrounded by phloem on all sides.
- Phloem in turn, is surrounded by a single-layered pericycle.

- Pith is absent.
- The stele is surrounded by a single - layered pericycle made of parenchymatous cells.

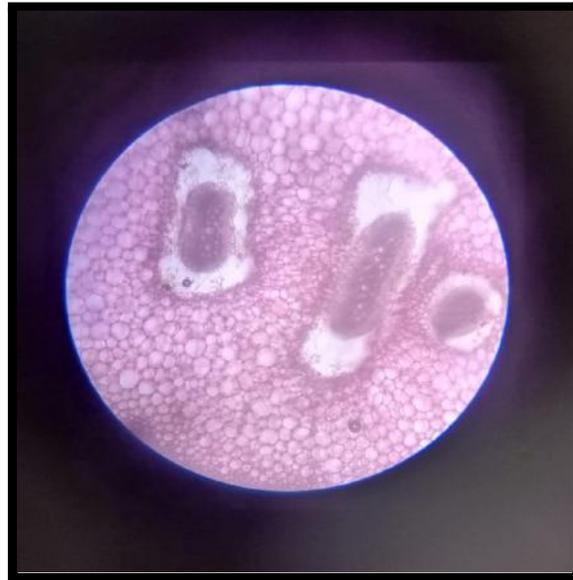


PLATE 2: T.S OF *SELAGINELLA* STEM

2. *LYGODIUM*

It is an evergreen fern with climbing fronds. They are epiphytic and perennial in nature. It grows thick into mats which smother the undergrowth and up over shrubby trees. It is found growing in moist and shady places which are rich in humus and other organic matters,



PLATE 3: HABITAT OF *LYGODIUM*

TAXONOMIC POSITION

Kingdom: Plantae
Class : Polypodiopsida
Order : Schizaeles
Family : Lygodiaceae
Genus : Lygodium

MORPHOLOGICAL EVALUATION OF *LYGODIUM*

SPOROPHYTE

- The plant body consists of leaves, stem and creeping rhizome

STEM

- Dichotomously branched, Indeterminate rachis growth

LEAVES

- Pinnately compound leaves

ANATOMICAL EVALUATION OF *LYGODIUM* STEM

A transverse section (T.S.) of the stem of *Lygodium* is circular in outline.

EPIDERMIS

- Single-layered and broader Parenchymatous cells

CORTEX

- The cortex is differentiated into outer and inner sclerenchymatous cells and middle parenchymatous cells.
- The whole of the cortex is made up of parenchymatous cells with small or large intercellular spaces and the sclerenchymatous cells, without intercellular spaces.

STELE

- Protostelic type. Stele comprises primary xylem and primary phloem.
- pith is absent and the stele is situated in the centre.



PLATE 4: T.S OF *LYGODIUM* STEM

3. *EQUISETUM*



PLATE 5: HABITAT OF *EQUISETUM*

TAXONOMIC POSITION

- Kingdom:** Plantae
Class : Polypodiopsida
Order : Equisetales
Family : Equisetaceae
Genus : Equisetum

MORPHOLOGICAL EVALUATION OF *EQUISETUM*

SPOROPHYTE

- The plant body is sporophyte and erect.
- The sporophyte is differentiated into root, stem and leaves.

STEM

- Stem consists of underground rhizome and upright green branches.

- Jointed stem with nodes and internodes with longitudinal ridges and furrows.

LEAVES

- Silica deposits in stem make it rough.
- Leaves nodes with small, sessile microphyllous scale leaves in whorls.
- Fertile branches bear strobili after some vegetative growth.

ROOTS

- Adventitious roots arise from nodes of rhizome.
- Roots are photosynthetic in nature.

SPORES

- *Equisetum* is homosporous and Eusporangiate.
- Strobili are borne terminally and singly on aerial fertile branches.
- Strobilus consists of a central axis on which stalked sporangiophores with sporangium are arranged in whorls.

ANATOMICAL EVALUATION OF *EQUISETUM* STEM

A transverse - section of the *Equisetum* stem is wavy in outline because of the presence of ridges and grooves. It includes Epidermis, Cortex and Stele

EPIDERMIS

- The epidermis is single-layered with stomata and heavily coated with silica deposits.

CORTEX

- Outer cortex is sclerenchymatous and chlorenchymatous
- Inner cortex is made up of large parenchymatous cells with vallecular
- Vallecular canals are large air-filled, intercellular spaces below the furrows in the inner cortex.
- It shows a hydrophytic character.
- Endodermis and pericycle single layered.

STELE

- Xylem is V-shaped.
- Protoxylem is endarch lying opposite to carinal cavity.
- Two strands of metaxylem are present.
- Phloem is present between two strands of metaxylem and made up of phloem parenchyma and sieve tubes.
- Pith is present in the form of pith cavity, located in the centre of the aerial shoot
- A water-containing cavity is present in each vascular bundle known as a carinal canal
- The Carinal canal is a water-filled region present in the vascular bundle.

PITH

- Pith is large central cavity filled with water.

XEROPHYTIC CHARACTERS

- Presence of ridges and furrows.
- Presence of sunken stomata.
- Presence of well-developed sclerenchymatous hypodermis.
- Presence of reduced and scaly leaves.
- Presence of well-developed vascular cylinder.

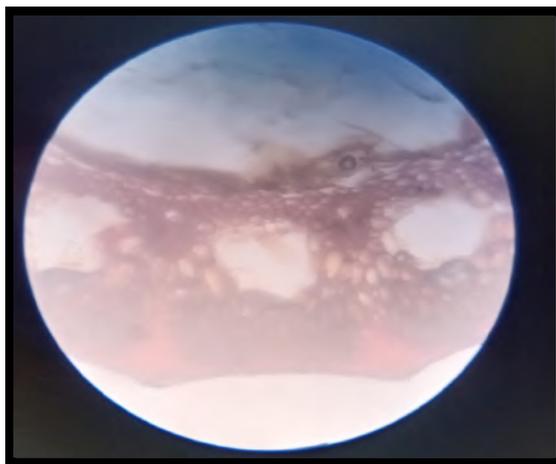


PLATE 6: T.S OF *EQUISETUM* STEM

4. *ANGIOPTERIS*

Angiopteris is an evergreen fern plant. They are unique among ferns in having explosively dispersed spores.



PLATE 7: HABITAT OF *ANGIOPTERIS*

TAXONOMIC POSITION

- Kingdom: Plantae**
- Class : Polypodiopsida**
- Order : Marattiales**
- Family : Marattiaceae**
- Genus : Angiopteris**

MORPHOLOGICAL CHARACTER

SPOROPHYTE

- The sporophytic plant body consists of an upright, tuberous, conical, fleshy rhizomatous stem.

STEM

- The stem is thick and inhabits the plant resembles a tree fern.

- The stem is often called a caudex or trunk which may be a foot or two in height and almost the same girth.

RHIZOME

- The upper surface of the rhizome bears a crown of graceful, stately leaves.

LEAVES

- Leaves are deciduous.
- The leaves are 5-6 metres long in a luxuriously growing plant, with a petiole as thick as a man's arm.
- The leaves are typically bi-pinnately compound.
- The venation is of the open dichotomous type Pinnae are glabrous (smooth).
- A pair of thick fleshy stipules at the base of the leaf is present.
- When the leaves fall off, these persist with the leaf bases and form a protective armour around the stem.
- The base of the petiole and the stipules together appear like a horse's hoof.
- The pinnae are long, dorsoventrally flattened and have a long drawn tip.
- The margin is serrate.
- The sori occupy a near-terminal position on the dichotomously branched veins.

ROOTS

- Roots are produced from the undersurface of the rhizome at the base of each leaf.
- Root hairs are peculiar in being multicellular.
- At the margins of the pinnae on the abaxial surface are borne, the sori.
- Roots are perennial, thick and have a mycorrhizal association.

ANATOMICAL CHARACTERS

Transverse section of petiole shows single layered epidermis which consists of thin walled cells .The bulk of petiole is composed of ground tissue. It is differentiated into three zones.

EPIDERMIS

- single-layered epidermis which consists of thin-walled cells.

CORTEX

- The bulk of petiole is composed of ground tissue and is differentiated into three zones.
- The outermost zones consist of 3-4 layers of cells which are made up of thin-walled parenchymatous cells.
- The middle zone consists of 3-4 layers of cells made up of thick-walled sclerenchymatous cells, being comparatively smaller in size than the cells of the outer and inner zone.
- Some of the cells of the middle and inner zone contain tannin.
- The endodermis is followed by a pericycle containing thin-walled cells, which are 1-3 layers in thickness.
- Xylem lies in the centre of the vascular strand. It is plate-like with several protoxylem points in exarch conditions.
- Xylem is surrounded by phloem.
- Phloem consists of sieve cells and parenchyma and xylem have simple tracheids of various sizes.
- Metaxylem tracheids have scalariform and pitted thickening while protoxylem tracheids have annular and spiral thickening.

STARCH GRAINS

- The inner zone consists of large and thin-walled polygonal cells filled with starch grains.
- Starch grains are usually large and spherical or oval in shape.
- The concentrations of these grains are more towards the base of the petiole and gradually decrease towards the apex.



PLATE 8: C.S OF *ANGIOPTERIS* PETIOLE

5. *MICROSORUM*

Microsorum is an epiphyte and perennial in nature. They grow from rhizomes, rather than roots.

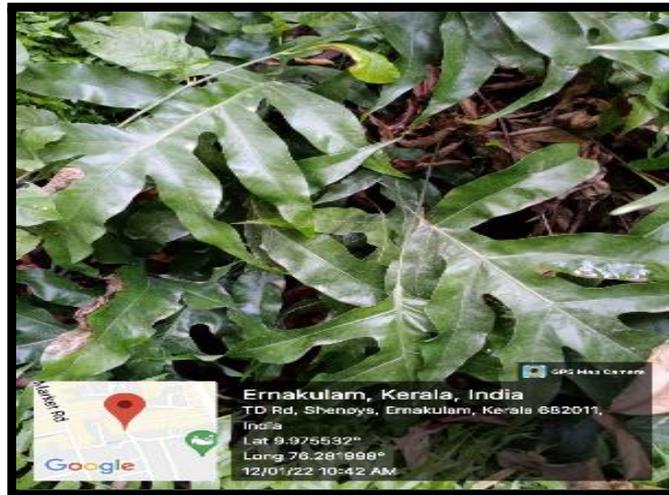


PLATE 9: HABITAT OF *MICROSORUM*

TAXONOMIC POSITION

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Polypodiaceae

Genus : *Microsorum*

MORPHOLOGICAL EVALUATION OF *MICROSORUM*

SPOROPHYTE

- The plant body consists of hard stem and long petiole.

LEAVES

- Lanceolate shape, pointed tip, the winged base of leaf, midrib is raised and highly prominent.

RHIZOME

- The underground horizontal stem is known as the rhizome

SPORES

- occur on the frond underside in small and dark brown spots

ANATOMICAL EVALUATION OF *MICROSORUM* PETIOLE

EPIDERMIS

- single-layered and parenchymatous

CORTEX

- It made up of parenchymatous cells.

STELE

- Vascular bundles are present.
- Endodermis is present



PLATE 10: T.S OF *MICROSORUM*_PETIOLE

6. *NEPHROLEPIS*

It is an epiphytic fern. Feather-like fronds in shades of green make these ferns valuable.



PLATE 11: HABITAT OF *NEPHROLEPIS*

TAXONOMIC POSITION

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Nephrolepidaceae

Genus : Nephrolepis

MORPHOLOGICAL EVALUATION OF *NEPHROLEPIS*

SPOROPHYTE

- The plant body is sporophytic differentiated into rhizomes, roots and leaves.

RHIZOME

- Rhizome is short, slender and wide creeping.
- It bears a close tuft of leaves and long, slender lateral branches called runners.
- Runners bear adventitious roots and in acropetal succession.

LEAVES

- The leaves are tufted, long, narrow and simply with fish tail.
- Margins are present.

ANATOMICAL EVALUATION OF *NEPHROLEPIS* STEM

The transverse section of the petiole has an adaxial groove.

EPIDERMIS

- It is composed of small thick walled cells.

CORTEX

- Epidermis is followed by 3-4 layers of sclerenchymatous cortex and compact parenchyma.
- Conducting strands are embedded in the parenchymatous cortex and are arranged in a horse - shoe like shape.

STELE

- Protoxylem lies towards the outside.
- Phloem completely surrounds xylem which in turn is surrounded by a layer of pericycle and endodermis.

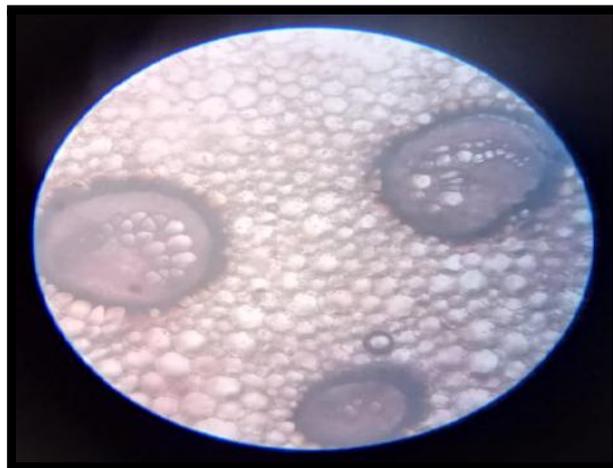


PLATE 12: T.S OF *NEPHROLEPIS* STEM

7. *PTERIS*

They inhabit shady and moist areas.



PLATE 13: HABITAT OF *PTERIS*

TAXONOMIC POSITION

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Pteridaceae

Genus : Pteris

MORPHOLOGICAL EVALUATION OF *PTERIS* .

SPOROPHYTE: The sporophytic plant body is differentiated into

- 1.Root
- 2.Rhizomatous stem
- 3.Leaves

ROOT

- The primary root is ephemeral, and is replaced by a large number of adventitious roots developed all over the surface of the rhizome.
- The roots are small and branched

RHIZOME

- The rhizome or stem may be creeping (*P. grandiflora*) or erect (*P. cretica*, *P. vittata*) which may or may not show branching.
- The rhizome is differentiated into nodes and internodes and its entire surface is covered with scales.
- The growing point of the rhizome is covered withramenta.
- Even the diversity is noted in different regions of the rhizome in the same species.

LEAVES

- The leaves are borne on the upper surface of the rhizome.
- When young the leaves are spirally coiled and show circinate vernation that is typical of true ferns
- The leaves are unipinnately or multi pinnately compound or decompound with a long rachis
- The pinnae are small near the base as well as towards the apex, while they are large towards the middle.

ANATOMICAL EVALUATION OF *PTERIS* PETIOLE

EPIDERMIS

- single-layered and covered with cuticle.
- Ramenta arise from the epidermis

CORTEX

- Differentiated into outer sclerenchymatous and inner parenchymatous zone

STELE

- Xylem has two adaxial hooks; xylem is surrounded by phloem.

- Pericycle and endodermis is present

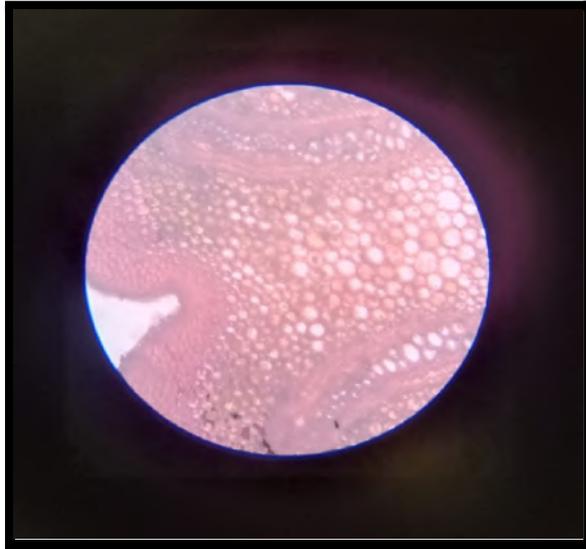


PLATE 14: T.S OF *PTERIS* PETIOLE

8. *MARSILEA*

Marsilea is a herbaceous plant. It is also known as a water clover plant and four-leaf clover plant. It does not resemble common ferns. They are either present above water or submerged.

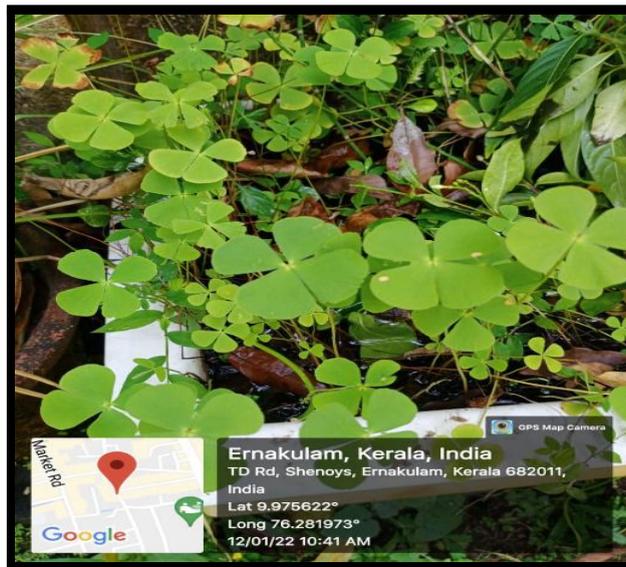


PLATE 15: HABITAT OF *MARSILEA*

TAXONOMIC POSITION

Kingdom: Plantae

Class : Polypodiopsida

Order : Salviniiales

Family : Marsileaceae

Genus : Marsilea

MORPHOLOGICAL EVALUATION OF *MARSILEA*

SPOROPHYTE

- The plant body is differentiated into rhizomes, leaves and roots.

RHIZOME

- It is slender, dichotomously branched with distinct nodes and internodes and is capable of indefinite growth in all directions.

LEAVES

- They are borne alternately on the upper side of the rhizome at nodes, in two rows.
- They show circinate vernation.
- In submerged plants the petiole is long and the lamina floats over the surface of the water but in muddy or marshy plants the petiole of the leaf is short and rigid with short lamina spreading in the air.
- The lamina consists of 4 leaflets/pinnae which are present at the apex of the petiole.
- Near the base of the petiole, the stalked bean-shaped sporocarps are borne.

ROOTS

- The roots are adventitious, arising from the underside of the node of the rhizome, either singly or in groups.

ANATOMICAL EVALUATION OF *MARSILEA* PETIOLE

EPIDERMIS

- The outermost layer is the epidermis which consists of rectangular cells.
- Below the epidermis is the hypodermis followed by the cortex.

CORTEX

- The cortex is differentiated into the outer and inner cortex.
- The outer cortex consists of aerenchyma having many air cavities or air chambers separated from each other with the help of one-celled thick trabeculae or septa.
- The inner cortex is parenchymatous and contains starch and tannin-filled cells.

STELE

- The stele is triangular in shape, the present shows the protostelic structure.
- The stele is bounded by a layer of endodermis and an unilayered pericycle.
- Xylem is V-shaped and the arms of 'V' contain a metaxylem in the center and protoxylem towards the ends.
- The xylem remains surrounded by the phloem.

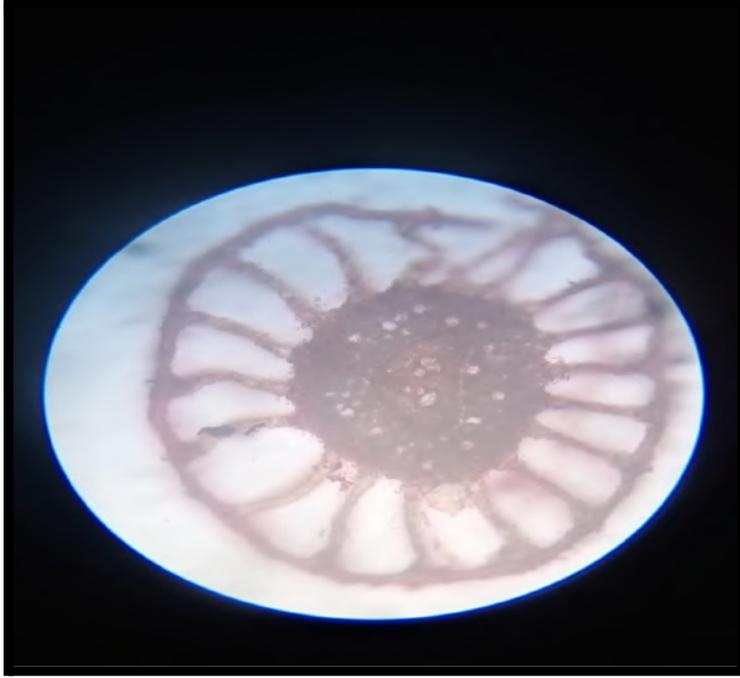


PLATE 16: ANATOMY OF *MARSILEA* PETIOLE

8. *ADIANTUM*

Adiantum is also known as maidenhair fern or walking fern. They are small, perennial and evergreen plant.



PLATE 17: HABITAT OF *ADIANTUM*

TAXONOMIC POSITION

Kingdom: Plantae

Class : Polypodiopsida

Order : Polypodiales

Family : Nephrolepidaceae

Genus : Nephrolepis

MORPHOLOGICAL EVALUATION OF *ADIANTUM*

SPOROPHYTE

- The plant is divided into stem, root and leaves.

RHIZOME

- Rhizome grows horizontally near the soil surface.
- Scales called palea covered the surface of rhizome.

LEAVES

- Leaves of *Adiantum* are called fronds.
- These leaves are large, about 4-6 inches in length and are bipinnately compound.
- Leaflets of the first order are called pinnae and leaflets of second order are called pinnules.
- Main axis of leaf on which leaflets are produced is rachis.
- The rachis is black in color and shiny.
- The leaves are produced in acropetalous succession on the creeping rhizome.
- They show circinate venation.
- The pinnae are stalked and have a dichotomous venation.
- There is no distinction between fertile and sterile leaves.
- The whole leaf may be sporangiferous or only certain pinnae may bear sporangia.
- The soral organisation is very evident.
- Sori are borne on the ventral surface of the pinnae.

ANATOMICAL EVALUATION OF *ADIANTUM* PETIOLE

EPIDERMIS

- The petiole in T.S. shows a single-layered epidermis with a thick cuticle.
- The epidermis is followed by a sclerenchymatous hypodermis which provides mechanical support.

CORTEX

- Consists of parenchymatous cells.
- The central region possesses a single large horseshoe-shaped stele.
- Xylem forms central core surrounded by phloem.

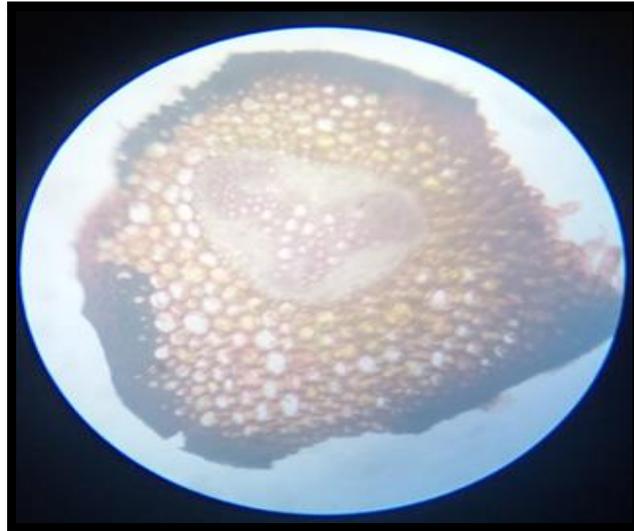


PLATE 18: ANATOMICAL EVALUATION OF *ADIANTUM* PETIOLE

DISCUSSION

Ferns and their allies are one of the Pteridophyta's oldest major divisions, with approximately 12 000 species scattered over 250 distinct genera (Baskaran et al., 2018). Sushruta (about 100 AD) and Charka (around 100 AD) of the biomedical and Ayurvedic schools of medicine, respectively, proposed the use of various ferns in the Samhita literature. Pteridophytes are also employed by physicians in the Unani medical system. Several ferns are suggested by native doctors in the traditional Chinese medical system (Kimura and Noro, 1965). Several researchers have recently conducted ethnobotanical and advanced pharmacological investigations on ferns and their companions. Most ferns and fern allies provided several health advantages to ancient civilizations that utilized them for food, tea, and medications. Modern techniques have merged interdisciplinary technology, as well as particular chemical components collected and identified, allowing the production of extremely particle medications from plant parts.

Plants that produce high quality and quantity of polysaccharides, steroids, terpenoids, flavonoids, alkaloids, and antibiotics are ideal for developing medications for a variety of ailments/diseases, including cancer therapies. Modern studies on the functional activities of pteridophytes for human health, such as the discovery of particular chemicals and their use in medications, have broadened the scope of pteridophytes, transforming these plants into a godsend for pharmaceutical businesses and associated industries. Plants that produce high levels of polysaccharides, steroids, terpenoids, flavonoids, alkaloids, and antibiotics are useful for producing drugs for a number of ailments/diseases, including cancer therapy. Modern research on the functional activities of pteridophytes for human health, such as the identification of specific compounds and their application in drugs, has widened the scope of pteridophytes, making these plants a boon to pharmaceutical companies and related industries. Earlier pteridophytes' pharmacological activity suggests an alternate therapy for treating human illnesses. Pteridophytes (ferns and fern allies) are an old lineage that humans have been discovering and exploiting for over 2000 years due to their beneficial characteristics as the earliest vascular plants. Previous research has demonstrated that the lycophyte *Selaginella* sp. has a wide range of pharmacological activity, including antioxidant, anti-inflammatory, anti-cancer, antidiabetic, antiviral, antibacterial, and anti-Alzheimer

capabilities. Among all the pteridophytes studied, taxa from the Pteridaceae, Polypodiaceae, and Adiantaceae showed the most therapeutic efficacy. According to our findings, several pteridophytes have characteristics that might be employed in alternative medicine to treat a variety of human ailments.

Ferns are common denominators of abundant and diverse biodiversity in practically every corner of the world. The comparison of evolutionary adaptations and natural innovations reveals the genetic underpinning for organism development. It is highlighted that good field stations with big greenhouses at the periphery of protected forests should work as 'Fernariums/ Mossariums/ and/or Lichenariums' to conserve and nurture rare, endangered, and medicinally outstanding species present in such areas/forests. Gene networks (DNA stretches) that preserve comparable wiring schematics (some or many similar DNA sequences) throughout related, distantly related, or completely different animals reveal how regulatory sections of the genome have developed. Without a doubt, comparative genomics can assist us in deciphering the evolvability of gene networks and conservation modes.

The potential use of Pteridophytes as Ecological Indicators is becoming more widely recognized. The use of pteridophytes as EIs is becoming more common. This demonstrates the group's significant potential as EIs, which is further backed by several studies using similar approaches, resulting in a huge number of species, genera, and families being proposed as EIs.

The ferns have also been shown to be having an important role in the bioremediation of wastewater. (Dudani et al., 2001) found the Chinese Bracken fern namely *Pteris vittata* L. to be a hyperaccumulator of the toxic metal Arsenic. Besides producing large biomass, they also found this fern to be efficient in accumulation with concentrations as high as 2.3% in the aerial portions of the fern. Later on, many researchers provided reports of the hyperaccumulation properties of *Pteris* as well as many other ferns also (Zhang, 2002) suggested that *P. vittata* could be an excellent model to study arsenic uptake, translocation, speciation, distribution and detoxification in plants and for phytoremediation of arsenic-contaminated soil and water.

Besides having all these wonderful properties, the pteridophytes are also greatly valued as ornamentals. Prior to the discovery of these benefits obtained from this group of plants,

ferns were used to enhance the beauty of the landscape and are continued to be used so till now. Ferns like *Adiantum sp.*, *Selaginella sp.*, *Lygodium sp.*, *Pteris sp.*, etc. are also grown in the gardens or in the pots.

The pteridophytes are moisture and shade-loving plants and are dependent upon the microclimatic conditions of the region for their successful survival in that region. Any kind of disturbance in these microclimatic conditions can hinder the growth and evolutionary processes occurring naturally in these plants thereby, leading to declining in their populations. Thus, factors like climate change, increasing urbanization, industrialization, encroachment of forest lands, unplanned developmental activities, and overexploitation of natural resources, pose a major threat to the survival of these groups of plants. Due to the unplanned felling of trees in the forests the members of epiphytic pteridophytes belonging to the families Polypodiaceae, Davalliaceae, Aspleniaceae, Vittariaceae, have been reduced day by day (Nambiar and Shimna, 2022). Large-scale collection of ferns from the forests by the visitors and local people for ornamental purpose, medicinal purposes and during excursions also increases the pressure on these plants.

Biodiversity conservation is the need of time and hence, it has become imperative to develop in situ and ex situ conservation methods for conservation of the diminishing biodiversity. The in-situ conservation is very beneficial as it allows the evolution of the species to continue within the area of natural occurrence. Hence, the steps for conserving the ferns in situ should be focused upon. The ex-situ conservation includes the development of botanical gardens or conservatories, germplasm banks, DNA banks, seed banks and involve the use of techniques such as tissue culture, cryopreservation; incorporation of disease, pest and stress tolerance traits through genetic transformation and ecological restoration of rare plant species and their populations (Pritchard et al., 2012). The conservation of flowering plants has been achieved to a good extent by developing conservatories and botanical gardens which also help in creating awareness among the local people. Developing a fern conservatory or fern garden is not preferred much and hence, such steps should be considered and implemented for conserving the rare and endangered species. The tissue culture is a very useful technique for the mass multiplication of the plant species in a short time and hence, research focusing on developing a protocol for in vitro regeneration of ferns and fern-allies should be encouraged. Parts of areas rich in abundant pteridophyte diversity can be declared

as pteridophyte biosphere reserves or small gene sanctuaries can be established to save the epiphytic pteridophytes.

CONCLUSION

The present study demonstrates the relevance of Pteridophytes in nature. Pteridophytes are an ancient lineage of plants, composed of ferns and fern allies, which are spread across the globe. There is also a long record of humans using pteridophytes to their benefit, which includes the broad categories of medicine, ornamentation, food, phytoremediation, and agriculture. Biopharmaceutical approaches can be used to preserve and even improve bioactive molecules for the development of anti-disease medications. Several studies have revealed that ferns have medicinal potential and an essential role in wastewater bioremediation.

There is a dearth of studies demonstrating their real usefulness and the criteria utilized to choose the Ecological indicators. The genetic foundations for organism development are revealed through evolutionary interactions and natural innovations. For the sake of the future, research into the development of pteridophytes should be supported. According to the current study, pteridophytes should be kept and safeguarded in the future. Pteridophytes are highly prized foliage ornamentals.

Documentation on the economic importance of pteridophytes is needed to reveal the importance of this plant group to the public and the indigenous knowledge about them. It is also important that field botanists should avoid the ruthless collection of rare species and make sure that they leave the bulk of plants to continue to grow and reproduce in the world. Providing proper awareness about the conservation of pteridophytes among the local people is needed. Further studies on pteridophytes can bring many more species that are of economic importance to light.

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**STUDIES ON THE ANTIBACTERIAL POTENTIAL OF
GRACILARIA CORTICATA (J. Agardh) J. Agardh IN ETHANOL AND
CHLOROFORM EXTRACTS**

**DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE AWARD OF THE DEGREE OF BACHELOR OF SCIENCE
IN
BOTANY**

By

MEREENA JOSE

AB19BOT041



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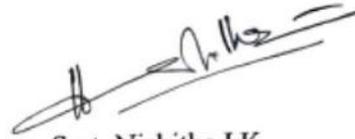
2022

CERTIFICATE

This is to certify that the dissertation entitled "Studies on the antibacterial potential of *Gracilaria corticata* (J. Agardh) J. Agardh in ethanol and chloroform extracts" is an authentic record of research work carried out by Miss Mereena Jose, AB19BOT041 under the supervision and guidance Smt. Nishitha I. K. Assistant Professor, Department of Botany and Centre for Research, St. Teresa's College (Autonomous), Emakulam, in partial fulfilment of the requirements for the award of the degree of Bachelor of Science in Botany. I further certify that no part of this work embodied in this project has been submitted for the award of any degree or diploma.

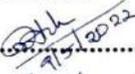


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- 1)  Anila N.
9/5/22
- 2)  Anisha S.
9/5/22



DECLARATION

I hereby declare that the project entitled “Studies on the antibacterial potential of *Gracilaria corticata* (J. Agardh) J. Agardh in ethanol and chloroform extracts” submitted to Mahatma Gandhi University, Kottayam, in partial fulfilment of the requirement for the Degree of Master of Science in Botany is an original project done by under the supervision and guidance of Ms. Nishitha I.K Department of Botany and Centre for Research, St. Teresa’s college (Autonomous), Ernakulam.

PLACE: ERNAKULAM

MEREENA JOSE

DATE:04/05/2022

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Place: Ernakulam

Mereena Jose

Date: 04/05/2022

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INTRODUCTION

Algae are diverse group of relatively simple, chlorophyll containing, photo-autotrophic and oxygen evolving aquatic thalloid (without differentiation into True roots, stems, leaves or leaf like organs) organisms. The word “algae” has its origin from Latin, where ‘alga’ means seaweed. The term algae was first used by Carolous Linnaeus in 1753. Most of them are photo-autotrophic but few are mixotrophic and myzotrophic (sucking through special feeding structure) study of algae is known as phycology (GK. Phykos- seaweed; logos= discourse Or study) or algology.

Algae are divided into nine main phylums, they are Phylum Rhodophycophyta, Phylum Xanthophycophyta, Phylum Chrysophycophyta, Phylum Phaeophycophyta, Phylum Bacillariophycophyta, Phylum Euglenophycophyta, Phylum Chlorophycophyta, Phylum Cryptophycophyta, and Phylum Pyrrophyta.

(<https://www.slideshare.net/BIYYANISUMAN/algae-suman-81289656>)

The term "seaweed" refers to a variety of marine plants and algae that can be found in the ocean, rivers, lakes, and other bodies of water. Some seaweeds, such as phytoplankton, are small and remain floating in the water column, providing the foundation for most aquatic food chains. Some are massive, such as the giant kelp that grow in dense forests. The large percentage are medium-sized, with red, green, brown, and black colours, and sometimes may wash up on beaches and shorelines. (Guiry, Michael D., 2014)

Marine algae from Indian coasts amounting to 844 species (including forma and varieties) are distributed among 217 genera. They grow in the intertidal, shallow and deep sea areas up to 180 meter depth and also in estuaries, backwaters and lagoons on solid substrates such as rocks, dead corals, pebbles, shells, mangroves and other plant materials (Anatharaman *et al.*, 2007; Sakthivel, 2007).

Mostly seen seaweeds are macro algae. They are of different types according to the colour of pigments present: brown algae (phylum Ochrophyta, class Phaeophyceae), red algae (phylum Rhodophyta, *Gelidium*), and green algae (phylum Chlorophyta, classes Chlorophyceae, Ulvophyceae etc. They differ significantly in many ultrastructural and biochemical functions, including photosynthetic pigments, storage molecules, cell wall composition, presence/absence of flagella, mitosis ultrastructure, linkage between cells, and structure of the chloroplasts, in addition to pigmentation.

Seaweed is high in vitamins, minerals, and fibre, as well as being palatable. The Japanese have a dish, 'sushi' which is nori seaweed wrapped with a mixture of fish, rice, and other ingredients for at least 1,500 years. Anti-inflammatory and anti-microbial compounds can be found in a variety of seaweeds. For thousands of years, their medicinal properties have been used; it was used to cure wounds, burns, and rashes by the ancient Romans. According to anecdotal evidence, the ancient Egyptians may have employed them to cure breast cancer. (McLachlan, J., and C. J. Bird, 1984).

In recent years, focus towards these organisms has increased due to their food and fuel production capability. In fuel industry algae biofuels have emerged as a clean, nature friendly, cost effective solution to other fuels. More recently algae have been identified and developed as renewable fuel sources, and the cultivation of algal biomass for various products is transitioning to commercial-scale systems. Large-scale cultivation of algae merges the fundamental aspects of traditional agricultural farming and aquaculture (Emily M Trentacoste *et al.*, 2014). Algae fuels are categorized into bio-ethanol, biogas, bio-hydrogen, biodiesel and bio-oil. Algae can be used in the preparation of Biodiesel, Bioethanol, Biobutanol and Hydrogen gas (Raja *et al.*, 2013)

They are considered as a potential source of bioactive substances such as proteins, lipids, and polyphenols possessing potent antibacterial, anticancer, antioxidant, antifungal, and antiviral properties (Sundaramurthy *et al.*, 2016). Seaweeds that are medicinal are rich in secondary metabolites which include alkaloids, glycosides, flavonoids, saponins, tannins, steroids, related active metabolites, which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry (Eluvakkal *et al.*, 2010). Recently, their value as a source of novel bioactive substances has grown rapidly and researchers have revealed that marine algal originated compounds exhibit various biological activities (Kim and Wijesekara, 2010; Wijesekara and Kim, 2010; Wijesekara *et al.*, 2010 and Wijesekara *et al.*, 2011). The secondary metabolites of seaweeds such as Isoprenoids (terpenes, carotenoids, steroids), polyketides, phlorotannins, amino-acid derived natural products (alkaloids), and shikimates (flavonoids) have always attracted the interest of biochemists because of their diversity is comparable with those present in the leaves of higher plants (Manilal *et al.*, 2009). Seaweeds were rich in dietary fibre (>50% dry weight), particularly in the soluble form.

RHODOPHYCEAE

Rhodophyta is a phylum of macroalgae that includes the classes Phaeophyceae and Chlorophyta, which are brown and green seaweeds, respectively.

Within Archaeplastida, Rhodophyta, or red algae, is a monophyletic lineage that contains glaucophyte algae, green algae, and terrestrial plants. Bangia-like species have been found in 1.2 billion-year-old strata, indicating that Rhodophyta has a lengthy fossil history. The morphology of red algae ranges from unicellular filamentous to multicellular thalloid forms, with certain species producing economically important products like agar and carrageenan. These species can be found in a variety of marine settings, ranging from the intertidal zone to deep oceans. There are also freshwater (e.g., *Batrachospermum*) and terrestrial lineages. A triphasic life cycle with one haploid and two diploid phases, with the carposporophyte borne on female gametophytes, is one of the Rhodophyta's significant advances.

Freshwater Rhodophyta has 66 species and 27 genera in North America, although these numbers will change as molecular investigations uncover more diversity. Freshwater red algae have a limited size range than marine species, with the majority (80%) of them measuring 1-10 cm in length. Gelatinous filaments, free filaments, and pseudoparenchymatous forms are the most prevalent types. (Yoon, Hwan Su, et al., 2017)

GRACILLARIA

In terms of the number of species, the genus *Gracilaria* is one of the largest genera of red algae. It's also a wide spread genus, with species found in all oceans except the Arctic. Nearly 28 species of *Gracilaria* have been reported from the Indian coast (Sahoo *et al.*, 2001). Because of its size and extensive range, it's suitable for biogeographic investigation. The greatest number of *Gracilaria* species can be found in tropical waters. Large beds of *Gracilaria* usually grow in the eulittoral zone, or just below it in the beginning of the sublittoral, on sandy or muddy sediments that are protected from waves. Sometimes it can be found free-floating in tidal lakes of salt or brackish water. It can adapt to large variations in growing conditions such as freshwater dilution, increase in fertilizer concentration from runoff, and raised temperatures. Large biomasses can grow when there is little competition from other species, and vegetative propagation may be a normal method of reproduction (McLachlan, J.,

et al., 1984). In tropical and subtropical oceans, these are frequently red, green, or greenish brown.

Gracilaria are found as branched thalli, terete to flattened, branching sub-dichotomous to irregular. It has holdfast a disc or crust giving rise to one to many erect axes. The thalli are red, olive, green to purple, Spermatangia are seen in pits or shallow depressions. Sporophytes with tetrasporangia are scattered in the outer cortex, cruciately divide (R Iyer, et al., 2004)

Because they have phycocolloids, the major source of agar- (1, 4)-3, 6-anhydro-1-galactose and -(1,3)-d-galactose with low esterification in the cell wall, *Gracilaria* species are essential for industrial and biotechnological uses. Agar and other polysaccharides are found in *G. confervoides*, *G. dura*, *G. chilensi*, and *G. secundata* among the carbohydrates.

The present work was undertaken to study the antimicrobial potential of *Gracilaria corticata*. The objectives of the study are

- Taxonomic description of the algae
- Assessment of antibacterial potential of the common seaweed *Gracilaria corticata* in its dried form extracted in two different solvents; Ethanol and Chloroform.
- Evaluate the difference in antibacterial potential shown by the alga in the two different solvents against Gram positive *Staphylococcus* and Gram negative *E. coli*.
- Estimate the extractive value of the plant, in both ethanolic and chloroform solvents.

LITERATURE REVIEW

In a study conducted by Inci Tuney (2006), antibacterial activity of extracts or components from various algae has been demonstrated in vitro against both gram-positive and gram-negative bacteria. The antibacterial susceptibility test was performed using the agar disc diffusion method, with 6 mm discs impregnated with 20 µl of extracts and placed in infected agar. *Gracilaria* chloroform extract was tested for antibacterial properties against *Staphylococcus aureus* bacterial strains. *Gracilaria* extract showed action in *S. aureus* extract. Ethanol extracts from *G. domigensis* and *G. sjoestedii* showed antibacterial activity against *E. coli* and *S. aureus*. (Tüney, İnci, et al., 2006)

Krishnapriya et al., (2013) conducted an antibacterial activity on the seaweed extracts, carried out by agar disc diffusion assay. The Muller Hinton agar (MHA) medium was used for this study using bacterial pathogens. Among the solvent extracts, methanol extract showed best results for both positive and negative strains. Chloroform extract of *G. verrucosa* gave the highest zone of inhibition measuring 21±1.0 mm. Ethanol extract of *G. acerosa* also showed a zone of inhibition of 12±1.0 mm. Ethanol and chloroform extracts of *G. verrucosa* gave clearly distinct zone of inhibition measuring 8±1.0 and 9±1.0 mm, with respect to control (25±1.0 mm) against *Staphylococcus*. (Varier, Krishnapriya Madhu, et al., 2013)

Saranraj, P. (2013) conducted a study and the methanol extract of *Gracilaria folifera* (5.0mg/ml) showed highest mean zone of inhibition (18±0.4mm) against the Gram positive cocci *Streptococcus pyogenes* followed by *Bacillus subtilis* (17±0.5mm), *Staphylococcus aureus* (17±0.3mm), *Streptococcus epidermis* (16±0.6mm) and *Bacillus cereus* (16±0.2mm). For Gram negative bacterium, the maximum zone of inhibition was recorded in methanol extract of *Gracilaria folifera* against *Klebsiella pneumoniae* (17±0.5mm) followed by *Salmonella typhi* (16±0.6mm), *Pseudomonas aeruginosa* (16±0.5mm), *Escherichia coli* (16±0.3mm). The zone of inhibition obtained from the Hexane extract of seaweed *Gracilaria folifera* against bacterial pathogens was comparatively very less when compared to the other solvent extracts. No zone of inhibition was seen in DMSO control and the positive control Ampicillin showed zone of inhibition ranging from 13±0.8 mm to 20±0.8mm against the test bacterial pathogens. (Saranraj, P., 2013)

The antibacterial properties of eight crude extracts of local *Acanthophora spicifera* obtained by two distinct extraction methods were investigated by Zakaria (2010) using soxhlet extraction and solvent partitioning. By using the Disc diffusion method, these extracts were

evaluated in vitro against 18 bacteria, 3 yeasts, and 6 fungal strains. The results demonstrated that the solvent partitioning extracts of methanol and ethyl acetate had a greater spectrum of action against the tested bacterial strains. *Bacillus cereus* ATCC 10876, *Bacillus licheniformis* ATCC 12759, Menthicilin Resistance *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* ATCC 27853, *Yersinia* sp., and *Citrobacter freundii* displayed inhibitory zones against these two extracts. While methanol extracts from Soxhlet extraction and butanol from solvent partitioning had no antibacterial activity against *P. aeruginosa* ATCC 27853, the other six extracts did. (Zakaria, et al., 2010)

In a study done by Ibraheem et al.; 2017; simplex extracts of *Acanthopora* showed potent inhibitory growth activities against three Gram positive bacteria [*Streptococcus agalactiae*, *pyogenes* and *Streptococcus sanguis* of inhibition ranging from [23.1±0.58 to 20.6±0.63 mm] and showed moderate activities with [*Corynebacterium diphtheriae*, *Bacillus subtilis* and *Staphylococcus aureus*] with inhibition zones ranging from [20.1±1.5 to 16.3±2.1 mm].

Also the crude extracts were found to be more active than the positive control Ampicillin, (22.3±1.5 mm), against *Streptococcus agalactiae* which showing inhibition zone. The hydro alcoholic extracts of the selected species were investigated for their antimicrobial activities using Agar well diffusion and Muller Henton against gram positive and gram negative bacteria. (Ibraheem, Ibraheem BM, et al., 2017)

In a study by Nurul Aili Zakaria et al.; (2011), the antimicrobial activities of the hexane extract were evaluated using disc diffusion method against 8 Gram-negative and 10 Gram-positive bacterial strains. Out of all bacterial tested, only a Gram-positive bacterium and a Gram-negative bacterium were susceptible to the extracts. The hexane extract showed antibacterial activity against both Gram-positive bacterium and Gram-negative bacterium (*P. aeruginosa* ATCC 27853). While, chloroform and ethyl acetate extract only showed inhibitory effect on *P. aeruginosa* ATCC 27853 with inhibition zone of 9.0 mm. No inhibitory effect was showed by methanol extract on bacteria tested. (Zakaria, Nurul Aili, et al., 2011)

MATERIALS AND METHODS

SPECIMEN COLLECTION

The specimen was collected by hand picking from Thikkodi beach, Calicut. The collected samples were washed immediately in seawater and then washed with fresh water and transported to the laboratory. It was again washed thoroughly to remove impurities and sand and rinsed with distilled water. The sample was identified taxonomically as *Gracilaria corticata*. Collected sample was taxonomically evaluated using the standard literature.

SAMPLE PREPARATION

For antimicrobial studies, the cleaned samples were then shade dried, cut into small pieces and powdered in a mixer grinder. The organic solvents Chloroform and Ethanol were used for the extraction process due to its higher efficiency using Soxhlet extraction method. 20g of samples were packed in a thimble and placed in the extractor. 200ml of the solvent was added into the flask and heated. The temperature was maintained at 80⁰C to 85⁰C throughout the extraction. The soluble active constituents of the extract remained in the flask and the process was repeated until the compounds were completely extracted. The liquid extract was then cooled and concentrated by using an evaporator.

The beaker with dried extract was weighed and noted. DMSO was used to dissolve the extracts from the beaker. Later the weight of the beaker alone was noted. Hence, the actual weight of the dried extract was obtained. Similarly, the weight of dried extract of *Gracilaria*, in ethanol and chloroform was 0.46g and 10mg respectively. From this the extractive value was calculated using the formula

Extractive value (%) = (Weight of dried extract/ Weight of plant material) X 100

PREPARATION OF EXTRACT IN VARIOUS CONCENTRATIONS

From the stock extract, concentrations of 10%, 20%, 40%, 60% (v/v) was made. The stock concentration of *Gracilaria* in ethanol and chloroform was 10mg/ml and 10mg/ml respectively. From the stock the appropriate amounts was pipetted out and made up to the required concentrations using DMSO.

ANTIBACTERIAL ACTIVITY IN *GRACILARIA*

PREPARATION OF BACTERIAL CULTURE

In the present study, the extracts were evaluated for antimicrobial activity against *Staphylococcus* strain and *E. coli*, a Gram positive and a Gram negative bacteria respectively. 3g of nutrient broth was dissolved in 100ml of distilled water in a conical flask. The broth is sterilized by autoclaving for 15 minutes. Both of the obtained bacterial stains were inoculated in the nutrient broth in laminar air flow and incubated in appropriate conditions for 24hrs.

PREPARATION OF PETRI PLATES

The selected two species of seaweeds were analysed for the antimicrobial activity for gram negative *Escherichia coli* and gram positive *Staphylococcus* by disc diffusion methods. Agar medium was prepared by dissolving 4g agar and 2.6g of nutrient broth in 100ml distilled water. The mixture is sterilized in an autoclave for 15 minutes. Just after sterilization the mixture was poured into petri plates in laminar air flow. The petri plates were allowed to solidify under aseptic conditions.

ANTIMICROBIAL TEST BY DISC DIFFUSION METHOD

Bacteria were inoculated onto the prepared agar petri plates using sterilized cotton swabs. Sterilized 6mm discs were taken from filter paper and autoclaved and is used for the method. The disc was then dipped in different concentrations of stock (10, 20, 40, 60) and placed on the agar plate using sterile forceps. Tetracycline was used as positive control and DMSO was used as negative control. This was done for both extracts of *Gracilaria* against the two strains of bacteria. The petri plates were incubated at 37°C for 24 hours and results were recorded.

OBSERVATION AND RESULTS

The current study aimed at the taxonomic description of the red seaweed *Gracilaria corticata* and the estimation of its extractive value and antimicrobial potential in two solvents, ethanol and chloroform. The antimicrobial potential activity was studied against Gram positive *Staphylococcus* and Gram negative *E. coli*, two non-pathogenic bacteria. The results obtained are described below.

TAXONOMIC DESCRIPTION

Kingdom: Plantae

Phylum: Rhodophyta

Class: Florideophyceae

Order: Gracilariales

Family: Gracilariaceae

Genus: *Gracilaria*

Species: *corticata*

Gracilaria corticata (J. Agardh) J. Agardh

Thallus erect, up to 14cm in length, arising singly from a discoid holdfast. Stipe very short, terete, up to 5mm long, often inconspicuous. Branching frequently, becoming denser in upper parts of the plant; mostly dichotomous, and producing a bushy appearance. Axes compressed, almost cartilaginous; constricted at the base in basal branches. Blades linear, up to 15cm long, up to 4mm wide; apices generally obtuse, acute in finer branches. Blade surface and margins smooth. Fresh specimens purple to green and firm but pliable (Iyer et al, 2004).



Gracilaria corticata (J. Agardh) J. Agardh

EXTRACTIVE VALUE

Extractive values of plant materials are used to evaluate extracts of the sample, in order to get an idea about the nature of chemical constituents present in it. It can also be used to assess quality, purity and detect adulteration of the extract.

In the present study, polar and non-polar solvents were used for eluting the valuable phyto-compounds present in the sample. Extractive values of ethanol and chloroform extracts of *Gracilaria corticata* used in the antibacterial study, are estimated in the table 1 given below;

Table 1: Extractive value of solvents administered for *Gracilaria corticata*

Solvent	Extractive value of the sample (%)
Ethanol	2.3
Chloroform	0.5

The extractive value was greater for the ethanolic extract than for chloroform suggesting that polar solvent was more efficient in extracting the phytochemicals from the algae.

ANTIBACTERIAL ACTIVITY

The extracts of the algae exhibited moderate to mild antibacterial activity against the two microorganisms. The activity observed can be described as being bacteriostatic showing very

mild zones of inhibition. The ethanol extract of the algae showed mild antibacterial activity against both the test organisms. *Gracilaria* shows mild action against gram negative bacteria at all concentrations of ethanol extract used in the current study against both test organisms. Table 2; Fig. 2

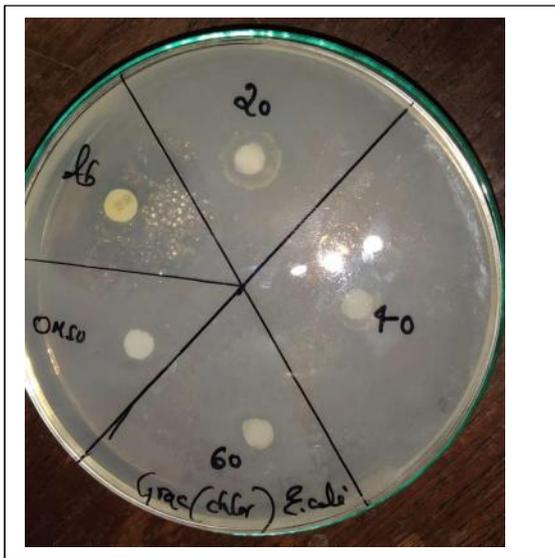
Table 2: Antibacterial activity of ethanolic extract of *Gracilaria corticata* against *E. coli* and *Staphylococcus* bacteria:

Concentration (%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	Mild action	Mild action
40	Mild action	Mild action
60	Mild action	Mild action

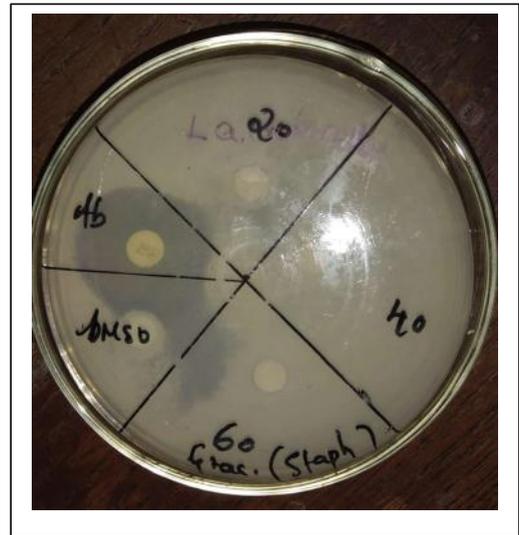
Table 3: Antibacterial activity of chloroform extract of *Gracilaria corticata* against *E. coli* and *Staphylococcus* bacteria

Concentration (%)	<i>E. coli</i>	<i>Staphylococcus</i>
20	No action	No action
40	No action	No action
60	No action	Mild action

The chloroform extract of *Gracilaria* has no significant effect on the bacterial growth. Mild bacteriaostatic activity is observed at higher extract concentrations on *Staphylococcus*. No potential activity could be observed on the growth of *E. coli* in any of the concentrations used for the current study Table 3: Fig. 3

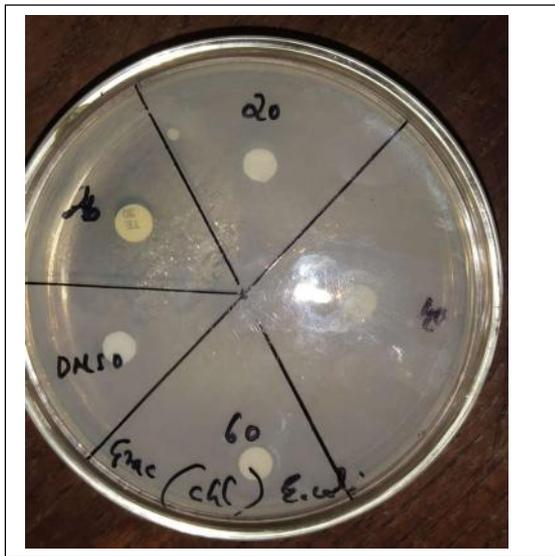


E. coli

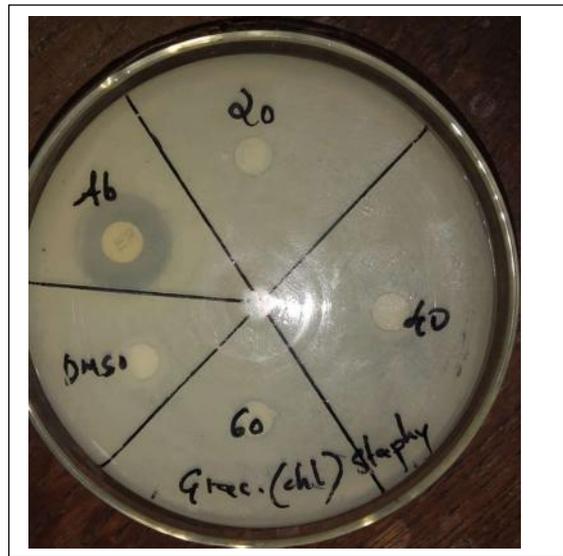


Staphylococci

Fig 2: Antimicrobial activity of Ethanol extract of Gracilaria



E. coli



Staphylococci

Fig 3: Antimicrobial activity of Chloroform extract of Gracilaria

DISCUSSION

Algae have attracted great importance in the recent years due to the large number and amounts of bioactive components in them. More than 600 trace elements are found in high concentration in the seaweeds compared to the terrestrial plants, because of which it has various pharmacological activities. The seaweeds offer greatest wealth in terms of biomass and Rhodophytes show the largest representation among them. The red sea weed *Gracilaria* is amongst the largest group with over 150 species world wide and nearly 28 species in India (Sahoo *et al.*, 2001).

Gracilaria has been identified as a rich source of various bioactive compounds as assessed by the studies on its different species such as *G. corticata*, *G. dentata*, *G. edulis*, *G. opuntia*, *G. pygmaea* and *G. verrucosa* carried out by various researchers. In a study carried out by Balakrishnan *et al.* in 2013, phytochemical screening of *G. corticata* was done using different solvents like methanol, ethanol, petroleum ether and acetone revealing the presence of most of the bioactive components of which alkaloids, phenols, quinones and steroids are most abundant. In a similar investigation led by Gnanaprakasam *et al.*, (2017), the hexane, chloroform, ethyl acetate, acetone and methanol extracts of *G. corticata* were used to analyse the phytochemicals, and results revealed that the terpenoids, tannins and phenolic compounds were present in the all the extracts and alkaloids were present only in the chloroform and ethyl acetate extracts.

Antibacterial activity refers to the process of killing or inhibiting the disease-causing bacteria. Several plants have been traditionally used for their antibacterial activity. Like plants some algae also exhibit antibacterial properties due to the presence of terpenoids, steroids, saponins, tannins, and flavonoids. There are numerous reports regarding the inhibitory activities of macroalgae against human pathogens, fungi and yeasts. So, the use of algae as an alternative for prevention and treatment of infectious diseases has been suggested by Abirami and Kowsalya (2012). In the present study ethanolic and chloroform extract were evaluated for activity against Gram positive *Staphylococci* and Gram-negative *E. coli*. It was found that ethanolic extract had bacteriostatic activity against both the bacteria at all concentrations treated and the effect was dose dependent. Sanaraj P., 2013, in his study on *G. edulis* also reported maximum activity against Gram positive bacteria in ethanol extracts. Rashida *et al.* (2019) also report ethanol extract of *Gracilaria* to have higher antibacterial activity than other solvents.

In the preliminary assay conducted to evaluate antibacterial activity of *Gracilaria* species against human pathogens by Susanth (2012), ten different organic solvents were considered. This study also reported that the extracts of ethanol and chloroform were the most potent of all.

Johnsi et al (2011), studied the antibacterial activity of aqueous extract of four seaweeds against ten pathogenic bacteria. This study reports the aqueous extract of *Gracilaria corticata* as having the highest potency against the pathogen *Proteus mirabilis*. In the current study however extracts in both solvents show bacteriostatic activity against *E. coli* and *Staphylococci*. Neither extracts are bactericidal and show a mild inhibitory zone of 7 - 8 mm.

Different solvent systems were used to extract bioactive principles from macroalgae with concomitant changes in the antibacterial activities (Thirupurasundari et al., 2008). The solvents such as acetone, benzene, butanol (Vanitha et al., 2003; Prakash et al., 2005), ethanol (Selvi et al., 2001) were used to extract antimicrobial compounds from macroalgae. The aqueous extracts prepared from seven macroalgal samples showed varying degrees of activity against tested pathogens, including the Gram positive and Gram negative bacteria. (Johnsi et al, 2011). Padmakumar (2002) is of the opinion that these differences are due to the different solubility behaviour of secondary metabolites which could be influenced by seasonal and geographical distribution of the species.

SUMMARY

Algae are an important constituent of the aquatic ecosystems and can be seen in water bodies like oceans, seas, lakes, estuaries and soon. They can be of different types and in different colours. Mostly seen seaweeds are macroalgae. They can be used as food and are a storehouse of bioactive components like vitamins, phenolics, terpenoids and other secondary metabolites. They also possess antibacterial, antioxidant, antifungal properties. Red algae is used for the extraction of agar (*Gracilaria*). It also shows few antibacterial properties.

The present study was done to estimate the difference in extractive yield, and the antimicrobial potential of the dried form of, *Gracilaria corticata* in polar and non-polar solvents, ethanol and chloroform respectively. The whole plant body was taken for the study. The cleaned, dried and powdered sample was extracted using the sohxlet apparatus. Extractive values of plant materials are often used to evaluate extracts of the sample, in order to get an idea about the nature of chemical constituents present in it. It can also be used to assess quality, purity and detect adulteration of the extract. *G. corticata* showed a better elution for polar solvent than non-polar solvent. The extractive yield obtained was more for ethanolic extract (2.3%) as compared to chloroform extract (0.5%).

In the present project, antibacterial potential of *Gracilaria corticata* was tested against two non pathogenic bacteria, the Gram negative *E. coli* and the Gram negative *Staphylococcus* by the disc diffusion method. It is concluded that the organic solvent extraction by ethanol and chloroform was suitable to verify the antimicrobial properties of *Gracilaria corticata* and they were supported by many investigations.

The current investigation showed that *Gracilaria corticata* has antimicrobial potential. The ethanol extract has better antimicrobial activity when compared to chloroform extracts. Ethanol extract was bacteriostatic for both gram negative (*E. coli*) and gram positive (*Staphylococcus*) bacteria at all concentrations studied. Whereas the extract in chloroform showed no significant activity except in the higher concentration (60%) and only against the Gram positive *Staphylococcus*.

The present study justifies the claimed uses of *Gracilaria corticata* in the traditional system of medicine to treat various infectious diseases caused by the microbes. These results suggest the possibility of using marine algae extracts in therapy as natural alternatives to antibiotics currently in the market, and clearly show that seaweeds are a valuable source of biologically active compounds.

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**REDIFINING POWER STRUCTURES IN TAMIL CINEMA:
A STUDY OF *JAI BHIM* AND *ASURAN***



*Project submitted to St. Teresa's College (Autonomous) in partial fulfilment of
the requirement for the degree of BACHELOR OF ARTS in English Language
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I hereby declare that this project entitled “Redefining Power Structures in Tamil Cinema: A Study of *Jai Bhim* and *Asuran*” is the record of bona fide work done by me under the guidance and supervision of Mrs. Athira Babu, Assistant Professor, Department of English.

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CERTIFICATE

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In the earlier Tamil movies it is obvious that Dalits are shown in a low light in which they are characters to be ridiculed. They are not given any identity and sometimes they are even seen as anonymous masses without their own individuality. There is a remarkable change in the depiction of Dalits in recent movies. Dalits are portrayed as resilient characters who unlike their older generations, thrive for their rights and try to live a dignified life. This project is an attempt to analyze two Tamil Movies- *Jai Bhim* (2021) and *Asuran* (2019) and to distinguish the prevailing ideology and power structures using the theories of Louis Althusser and Michel Foucault. In these films, there is a realistic portrayal of Dalits and a reversal of power structures as the narrative is from the point of view of Dalits. This project consists of two chapters in which the first chapter gives an idea of the theories used. The second chapter consists of a detailed analysis of the two movies in relation to ideology, power and knowledge.

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Tess Toni

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Introduction

Caste System is a reality of India though it has been abolished by law for almost 70 years now. The gap between those of the upper caste and those of the lower caste is quite wide. Caste system is only a social construct. But as people have been following this system for time immemorial there is no end to this system. Differences in culture of various castes are quite acceptable. But discrimination based on caste differences is certainly not acceptable and justifiable. Though India has laws and committees to make sure there is no caste discrimination or any other problems related to it, it is all in vain.

Tamil Nadu has a very rigid caste system and media has always depicted it in various forms. There is discrimination in every field and also in all forms. Many of the practices abolished by the Government are still in practice in the state. There are honour killings even today when an upper caste member gets married to someone of a lower caste. Like in real life, Tamil movies clearly show that Dalits have had no power and no representation. They mostly did not have representation in Tamil media until recently. And in case of representation, they were just seen as a huge mass without individuality. They were found in cliché roles such as servants or even as a dirty lot. There was always an assumption that they were not important.

The aim of this project is to analyse the changes in representation of the Dalits in the Tamil movie industry. Cinema has always been a vital part of the life of Tamilians and it has influenced the Tamil culture and politics. Tamil Cinema for a long time was dominated by caste pride through depicting life of 'thevar', 'goundar', 'nattammai' and other upper caste group. In certain recent movies, there is a realistic portrayal of the lower caste members. A reversal of power structures is seen as the central characters in

these movies are members of the Lower Caste. The ideological structures and torturous life is enacted realistically. Recent Tamil media acts as a medium of empowerment. It elaborates on the importance of education thereby empowering the downtrodden. Dalits are using media as a form of resistance/retaliation expecting to empower their entire community.

Being a member of the Dalit Society Pa Renjith renowned Tamil Director has often faced ignorance and discrimination many times. This inspired him to start a new wave of movies which aimed at the upliftment of Dalits and to open to the world the real problems of the Tamil. The happenings of Tamil Nadu even in this 21st century are quite shocking. With powerful movies like *Kabaali* and *Kaala* he started a revolution which actually started making changes for the unfortunate and voiceless. Vetrimaaran is yet another powerful director who has made movies like *Asuran* and *Paavai Kadhagal* which aims at changing the society by making them realize their mistakes. Maari Selvaraj with his *Karnan* supported this movement. The latest addition to this list is *Jai Bhim* which has sparked many discussions in the media. It's high time the people actually get inspired by this and continue with this wave of change. They should truly open their eye to basic human dignity.

Asuran and *Jai Bhim* are two movies which show the plight of two families who face severe oppression from the upper caste and those in power. By using the concepts of Althusser such as Ideology, Ideological and Repressive State Apparatuses, these movies can be analysed and the social constructs can be identified. People are interpellated to believe in caste system as their reality. Foucault's ideas of power also support these concepts of how people are enslaved and made to believe those in power wants them to

believe. And it's always the people in power who get to make the laws and perpetuate them. It becomes dangerous when people accept all of these unconsciously without even the slightest clue that they are becoming interpellated.

In chapter one, there is a detailed view on the theoretical aspect of Ideology, Hegemony, Interpellation, Ideological State Apparatus and Repressive State Apparatus. The concepts of Knowledge and Power and its relation with each other are also looked at in detail. Chapter two is a detailed evaluation of *Jai Bhim* and *Asuran* based on the above mentioned theories.

Chapter 1

Theoretical Framework of Ideology and Power

Ideology can be defined as a system of ideas and ideals internalized by a person especially one which forms the basis of economic or political theory and policy. This is a social process. In simple words, Ideology is the attitude of a person towards gender, class, race and all such things. Ideology is of great importance, since it is that one thing that affects every walk of a person's life. What a family follows is what is internalized by a child born there. A child observes whatever he sees and this unknowingly starts accumulating in his mind. He therefore behaves the way he has learnt to. Althusser defines ideology as "Ideology represents the imaginary relationship of individuals to their real conditions of existence"; and "Ideology has a material existence".

It was Antonio Gramsci who gave clarity to the concept of hegemony. He was a politician and an Italian Marxist Intellectual. In his Prison Notebooks, Gramsci developed hegemony. Hegemony is the dominance of one social group over another. They are mostly dominant politically, economically and socially. They have authority over the weaker sections of the society. In Marxist Philosophy, Cultural Hegemony is the domination of a culturally diverse society by the ruling class which manipulates the culture of that society- the beliefs and explanations, perceptions, values and mores- so that the imposed, ruling-class worldview becomes the accepted cultural norm. This has many bad effects as it instills a feeling that the members of the weaker sections of the society have no culture and tradition of their own. They are not known in their own name. They exist only in relation to the dominant people of the society. The ideology of the powerful is instilled in the minds of the oppressed. The oppressed are bound to obey the

whims of the upper strata of the society.

Hegemony is basically a defined social group ruling society. They have the power in their hands and it is their ideology that spread through that particular society. Since they have enough power and money, the people of the lower strata have no other option but to accept and follow the ideology of the people who rules the society. Hegemony weakens the cultural system. It is actually an unconscious control system. The hegemonic values and components are seen as essential part of a society and its values rather than as the part of a dominant ideology or culture. Due to this, minorities identify these as a part of their values. They see it as their own and therefore accept their subservience as a part of their social life. They fail to an extent to realize it as a trap.

Interpellation is a process in which individuals born in a society encounter with its culture and ideology and internalizes them. It further gives the idea that a certain way of living or certain thoughts you have is not really yours but that what has been presented to you for years for you to accept and believe that it's yours. It is a social process.

Interpellation gives a particular identity to people and it encourages people to accept it. This is not done by force or violence. When a person or a child looks around, he observes these roles as the accepted ones or even the ones that is offered to us by the culture; he tends to see it as the normal one and accepts it subconsciously. Everywhere he is encouraged to accept them. When something is widely accepted, the tendency to question it reduces. Everyone is interpellated from the day they are born into specific roles that society has created for them. According to Althusser, people are interpellated from the day of their birth or sometimes even before as parents and others conceive of the role and identity that their child after birth will assume. Althusser argues that the process of

interpellation works best when it is invisible. When this happens, individuals tend to accept cultural notions as though they are natural and obvious. For example: Men act in a certain way and women act in another way. Interpellation is at its best when people give no thought or remain unknown to the fact that they are being interpellated.

Louis Pierre Althusser is an exceptional French Marxist Philosopher of the twentieth century. He took part in war and he was imprisoned as a prisoner of war. His experiences in the camp, especially that of solidarity, community and political action served as an opening to his ideas in communism. Some of his major works are Ideology and Ideological State Apparatuses, Reading Capital and for Marx.

According to Althusser, to properly place people in a society, assimilation happens. This occurs in two ways:

1. Repressive State Apparatus
2. Ideological State Apparatus

One happens by means of force and the other using ideas. Let us see in detail what these terms mean.

Repressive State Apparatus:

This is concept formed by Althusser where people are suppressed using power and violence. These mechanisms work through force. They operate through punishment or threats of punishment, or may even by power demonstrations. In simple words it can be said that, it works primarily by means of physical and mental coercion and violence.

Repressive State Apparatus works through the Police, the Army, the Judiciary, the Power Systems etc. It works in a public domain. It is very rare and infrequent. It primarily works by repression. This instills fear in the minds of the people and they are forced to accept

that certain things will always be like that. They have no means to protest against injustice.

Ideological State Apparatus is a very important concept by Althusser. Ideological State Apparatus works through ideas and attitudes. One encounters this throughout one's life. There's a continuous process of subconscious training and conditioning so as how to lead one's life. These processes happen so natural that one ends up believing that it is a natural process. Some are even unaware of such processes. Ideological State Apparatus are large social institutions that train people in thinking in a particular way and they bring in an ideology. The social institutions include families, schools, churches, art, movies, books, toys, advertising, music, television, fashion, technology, games etc. These ideologies are commonly accepted in a society. All of these sources of power successfully instills in a person certain attitudes, ideas, perceptions, behaviors, values etc. This is something present in a society very commonly. School is a particularly important ISA, as the students give their undivided attention to the teacher and get influenced by the teachers. It is very easy for a teacher to instill something into the minds of young children if that is a part of their agenda. ISA s help in reinforcing the hegemonic rule by replicating the dominant ideology present in the society then. In a society, there are always certain social groups and people who are dominant. Their ideology and culture is forced upon the people. Initially people might be a little confused so as to what is happening. But later on they tend to internalize it with their own consent. As far as the caste system in India is concerned, people tend to accept the fact that they are lower than some other people in the society. They do not think about it objectively. Rather they tend to look at it emotionally and sometimes confuse it be a part of their tradition to be

downtrodden. Sometimes people of the lower community even agree to do things even though it hurts their dignity simply because they believe that they have no dignity and that they have to do certain actions.

The dominant groups in a society usually perpetuate what is basically just their own culture or whatever is in their interests. They make rules and norms according to their own wishes and ask the others to follow that what they make. Often the poor and the voiceless in the society have no way but to live according to whims of the powerful. The powerful make it just for their comfort and to make the weaker sections of the society submissive. These are concepts which still exist in society no matter how much the each society has progressed. In certain societies, even today their dominance is perpetuated. There are dictators who use power to suppress people today using RSA. They torture people for them to accept what they feel is right. Even after so many rights called human rights are introduced, people in prison suffer due to their inability to get rights. In certain societies, the upper strata of the society even today spreads message of hatred and separation with the people of the lower strata of the society. Equality is not even worth mentioning in certain countries. Though a lot of people struggle for their rights and freedom, sometimes ISA and RSA works together to deprive them of anything that will supposedly improve their living conditions. A society is very powerful. It is actually society that decides how a human being gets to live. Society influences every walk of a human life. A child born into a society becomes what he is, depending on how a society is.

Michael Foucault is a French philosopher whose theories primarily concern about the relationship between power and knowledge and how they are used as forms of social

control. Foucault is of the opinion that power is dispersed and pervasive. 'Power is everywhere' and 'comes from everywhere' so in this sense is neither an agency nor a structure (Foucault 1998: 63). It is a regime of truth that exists in society and is in constant negotiation. Foucault is of the opinion that power-knowledge discourse does not always mean that knowledge is power. He tries to show us that those in power suppress certain knowledge and produce what they want to show to the world. They interpret texts the way they want and they spread word the way they wish. They try to bend knowledge in a way that is beneficial for them. They spread knowledge in their interests and it might not be the actual truth. The caste system and how it should be exercised in a society was similarly spread. The dominant social groups other the weaker group. The dominant group need not be the one with more members. They might be the smaller group, yet they have more power in their hands and they make sure they do not lose that grip they have over the weak. They sometimes don't even treat the othered people as human beings. They treat them as lesser people and they don't look at them as individuals. They are looked upon as a mass of people without their own identity or life.

Chapter 2

Ideology and Power Structures in *Jai Bhim* and *Asuran*

Until recently, Tamil movies portrayed normal stories featuring upper caste actors and almost the entire crew were members of the upper caste. Dalits, if at all represented, were shown as a huge mass of people. They had this cliché representation which included showing them as dirty and shabby, and they never had a voice. They had no individuality and played roles like servants, workers, cleaners etc. who were sidelined. Recently the trend has changed and there are Dalits who play central characters in movies. The entire movie is from their point of view and it revolves around them and their family, and focuses on the problems faced by them. The Dalits are not represented as meek sufferers but as strong fighters to all their problems. The concern is about the fact that they put up a strong fight whether or not they find relevant success.

Jai Bhim is a movie directed by T.J. Gnanavel. *Jai Bhim* shows the story of Rajakannu, a poor lower caste brick maker, who is also a snake catcher. He is wrongly trapped in a robbery as he had gone to the house of the Ramapuram Panchayat President to catch a snake and is forced to own up the crime. He stays strong and is finally beaten to death. The police say that Rajakannu and his two relatives, Mosakutty and Irutappan have fled the jail to escape the wrath of the court and their higher officials. The movie shows the detailed struggle of Rajakannu's wife Sengini, Chandru, a High Court Lawyer and Mythra, the village teacher to find the truth behind the disappearance of the three and to prove the guilt of the Police. The Police use various forms of torture to make the Irulas confess the crime, all the while knowing who the real thief is. In the movie, the court

scenes have brighter light which represents the hope that the unfortunate possess. The jail scenes have dimmer light which is representative of the cruelty that is happening there. In comparison to the upper castes, the Irulas are very poor and they dress accordingly. Sengini and her people are better dressed in the last scene, in which their house warming occurs. This might be symbolic of the new house and the better living conditions they have now got.

From the beginning of *Jai Bhim* one gets to see a kind of discrimination which is normalized in the society. This ideology which supports discrimination and inequality is perpetuated in the society. Everything is fixed for the members of the lower caste and they have no say in what happens to their life. The movie begins with a scene in which people of the lower caste are rearrested on false claims. The people are asked their caste as they are released and those from the upper caste are set free while those of the lower caste are made to stand aside and then taken to prison once again. The notion that the lives of the lower caste members don't matter as much as that of the upper caste members is deeply entrenched in the society.

This ideology present makes many of the police and other powerful officers casteist and they are dishonest. They accuse the lower caste people of crimes they haven't committed and arrest them either with evidence they have forged or without evidence at all. "My Lord, Kolanji is not an innocent tribal. He is a habitual offender. A serial offender." (00:32:17-25)The public prosecutor says this on the basis of the false records made by the police. A deeper look into the case by Advocate Chandru points out that Kolanji, a tribal rearrested was actually innocent. "On the occurrence of both the thefts, Kolanji had been in prison, My Lord. Cops who foist false cases are the habitual

offenders. Serial fake-case makers.” (00:33:07-19) Advocate Chandru further goes on to open the eyes of the law practitioners, “It’s not the only foisted case, My Lord. Twelve tribals who were released from jail the same day were falsely charged and rearrested right outside Cuddalore Jail.” (00:33:22-31). Many of those in power believe that there is no one to question the injustices done against Dalits as a result of which shockingly 7000 lower caste people were arrested on false claims of robbery. Unlike the upper castes, the people of the lower castes are not seen as individuals but as a collective whole like Irulas, Koravoors, Ottars etc. “Great, Chandru. 7000 birds with one stone!” (00:34:38-39) Chandru corrects his colleague telling “7000 humans, sir.”(00:34:40-41) It is such a pity that the ideology sees them as lesser humans born merely to be used for their comfort by the members of the upper caste. The members of the lower society are quite powerless on their own and need external help for anything of this sort. “Thanks to a social activist, Kaliappan (a relative of Kolanji) made it to this courtroom. The other eleven had no such opportunity.” (00:33:30-37) This statement by Chandru shows the pathetic situation of the jailed tribals.

Even though the judges know that mishaps have occurred when the lower caste people were wrongly arrested, they are reluctant to look into the case. They take actions only due to the coercion by Chandru “Whoever is affected whatever their number, they deserve justice! This court has a moral duty to ensure that!” (00:34:07-12). The society runs on an ideology which is perpetuated by the upper caste which gives importance only to them and their wishes.

There is clear hegemony present in this case as the upper caste people have enough and more of everything. They are both rich and influential and are able to do

anything they wish to do. On the other hand, the people of lower caste lack basic amenities and are denied permission to construct a brick house owing to their lack of land. Rajakannu laments “How many bricks have I cut out with these hands! Yet I cannot build one brick house of my own”. (00:10:35-39) They live in a ramshackle house which puts their life in danger. According to the laws of India, the members of scheduled castes and scheduled tribes are entitled to land and other reservations. The authorities keep denying it to them stating their lack of certificates. They do not get help with the certificates either. Even though education is supposed to be free and mandatory, and is provided by the government, most government officials turn a blind eye towards tribals and their desire to study. “Who cares if you learn to read or not. Tribals should stay in the hills. Instead you (tribals) come into town and nag about Tribe Certificates and what not” (00:14:32-40). He continues, stating “You have no place to live on, You have no ration card, Your names aren’t there on the voter list. On what grounds do I give you a ST certificate” (00:15:02-10). He mercilessly sends them away totally refusing to help them. At the next moment, the official is seen welcoming two others of the upper caste warmly.

Their village headman refuses to look into their case, even though he is well aware of their conditions. “Isn’t it bad enough to plead with folks from lower castes to get their votes? Must we bend low in the hovels of these too?” (00:23:52-59) The Irulas have been staying at that place for so long and yet are not considered a part of that village. This is symbolic to them being seemingly inexistent to the higher caste people and officials. When Rajakannu catches the snake which was in the house of the Ramapuram Panchayat President and refuses to take money on the context of the his wife hailing from Rajakannu’s village, the wife retorts “Since when is your place a part of the

village” (00:13:37-39)

The existence of the caste system still owes to the interpellation happening in the society. It is interpellation that has weighed down the members of lower caste for centuries. Certain ideologies still exist in society because they are passed on from one generation to the next. In *Jai Bhim* it can be seen that all the lower caste people initially believe that they are lower than the upper caste. From the time children start to understand, they see how their elders are treated and they come to believe that this is how their life is going to be. Though they know that their situations are tough, they don't really get to question it. People are interpellated to believe things which are always in favor of the ruling caste. Generations of Dalits end up being helpless and downtrodden as they are also born into this same ideology and interpellated.

In many instances throughout the movie, those in power are further trying to tarnish the name of the tribals by tagging them as thieves. Gradually this interpellates the entire society to believe that the people of the lower castes are thieves and are not to be trusted. The Police exploit the helplessness of Rajakannu to tag him as a thief and they turn a deaf ear to his pleas of innocence. Rajakannu is a very strong man who wants to stand up for the innocence of their tribe and this costs him his life. When Irutappa suggests out of pain that they give in to the whims of the Police, Rajakannu says, “No, Irutappa. These wounds will heal in a few days. The stamp as thieves will stay forever. Don't break.”(00:55:07-14).

The Police along with the upper caste and those in power randomly put up cases of theft on them which makes the society in large to believe that there must be some truth

in it. The officials treat them very cruelly which makes them internalize the fact that they should fear all the systems of power. Many tribals pathetically explain their fate. “Sir, against my father, against my husband and now against my son, false charges are going on.”(01:49:27-29). “On my way back from hunting at night, I saw some cops. I greeted them with folded hands. Claiming I no longer feared them as I dared to greet them, they put a theft case on me and had me jailed for three years.”(01:49:29-44). “I was walking down the road. Cops were standing ahead of me. Wanting no trouble I kept to the other side. Noticing that, they claimed I was trying to get away from the police and charged me with motorcycle theft. I can’t even ride a bicycle!” (01:49:45-57). “They took me to the forest and strung me up by the thumbs like this. Stuck a rod in the back of my neck and beat me half to death! I still didn’t admit to any wrongdoing. I’m afraid to even think about it!” (01:50:00-13) His wife explains the rest of it weeping, “My husband, he bore the pain, Sir. They brought me to the station and did horrible things. Unable to bear it, my husband admitted to the crime.” (01:50:13-20) A young school boy narrates his pathetic fate “When they couldn’t find my father, the cops took me in and beat me up. Since then, even if an eraser goes missing at school, the first thing which is checked is my bag. Fearing that shame, I don’t even go to school.” (01:50:39-52). All of these were instances where the Tribal people were wrongfully detained.

Jai Bhim is a movie in which Repressive State Apparatus and Ideological State Apparatus work together to deprive the poor people of a good life. These unjust systems initially work together tyrannically to pathetically murder Rajakannu. Repressive State Apparatus usually consists of the army, the police, the judiciary and the prison system. In this case, the police, the judiciary and the prison system have an important role. It has

operated primarily through violence, and mental and physical coercion. In the entire movie, the violence and brutality by the officials are quite evident. Yet the movie can be divided into two halves of which in the first part, Repressive State Apparatuses have ruined everything for Sengini. But in the second half, Judiciary, which is an agency of RSA, has helped Sengini in the right way. This movie undermines the usual idea of RSA by using one of the systems of RSA, ie, the judiciary to bring justice. Sengini has to go through innumerable torture in the first half due to her lack of knowledge about the judiciary and how it could help her. In the instance when her daughter is abducted by the Police Officer to the Police Station to force Sengini to withdraw the case, she informs this to Chandru and this forces the Judiciary to put pressure on the Police officers. When the Inspector is about to torture Sengini, he gets a call from the higher authorities to safely take them back to Sengini's place. The judiciary which she once considered as her biggest fear has now protected her. This changes a lot of her perspectives and influences a lot of her later actions which includes her remarkable reply to the Police Chief when he approaches her for an out-of-court settlement. Further the Judiciary appoints Perumalsamy, a just Police Officer to investigate the case, grants Chandru more time and also asks the Attorney General to report instead of the Public Prosecutor which makes Sengini realize the difference from her earlier experiences.

At many instances, there is severe brutality by the Police who torture the voiceless for their own selfish needs. "Two sets of fingerprints were found at the crime scene. One belonged to Rajakannu, who went there to catch a snake. The other belonged to Ravi. The actual thief."(02:30:15-24) On further enquiry by Perumalsamy, the investigating officer it was found out that the actual thief, the goldsmith and the police all shared what they

got from the loot. “Despite knowing who stole it, the cops took a bribe from the guilty and made a scapegoat out of the innocent Irulas.”(02:30:54-31:00) Unlike most Irulas who would agree helplessly to become the scapegoats, Rajakannu and his people stayed stout which made the torture further unbearable.

Sengini, Rajakannu’s heavily pregnant wife is dragged out of their little hut by being held on to the hair. She is put in jail along with her other male relatives, Mosakutty and Iruttappan and is beaten up just like they are. She is tortured in filthy conditions until Rajakannu is found and her pain is inexpressible. Brutality is at its peak when Mosakutty is raised on his thumbs and beaten. Also, they are deprived of food and drinks for a long time.

Panchammai, Rajakannu’s sister is also brought to the police station and beaten up. Panchammai is seen moving around without her saree, which was taken away by the police. Later, Constable Kirubakaran torments her by stripping her and asking her relatives to approach her sexually which makes her lose almost all her senses. They all cry aloud and the helplessness of the downtrodden is explicitly exposed. Here an honest and kind police officer tries to help Panchammai but is mostly powerless as he’s alone and the others and their bias are strong.

When Rajakannu is finally found, he is persecuted beyond limits. He is dragged into the Police Station, tied up in various forms and beaten up cruelly. Chili essence is rubbed into all of their wounds which make them cry out. Rajakannu is very adamant not to confess a crime he has not committed which irks the police officers. When he further says that he did not steal, a baton is kept on his thighs after being tied up. The station

inspector Guru stands on it and screams at Rajakannu “How many times do I tell you to confess? Confess to the theft! Won’t you bloody confess? ” (02:23:01-13) To this Rajakannu begs “I did not steal!” (02:23:13) Inspector Guru still standing on his thighs screams “Won’t you confess? Should I beg you?” (02:23:15-17)

Rajakannu keeps crying and struggling saying that he did not steal. The Inspector threatens to beat him to death and kicks him on his torso and chest innumerable times which makes him unable to get up. The Police speak among themselves that he won’t confess no matter what. Rajakannu on hearing this, struggling very hard to even raise himself a little half begs, half cries “I...I didn’t steal sir.”(02:24:17-18)

On hearing this Inspector Guru gets really angry and runs towards him. He with his strong boots on forcefully, with all his might, stamps into the chest of Rajakannu screaming “Bloody die” (02:24:22). Rajakannu falls to the ground shivering and coughs slowly but with much pain. Agonizingly these were his last moments. Mosakutty remembers that night’s happenings with much pain and fear “He stomped my uncle right in his chest. He coughed up hard and fainted”. (02:24:45-53) Inspector Gurumoorthy, Head Constable Veerasamy and Constable Kirubakaran, after knowing Rajakannu has died, secretly lay him on the borders trying to show that he has escaped prison and shockingly they still have no remorse for what they have done.

Ideological state apparatuses work quite evidently though invisibly in the society. While Police and certain other powerful institutions use violence to make people submissive, ISAs through thoughts, ideas and plots make all the difference. For instance, when Rajakannu and his relatives go to the government office for ST certificates, they are

continuously made to believe that they do not have any rights and that there is nothing to improve their lives. From all sides all that they hear is that they are so worthless and that they don't belong there. Their village chief condescendingly speaks "Big mistake letting you all into the village! Offer a place to sit, you'll ask for a place to lie down!" (00:07:44-47), while his wife questions their very existence in the village.

Usually in Ideological State Apparatuses, educational institutions work to instill the hegemonic ideology, but in this case it is not so. Infact education has made people aware about the inequality around them, as in the case of Chandru and Mythra and has enabled them to fight back. Education brings about major changes in one's life. In Sengini's case, none of her family members have had education and so they had no idea what education could do for them. It is two people with education that made all the difference in their lives. Therefore, educational institutions instead of perpetuating the wrong ideology have helped them in their lives by providing them the required knowledge.

As Rajakannu returns from the brick factory unaware of the theft, many of the upper caste villagers pounce upon him ruthlessly and hunt him down. Nobody questions their behavior and they know it is so. Since Rajakannu believes that he is lower to them, he weeps to be freed, pleading innocence. Everyone, especially the members of the upper caste is aware of the caste differences, and they make sure that it remains so. When Rajakannu touches the person riding the bike, the rider makes a sharp glance at Rajakannu which makes him take his hand away at that instant. When Mosakutty begs the Police Officers to stop hurting Rajakannu, Guru is displeased by the fact that they are talking back.

They are so insignificant that they are not even given a piece of land to live on. Rajakannu, representing all of their kin speaks “No matter what we do, none of us can get a land title deed.” (00:14:22-24) The people of the lower castes are not welcome to the houses of the higher castes. When Sengini comes to the village head’s place to plead for her husband he scolds her telling “You come wail here after the evening lamp has been lit?” (00:57:05-08). The Attorney General who is expected to strive for equality and upliftment of all makes a very casteist statement “You let a tribal woman enter the High Court. Why the hell are you even a cop? ” (01:24:22-23). Another important thing which is gradually internalized by the tribals in *Jai Bhim* is obviously the requirement of education for their upliftment.

Foucault power and knowledge tries to bring out the fact that the dominant sections in any society modify knowledge in such a way as to make it beneficial for themselves. They are quite aware of the fact that knowledge is power and therefore close the path of knowledge to the people they want to suppress. Power in the wrong hands is dangerous. In case that the court had not intervened, the normal path for Sengini would be to deep poverty with her 2 children. Naturally Alli would have to go to work in a small age and this is a vicious cycle which makes people like her illiterate and unable to get better jobs. They would end up doing the cliché jobs they would get. Instead of this usual ending, this movie which actually represented a real life incident ends with empowerment which is a hope to all the affected people. When Rajakannu, Mosakutty and Iruttappan are missing from the jail, Sengini is clueless on how to approach it. As she has always been denied knowledge, she has no idea about the legal side of it. Inspector Gurumoorthy with Head Constable Veerasamy and Constable Kirubakaran, deny all forms of justice by

bringing in false witnesses according to their needs, all of which comes under the pretext of bending knowledge the way they want. The same has happened when the other lower caste members were falsely imprisoned.

Proper education is the only solution to the problems of the Irulas. When education is introduced to them, they receive it quite well. “Learn to read, everything else will follow.” (00:14:30) They believe their teacher when she tells this and realize the fact that only education can empower them and help them get their rights. In many instances one can see the importance these poor uneducated people give to education. Rajakannu convinces Sengini to stay back for the better future of their kids. “If you (Sengini) come there as well, Alli can’t go to school. Won’t her studies be spoiled?” (00:20:55-59) “So as not to catch snakes all his life, he wants to study. He’s bright. If you get him a caste certificate.....” (00:14:48-52) Despite their persistent efforts for education, they are deliberately denied opportunity and are forsaken by those in power. It is ironical when the government officials do not provide them with the necessary documents even at their request when it is actually their duty to do so.

Though they have always valued education, Sengini and her family realize the actual value of education and its benefits only after they are helped by educated people. As the legal battle progresses, Sengini and their other family members now witness Chandru and Mythra, who are well educated, fight oppression and injustice. The scene where Alli mimics Chandru in reading is quite revolutionary since education is their only hope to shatter the barriers built to them by the upper caste.

As the movie progresses, a changing ideology can be noted in the movie. Initially

the Irulas are submissive and do not raise their voices against the oppressions they have to face. But slowly they find a voice of their own. Even at the peak of torture, Rajakannu does not own up to the crime, which shows his perseverance and willpower. In the first half of the movie, Sengini is moved by what's happening to her husband and is seen begging for her husband to the village chief, "Sir, What do we do when even you accuse us so? You are the President of this village. Please tell the cops to not beat them. That's all I ask of you. Please!"(00:57:12-22) But in the latter half, Sengini has evolved to become tough and even ignores the threatening of the village head. "We give you a place to live and work and it gets to your head? How long will it take for us to burn down your huts? Bloody withdraw the case!"(01:26:52-27:01). She refuses to go to the Police Station when illegally called and goes there only Chandru asks her to do so, to bring back Alli. She now trusts the Judiciary which empowers her to not fear the Police and so doesn't meekly listen to the Police like before.

It is revolutionary when the police jeep treads at the heels of Sengini and Alli with police begging behind them to get into the jeep. She doesn't bother about the following Policeman and doesn't care to get into the jeep. The village headman is shocked to know that the DGP has ordered the Police to take back Sengini and Alli safely to their house in a Police Jeep, in a society which still practices untouchability. This incident opens her eyes to her possibilities of safety and protection from the state. In a later scene when the DGP calls them for negotiation outside court, Sengini is not lured by the money offered by the DGP and stands up for their new found sense of honor.

"If they (Alli and the unborn child) ask me how I got the money for food, 'It was given by the same people who beat your father to death, that's how we survive!' Should I tell

them that, Sir? There's no one that cares when we get killed. But we won't live on the alms from the killers. I don't care if I lose this case. I'll tell my children we went down fighting. If you can, punish those murderous cops!" (02:19:17-55)

Their readiness to fight is seen here even though they may lose the battle. It is a great moment when she is awarded a piece of land at the heart of their village in which a brick house will be built for her. This is not something that will ever replace Rajakannu, but this was his biggest dream and through the fulfillment of his dream, it is surely a quite a moment for Sengini and Alli, and all the other members of lower caste. There is a song of hope and courage soon after the verdict is given "Even in a rocky field, there is moisture. Even in a thorny jungle, there are flowers. If you trust there will be another tomorrow. To hold you up, there will be another soul. Wherever you may go, the golden sky will be by you. There are no limits... come... time is ours" (02:37:05-35)

Asuran is directed by Vetrimaaran. It is a movie which is an adaptation of the novel *Vekkai* (Heat) written by Poomani. *Asuran* is a movie that revolves around the problems faced by Sivasami and his family due to the resistance they put up in selling their land to the rich higher caste landowner Vadakooran and his family, who wish to extend their cement factory onto this land. Land here is a symbol of pride and belonging and also the only source of income for Sivasami and his family. Murugesan remarks "Why do we have better stature than other people from our community? Because we own that farm." (00:12:57-13:02) Vadakooran is determined to harm Sivasami and his family to coerce them into giving up their land. Soon, Murugan, Sivasami's elder son is brutally murdered as he had slapped Vadakooran privately for making Sivasami beg pardon by falling on people's feet. Later Chidambaram, Sivasami's second son makes an attempt to

kill Vadakooran and succeeds which pressurizes Sivasami and his family to flee. Finally they reach a compromise that Sivasami would give their land to Vadakooran's family for Chidambaram's safety. Though this is agreed on by everyone, Vadakooran's family tries to hunt down Chidambaram, who is saved by his father. Sivasami ends up killing Rajesh, Vadakooran's son and Venkatesan, Vadakooran's brother as they continuously attack Chidambaram. Sivasami goes to the jail for his crimes and advises his son to study well.

In *Asuran*, one is introduced to a society where there is a huge division based on caste which is the ideology of the people. One section of the society consists of the people living in the north of the village who are the rich upper caste members. The other section of the society is the poor lower caste people who live in the other part of the village. They are expected to be subservient and to give up their land when demanded by the upper caste. The upper caste members do not give any importance to the life or living conditions of the Dalits. When Vadakooran is enquired about the electrically charged fencing that killed Sivasami's dog, his words reflect his attitude "My land is important to me. I will electrify it. Who cares if your dog dies or your people die?" (00:20:29-32).

In *Asuran*, there is visible hegemony by the upper caste people, who are very powerful and are backed by the police. "I will do everything you just said. Officially. I will come and see you after I'm done with him (Chidambaram)" (00:08:53-58). "I will have the entire family locked up" (00:08:20-21). The narrator explains that this constant domination by the upper class landowners is overbearing and quite common. "The problems faced by Chidambaram's family are not unique. Their problems were typical to any small landowner amidst big landlords." (00:12:15-23)

The lands owned by these lower caste small landowners are known as Panchami lands. Panchami lands are properties which were given to the lower caste members by the British. This was done by the then acting Collector of Chengleput, James Henry Apperley Tremeneere after he was reported about the poor living conditions of the Pariahs. The British gave 12 lakh acres to the members of the depressed classes of the Madras Presidency in 1892. This was done by the British to empower them both economically and socially. To make sure that these lands actually stayed with the underprivileged, the British Administration created rules that made it impossible for the rich upper class people to confiscate these lands. This land was non transferrable for 10 years and in case of a transfer, it could only be transferred to the members of the depressed classes. Transaction and sale of these lands with the upper caste was strictly prohibited and it was literally impossible. These lands are called Panchami lands as they belonged to Panchamars or Dalits.

Though these were the rules, the upper caste did find loopholes to confiscate these lands as seen in *Asuran*. Vadakooran's family asks Sivasami's family to transfer their land to one of their aides from the lower caste. Another illegal land acquisition can be noted in Sivasami's youth. The rich landlords who are greedy have acquired over 2500 acres of Panchami lands by illegally making the poor Panchamars give them their land in return for small amounts they have borrowed due to utter poverty. It was to avoid this dependence of the lower caste people on the upper caste that the British gave them the Panchami lands. The British had envisioned that this would help them live a life of dignity. The Dalits have toiled hard on this land and has ended up with nothing. Even when the upper caste landowners are called in for a negotiation with Advocate Sheshadri,

they refuse to give in, confident in their money and power. Viswanathan, the rich landlord smirks and asserts that they run the government. When they realize that Advocate Sheshadri is a threat to them, they conspire to deny him entry to the village on the day of strike and butcher Sivasami's family brutally. At present, what remain of the initial 12 lakh acres of Panchami lands are just 1.86 lakh acres. This is solely due to unlawful activities of the rich upper caste in power.

There is clear interpellation happening in the society as Rajesh is as biased as his father Vadakooran towards Dalits. The younger generations of the upper caste are interpellated to believe that the lower caste members should stay lower than them. When Sivasami is made to beg pardon in the north village, he falls on the feet of a small child. This is what the child will expect from the lower caste when he grows up as this is what he is exposed to.

When a child is born into an upper caste or lower caste family he/she is briefed on how their society works. Briefing doesn't essentially have to be in the form of the elders teaching the kids on what happens in the society; they are unconsciously briefed every now and then observing that what happens around them. When these kids see the way their parents and elders treat the lower caste members cruelly, they naturally tend to think that the lower caste members are not be valued and that they have no say in anything, as home is considered the first school. In the Dalit family when a child is born, the child sees and understands that life is actually tough for them as they are mostly poor and face serious discrimination. But these days, they are aware of the fact that they do not have to remain subservient to the upper castes as they know they are free. This new found sense of freedom is what is irking the upper caste the most.

The Police is much supportive of the rich landlords and are willing to do anything to the poor expecting a fat reward in return for their service. It is shocking as even the institutions which are supposed to provide justice actually support those in power and help in harming the people. Repressive State Apparatus is quite evident when they beat up Murugan when he is arrested for having a fight with Vadakooran's son. Though a lot of people were involved in this brawl, only Murugan is arrested. When Murugan is missing, the Police is quite hesitant to register a case. Gradually when Murugan's body is found burnt without a head, the Police refuse to recognize it as his body. They register a case only after much uproar from Sivasami, Murugesan and Pachaiyamal. And even when they register the case, they register it as two separate cases, one a man missing case and the other a body being found out without head. Chidambaram fails to reach the court due to the police guard in support of Rajesh at the court gate. In Sivasami's past life, the Police had conspired with the rich upper caste to harm the lower caste members during the strike organized by them to get their land back. They had arrested the lawyer and had sent away Sivasami to help the rich bring in violence into the strike and cause havoc.

The Police which is an agency of Repressive State Apparatus misuse their power not just by literal torture but with other forms of torture which includes confiscating what they do not own. The Inspector tries to take bribes from the people in the forest. The inspector asks a constable to catch a goat they see on the forest path, he says "It's a tender little one. It will make a great curry." (00:06:48-50) Murugesan smirks and makes a remark "These scoundrels (the Police) are the people in charge of upholding justice." (00:06:56-59)

Ideological state apparatus works when this very idea of the caste system gets

continuously perpetuated in the society as the right way of everything. The message that the lower caste should remain subservient is perpetuated in the actions and words of many people in the society. The fact that it is normal and okay for Sivasami to fall on the feet of people to beg pardon is a very dangerous form of ISA. Mariyamma, Sivasami's girlfriend was humiliated for wearing slippers. Pandiyan forced her to put the slippers on her head and he kept physically torturing her just for wearing slippers. The message that the upper caste wanted to give the society was to obey them and live according to what they say or that they would be mistreated.

This incident shown in *Asuran* is not fictitious. As recent as in 2019, real life incidents of caste atrocities similar to this have been reported in Tamil Nadu. An offensive school circular was circulated which urged students to be wearing caste bands in school. This was for sure met with much protest, but what is shocking is that the Tamil Society is still quite prejudiced. In 2013, A Dalit young school boy in Vadugapatti was made to put the slippers on his head and was paraded all round the school when he enraged the Upper Caste by apparently breaking the custom of the Lower Caste remaining barefooted. He had come to check the results wearing a slipper. In 2011, the house of G Thangapandian, a Dalit youth was attacked as he bought a bike which was looked upon as a luxury exclusive to the members of the Upper Caste. This happened in the Villur village in Madurai.

The family of Vadakooran doesn't pay heed to the Panchayat, while Sivasami and his family obey and follow exactly what the Panchayat decides. Sivasami has to listen to it since that is his only ray of hope for justice. Vadakooran and his family take law in their hands as they believe they have the power to do so. After Sivasami begs pardon to

Rajesh, Vadakooran ignores the objection of the Panchayat when he makes him fall on the feet of all the upper caste members. When the Panchayat head warns Venkatesan “You’re violating the council’s order” (02:08:30-32), he retorts “If we abide by it, what about our family’s honor? He (Chidambaram) has to die.” (02:08:33-36)

Foucault’s knowledge and power can be applied in this society too. In *Asuran*, there is a set of people who are members of the upper caste and they are rich, influential and powerful. They are actually the ones in power and they decide what is to happen in the village. The Policemen are mere puppets in their hands and works according to their wishes. After Vadakooran is killed, his son Rajesh is seen commanding the Police “You stay out of this. We know what to do and how to do it.” (00:08:32-35) Rajesh can also be seen hitting another man in front of the Inspector and he doesn’t even bother about it.

Vadakooran and his family are the most powerful in the village and they have all means to control the knowledge that spreads there. They make it clear to the Dalits that, if at all they raise their voice, they would be butchered like Murugan and that they would have to watch it helplessly. Chidambaram once makes a statement that, no matter what they do they will just end up nothing, and therefore there is no use studying. He has seen the fate of his family members and thinks that the same awaits him. In Sivasami's youth, the upper caste is seen strictly enforcing certain rules and they decide what is to be perpetuated in their society. The people in these places have no clue about their own land and it is clear that this knowledge is being kept from them. When Advocate Sheshadri is trying to create awareness and empower the villagers, the police help the upper caste in killing their efforts as the rich and powerful landlords fear the poor getting the right knowledge.

In multiple places, Sivasami is seen making sure that his kids attend school. This is to make sure that they get acquainted with the right knowledge and reach great heights Sivasami and his generation could never imagine. In his youth, he is seen to provide Mariyamma, his fiancée with everything for her to go for higher education. Though he had promoted education he didn't know its real importance until he met Advocate Sheshadri for the land strike and visualized how much capable education has made him. Advocate Sheshadri has played an important role in Sivasami's life by helping him with with multiple court cases of theirs. His acquaintance with Advocate Sheshadri opens Sivasami's eyes and made him realize that the right way to fight back is not through violence but by getting educated.

Chidambaram realized the importance of education when Advocate Sheshadri uses his knowledge to send Sivasami to the jail instead of Chidambaram, owing to Sivasami's requests for his son's better future. Chidambaram acknowledges that education will give them freedom and empowerment and understands what his father was always trying to tell him. School, which is usually a place where ISA works, is not seen to be perpetuating the wrong ideology in this case.

“If we own farmlands, they will seize it. If we have money, they will snatch it. But if we have education, they can never take it away from us. If you really want to win against them, study. Study hard and become a powerful man. But when you have power, don't do to anyone what they did to us. It's easy to deepen hate. But we must rise above it.” (02:14:29-45) These remarkable words of Sivasami on the importance of education is the message the movie gives to all the downtrodden.

There is a negation in the ideology in the movie when the lower caste people quit being subservient and actually start raising their voices as a form of resistance. By fighting through words and actions, they have started a movement against the existing ideology. The lower caste people have realized that the more they bend the more they are pushed. After destroying the electric fence Murugan bravely speaks “We won’t let him get away with it. We will retaliate too. Those times (of subservience) are gone. Unlike you (the older generation), we can’t fold our hands and bow our heads and take handouts from him. How will he give us our due? We must fight him for it. Why are you so afraid?” (00:22:06-22) They have gradually realized that effective changes can be brought about only by gaining knowledge and not by their aggressive actions or words.

The term ‘Asuran’ means ‘Demon’. Demon doesn’t essentially have to mean evil, it has a human side. When Sivasami rushes into the house of Viswanathan to seek revenge, he can be looked upon as a demon, but he too has a human side. His brother is stabbed and his remaining family is all burnt down. When a little baby is thrown out Pandiyan throws his back in to burn to death. Ironically, it was Sivasami that had helped Pandiyan to reach his present status as he was very poor though an upper caste member. It’s this brutality that makes Sivasami a ‘demon’. In his later life, Sivasami lives with immense patience and tolerance. He is seen to turn a blind eye towards all the atrocities committed towards his family until he can no longer stay calm or his son would be killed. The great Babasaheb Ambedkar had asserted that Asuras are human beings who were dehumanized for long. In this movie, Sivasami and his family, who represent Dalits in general, are dehumanized in many instances one of which is when they are hunt down by dogs and men. Another instance is when they are told that their life or their dog’s have no

sympathy from Vadakooran and his family. This implies that they are of no greater value than their dog which has been electrocuted by the fence put up by Vadakooran and his men. This movie exhibits that no matter how tolerant one is, injustice will somehow compel that person to resort to violence to live on, let alone fight back. When Sivasamy fights back he is seen as Asuran, a strong fighter who will fight for the lower caste and all their losses. Through the theme song 'Asuran', the message of resistance and fighting back is clearly delivered.

“Rise against all odds;

Those at odds- off with their heads

What is justice? It's yours to profess

What's the origin? That's your quest.

Rise Asuran! Rise Asuran! Rise Asuran!

Make them tremble in fright

Let their wicked blood splatter” (01:46:14-48:30)

Initially, Sivasami can be seen as a man who is very calm and subservient.

Though his sons always try to fight the upper caste people, he's always seen advising them to stay calm. Sadly, their resistance finally leads to the death of Murugan and his family is left heartbroken. Sivasami had realized that violent fighting is not the right way to retaliate as his brother was killed for leading a movement against the rich upper caste landlords and his family was burnt to death for his violent rebellions. But it took some

time for his family to perceive the same and understand the importance of education. What is enthralling here is that, while the lower caste people fight for their very survival, the upper caste people fight for their selfish desires. Murugan was killed just for Vadakooran to assert his power on them, the lower caste. Even Chidambaram is trying to be beaten to death for their family's apparent honor. Sivasami never starts a fight, and he fights only when it's quite necessary as protecting his son's life. There is a change in ideology taking place even in the minds of the upper caste people. When Sivasami is at the village falling at the feet of the upper caste, an upper caste man behaves quite differently from others. He addresses Sivasami as brother and speaks with much pity "Brother, you don't have to do that! Muthu, please give him some water. No one has the guts to stand up to their atrocities. Don't worry. Your son will return home safe." (00:29:39-47) This might be a hint that the members of the present generation are more humane and less biased. This is a bigger change, since its origin is the minds of the upper caste and not the downtrodden.

In both *Jai Bhim* and *Asuran*, it is the presence of an Advocate and his knowledge that helps them crucially in a critical time. The timely interference of the advocate has saved many lives in both the cases. In *Jai Bhim*, Advocate Chandru helped in the release of Mosakutty and Iruttappan and also his powerful words helped serve justice to the loss of Sengini and her children. In the case of *Asuran*, it is the timely interference of Advocate Sheshadri which helped in the release of Sivasami, as he mentioned in the court that it was a communal riot and later Chidambaram. Advocate Sheshadri's also aided the lower caste people in understanding that the land is legally theirs (Panchami lands). He takes it as his job to spread awareness and help them fight for their land. In *Jai Bhim*,

Advocate Chandru fights for the rights of people belonging to various castes and classes who work in different fields. What is common between them is their sense of justice and their willingness to fight for people selflessly. They are fundamentally the voice of the voiceless and have a common goal of uplifting the downtrodden.

There is a similar story line in both the movies; helpless people from depressed classes are exploited physically and mentally on no particular faults of their own. In *Jai Bhim*, their rights are brutally denied and they are being tortured for the selfish desires of those in power which results in Rajakannu losing his life. In *Asuran*, Vadakooran and his family hold a grudge towards Sivasami and his family since they didn't give up their land to Vadakooran for their factory. Therefore they torture different members of Sivasami's family, to coerce them to give up their land. These two cases represent the Tamil Society where various members of the depressed castes still face serious atrocities. And the only way out for them is education, as knowledge is power and their only way out.

From no representation to stories being centered on Dalits, the Tamil Media has gone a long way. Tamil Society is one which works quite in par with what happens in movies. As movies influence Tamilians a lot, this revolutionary change in the movies might bring a drastic change in the society too. What is getting represented in movies are mostly the real life happenings in the Tamil society. This change in the film industry is expected to bring a change in the entire system which makes up the society. These movies are a ray of hope for the downtrodden who have now realized the importance of education. The right sense of justice is something which is being promoted by these movies. If these movies influence the members of the upper caste to become 'just' in addition to the empowerment it gives to the members of the lower caste, it will surely

make life better for the latter.

Conclusion

Caste system had prevailed in Indian Society for a long time and its ill effects were very profound from then. Even in the twenty-first century, different states of the country have visible discrimination based on caste. Movies are a way of representing society and what happens there. Dalits earlier had less representation and were shown in roles with no identity and individuality.

In *Jai Bhim*, in the first half, Sengini is a woman who doesn't have any idea of laws or her rights. But as the film progresses, she grows to become a wise woman who is well aware of her rights and the power of education. She gets a better life with help from the government, who's one institution, the Police was the initial cause of her misery and also understands the value of education which will make her educate her children for sure.

In *Asuran*, Sivasami and his family were tortured in different ways to give up their land to the upper caste. Here too, Sivasami and his family are helpless at the taunts of the upper caste who are supported by those in power. The family realizes the actual importance of education and the power that knowledge provides.

By looking at the concepts of Althusser and Foucault, it can be seen that the caste system is just a perpetuated ideology which the upper caste or the powerful has held on to make life easy for them. By using various state apparatuses people are forced to adhere to this ideology to exist in a society. They are made to believe that this is the reality, wherein this which is created is just an illusion which a person follows unconsciously.

People are interpellated from the day they are born to believe in what the society wants them to believe.

There have been significant changes in the representation of the Dalits and they are taught ways to be strong. From having no idea about what happens in the society, the characters have evolved to realize the power of knowledge. They have come to know about their rights and the protection they are assured by law. Both these movies assert that education is power and the right form of resistance.

Over the past few years, there are huge waves of changes in Tamil Media. The spirit of revolution ignited in the field of movies paved way to the creation of The Casteless Collective, which is a sensational band. They create songs backed by powerful music which brings a sense of hope to the downtrodden. Their songs are being widely accepted, followed and encouraged by lots of people which show that people are looking forward for a change. There is a wide scope for further research on these phenomenal changes occurring in Tamil Media. These changes can be further explored with the help of Subaltern study, Post Colonial study, Neo Colonial study or even Post Human study.

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LEARNING PROCESS IN FIGHTER FISH (BETTA SPLENDENS)



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Affiliated to Mahatma Gandhi University, Kottayam in partial
Fulfilment of requirement for the degree of Bachelor of science
In Zoology
2021-2022

CERTIFICATE

This is to certify that the project entitled "LEARNING PROCESS IN FIGHTER FISHES (Betta splendens)" submitted by Ms. Ann Catherine Hridya, Reg No. AB19ZOO002 in partial fulfillment of Bachelor of Science Degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Reema Kuriakose and this is her original effort.

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Examiners

1)

2)

DECLARATION

I, hereby declare that this project work entitled “LEARNING PROCESS IN FIGHTER FISH (Betta splendens)” is submitted to St.Teresa’s College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science degree in Zoology. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own.

Name:Ann Catherine Hridya

Signature:

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Ann Catherine Hridya

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ABSTRACT

The project entitled "Learning process in Fighter fishes (*Betta splendens*)" was conducted to evaluate the memory of fighter fishes and its ability to distinguish between colours. During the experiment fishes are conditioned in such a way that they are fed just after showing the pink-coloured indicator and was not rewarded with anything after showing the green-coloured indicator. The fishes gradually started showing responses to pink coloured indicator avoiding the green. Later the activity was stopped for a week and repeated again to test its memory, and they responded positively. From the present study it can be concluded that fighter fishes were able to distinguish between colours and they have got enough memory.

INTRODUCTION

Animal learning is an alternation of behaviour as a result of individual experience. When an organism can perceive and change its behaviour, it is said to learn. The cat that runs to its food when it hears the sound of the cupboard opening; the rat that solves a maze, the bird that acquires the song of its species etc, are common examples that demonstrate animals can learn.

Learning produces changes in the behaviour of an individual due to experience. Learning is adaptive because it allows an animal to respond quickly to changes in its environment. Once an animal learns something, its behavioural choices increase. An animal's ability to learn may correlate with the predictability of certain characteristics of its environment where certain changes in the habitat occur regularly and are predictable, the animal may rapidly respond to a stimulus with an unmodified instinctive behaviour. An animal would not necessarily benefit from learning in this situation. However, where certain environmental changes are unpredictable and cannot be anticipated, an animal may modify its behavioural responses through learning or experience. This modification is adaptive because it allows an animal to not only change its response to fit a given situation, but also to improve its response to subsequent, similar environmental changes. The ability to learn is common to most animal species: the need to exploit past experience being obviously extremely important for survival, many animals have evolved ways of coping with it. Although the complexity of learning needed for optimal survival may be different in different species, the basic mechanisms appear to be fairly constant even in phylogenetically distant ones.

Learning ability in fishes is one of the most experimented topics worldwide. It is noticed that the more fish present in the group, the faster they learn (Brown and Warburton, 1999). Social learning occurs when information passes from one individual to another by observation or interaction. This can lead to a transfer of information through generations (vertical transmission) resulting in cultural traditions. This has implications in migration, shifts in spawning or foraging grounds that is happening in commercial fish stocks.

Many acts of discrimination are based on conditioning of the fish to single or multiple stimuli. The ability to associate stimulus and response is present even in simple invertebrates; but levels of “intelligence” can be distinguished among other criteria, based on the speed of learning simple tasks and on the retention span of learned responses.

Fish can communicate in their underwater environments through the use of acoustic communication. Acoustic communication in fish involves the transmission of acoustic signals from one individual of a species to another. The production of sounds as a means of communication among fish is most often used in the context of feeding, aggression or courtship behaviour. The sounds emitted by fish can vary depending on the species and stimulus involved. They can produce either stridulatory sounds by moving components of the skeletal system, or can produce non-stridulatory sounds by manipulating specialized organs such as the swim bladder.

Betta fish are most beautiful when they are “flaring” and showing off their colorful fins. However, this display is actually a sign of aggression and means that trouble can be on the way if there are other fish in the tank.

They are capable of learning and remembering. It makes evolutionary sense that an organism has to possess some degree of intelligence to navigate its surroundings and avoid predation. The Betta Fish is no exception. It lives in a dynamic world in which it must constantly respond appropriately to environmental stimuli.

A number of studies have been conducted on learning habits in various fishes, but studies related to fighter fish are very less.

REVIEW OF LITERATURE

Animal cognition is the process by which animals acquire, process, store and act on information gathered from the environment (Shettleworth, 2010). It also connotes that intelligence is about learning from past experiences and applying this knowledge to solve novel problems, in other words intelligence is about behavioural flexibility (Roth and Dicke, 2005). The complexity of the environment in which many fish live (both social and physical), is often the cause of such high degree of behavioural plasticity, a good memory certainly indicates association between positive and negative stimuli and the consequent responses of the organism. Animal cognition encompasses the mental capacities of non human animals. The study of animal conditioning and learning used in this field was developed from comparative psychology. It has also been strongly influenced by research in ethology, behavioural ecology, and evolutionary psychology; the alternative name cognitive ethology is sometimes used. Many behaviours associated with the term animal intelligence are also subsumed within animal cognition.

Several fish species are capable of learning complex spatial relationships and forming cognitive maps (A cognitive map is a type of mental representation which serves an individual to acquire, code, store, recall, and decode information about the relative locations and attributes of phenomena in their everyday or metaphorical spatial environment). They can orient themselves using multiple landmarks or symbols and they are able to integrate experiences which enable them to generate appropriate avoidance responses. Fish is known to have quite small brains relative to body size compared with other vertebrates, typically one-fifteenth the brain mass of a similarly sized bird or mammal. However, some fish have relatively large brains, most notably mormyrids and sharks, which have brains about as massive relative to body weight as birds and marsupials.

Fish intelligence is the result of the process of acquiring, storing in memory, retrieving, combining, comparing, and using in new contexts information and conceptual skills, as it applies to fish. In many areas, such as memory, their cognitive powers match or exceed those of higher vertebrates including non-human primates. Fish hold records for the relative brain weights of vertebrates.

Most vertebrate species have similar brain-to-body mass ratios. The deep sea bathypelagic bony eared assfish, has the smallest ratio of all known vertebrates. At the other extreme, the electrogenic elephantnose fish, an African freshwater fish, has one of the largest brain-to-body weight ratios of all known vertebrates (slightly higher than humans) and the highest brain-to-body oxygen consumption ratio of all known vertebrates (three times that for humans).

Fish can remember the attributes of other individuals, such as their competitive ability or past behaviour, and modify their own behaviour accordingly. For example, they can remember the identity of individuals to whom they have lost in a fight, and avoid these individuals in the future; or they can recognize territorial neighbours and show less aggression towards them as compared to strangers. They can recognize individuals in whose company they obtained less food in the past and preferentially associate with new partners in the future. Fish can learn how to perform a behaviour simply by watching other individuals in action. This is variously called observational learning, cultural transmission, or social learning. For example, fish can learn a particular route after following an experienced leader a few times.

Solving an environmental problem may need the retrieval of learned and memorized information that is suitable for the specifics of the situation (Shettleworth, 1993). For instance, recalling mate location in a particular area or making the association between specific events and negative stimuli, such as predator presence, may help individuals to make beneficial decisions (Gerber et al., 2014). A memory to enhance future survival and reproduction is hence essential in a variable and challenging environmental condition (i.e., food availability, predation risk, contextual information, etc.) (Brom et al., 2014; Dunlap et al., 2009; Templar and Hampton, 2013)

Long term memories that can last at least a few months to decades have been documented in non-human animal species. As it has been shown that the neural basis of learning and memory is well conserved across vertebrate species (O'Connell & Hofmann, 2011; Salas et al., 2006), there are also widespread examples of learning and memory abilities in fish that are similar to other vertebrate species (Brown, 2015).

Concerning long-term memory, various studies provided evidence that fish can retain information in their memory for a few weeks to several months. As such, rainbow trout retrieved an information consolidated 3 months ago, wherein they had to press on a trigger to get food delivered to them (Adron et al., 1973); gobies returned to their home pools 40 days after being translocated to neighbouring pools (Aronson, 1971); anemonefish still remembered landmarks of their anemones neighbourhood after 6 months (Fricke, 1974). What may strengthen memory retention capacities is the valence of the event, where in long-term memories documented in the literature are often memories of adverse events (Brown, 2001; Gerber et al., 2014; Schafe et al., 2001). For instance, salmon and carp that had been caught with hook showed hook-shyness 1 year later without any exposure in between (Beukema, 1970; Tarrant, 1964). Similarly, crimson spotted rainbowfish retrieved the memory of an escape strategy learned in a laboratory experiment 11 months earlier. Familiarity with the test environment could have been a potential confounding factor (Brown, 2001).

Cleaner fish can remember a single negative experience (i.e., being caught in a barrier net) about 11 months later in the wild (Triki & Bshary, 2019). Their hiding response suggests that this particular behaviour is the result of long-term memory retention of being caught in a barrier net. The fish that went into hiding reacted to the barrier net instead to the mere presence of divers. This is because when a fish went into hiding, the very same individual re-emerged when the barrier net had been removed. However, it is possible that the combination of the presence of a barrier net and the close proximity of the diver triggered the hiding response. The ability of cleaners to remember for at least 11 months a single event of being caught in a barrier net is similar to long-term memory retention proposed to cause escape response from a trawl in crimson spotted rainbow fish (Brown, 2001), and hook-shyness in salmon and carp (Beukema, 1970; Tarrant, 1964). These memories, being caught in a barrier net, trawl or a fishhook, have in common that both are consequences of highly aversive events linked to life threatening events. It is hence crucial to retain information after a single exposure and to adjust behaviour in a similar future event. A recent study on sharks, for instance, has shown that individuals may learn to avoid being caught after the catch-and-release experience (Mourier, Brown, & Planes, 2017).

Colour and shape perception allows the animal's visual environment discrimination and brings advantages for feeding, defense, life in groups, migration, and mate choice (Hughes and Blight, 2000; Blackiston et al., 2011). In fact, fishes are able to see colour from blue to infrared (Levin and Mac Nichol, 1982) and can discriminate a variety of geometrical forms (Petrazzini et al., 2012). The advantage of visual cues recognition is the straight signal source, while olfactory and auditory signals show scattered routes of dispersion (Litherland and Wallis, 2009).

Colour perception preference directly affect fish learning (Spence and Smith, 2008; Sison and Gerlai, 2010), memory formation (Colwill et al., 2005), and decision-making (Avdesh et al., 2010). Many studies show ambient colour and hint colour influence on fish navigation (Hughes and Blight, 2000; Cognato et al., 2012), spatial location (Petrazzini et al., 2012; Parker et al., 2012) and welfare (Serra et al., 1999; Luchiari et al., 2007). However, studies approaching the fish's ability to discriminate different colours and shapes are still lacking and may indicate an animal's cognitive faculty, plus allowing neural studies, such as neurological diseases or neural disabilities caused by drugs (White, 2000; Gerlai, 2010).

Fighter fish have memories that last several months, not a few seconds like many believe. Scientists say that popular pets can remember things that happened as long as five months ago. To prove this, researchers conducted a couple of studies that have concluded that fishes are not as forgetful as many thinks.

METHODOLOGY

Materials Required

Glass bowl having a mouth width 15 cm, two different colour cues (which can be made from cardboard pieces), 2 fighter fishes, and fish feed.

FISH USED: Fighter fish

SCIENTIFIC NAME: Betta splendens

FAMILY: Osphronemidae



Indicators used



Fighter fish used



fish feed used

PROCEDURE

The project entitled "Learning process in fighter fishes(*Betta splendens*) " was carried out for a period of one month. In this experiment, two different colours used for detection, are pink and green. At the beginning of the experiment, the fishes were allowed to observe the green colour cardboard piece for about 20 seconds exactly one hour before feeding the fish. The fish is then allowed to observe the pink colour indicator just or immediately before feeding the fish. This procedure was repeated in the similar way for about 24 days. Later the activity is stopped and repeated after taking a break of one week to analyze its memory. Light conditions were maintained constant inside the bowl throughout the experiment. The fishes were fed twice a day with approximately equal quantities every time.

OBSERVATION

Evaluating the memory of fighter fish and its ability to distinguish between colours.

Number of days	response to pink indicator	response to green indicator
0 to 7	Nil	Nil
7 to 14	Nil	Nil
14 to 21	Yes (from 15 th day)	Nil
21 to 24	Yes	Nil
25 to 31	indicator not shown	indicator not shown
32 nd day	Yes	Nil

RESULT

In this project entitled "Learning process in fighter fishes " fishes are conditioned in such a way that they are fed just after showing the pink colour and was not rewarded with anything after showing the green-coloured indicator. At first the fishes did not respond to either of the colours and responded only to the food but as the process was continued after almost 15 days the fishes started showing responses whenever the pink-coloured indicator was brought to their sight and no responses were shown to the green colour. As they realized that, rewards were not given with green, they gradually stopped the response. Later they started showing responses only to pink coloured indicator. As soon as the pink colour was shown they were excited, and they moved towards the corner of the bowl opening their mouths, anticipating the food. This process was repeated for a few days purposely so that the colour gets stored in their memory and to get the relationship between colour and food. Then the activity was stopped and the fishes were not exposed to either of these colours and were fed normally. When the pink colour was shown exactly after one week, surprisingly the fishes responded to pink colour which points to the fact that the fishes were able to store it in their brain and recall the similar situation from their memory when repeated again.

DISCUSSION

The project was based on the learning ability and memory capacity exhibited by fighter fishes. These fishes were able to distinguish between the colours provided and were also able to recollect it later. Fish intelligence is the result of the process of acquiring, storing in memory, retrieving, combining, comparing, and using in new contexts information and conceptual skills". According to Culum Brown, "Fishes are more intelligent than they appear."

Fish can also learn to avoid aversive stimuli rapidly and retain the information for extensive periods, for example pike that have been hooked often show hook shyness over a year (Beukemaj, 1970). Similarly, rainbowfish taught to swim through a hole in a net that travelled down the length of their aquarium took just five runs to figure out the location of the escape route. When tested almost a year later, they still recalled how to escape the net even though they had not seen the net in the intervening period (Brown, 2001).

Zebrafish have shown Pavlovian learning in several experiments. The experiment illustrates a "place-preference" task used by Darland and Dowling to screen zebrafish for cocaine sensitivity. During training, the screen is sealed and a zebrafish is exposed to cocaine in one of the chambers. In subsequent preference tests, the fish showed an appetitive conditioning effect by approaching and staying in the chamber in which they had previously received cocaine.

The scientific potential of the zebrafish was discovered by George Streisinger. George Streisinger (1927) was the father of zebrafish research. He established a zebrafish research colony and developed the first methods for mutagenesis and mutant screening with the goal of studying the development of the nervous system through genetic analysis. In another experiment conducted on goldfishes the average time for the goldfish to find food in the maze after an absence of six months was 12.82 seconds, which was less than half of the time taken on the last day of training (30.19 seconds) 16 months earlier. These results suggest that the ability of goldfish to retain and recall an explicit memory is impressive considering its small brain size, short life span, and lowest place on the vertebrate hierarchy (Katie, Michigan, 2015)

The literature contains several examples of specialized cognitive abilities, but few regarding fish. The guppy, *Poecilia reticulata*, is a freshwater fish in which females choose their mates based on colouration, and orange-coloured fruits are important diet enrichments for both sexes. For these reasons, we expect that this species has evolved enhanced learning abilities in colour discrimination compared to other types of discrimination.

A number of studies have shown that fish can retain information for months or years. Anecdotally, channel catfish (*Ictalurus punctatus*) can remember the human voice call announcing food, five years after last hearing that call. Goldfish remember the colour of a tube dispensing food one year after the last tube presentation. Sockeye salmon still react to a light signal that precedes food arrival up to eight months since the last reinforcement. Crimson-spotted rainbowfish can learn how to escape from a trawl by swimming through a small hole in the center and they remember this technique months later. Rainbow trout can be trained to press a bar to get food, and they remember these three months after last seeing the bar. Red Sea clownfish can recognize their mate 30 days after it was experimentally removed from the home anemone.

Individual carp captured by anglers have been shown to become less catchable thereafter. This suggests that fish use their memory of negative experiences to associate capture with stress and therefore become less easy to catch. This type of associative learning has also been shown in paradise fish (*Macropodus opercularis*) which avoid places where they have experienced a single attack by a predator and continue to avoid for many months. Red Sea clownfish can recognize their mate after 30 days separation.

Through social learning, fishes might learn not only where to get food, but also what to get and how to get it. Hatchery-raised salmon can be taught to quickly accept novel, live prey items similar to those they will encounter once they will be released in the wild, simply by watching an experienced salmon take such prey. The same is true of young perch. In the laboratory, juvenile European seabass can learn to push a lever in order to obtain food just by watching experienced individuals use the lever.

CONCLUSION

Many studies in fishes have shown that fishes can retain information for months or years and also, they have the ability to depend on visual cues for feeding, mating, migration etc. In this study the main objective of the project was to evaluate the memory and its ability to distinguish between colours in fighter fishes.

From the present study it can be concluded that fighter fishes were able to distinguish between colours and they have enough memory to relate between colour and the feed.

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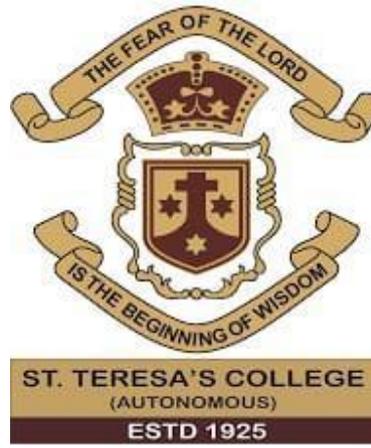
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DNA BARCODING IN INDIAN MARINE FISHES

- *Cynoglossus macrostomus* and *Decapterus russelli*



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Affiliated to Mahatma Gandhi University, Kottayam

In Partial fulfilment of requirement for the Degree of Bachelor of Science in

Zoology

2021-2022

CERTIFICATE

This is to certify that the project report entitled “DNA BARCODING IN INDIAN MARINE FISHES- *Cynoglossus macrostomus* and *Decapterus russelli*” submitted by Ms. ANNA SEBASTIAN, Reg. No: AB19ZOO003 in partial fulfilment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under my guidance and supervision and to my best knowledge, this is her original effort.

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EXAMINERS

1)

2)

DECLARATION

I, hereby declare that this project work entitled “DNA BARCODING IN INDIAN MARINE FISHES- *Cynoglossus macrostomus* and *Decapterus russelli*” is submitted to St. Teresa’s College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science degree in Zoology. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report are entirely my own.

NAME: ANNA SEBASTIAN

SIGNATURE

REGISTRATION NUMBER: AB19ZOO003

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ANNA SEBASTIAN

DNA BARCODING IN INDIAN MARINE FISHES-

Cynoglossus macrostomus and *Decapterus russelli*

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ABSTRACT

The present study reflects DNA barcoding in two Indian Marine fishes. DNA barcoding involves sequencing a fragment of the mitochondrial cytochrome oxidase I (COI) gene, known as DNA barcodes, from taxonomically unknown specimens. The following study has been undertaken to research and analyse the barcode of Indian Marine fishes and their DNA sequences. DNA barcoding is a method of species identification using a short section of DNA from a specific gene. This study focuses on barcoding the DNA of 2 genera of fishes collected from Beypore Fishing harbour, Kozhikode. The study was completed in 4 phases. The first two phases were collection and preservation of specimens for later analysis. Third phase consisted of DNA isolation from the collected samples and amplification of the sequence. Since DNA extraction is a sensitive process, buffers are added to stabilise the pH over cell lysis and isolation. The final phase consisted of PCR and barcoding the isolated DNA samples. From the 21 samples collected, fishes belonging to 8 genera were identified which include Pomadasys, Carangoides, Decapterus, Cynoglossus, Nemipterus, Selar, Mene, Leiognathus and Pericanthus out of which 2 genera were specifically analysed namely *Cynoglossus macrostomus* and *Decapterus russelli*.

INTRODUCTION

Identification of fishes contributes to the classification of each fish and the crucial function they play in the biological world, as well as the discovery of important food and medicinal ingredients from them. Fishes are the primary food source of humans, hence research on them has a significant impact on human life. Various approaches can be used to identify an unknown specimen. Identifying it with the use of morphology was a typical method employed previously, but morphology only provides external structural features, fishes have undergone multiple ontogenetic development, convergent and divergent evolution, identifying them only based on their morphological characteristics will be very challenging. As a result, morphological features alone are not sufficient for unknown specimens; in these cases, DNA barcoding is used.

DNA barcoding, or sequence-based specimen identification, was developed by Paul Hebert in 2003 to identify a broad range of taxa by sequencing a standardised short DNA fragment, the “DNA barcode”. DNA Barcoding is a technique for identifying different species. It was first used in fishes by Ward in 2005. It functions by examining a specific DNA region and this specific area is known as the DNA barcode. For animal identification, the most broadly used barcode marker is mitochondrial Cytochrome C Oxidase subunit I (COI), which is highly conserved across species employing oxidative phosphorylation for metabolism. After that, the sequence of this DNA barcode is compared to a reference library that contains information on many different species and their barcodes. DNA Barcoding consists of the following steps: DNA Isolation, Amplifying the isolated DNA using Polymerase Chain Reaction, Gel Electrophoresis and Sequence Analysis. DNA isolation is a key step because, without high quality DNA, the PCR amplification will not be optimal. The following statements are some of the benefits of DNA barcoding over previous classification systems: DNA barcoding help in accurately distinguishing some species that are similar in morphology, cryptic species are indistinguishable biological groups that are incapable of interbreeding and cannot be distinguished using traditional methods of classification because traditional methods classify cryptic species as a single group, despite the fact that these species show genetic variation. (Lakra et al., 2011)

They can be easily distinguished through DNA Barcoding, Barcoding methods can often give information without causing harm to the animal studied. In cases of morphological ambiguity, such as with larval stages, DNA barcoding technology can help identify species. The applications of DNA barcoding includes ecological monitoring, early detection, control and removal of non-indigenous species, fisheries management, food safety and protection of endangered species. The results generated by DNA barcoding are limited or biased to the frequency of occurrence, and quantifying fish abundance from molecular data is another key problem for this approach.

There are several different types of DNA extraction methods. A few of them include Phenol-Chloroform Isoamyl Alcohol, Proteinase K, CTAB Method, Spin Column-based Methods and Magnetic Bead-based Technique. However the method implied on each specimen depends on sample type and purity and the yield of DNA that is to be obtained. DNA extraction is completed in 4 steps: lysis, separation, precipitation and purification. There are 2 main types of DNA sequencing methods. The classical method is known as Chain Termination method or Sanger Sequencing Method. Other modern methods that can process a large number of DNA molecules swiftly are collectively called High-Throughput sequencing (HTS) and Next-Generation sequencing methods (NGS). In the conducted study, DNA isolation was done using the DNeasy Blood and Tissue Kit and respective buffers namely Proteinase K, Buffer ATL, Ethanol, Buffer AL, Buffer AW1, Buffer AW2, Buffer AE and Buffer AP. Polymerase Chain Reaction (PCR) is a powerful method for amplifying particular segments of DNA, distinct from cloning and propagation within the host cell. Out of the various PCR techniques, amplification of the isolated sequence was done using normal Polymerase Chain Reaction. DNA sequencing was done to the amplified sequence using Sanger sequencing method. It is done to determine the nucleic acid sequence or order of nucleotides in DNA. Lastly, a phylogenetic tree was drawn with the help of the software MEGA 11 to identify the species of the specimen.

AIM AND OBJECTIVE

AIM

To identify two types of fishes using DNA barcoding.

OBJECTIVE

- To barcode the various types of Indian Marine Fishes.
- To provide the statistics, characteristics and presence of types of fishes in certain areas.
- To compare and classify the varieties of fishes in marine habitat.

REVIEW OF LITERATURE

DNA Barcoding is a molecular diagnostic method used for identification of species by using a standardised DNA sequence or genetic region which acts as the 'barcode'. 'DNA barcoding' is a new identification tool proposed by Hebert et al. (2003), and is a valuable addition to the taxonomic tool box. They advocated the use of short DNA sequences from the specified region of the genome termed as DNA barcode for biological identification. It implies sequencing of a standard DNA locus as a tool for identifying species. An ideal DNA barcode should be easily retrievable with a single primer pair, be amenable to bidirectional sequencing and effectively provide high discrimination among species.

According to Savoleinen et al., (2005), the scientific benefits of DNA barcoding include: (i) enabling species identification, including any life stage or fragment, (ii) facilitating species discoveries based on cluster analyses of gene sequences, (iii) promoting development of handheld DNA sequencing technology that can be applied in the field for biodiversity inventories and (iv) providing insight into the diversity of life.

Based on the works of Jeremy C. Andersen et al., (2019), the collection of DNA barcode sequences from unidentified specimens provides useful genomic data and at the same time DNA barcoding techniques are being used with increasing frequency to guide management decisions, particularly for the identification of alien invasive species. (Dejean et al., 2012)

This study utilises the standard Cytochrome C Oxidase subunit I (COI) which is found in most eukaryotes and highly conserved and so can be copied from unknown organisms. They also have less intraspecific (within species) variation than interspecific (between species) variation, known as the "Barcoding Gap". When fully developed, a COI identification system will provide a reliable, cost-effective and accessible solution to the current problem of species identification. Its assembly will also generate important new insights into the diversification of life and the rules of molecular evolution (Hebert et al., 2003). The mitochondrial genome of animals is a better target for analysis than the nuclear genome because of its lack of introns, its limited exposure to recombination and its haploid mode of inheritance (Saccone et al.,

1999). Robust primers also enable the routine recovery of specific segments of the mitochondrial genome (Folmer et al., 1994).

The major goal of DNA-barcoding efforts is to aid identification of specimens by matching sequences to a sequence library. The revolution introduced by DNA barcoding resides in the molecularisation, computerisation and standardisation of taxonomic approach. The identification and then the interpretation of molecular entities is the main goal of DNA barcoding that could be reached only by users with a sound theoretical background on what is identifiable by this technique (Casiraghi et al., 2010). Many authors have proposed DNA barcoding as an integrated approach with classical taxonomy for species identification and authentication. Modifications in extraction methods, primer sequences, use of an engineered polymerase and even the combining of barcodes from multiple loci has been used successfully to clear any issues related to DNA Barcoding in vertebrates.

In order to test the utility of DNA Barcoding in forensic vertebrate species identification, COI sequences from previously identified samples from humans and a variety of domestic and wild specimens of Brazilian mammals, birds, fishes were compared against the Barcode of Life Database (BOLD). BOLD provided a correct species-level identification for 12 out of the 20 queried sequences (60%) and presented the correct species as the best matched one for 17 out of 18 samples morphologically identified to this level (94%). (Carvalho, 2014)

Barcoding can be used as an alternative to traditional sampling methods in fish research. Barcoding procedures can often give information without causing harm to the animal being investigated. Hebert et al., (2003) proposed using DNA barcoding to help fish identification, which prompted the formation of Fish Barcode of Life (FISH-BOL), which aims to barcode all taxonomically documented fish species (Ward et al., 2009). The FISH-BOL project began in 2005, and roughly 8,000 of the 31,000 fish species recognised have been barcoded for the COI gene. According to the initial report, around 98 percent and 93 percent of marine and freshwater species may be distinguished using barcodes, respectively.

According to the work of Zhou et al., (2009), it is clear that the limited access to taxonomic expertise is an issue for large-scale biodiversity surveys. Their study shows that a

comprehensive DNA barcode library built on expertly identified specimens enables fast and accurate species identification. There will be easier ways of analysing bulk environmental samples which will become more widespread and less expensive over time, facilitating ecological and monitoring applications of the barcode library. Continued interaction with the taxonomic community during barcode-based biodiversity and monitoring studies, involving submitting specimens with novel sequences for determination or revision, will ensure the growth and maintenance of a high-quality database.

The analyzed fish types, *Decapterus russeli* and *Cynoglossus macrostomus* were ray-finned fishes belonging to two different orders Carangiformes and Pleuronectiformes. Carangiformes and Pleuronectiformes are part of the 5 sister clade Ovalentaria.

The russeli species, commonly known as Indian scad was discovered by Rüppell, 1830. They are marine, benthopelagic tropical fishes occurring in the Indian Ocean. They are an important species in coastal fisheries throughout its range. Their main prey are smaller planktonic invertebrates. They are host to the ectoparasitic copepod *Lernanthropus decapteri*. They have elongate slender and slightly compressed body. The lateral line shows slow regular arch towards the head. There are two widely separate dorsal fins with 8 dorsal spines and 28-33 dorsal soft rays. They have 2 anal spines and 25- 29 anal soft rays. They are large schooling fish found in deeper waters (Froese, R., & Pauly, D., 2022)

The macrostomus species commonly known as Malabar tonguesole was discovered by Norman, 1928. They are marine, brackish, benthopelagic, non-migratory and tropical fishes restricted to the coast of Indian ocean. They feed mainly on benthic invertebrates, especially worms. They are vulnerable species (VU) according to the IUCN red list. They are commonly found in shallow waters on a muddy or sandy bottom. A few species are restricted to fresh water.(Froese, R., & Pauly, D.,2022)

METHODOLOGY

SPECIMEN COLLECTION

The fishes were collected from Beypore Fishing Harbour, Kozhikode. A total of 21 fish types were selected and labelled for analysis by DNA barcoding. The specimens were stored in the Museum at Kerala University of Fisheries and Ocean Studies, Panangad, Kerala.

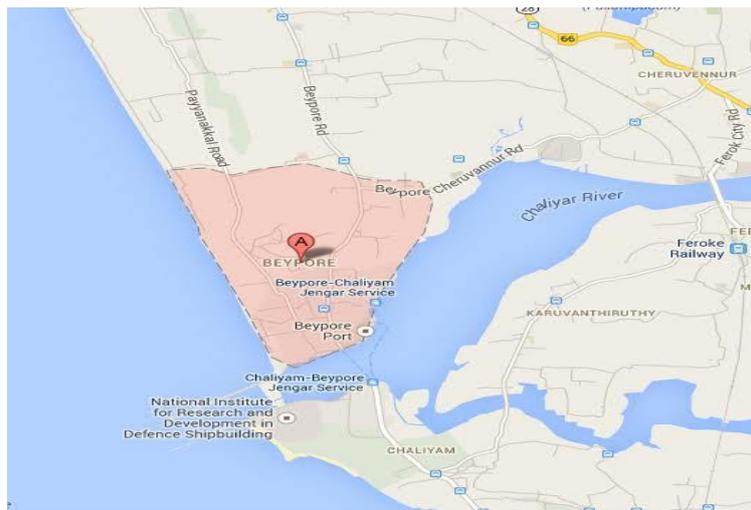


Fig. 1: Map showing Beypore Fishing Harbour, Kozhikode



Fig. 2: Fish samples collection

PROCEDURE

PREPARATION OF SAMPLE

The selected mature fish specimens were measured and mounted on a hard surface to observe the characteristic features. Muscle tissue samples were dissected using sterilised tools, preserved in 10% Formalin, properly labelled and stored in the refrigerator.

The protocol for DNA extraction, PCR amplification of CO1 gene, product purification and sequencing follow the study of Lakra et al. (2011).



Fig. 3: Preparation of fish samples

DNA ISOLATION

The preserved tissue samples were taken out of the vial and a portion (<25mg) of the flesh was transferred to a centrifuge tube and labelled. 200 μ L of Buffer ATL (Lysis Buffer) was added to the centrifuge tube followed by 20 μ L of Proteinase K. The mixture was vortexed to homogenise the contents and incubated in a thermomixer at 56°C for 2 hours until the whole tissue was completely digested. 200 μ L of Buffer AL (Lysis Buffer) was added to the centrifuge tube and incubated for another 10 minutes at 56°C. After removing the centrifuge tubes from the thermomixer, 200 μ L of 95% chilled Ethanol was added to it and then incubated at room

temperature for 5 minutes. The contents of the centrifuge tube were then transferred into labelled spin columns taken in 2ml collection tubes. The tubes were placed in a balanced configuration and centrifuged for 1 minute at 8000 rpm.



Fig. 4: Vortexing microtubes

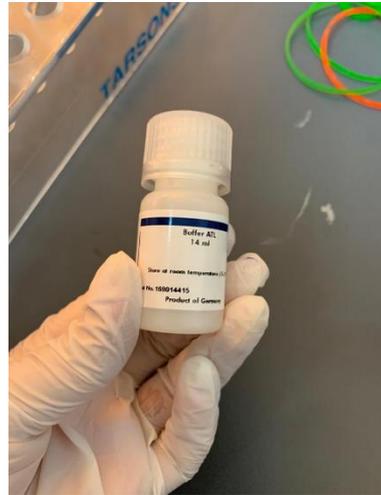


Fig. 5: ATL Buffer



Fig. 6: Thermomixer



Fig. 7: Centrifugation

The collection tubes were then replaced with new 2ml tubes. 500 μ L of Buffer AW1 (Wash Buffer) was added to the centrifuge tube and centrifuged for 3 minutes at 14000 rpm. The process was repeated with Buffer AW2 (Wash Buffer). The collection tubes were replaced with labelled centrifuge microtubes. 50 μ L of Buffer AE (Elution Buffer) was added to the centrifuge tube (incubated at room temperature for 1 minute) and then centrifuged for 1 minute at 8000 rpm. Another 50 μ L of Buffer AE was added to the centrifuge tube and incubated at room temperature for 1 minute and centrifuged at 1 minute at 8000 rpm. The Buffer AE elutes the DNA from the spin column membrane into the centrifuge tube. The eluted DNA in the labelled centrifuge microtube was stored at -4°C.

PCR

Species-specific variations or polymorphisms in the DNA sequence that are spread randomly over the entire genome and result in characteristic DNA fingerprints have been exploited through use of polymerase chain reaction (PCR) and its variants. (Priyanka Mishra et al., 2015). The procedures are followed using INVITROGEN Genomic DNA Mini Kit.

24 µL Master Mix (312.5 µL Emerald Amp GT PCR, 31.25 µL Forward F1 Primer, 31.25 µL Reverse R1 Primer and 225 µL dH₂O) was added to new individual vials which were properly labelled. The primer pair LCO1490(59-GGTCAACAAATCATAAAGATATTGG-39) and HCO2198(59-TAAACTTCAGGGTGACCAAAAAATCA-39) was subsequently used to amplify a 658 bp fragment of the COI gene. The samples were taken out of storage and added to the Master Mix vials.

The next step in the process involved 35 cycles of PCR (involving Denaturation, Annealing and Extension followed by Final Extension) maintained at 4°C. The vortexed vials were kept in the PCR Machine until a temperature of 105°C was attained after which the process started and took around two and a half hours to complete.

- DENATURATION
Done at 95°C for 5 minutes

- ANNEALING
Done at 58°C (but may vary depending on Primers used)

- EXTENSION
Done at 72°C



Fig. 8: Genomic DNA Mini Kit

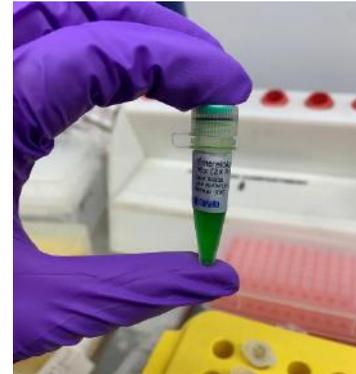


Fig. 9: Master Mix



Fig. 10: PCR Machine

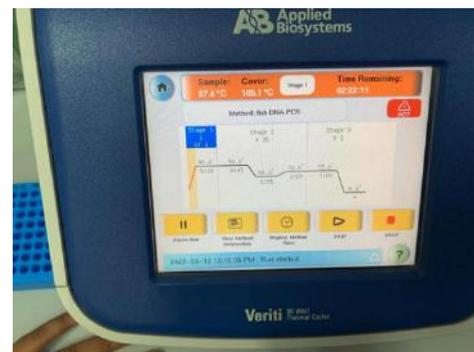


Fig. 11: Progress of PCR

PREPARATION OF GEL

0.4g Agarose Special Powder was added to 100mL Borosil and mixed well. This was transferred to a gel tray with wells/pits.

GEL ELECTROPHORESIS

Anode and Cathode electrodes were placed in a container and TE Buffer was added until the electrodes were completely immersed. The amplified sample mixtures were poured into the wells/pits. The Electrophoresis Machine was set to 90-91 Volts and run for about 20 minutes. The gel is then transferred onto a Bio Rad Imager for analysis of results of DNA imaging on the computer. The isolated DNA samples were given for sequencing.

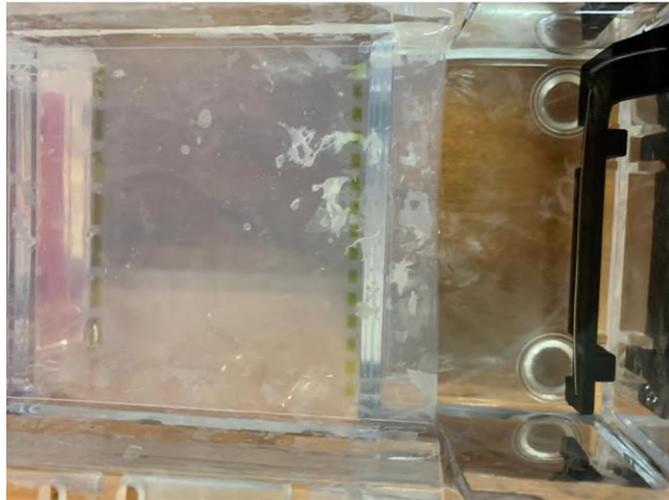


Fig. 12: DNA Bands separation after Gel electrophoresis



Fig. 13: Analysis of DNA Bands

PHYLOGENETIC TREE

A phylogenetic tree is made and analysed by using various softwares. In the field of genome analysis, biologists seek to identify important genes or chromosome regions by comparing phylogenetic trees and analysing the mutation at which locus might affect phenotypic traits (Ge et al., 2020).

The dataset of 646 basepairs of *Cynoglossus macrostomus* and 645 basepairs of *Decapterus russelli* are acquired from the ICBN nucleotide sequences of COI. These sequences were then aligned using the MUSCLE (Edgar, 2004) sequence algorithm implemented in MEGA 11. From

the aligned sequences a phylogenetic tree was constructed by using the Maximum Likelihood (ML) method. The most common way to estimate the reliability of a phylogenetic tree is by the bootstrap method (Hall, 2013).

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc Len	Accession
<input checked="" type="checkbox"/> Cynoglossus macrostomus voucher PMNH-PAK> 55253 cytochrome oxidase subunit 1 (COI) gene, partial cds...	Cynoglossus ma...	1157	1157	93%	0.0	99.22%	655	MN511874.1
<input checked="" type="checkbox"/> Cynoglossus macrostomus voucher PMNH-PAK> 55252 cytochrome oxidase subunit 1 (COI) gene, partial cds...	Cynoglossus ma...	1157	1157	93%	0.0	99.22%	655	MN511873.1
<input checked="" type="checkbox"/> Cynoglossus macrostomus voucher NBFGR-Cym05-3 cytochrome oxidase subunit 1 (COI) gene, partial cds, mit...	Cynoglossus ma...	1157	1157	93%	0.0	99.22%	655	FJ347954.1
<input checked="" type="checkbox"/> Cynoglossus macrostomus voucher NBFGR-Cym05-1 cytochrome c oxidase subunit 1 (COI) gene, partial cds, m...	Cynoglossus ma...	1151	1151	93%	0.0	99.07%	655	FJ347911.1
<input checked="" type="checkbox"/> Cynoglossus macrostomus voucher NBFGR-Cym05-4 cytochrome oxidase subunit 1 (COI) gene, partial cds, mit...	Cynoglossus ma...	1151	1151	93%	0.0	99.06%	655	FJ347955.1
<input checked="" type="checkbox"/> Cynoglossus macrostomus voucher NBFGR-Cym05-2 cytochrome c oxidase subunit 1 (COI) gene, partial cds, m...	Cynoglossus ma...	1146	1146	93%	0.0	98.91%	655	FJ347912.1
<input checked="" type="checkbox"/> Cynoglossus puncticeps isolate INAPKKD-SIFT-99 cytochrome oxidase subunit 1 (COI) gene, partial cds, mitoch...	Cynoglossus ru...	1138	1138	94%	0.0	98.45%	664	EU541318.1
<input checked="" type="checkbox"/> Cynoglossus macrostomus isolate ENNOUR ESTUARY cytochrome c oxidase subunit 1 (COX1) gene, partial cds...	Cynoglossus ma...	1024	1024	83%	0.0	99.12%	575	OL587637.1
<input checked="" type="checkbox"/> Cynoglossus lingua isolate INAPKKD-SIFT-96 cytochrome oxidase subunit 1 like (COI) gene, partial sequence...	Cynoglossus ling...	987	987	83%	0.0	98.07%	567	EU541315.1
<input checked="" type="checkbox"/> Cynoglossus lingua isolate INAPKKD-SIFT-97 cytochrome oxidase subunit 1 (COI) gene, partial cds, mitochondrial	Cynoglossus ling...	874	874	71%	0.0	98.78%	491	EU541316.1
<input checked="" type="checkbox"/> Cynoglossus lingua isolate WJ/C1733 cytochrome oxidase subunit 1 (COI) gene, partial cds, mitochondrial	Cynoglossus ling...	870	870	71%	0.0	98.77%	489	MK617142.1
<input checked="" type="checkbox"/> Cynoglossus puncticeps mitochondrion, complete genome	Cynoglossus ru...	719	719	96%	0.0	86.25%	17142	JQ349003.1
<input checked="" type="checkbox"/> Cynoglossus cf. puncticeps FOA/011-09 voucher RW-A696.1 cytochrome oxidase subunit 1 (COI) gene, partial...	Cynoglossus cf...	713	713	92%	0.0	86.95%	651	GU674223.1
<input checked="" type="checkbox"/> Cynoglossus cf. cynoglossus FOAK854-10 voucher BW-A8837 cytochrome oxidase subunit 1 (COI) gene, partial...	Cynoglossus cf...	701	701	92%	0.0	86.59%	647	HQ564330.1
<input checked="" type="checkbox"/> Cynoglossus puncticeps isolate 2296 cytochrome c oxidase subunit 1 (COX1) gene, partial cds, mitochondrial	Cynoglossus ru...	697	697	94%	0.0	86.09%	672	OM142889.1
<input checked="" type="checkbox"/> Cynoglossus cf. cynoglossus FOAK855-10 voucher BW-A8838 cytochrome oxidase subunit 1 (COI) gene, partial...	Cynoglossus cf...	695	695	92%	0.0	86.44%	647	HQ564331.1

Fig. 14: BLAST Results of *Cynoglossus macrostomus*

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc Len	Accession
<input checked="" type="checkbox"/> Decapterus russelli voucher st2 N3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds, mitochondrial	Decapterus russelli	1106	1106	94%	0.0	100.00%	642	QNI162496.1
<input checked="" type="checkbox"/> Decapterus russelli voucher ID75 cytochrome c oxidase subunit 1 (COX1) gene, partial cds, mitochondrial	Decapterus russelli	1104	1104	96%	0.0	99.09%	660	MT803694.1
<input checked="" type="checkbox"/> Decapterus russelli voucher NaID2 cytochrome c oxidase subunit 1 (COI) gene, partial cds, mitochondrial	Decapterus russelli	1182	1182	93%	0.0	100.00%	647	KT800387.1
<input checked="" type="checkbox"/> Decapterus russelli voucher NaID1 cytochrome c oxidase subunit 1 (COI) gene, partial cds, mitochondrial	Decapterus russelli	1182	1182	93%	0.0	100.00%	647	KT800386.1
<input checked="" type="checkbox"/> Decapterus russelli mitochondrion, complete genome	Decapterus russelli	1179	1179	96%	0.0	98.37%	16542	MN711693.1
<input checked="" type="checkbox"/> Decapterus macrostoma voucher DMCHNT cytochrome oxidase subunit 1 (COI) gene, partial cds, mitochondrial	Decapterus mac...	1173	1173	93%	0.0	100.00%	642	KT326329.1
<input checked="" type="checkbox"/> Decapterus russelli voucher CEW0211 cytochrome c oxidase subunit 1 (COI) gene, partial cds, mitochondrial	Decapterus russelli	1164	1164	96%	0.0	98.64%	678	KU499593.1
<input checked="" type="checkbox"/> Decapterus maraudsi haplotype Hag21 cytochrome oxidase subunit 1 (COX1) gene, partial cds, mitochondrial	Decapterus mer...	1164	1164	94%	0.0	99.22%	651	KX610908.1
<input checked="" type="checkbox"/> Decapterus russelli voucher CEW0210 cytochrome c oxidase subunit 1 (COI) gene, partial cds, mitochondrial	Decapterus russelli	1162	1162	96%	0.0	98.63%	678	KU499592.1
<input checked="" type="checkbox"/> Decapterus russelli cytochrome c oxidase subunit 1 gene, partial cds, mitochondrial	Decapterus russelli	1162	1162	92%	0.0	99.84%	634	MF541369.1
<input checked="" type="checkbox"/> Decapterus russelli voucher CEW0214 cytochrome c oxidase subunit 1 (COI) gene, partial cds, mitochondrial	Decapterus russelli	1158	1158	96%	0.0	98.48%	678	KU499596.1
<input checked="" type="checkbox"/> Decapterus russelli voucher EADF_306 cytochrome c oxidase subunit 1 (COX1) gene, partial cds, mitochondrial	Decapterus russelli	1155	1155	95%	0.0	98.63%	663	MT076577.1
<input checked="" type="checkbox"/> Decapterus russelli voucher EADF_196 cytochrome c oxidase subunit 1 (COX1) gene, partial cds, mitochondrial	Decapterus russelli	1155	1155	95%	0.0	98.63%	663	MT076575.1
<input checked="" type="checkbox"/> Decapterus russelli voucher EADF_197 cytochrome c oxidase subunit 1 (COX1) gene, partial cds, mitochondrial	Decapterus russelli	1155	1155	95%	0.0	98.63%	663	MT076574.1
<input checked="" type="checkbox"/> Decapterus russelli voucher EADF_122 cytochrome c oxidase subunit 1 (COX1) gene, partial cds, mitochondrial	Decapterus russelli	1155	1155	95%	0.0	98.63%	663	MT076573.1
<input checked="" type="checkbox"/> Decapterus maraudsi haplotype Hag28 cytochrome oxidase subunit 1 (COX1) gene, partial cds, mitochondrial	Decapterus mer...	1155	1155	94%	0.0	98.92%	651	KX610915.1

Fig. 15: BLAST Results of *Decapterus russelli*

OBSERVATION AND RESULT

The amplified Cytochrome Oxidase I (COI) sequences were identified using BLAST. The fishes identified based on matches of $\geq 97\%$ similarity to a published sequence in the NCBI GenBank database were *Cynoglossus macrostomus* and *Decapterus russelli*.

Cynoglossus macrostomus Dendrogram



Fig. 16: *Cynoglossus macrostomus*

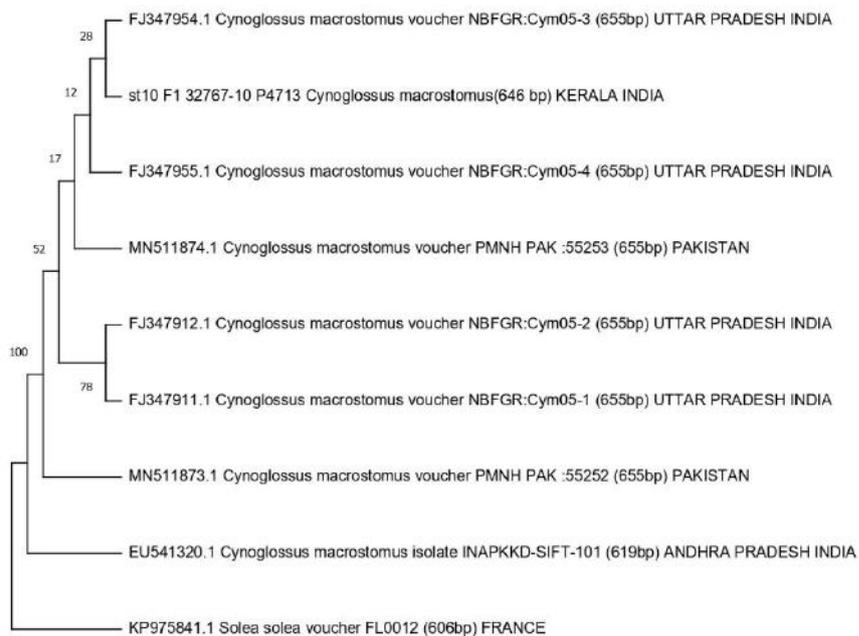


Fig. 17: Phylogenetic Tree of *Cynoglossus macrostomus*

Voucher Specimen- F132767

Outgroup- KP975841

Decapterus russelli Dendrogram



Fig. 18: *Decapterus russelli*

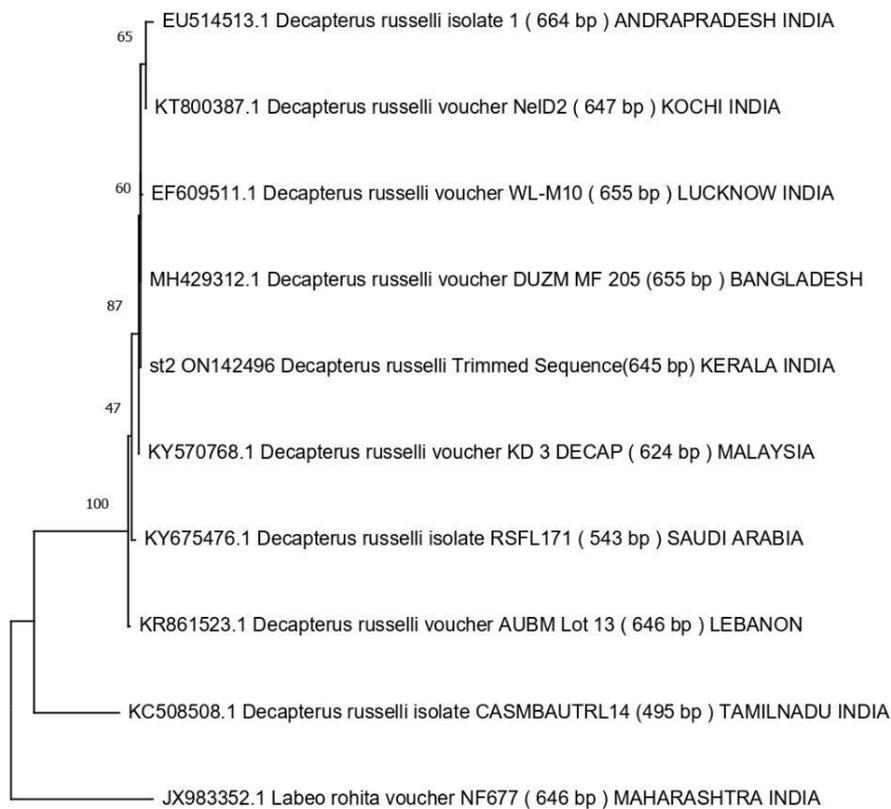


Fig. 19: Phylogenetic Tree of *Decapterus russelli*

Voucher Specimen- ON142496

Outgroup- JX983352

DISCUSSION

The analysis of morphological traits has been the most traditional method used for species identification and taxonomy. However, when morphological identification is compromised, genetic identification can be used to associate sequences from unknown samples to a sequence from a reference sample. Based on a standard region of 650 base pairs of the subunit I of Cytochrome C Oxidase mitochondrial gene (COI) and using a validated reference database, the DNA Barcoding system for cataloguing and identifying animal species has been proposed.

Present study suggests that DNA barcoding has been successful in identifying and discriminating the vast majority of marine ichthyofauna (fishes). The DNA barcoding method is an effective tool for species identification, particularly with specimens that are damaged, incomplete, unknown or consisting of several morphologically distinct stages.

The objective of DNA barcoding analyses is simple- to assign each unknown sequence to a set of referenced (tagged-specimen) sequences extracted, for instance, from databases like BOLD (Casiraghi, 2010). Many different bioinformatics approaches are available to reach this aim. Although it has many advantages, it also has its limitations. In some cases, related species may present identical sequences making DNA barcodes useless for species discrimination.

DNA Barcoding is already effective for species identification in many cases and, although presenting some limitations, the use of the tool must be improved and widespread in forensic casework. DNA sequences are increasingly being used for the rapid quantification of biodiversity and for some taxonomic groups, which may provide a more accurate overview of species diversity than traditional (i.e., morphological) methods. While these approaches can be used to highlight biogeographic regions that are risks due to anthropogenic disturbances and have great potential for use by ecologists, evolutionary, and conservation biologists, frequently the utility of these methods are constrained by the presence of incomplete reference libraries. Here, we have presented a technique that can be utilised by any community DNA sequencing project to rapidly categorise unidentified specimens or specimens from

species whose biogeographic status is unknown as representing either likely native or non-native species.

A phylogenetic tree is an estimate of the relationships among taxa (or sequences) and their hypothetical common ancestors. Today most phylogenetic trees are built from molecular data: DNA or protein sequences which are orthologous to infer the evolutionary relationship among organisms.

Originally, the purpose of most molecular phylogenetic trees was to estimate the relationships among the species represented by those sequences, but today the purposes have expanded to include understanding the relationships among the sequences themselves without regard to the host species, inferring the functions of genes that have not been studied experimentally, and elucidating mechanisms that lead to microbial outbreaks among many others.

The most basic assumption of phylogenetic analysis is that all the sequences on a tree are homologous, that is, descended from a common ancestor. Alignment programs will align sequences, homologous or not. All tree-building programs will make a tree from that alignment. However, if the sequences are not actually descended from a common ancestor, the tree will be meaningless and may quite well be misleading. The most reliable way to identify sequences that are homologous to the sequence of interest is to do a Basic Local Alignment Search Tool (BLAST) search using the sequence of interest as a query.

Building a phylogenetic tree requires four distinct steps namely, identification, alignment estimation and presentation. The program MEGA was used to implement the above steps in a phylogenetic tree construction. The same method was used for the construction of the phylogenetic trees of the fishes identified (Hall, 2013).

Bootstrap values are used to distinguish how many times out of 100 the same branch was observed when repeating the phylogenetic reconstruction on a re-sampled set of data. Interpretation of Bootstrap value is controversial. Felsenstein (1985) proposed that Bootstrap value of 95% or greater be considered statistically significant and indicate support for a clade: alternative nodes can be rejected if they occur in less than 5% of the Bootstrap estimate. There

are generally accepted thresholds for "good" and "moderate" support, e.g. $BS > 70/80$ is considered good, and $50/60 < BS < 70/80$ is considered moderate.

The Bootstrap proportion has variously been interpreted as:

1. A measure of reliability, telling us what would be expected to happen if we repeated our experiment;
2. A measure of accuracy, telling us about the probability of our experimental result being true; and
3. A measure of confidence, interpreted as a conditional probability similar to those in standard statistical hypothesis tests (i.e. measuring Type I errors or false positives).

(Morrison, 2014)

The phylogenetic tree of fishes *Cynoglossus macrostomus* and *Decapterus russelli* were specifically analysed in the present study.

In the phylogenetic tree of *Cynoglossus macrostomus*, the voucher specimen is named as F132767. The native place of *Cynoglossus* is Andhra Pradesh making the region its type locality. The voucher specimen is collected from Beypore Kerala. The dendrogram is analysed based on the similarities and differences between clades and Bootstrap values. The final dataset of 10 sequences were aligned using MUSCLE (Edgar 2004) in MEGA 11. Once the sequences are muscle aligned and a dendrogram is drawn, the species are shown in branches or clades in accordance with their similarities. The type locality voucher is named as EU541320. Here an out group is chosen to show the significant difference between the two genus of fishes. The out group chosen is *Solea solea* and it has no similarities to the genus *Selar*. The type locality voucher and voucher specimen are on two different clades in two different branches. There is a chance that the voucher specimen comes under different species since they don't align with the type locality specimen. This could be because they are cryptic species or due to taxonomical ambiguities. Even though they are taxonomically identified as *Cynoglossus macrostomus*, the dendrogram drawn shows that they are different. Sequences generated in the present study have been submitted to GenBank under accession number F132767.

In the dendrogram of *Decapterus russelli*, the specimen obtained is named as ON142496. The type locality is Bangladesh. The tree is analysed based on the similarities and differences between clades and Bootstrap values. The final dataset of 10 sequences were aligned using MUSCLE (Edgar 2004) in MEGA 11. Once the sequences are muscle aligned and a dendrogram is drawn, the species are shown in branches or clades in accordance with their similarities. Species of similar interests or species with most similarities are shown in a clade. The ones with least similarities are shown in different branches. Here the type locality specimen is named MH429312. The specimen under study is obtained from Kerala. Both the voucher specimen and type locality specimen are in a single clade indicating that they have very little differences. The out group chosen to highlight the difference is *Labeo rohita* which shows zero similarities to Carangoides. Since the similarities between the type locality voucher and voucher specimen is above 40, it can be said that they belong to the same specimen of fishes. The variations are vague and similarities are more prominent making it a less divergent species of Decapterus. Sequences generated in the present study have been submitted to GenBank under accession number ON142496.

By analysing the phylogenetic tree or dendrogram, it is observed, that the voucher specimen of *Cynoglossus macrostomus* having accession number F132767 may be a cryptic species whereas the voucher specimen of *Decapterus russelli* having accession number ON142496 may belong to the same specimen of fishes.

Through this study, a reliable DNA barcode reference library for the marine fish in south India has been established, which could be used to assign fish species by screening sequences against it in the future. This could contribute to achieving better monitoring, conservation, and management of fisheries in this overexploited region.

CONCLUSION

DNA barcoding is a very useful tool for identifying unknown specimens. Identification of a fish can be done by taking a small sample from the specimen without causing much damage to them. Even with a small sample, it will be adequate to find the sequence and thus the unknown specimens can be identified. The DNA Barcoding method of identifying unknown species is extremely accurate. DNA barcoding uses genetic-level identification of species to get insight into their evolutionary relationships. Genetic material is the only thing that is transferred down to the next generation. So, as compared to traditional methods such as identifying fish using morphology, which can be misleading and has a higher possibility of error, analysis involving genetic material is significantly better. A phylogenetic tree might be used to interpret the results. A single glance reveals the clade or group to which the unknown species belongs. Phylogenetic trees are the most basic and easiest approach to understanding the results of DNA Barcoding.

The discriminatory power of COI barcodes is emphasized and their application to cases requiring species level resolution starting from unknown sequences. There are high results of reliability to DNA barcodes from public reference libraries, to identify species from different geographical origins. The ability to assign species with high precision from DNA samples of disparate quality and origin has major utility in several fields, from forensics, fisheries and conservation programs to control of fish products authenticity.

It is well known that no identification method (morphological, biochemical, genetic or whatsoever based) can truly identify species, because species are entities in continuous evolution and it is theoretically impossible to statically define such dynamic matter. The results of the present study of DNA Barcoding will facilitate other lines of research in biodiversity assessments of unknown fishes.

The present study has also demonstrated that DNA barcoding holds great promise as a tool for rapid biodiversity assessment in unknown fishes.

From the dendrogram for *Cynoglossus macrostomus*, it can be observed that there is variation between the Bootstrap values of the type locality specimen and voucher specimen. There is a possibility that the voucher specimen comes under different species since they don't align with the type locality specimen. This could be because they are cryptic species or due to taxonomical ambiguities. Even though they are taxonomically identified as *Cynoglossus macrostomus*, the dendrogram drawn with the sequence obtained from GenBank shows that they are different. But in the case of *Decapterus russeli*, since the similarities between the type locality voucher and voucher specimen is above 40, it can be said that they belong to the same specimen of fishes. The variations are vague and similarities are more prominent making it a less divergent species of Carangidae. In the present study, one species is similar but the other, even though they are taxonomically identified within the same genera Carangoides, their similarities are very vague.

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**IN VITRO BREATHING ANALYSIS OF COLD AND FRESH EGG IN WATER AND
CARBONATED MEDIUM**



Project work by

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Affiliated to Mahatma Gandhi University, Kottayam in partial

Fulfilment of requirement for the degree of Bachelor of science

In Zoology

2019-2022

CERTIFICATE

This is to certify that the project report entitled, “***In vitro* breathing analysis of cold and fresh egg in water and carbonated medium** ” submitted by **Ms. ANU CLEETUS** Reg.No.AB19ZOO004 in partial fulfilment of the request of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under my guidance and supervision and to the best of my knowledge, this is her original effort.

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Ernakulam

Examiners

1).....

2).....

DECLARATION

I, hereby declare that this project work entitled “IN VITRO BREATHING ANALYSIS OF COLD AND FRESH EGG IN WATER AND CARBONATED MEDIUM” is submitted to St. Teresa’s collage (Autonomous), Ernakulum affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science degree in Zoology. This work has been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own

Name: Anu Cleetus

Signature

Reg. No: AB19ZOO004

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Anu Cleetus

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ABSTRACT

Every animal needs oxygen to survive. Animals that grow inside their mother get oxygen from their mother's blood. But those that grow in a shell must get air somehow. This study aims on how a shelled animal get oxygen from outside. It is carried out with a view to analyse how the chick breath inside in fresh and cold egg in different mediums for different hours. The breathing rate of the chick embryo gradually increases, after a particular time the breathing activity decreases. The embryonated eggs faces difficulty to survive.

INTRODUCTION

Every animal requires oxygen to live. When animals, including humans, breathe in, oxygen enters the lungs, where it is shuttled into the blood stream and distributed to all the different parts of the body. The oxygen is used in an internal chemical reaction called metabolism to provide the animal with energy. The process of metabolism also produces a waste gas called carbon dioxide. In order to get rid of this waste gas, the blood stream carries the carbon dioxide back to the lungs where it is collected and finally breathed out.

Animals that grow inside their mothers, like humans, get their oxygen from their mothers. The blood stream of the baby animal and the mother are connected through an umbilical cord, which allows the baby to collect oxygen that his or her mother breathes in and use the mother's lungs to get rid of the carbon dioxide. But how do animals that grow in a shell and do not have umbilical cords, like chickens, take in oxygen and get rid of carbon dioxide?

Bird and reptile eggs have a hard shell. Directly under the shell are two membranes. When the eggs are laid by the mother, they are warmer than the air, and as they cool, the material inside the egg shrinks a little bit. This shrinking pulls the two membranes apart, leaving behind an air cell, also called an air sack, that is filled with oxygen. As the animal develops, it needs the oxygen replenished so it can continue to grow. So, how does this happen? Well, when a chicken egg is examined carefully with a magnifying glass, we can see that there are tiny little holes, called pores, in the shell. A chicken eggshell has more than 7,000 pores. It was found that through these pores gas exchange happens. To identify and record the breathing rate of chicken's egg, dye and detergent are used.

The role of the detergent is to help break through the membranes of the egg so that the dye can make a concentrated, visible mark on the inside of the eggshell, rather than a light smear all over it. The detergent does not affect the eggshell.

REVIEW OF LITERATURE

Rahn & Paganelli (1979) studied that the bird egg is a self-contained life support system for the developing bird embryo. All the nutrients, minerals, energy sources and water utilized by the embryo during its incubation are already present in the freshly laid egg, so that the egg requires only warming by the parents and periodic turning to prevent the adhesion of the embryo to the shell membranes. Still, the egg lacks one crucial requirement: oxygen, which drives the metabolic machinery of the embryonic cells so that they can execute the complex maneuvers of development.

Windle,et.al (1938) found that small amounts of carbon dioxide in an atmosphere rich in oxygen initiated rhythmic respiration-like movements in incubating chick embryo several days before these movements occurred normally. Greater concentration of carbon dioxide or more complete anoxemia led to the establishment of stronger respiratory activity resembling the gasping of acute air-hunger. Near the end of 20th or beginning of 21st day of incubation the chick prick its enclosing membranes and begins to breathe the air within the shell. Soon thereafter it chips a hole in the shell admitting atmospheric air. The respiratory allantois continues to function until hatching has been completed".

Visschedijk,et.al (1980) studied that the gas moves through the pores of the egg shell by diffusion in the gas phase. The gas flux is therefore determined by the product of the effective conductance of the shell and the partial pressure gradient of the gas between the ambient air and the inner side of the shell. Chicken embryos aged 16-19 days were exposed to various oxygen concentrations and carbon dioxide production was measured. At subnormal oxygen concentrations carbon dioxide output diminished as the oxygen concentration was lowered and the duration of exposure was prolonged. At oxygen concentrations above normal a small but significant increase in carbon dioxide production was found. This difference is ascribed to the fact that a reduction of barometric pressure not only decreases oxygen partial

pressure in the ambient air but also increases effective conductance of the egg shell, the latter being inversely proportional to the barometric pressure".

Kuo & Shen (1937) studied that the development of respiration was studied by observing movements through transparent shell membranes and by recording them graphically in chicks taken out of the shell. The data indicate that development of respiration is a slow and continuous process. No abrupt changes are evidenced either before or after hatching. Drying of the skin and CO₂ tension are factors involved in stimulation of pulmonary respiration. Allantoic circulation remains for a time after breathing is established".

Wangensteen, et.al (1974) studied that the gas exchange by embryos from chickens acclimatized to an altitude of 3800 m (pB = 480 torr) was studied in order to ascertain the nature of the altitude adaptation in this species. The PO₂ and PCO₂ differences across the egg shell were measured and found to be less than the values previously reported for sea-level eggs by about a factor of two. Further measurements of embryonic oxygen consumption (O₂) and shell conductivity to oxygen (GO₂) indicated that, compared to eggs at sea level, O₂ was reduced by a factor of 0.58 while GO₂ was increased only by a factor of 1.07 in the high-altitude eggs. These independent measurements predict the Δ PO₂ across the egg shell of the high-altitude eggs to be only 0.54 times that of sea-level eggs; the directly measured factor was 0.53. The authors conclude that at high altitude a major adaptation of the chick embryo is a reduced metabolism which decreases the Δ PO₂ across the egg shell since its gas conductivity remains essentially unchanged".

Tazawa (1980) studied that the fertile chicken eggs belonging to the same flock of hens were divided into two groups and incubated for 16 days. During incubation, group 1 eggs were turned twice a day and group 2 eggs were left unturned. Blood sampled from the allantoic vein or artery was analyzed for gas tensions (PO₂ and PCO₂), pH and Hct. These values were compared by unpaired t-test for significance differences between the two groups. While the differences of PCO₂ and pH were found insignificant, failure to turn the eggs caused a pronounced fall in the arterialized PO₂ which was accompanied with an increase in Hct. In addition, the embryo weight was reduced in unturned eggs. Lack of turning retarded the

absorption of albumen. The unabsorbed albumen interposed between the chorioallantoic membrane and inner shell membrane, impeding the blood oxygenation through the chorioallantois. Little change in PCO₂ might be attributed to a large diffusive conductance of the chorioallantois for CO₂. The present results suggest that the eggs must be turned periodically during incubation to prevent the distortion of normal oxygen exchange especially for the study of egg respiration".

Hagelin, et al (2013) studied that we know almost nothing about the chemosensory experiences of birds as they develop within an egg's fluid-filled environment. Given well-established literature on chemical detection of mammals in utero, we explored whether domestic chickens (*Gallus gallus domesticus*) exhibit a similar ability to detect or learn about chemical stimuli prior to breathing air. We incubated 18 eggs from embryonic day (E)9-18 in scented air containing Z-4-decenal and octanal, two key components of a citrusy-scented avian social odour (from Crested Auklets [*Aethia cristatella*]; Proc R Soc Lond 270:1323-1329, 2003). Control eggs were not exposed to scent. Behavioural responses of embryos were quantified by opening the shell and exposing embryos to three different test scents (Crested Auklet, wintergreen [novel scent], and water [unscented control]).

They conclude that embryos modified their behaviour after experiencing air-borne compounds that were transmitted into the egg's fluid environment".

Beattie (1964) studied that the measurements of the oxygen uptake and carbon dioxide output during the last 36 hr. of incubation show that the exchange of these two gases increases progressively from the start of pulmonary respiration until hatching. The evidence indicates that prior to the penetration of the shell by the beak of the embryo ("pipping") there is marked hypoxia and hypercapnia which are relieved when the embryo gains access to atmospheric air. At the time of hatching there is a very rapid release of carbon dioxide from the body surface and the internal surface of the allantoic membranes. There is no evidence to suggest that its release indicates either hypoxia or hypercapnia in the embryo immediately before hatching".

Burton & Tullett studied that this review is concerned with how bird embryos breathe. The first part discusses the ontogeny and mechanism of gaseous exchange, describing briefly the development of the extra-embryonic membranes, the movement of oxygen across the shell and shell membranes into the blood and the elimination of carbon dioxide and the transition from respiration via the extra-embryonic membranes to pulmonary respiration. The discussion concerns mainly the domestic fowl, it can in general terms be applied to most birds. Egg weight, shell porosity and special circumstances, such as high altitude, unusual nesting or incubation regimes, have important effects on embryonic respiration. These are considered in the second part of the review".

Gabrielli & Accili (2010) studied that the chick chorio allantoic membrane is a very simple extraembryonic membrane which serves multiple functions during embryo development; it is the site of exchange of respiratory gases, calcium transport from the eggshell, acid-base homeostasis in the embryo, and ion and H₂O reabsorption from the allantoic fluid. All these functions are accomplished by its epithelia, the chorionic and the allantoic epithelium, by differentiation of a wide range of structural and molecular peculiarities which make them highly specialized, ion transporting epithelia".

Burton, et.al (1989) studied that the covering the whole shell or the shell over the chorioallantoic membrane with liquid paraffin resulted in the diffusion of oxygen through the shell over the air space before the onset of pulmonary respiration being reduced to a level insufficient to keep the embryo alive. During the parafoetal period respiratory exchange through the air space becomes progressively more important than that through the allantois with the result that the embryo can survive a blocking of the whole shell or allantoic shell during this period, provided that pulmonary respiration has been developed sufficiently and that the shell is pipped within a certain time limit. After pipping respiratory exchange can be effected by pulmonary respiration alone, without great danger to the embryo. It may therefore be concluded that the respiratory exchange through the allantoic shell is greater than through the air space shell up to the time of pipping. By measuring directly the gaseous exchange through the shell over the air space and allantoic shell it was shown that the amount of the diffusion through the shell over the air space was in proportion to the total diffusion, at least until the onset of pulmonary respiration. After that moment the absolute as well as the

percentage exchange via the air space increased. The allantois did not begin to degenerate until pipping had taken place. Lung function then increased and soon took over completely the function of the allantois. As soon as the allantoic respiration had been practically abolished hatching activities started with the result that hatching often followed within half an hour

MATERIALS AND METHODS

The present study was carried out with a view to analyse the breathing of hen's egg inside (fresh and cold egg) in different medium, ie; Water and Carbonated drink (soda).

1. Selection of egg:

Twenty eggs were taken, from same type.

- Ten are fresh eggs
- Ten are refrigerated for 24hrs.

2. Selection of medium:

Two different medium are taken, namely: water and carbonated drink (soda).

3. Preparation of medium:

3.1 Preparation of first medium

Take 3 ½ cup of water in a container box. Add (1 tsp) detergent and add (5 drops) red food colour to it. Mix well for sometimes without forming foam on water.

3.2 Preparation of second medium

Take 3 ½ cup of carbonated drink in a container box. Add (1tsp) detergent to it. Now, add food colour (5 drops) and mix well for sometimes.

4. Analysis of chick breath inside its shell:

Take two container boxes with water and carbonated drink on each. Pour 3 ½ cups of water in one container box and 3 ½ cups of carbonated drink in another container box. Add (1tsp) liquid dishwashing detergent and (1tsp) red food colour each to the container boxes.

Carefully set the fresh and cold eggs in each container box. Make sure that the eggs are submerged in the liquid, if any part of the egg is above the surface of the liquid, repeat the steps and add the extra liquid to the container box until the eggs are submerged. Note the starting time and soak the eggs for 5hrs. After the eggs have soaked in the liquid for 5hrs. carefully lift the eggs each from the two medium in every hour using a large spoon. Wipe it with a cotton towel. Now, crack the raw egg into a cup being carefully not to damage or crush the shell much. Set the empty egg shells on a plate and carefully inspect the inside of the shell.

OBSERVATION AND RESULT

The detergent is used to break the membranes of the egg so that the dye can make a concentrated, visible mark on the inside of the eggshell.

The present study was undertaken to analyse the breathing activity of embryonated eggs in water and carbonated medium for different hours.

Embryonated eggs in water medium

Analysing the breathing activity of the fresh embryonated egg in five hours it was observed that the first three hours the intake of oxygen is high whereas in fourth and fifth hour oxygen intake decreases gradually due to the intake of water from the medium. The embryonated egg faces difficulty to survive.

In the cold egg the breathing activity is absent due to the eggs treated 24 hours in refrigerator so that the embryonated eggs die.

Embryonated egg in carbonated medium

Analysing the breathing activity of the fresh embryonated egg in 5 hours it was observed that the first 4 hours the intake of oxygen is gradually increases due to the carbondioxide concentration in the carbonated medium decreases over time whereas in 5th hour the breathing activity of the embryonated eggs starts decreasing due to the intake of water from the medium. The embryonated eggs faces difficulty to survive.

As mentioned above, in the cold eggs the breathing activity is absent.

Colour grade chart

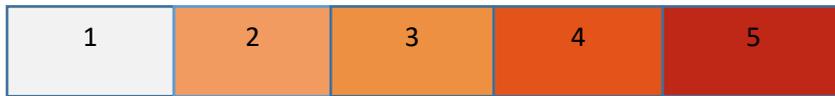
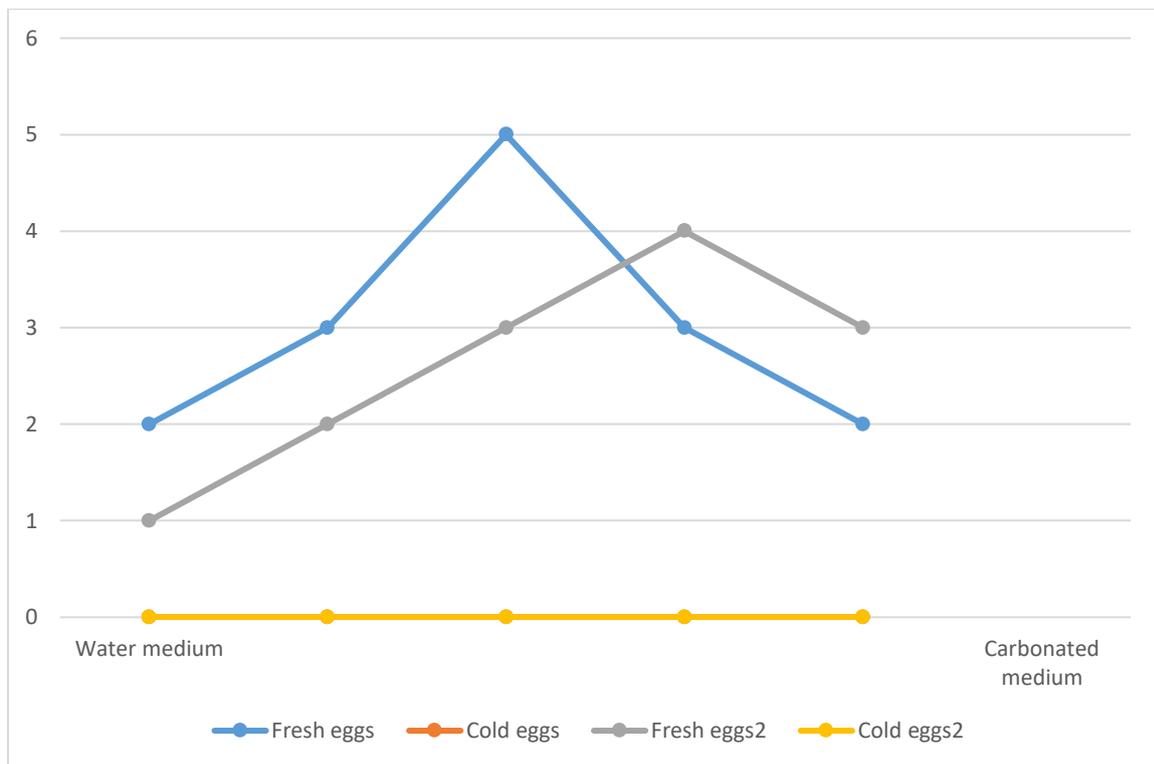


Table showing breathing activity of embryonated eggs in water and carbonated medium by colour gradient

Time	Water medium		Carbonated medium	
	Fresh eggs	Cold eggs	Fresh eggs	Cold eggs
1 hour	2	Nil	1	Nil
2 hour	3	Nil	2	Nil
3 hour	5	Nil	3	Nil
4 hour	3	Nil	4	Nil
5 hour	2	Nil	3	Nil

Graphical representation of breathing activity of embryonated eggs in water and carbonated medium by colour gradient



Water medium



Fresh egg in 1 hour



Fresh egg in 2 hours

Carbonated medium



Fresh egg in 1 hour



Fresh egg in 2 hours



Fresh egg in 3 hours



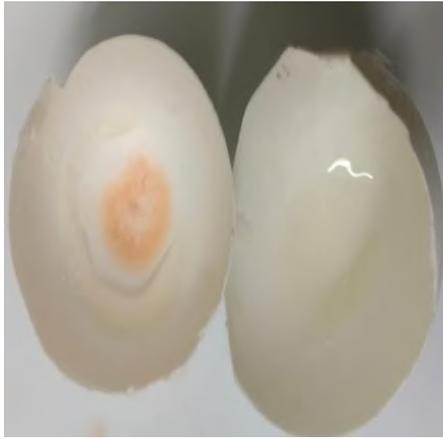
Fresh egg in 3 hours



Fresh egg in 4 hours



Fresh egg in 4 hours



Fresh egg in 5 hours



Fresh egg in 5 hours



In the cold egg the breathing activity is absent

DISCUSSION

Every animal including embryonated eggs needs oxygen to live.

Embryos developing inside the mother's uterus have an umbilical cord that takes in oxygen from their mother's bloodstream, and gets rid of carbon dioxide that is produced as waste but embryonated eggs that develop inside shells have no umbilical cord. Instead, they depend upon an air sac filled with oxygen that lies between two membranes which are directly under the hard shell of the egg. Oxygen enters the eggs through pores in the cuticle and passes through columns of crystals to the permeable shell membranes. Carbon dioxide and water vapour escape to the outside environment through these same pores.

The present study was undertaken to analyse the breathing activity of cold and fresh egg in water and carbonated medium. It also helpful in study the rate of oxygen and carbon dioxide in the medium which effect the breathing activity of embryonic chick. The gas moves through the pores of the egg shell by diffusion in the gas phase (Visschedijk, et.al (1980)).

According to the previous report it was demonstrated that small amounts of carbon dioxide in an atmosphere rich in oxygen initiated rhythmic respiration-like movements in incubating chick foetus several days before these movements occurred normally. Greater concentration of carbon dioxide or more complete anoxemia led to the establishment of stronger respiratory activity resembling the gasping of acute air-hunger. Near the end of 20th or beginning of 21st day of incubation the chick perforates its enclosing membranes and begins to breathe the air within the shell. Soon thereafter it chips a hole in the shell admitting atmospheric air. The respiratory allantois continues to function until hatching has been completed (Windle,et.al(1938)).

Kuo, Z. Y., & Shen, T. C(1937) reported that the development of respiration was studied by observing movements through transparent shell membranes and by recording them graphically in chicks taken out of the shell. The data indicate that development of respiration is a slow and continuous process. No abrupt changes are evidenced either before or after hatching. Drying of the skin and CO₂ tension are factors involved in stimulation of pulmonary respiration. Allantoic circulation remains for a time after breathing is established.

In the present work, water medium has hydrogen and oxygen. In the study embryonic chick in the water medium breath easily due to the presence of oxygen. But in carbonated medium the embryonic chick face difficulty in breathing due to the absence of oxygen.

On comparison of the breathing activity of fresh and cold embryonic chick in both water and carbonated medium the fresh egg in water medium shows high coloration inside the shell but no coloration in the cold egg. Where as in carbonated medium the fresh egg shows slight coloration inside the shell and no coloration in cold egg. The role of the detergent is to help break through the membranes of the egg so that the dye can make a concentrated, visible mark on the inside of the eggshell, rather than a light smear all over it. The detergent does not effect the eggshell.

CONCLUSION

Bird and reptile eggs have a hard shell. Shelled egg must get oxygen somehow for their growth and development. The breathing activity of chick inside its fresh and cold egg in different medium, ie; water and carbonated medium (soda).

The breathing activity of fresh embryonated egg in 5 hours was that the first 3 hours intake of oxygen is high were as in 4th and 5th hour oxygen intake decrease gradually. The embryonated eggs faces difficulty to survive.

In cold eggs breathing activity is absent.

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LEARNING PROCESS IN GUPPY FISH (POECILIA RETICULATA)



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Affiliated to Mahatma Gandhi University, Kottayam in partial
Fulfilment of requirement for the Degree of Bachelor of Science
In Zoology
2021-2022

CERTIFICATE

This is to certify that the project entitled "LEARNING PROCESS IN GUPPY FISHES (Poecilia reticulata)" submitted by Ms. Ashwika Maria , Reg No. AB19ZOO005 in partial fulfillment of Bachelor of Science Degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Reema Kuriakose and this is her original effort.

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Ernakulam

Examiners

- 1)
- 2)

DECLARATION

I, hereby declare that this project work entitled "LEARNING PROCESS IN GUPPY FISHES (Poecilia reticulata)" is submitted to St.Teresa's College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science Degree in Zoology. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own.

Name: Ashwika Maria
Reg.No: AB19ZOO005

Signature

ACKNOWLEDGEMENT

The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my project. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

I owe my deep gratitude to my project guide Dr.Reema Kuriakose , who took keen interest on my project work and guided me all along, till the completion of my project work by providing all necessary information required.

I take this opportunity to express my profound gratitude to my parents and friends who helped me a lot to finish the project within the limited time. Also, I would like to extend my sincere gratitude to all staff in laboratory for their timely support.

Last but not least I would like to thank God Almighty for the successful completion of my project.

Ashwika Maria

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ABSTRACT

The project entitled "Learning process in guppy fishes (*Poecilia reticulata*)" was conducted to evaluate the memory of guppy fishes and its ability to distinguish between colours. During the experiment fishes are conditioned in such a way that they are fed just after showing the red - coloured indicator and was not rewarded with anything after showing the blue coloured indicator. The fishes gradually started showing responses to red coloured indicator and began to avoid the blue indicator. Later the activity was stopped for a week and repeated again to test its memory, and they responded positively. From the present study it can be concluded that guppy fishes were able to distinguish between colours, they have got enough memory and were able to respond to it in the subsequent trials.

INTRODUCTION

Animal learning is an alternation of behaviour as a result of individual experience. When an organism can perceive and change its behaviour, it is said to learn. The cat that runs to its food when it hears the sound of the cupboard opening; the rat that solves a maze, the bird that acquires the song of its species etc, are common examples that demonstrate animals can learn.

Learning produces changes in the behaviour of an individual due to experience. Learning is adaptive because it allows an animal to respond quickly to changes in its environment. Once an animal learns something, its behavioural choices increase. An animal's ability to learn may correlate with the predictability of certain characteristics of its environment where certain changes in the habitat occur regularly and are predictable, the animal may rapidly respond to a stimulus with an unmodified instinctive behaviour. An animal would not necessarily benefit from learning in this situation. However, where certain environmental changes are unpredictable and cannot be anticipated, an animal may modify its behavioural responses through learning or experience. This modification is adaptive because it allows an animal to not only change its response to fit a given situation, but also to improve its response to subsequent, similar environmental changes. The ability to learn is common to most animal species: the need to exploit past experience being obviously extremely important for survival, many animals have evolved ways of coping with it. Although the complexity of learning needed for optimal survival may be different in different species, the basic mechanisms appear to be fairly constant even in phylogenetically distant ones.

Learning ability in fishes is one of the most experimented topics worldwide. It is noticed that the more fish present in the group, the faster they learn (Brown and Warburton, 1999). Social learning occurs when information passes from one individual to another by observation or interaction. This can lead to a transfer of information through generations (vertical transmission) resulting in cultural traditions. This has implications in migration, shifts in spawning or foraging grounds that is happening in commercial fish stocks

Many acts of discrimination are based on conditioning of the fish to single or multiple stimuli. The ability to associate stimulus and response is present even in simple invertebrates; but levels of "intelligence" can be distinguished among other criteria, based on the speed of learning simple tasks and on the retention span of learned responses

Fish can communicate in their underwater environments through the use of acoustic communication. Acoustic communication in fish involves the transmission of acoustic signals from one individual of a species to another. The production of sounds as a means of communication among fish is most often used in the context of feeding, aggression or courtship behaviour. The sounds emitted by fish can vary depending on the species and stimulus involved. They can produce either stridulatory sounds by moving components of the skeletal system, or can produce non-stridulatory sounds by manipulating specialized organs such as the swim bladder.

Guppies have been known to remember and recognize other fish and have even been shown to possess learning retention. In addition, guppies can successfully complete mazes and even remember which of their counterparts were better competitors.

A number of studies have been conducted on learning habits in various fishes but studies related to guppy fish are very less.

REVIEW OF LITERATURE

Fishes can be taught all kinds of things and they remember such things for many days at least, and often many weeks.

In his book “Fish Cognition and Behaviour”, Tony J Pitcher mentions some goldfish in his lab who were trained to choose tubes of one colour over another in order to get food. Then for one year the fish were fed normally in the absence of tubes. When, at the end of that year, the tubes were presented again, the goldfish immediately selected the one with the correct colour.

The discovery of long-term olfactory memory in salmon took place in the 1960s and 1970s and is mainly due to the work of Arthur Hasler at the University of Wisconsin.

Fish can also learn to avoid aversive stimuli rapidly and retain the information for extensive periods, for example pike that have been hooked often show hook shyness over a year (Beukemaj, 1970). Similarly, rainbowfish taught to swim through a hole in a net that travelled down the length of their aquarium took just five runs to figure out the location of the escape route. When tested almost a year later, they still recalled how to escape the net even though they had not seen the net in the intervening period (Brown, 2001).

Zebrafish have shown Pavlovian learning in several experiments. The experiment illustrates a “place-preference” task used by Darland and Dowling to screen zebrafish for cocaine sensitivity.

A microspectrophotometry (MSP) study of the Trinidad guppy revealed cone photoreceptor cells in the retina with λ_{\max} values of 389 nm (“ultraviolet”), 408 nm (“violet”) and 464 nm (“blue”) as well as rod cells of 501 nm (Archer & Lythgoe, 1990).

Another recent study of the Venezuelan (Cumana) guppy reported a similar result [359 nm (ultraviolet), 406 nm (violet), 465 (blue) and 503 nm (rod)] (Watson et al., 2011). It has long been known through MSP studies that, in comparison to the short-wavelength cone classes and the rod cells, the cone classes in the middle-to-long wavelength range are highly variable in spectral sensitivity among individual guppies (Archer et al., 1987, Archer and Lythgoe, 1990, Watson et al., 2011). When pooling λ_{\max} values recorded from multiple cells in multiple individuals, λ_{\max} distribution centers around three peaks, 533, 548 and 572 nm, in a study of

the Trinidad guppy (Archer & Lythgoe, 1990). A study of the Cumana guppy reported a consistent result in which λ_{max} distributions are centered at 525 nm (“green”), 541 nm (“green-yellow”) and 560 nm (“yellow”), slightly shorter than in the former study (Watson et al., 2011).

Fish kept in an artificial environment may impact its physiology and behavior. Fish have chromatic vision and their ability to acknowledge feed and consequently their food intake, growth and survival rate might be influenced by background color (Papoutsoglou et al. 2005, Strand et al. 2007). Moreover, different background colors could cause differences in physiological state, skin pigmentation (Van der Salm et al. 2004, Doolan et al. 2008). Thus, the fish reactions to various background colors differ considerably counting on fish species and life stage (Papoutsoglou et al. 2005).

In ornamental fish sector several fish species like gold fish have been studied to show the effects of background color (Eslamloo et al. 2013).

Jentoft et al. (2006) reported the significant increase in the growth performance of fish raised in transparent background could be due in part to the high contrast between feed and background color and consequently improving the visibility of feed in light tanks.

A recent study on sharks, for instance, has shown that individuals may learn to avoid being caught after the catch-and-release experience (Mourier, Brown, & Planes, 2017).

Guppies have four double-cone classes. The shortest sensitive is always found as the shorter member of a double cone (a.k.a. the accessory cone), and has been reported as having a λ_{max} value between 464 nm and 472 nm (MacNichol et al. 1978; Levine and MacNichol 1979; Levine et al. 1979; Archer et al. 1987; Archer and Lythgoe 1990; Watson et al. 2011).

Colour and shape perception allows the animal's visual environment discrimination and brings advantages for feeding, defense, life in groups, migration, and mate choice (Hughes and Blight, 2000; Blackiston et al., 2011). In fact, fishes are able to see colour from blue to infrared (Levin and Mac Nichol, 1982) and can discriminate a variety of geometrical forms (Petrazzini et al., 2012). The advantage of visual cues recognition is the straight signal source, while olfactory and auditory signals show scattered routes of dispersion (Litherland and Wallis, 2009).

Colour perception preference directly affect fish learning (Spence and Smith, 2008; Sison and Gerlai, 2010), memory formation (Colwill et al., 2005), and decision-making (Avdesh et al., 2010). Many studies show ambient colour and hint colour influence on fish navigation (Hughes

and Blight, 2000; Cognato et al., 2012), spatial location (Petrazzini et al., 2012; Parker et al., 2012) and welfare (Serra et al., 1999; Luchiari et al., 2007). However, studies approaching the fish's ability to discriminate different colours and shapes are still lacking and may indicate an animal's cognitive faculty, plus allowing neural studies, such as neurological diseases or neural disabilities caused by drugs (White, 2000; Gerlai, 2010).

METHODOLOGY

MATERIALS REQUIRED

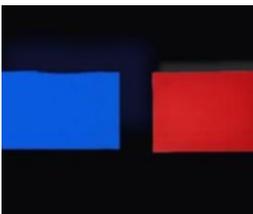
Glass bowl having a mouth width of 15cm, two different colour cues – red and blue (which can be made from cardboard pieces or cloth pieces), 8 guppy fishes (*Poecilia reticulata*) and fish feed (normal feed).

FISH USED: Common guppy fish

SCIENTIFIC NAME: Poecilia reticulata



Fish feed used



Indicators used



Guppy fish used

PROCEDURE

The project was carried out for a period of one month. In this two different colors used for detection are red and blue . At the beginning of the experiment, the fishes were allowed to observe the blue colour cardboard piece for about 20 seconds exactly one hour before feeding the fish. The fish is then allowed to observe the red colour indicator just or immediately before feeding the fish for 20 seconds.

This procedure was repeated in a similar way for about 21 days. Later the activity is stopped and repeated after taking a break of one week to analyze its memory. Light conditions were maintained constant inside the bowl throughout the experiment. The fishes were fed twice a day with approximately equal quantities every time.

OBSERVATION

Number of days	response to red indicator	response to blue indicator
----------------	---------------------------	----------------------------

0 to 7	Nil	Nil
8 to 14	Nil	Nil
15 to 21	Yes	Nil
22 to 28	indicator not shown	indicator not shown
29 to 30	Yes	Nil

RESULT

The fishes are conditioned in such a way that they are fed just after showing the red colour and was not rewarded with anything after showing the blue -coloured indicator.

At first the fishes did not respond to either of the colours and responded only to the food but as the process continued after almost 15 days the fishes started showing responses as soon as the red-coloured indicator was brought to their sight and no responses were shown to the blue colour. As they realized that rewards were not given with blue, they did not give any response. Gradually they started showing responses only to red coloured indicator. As soon as the red colour was shown they were excited, and they moved towards the corner of the bowl opening their mouths, anticipating the food.

This process was repeated for a few days purposely so that the colour gets stored in their memory and to get the relationship between colour and food.

Then the activity was stopped for a few days and the fishes were not exposed to either of these colours and were fed normally.

When the red colour was shown exactly after one week, it was observed that the fishes responded to red colour which points to the fact that the fishes were conditioned and able to store it in their brain and recall the similar situation from their memory when repeated again even after an interval.

DISCUSSION

The project was based on the learning ability exhibited by guppy fishes. These fishes were able to distinguish between the colours provided and were also able to recollect it later. Fish intelligence is the result of the process of acquiring, storing in memory, retrieving, combining, comparing, and using in new contexts information and conceptual skills.

Fish are an ideal system to study color vision because of their diverse set of visual pigments and the highly variable light environments they inhabit.

Guppy fishes have a visual system with five cones that allow them to see colors ranging from ultraviolet (UV) to red, and they are often used to study vision and behavior.

Guppies preferred orange and red and paid much less attention to other colors like yellow, green, and blue.

A number of studies have shown that fish can retain information for months or years (Beukemaj, 1970).

George Streisinger (1927) was the father of zebrafish research. He established a zebrafish research colony and developed the first methods for mutagenesis and mutant screening with the goal of studying the development of the nervous system through genetic analysis.

In another experiment conducted on gold fishes the average time for the goldfish to find food in the maze after an absence of six months was 12.82 seconds, which was less than half of the time taken on the last day of training (30.19 seconds) 16 months earlier. These results suggest that the ability of goldfish to retain and recall an explicit memory is impressive considering its small brain size, short life span, and lowest place on the vertebrate hierarchy (Katie, Michigan, 2015). In this experiment, it was inferred that guppies responded to colour cues within 10-15 seconds.

The literature contains several examples of specialized cognitive abilities, but few regarding fish. The guppy, *Poeciliareticulata*, is a freshwater fish in which females choose their mates based on colouration, and orange-coloured fruits are important diet enrichments for both sexes. For these reasons, we expect that this species has evolved enhanced learning abilities in colour discrimination compared to other types of discrimination.

Individual carp captured by anglers have been shown to become less catchable thereafter. This suggests that fish use their memory of negative experiences to associate capture with stress and therefore become less easy to catch. This type of associative learning has also been shown in paradise fish (*Macropodus opercularis*) which avoid places where they have experienced a single attack by a predator and continue to avoid for many months. Red Sea clownfish can recognize their mate after 30 days of separation.

Through social learning, fishes might learn not only where to get food, but also what to get and how to get it. Hatchery-raised salmon can be taught to quickly accept novel, live prey items similar to those they will encounter once they will be released in the wild, simply by watching an experienced salmon take such prey. The same is true of young perch. In the laboratory, juvenile European seabass can learn to push a lever in order to obtain food just by watching experienced individuals use the lever.

CONCLUSION

Many studies in fishes have shown that fishes can retain information for months or years and also they have the ability to depend on visual cues for feeding, mating, migration, etc.

In this study the main objective of the project was to evaluate the memory and its ability to distinguish between colours in guppy fish. From the present study it can be concluded that guppy fishes were able to distinguish between colours and they have enough memory to relate between colour and feed.

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**IN VITRO BREATHING ANALYSIS OF COLD AND FRESH EGG IN WATER AND
CARBONATED MEDIUM**



Project work by

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Affiliated to Mahatma Gandhi University, Kottayam in partial

Fulfilment of requirement for the degree of Bachelor of science

In Zoology

2019-2022

CERTIFICATE

This is to certify that the project report entitled, “***In vitro* breathing analysis of cold and fresh egg in water and carbonated medium** ” submitted by **Ms.Bitty Mol K B** Reg.No.AB19ZOO007 in partial fulfilment of the request of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under my guidance and supervision and to the best of my knowledge, this is her original effort.

Dr. Soja Louis

Head of the department

Department of Zoology

St.Teresa’s College

Ernakulam

Dr. Reema Kuriakose (Supervising teacher)

Controller of examination

Department of Zoology

St.Teresa’s College

Ernakulam

Examiners

1).....

2).....

DECLARATION

I, hereby declare that this project work entitled “IN VITRO BREATHING ANALYSIS OF COLD AND FRESH EGG IN WATER AND CARBONATED MEDIUM” is submitted to St.Teresa’s collage (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science degree in Zoology. This work has been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own

Name: Bitty Mol K B

Signature

Reg. No: AB19ZOO007

ACKNOWLEDGEMENT

The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my project. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

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Bitty Mol K B

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ABSTRACT

Every animal needs oxygen to survive. Animals that grow inside their mother get oxygen from their mother's blood. But those that grow in a shell must get air somehow. This study aims on how a shelled animal get oxygen from outside. It is carried out with a view to analyse how the chick breath inside in fresh and cold egg in different mediums for different hours. The breathing rate of the chick embryo gradually increases, after a particular time the breathing activity decreases. The embryonated eggs faces difficulty to survive.

INTRODUCTION

Every animal requires oxygen to live. When animals, including humans, breathe in, oxygen enters the lungs, where it is shuttled into the blood stream and distributed to all the different parts of the body. The oxygen is used in an internal chemical reaction called metabolism to provide the animal with energy. The process of metabolism also produces a waste gas called carbon dioxide. In order to get rid of this waste gas, the blood stream carries the carbon dioxide back to the lungs where it is collected and finally breathed out.

Animals that grow inside their mothers, like humans, get their oxygen from their mothers. The blood stream of the baby animal and the mother are connected through an umbilical cord, which allows the baby to collect oxygen that his or her mother breathes in and use the mother's lungs to get rid of the carbon dioxide. But how do animals that grow in a shell and do not have umbilical cords, like chickens, take in oxygen and get rid of carbon dioxide?

Bird and reptile eggs have a hard shell. Directly under the shell are two membranes. When the eggs are laid by the mother, they are warmer than the air, and as they cool, the material inside the egg shrinks a little bit. This shrinking pulls the two membranes apart, leaving behind an air cell, also called an air sack, that is filled with oxygen. As the animal develops, it needs the oxygen replenished so it can continue to grow. So, how does this happen? Well, when a chicken egg is examined carefully with a magnifying glass, we can see that there are tiny little holes, called pores, in the shell. A chicken eggshell has more than 7,000 pores. It was found that through these pores gas exchange happens. To identify and record the breathing rate of chicken's egg, dye and detergent are used.

The role of the detergent is to help break through the membranes of the egg so that the dye can make a concentrated, visible mark on the inside of the eggshell, rather than a light smear all over it. The detergent does not affect the eggshell.

REVIEW OF LITERATURE

Rahn & Paganelli (1979) studied that the bird egg is a self-contained life support system for the developing bird embryo. All the nutrients, minerals, energy sources and water utilized by the embryo during its incubation are already present in the freshly laid egg, so that the egg requires only warming by the parents and periodic turning to prevent the adhesion of the embryo to the shell membranes. Still, the egg lacks one crucial requirement: oxygen, which drives the metabolic machinery of the embryonic cells so that they can execute the complex maneuvers of development.

Windle, et.al (1938) found that small amounts of carbon dioxide in an atmosphere rich in oxygen initiated rhythmic respiration-like movements in incubating chick embryo several days before these movements occurred normally. Greater concentration of carbon dioxide or more complete anoxemia led to the establishment of stronger respiratory activity resembling the gasping of acute air-hunger. Near the end of 20th or beginning of 21st day of incubation the chick pricks its enclosing membranes and begins to breathe the air within the shell. Soon thereafter it chips a hole in the shell admitting atmospheric air. The respiratory allantois continues to function until hatching has been completed".

Visschedijk, et.al (1980) studied that the gas moves through the pores of the egg shell by diffusion in the gas phase. The gas flux is therefore determined by the product of the effective conductance of the shell and the partial pressure gradient of the gas between the ambient air and the inner side of the shell. Chicken embryos aged 16-19 days were exposed to various oxygen concentrations and carbon dioxide production was measured. At subnormal oxygen concentrations carbon dioxide output diminished as the oxygen concentration was lowered and the duration of exposure was prolonged. At oxygen concentrations above normal a small but significant increase in carbon dioxide production was found. This difference is ascribed to the fact that a reduction of barometric pressure not only decreases oxygen partial pressure in the ambient air but also increases effective conductance of the egg shell, the latter being inversely proportional to the barometric pressure".

Kuo & Shen (1937) studied that the development of respiration was studied by observing movements through transparent shell membranes and by recording them graphically in chicks taken out of the shell. The data indicate that development of respiration is a slow and continuous process. No abrupt changes are evidenced either before or after hatching. Drying of the skin and CO₂ tension are factors involved in stimulation of pulmonary respiration. Allantoic circulation remains for a time after breathing is established".

Wangensteen,et.al (1974) studied that the gas exchange by embryos from chickens acclimatized to an altitude of 3800 m (pB = 480 torr) was studied in order to ascertain the nature of the altitude adaptation in this species. The PO₂ and PCO₂ differences across the egg shell were measured and found to be less than the values previously reported for sea-level eggs by about a factor of two. Further measurements of embryonic oxygen consumption (O₂) and shell conductivity to oxygen (GO₂) indicated that, compared to eggs at sea level, O₂ was reduced by a factor of 0.58 while GO₂ was increased only by a factor of 1.07 in the high-altitude eggs. These independent measurements predict the Δ PO₂ across the egg shell of the high-altitude eggs to be only 0.54 times that of sea-level eggs; the directly measured factor was 0.53. The authors conclude that at high altitude a major adaptation of the chick embryo is a reduced metabolism which decreases the Δ PO₂ across the egg shell since its gas conductivity remains essentially unchanged".

Tazawa (1980) studied that the fertile chicken eggs belonging to the same flock of hens were divided into two groups and incubated for 16 days. During incubation, group 1 eggs were turned twice a day and group 2 eggs were left unturned. Blood sampled from the allantoic vein or artery was analyzed for gas tensions (PO₂ and PCO₂), pH and Hct. These values were compared by unpaired t-test for significance differences between the two groups. While the differences of PCO₂ and pH were found insignificant, failure to turn the eggs caused a pronounced fall in the arterialized PO₂ which was accompanied with an increase in Hct. In addition, the embryo weight was reduced in unturned eggs. Lack of turning retarded the absorption of albumen. The unabsorbed albumen interposed between the chorioallantoic membrane and inner shell membrane, impeding the blood oxygenation through the

chorioallantois. Little change in PCO₂ might be attributed to a large diffusive conductance of the chorioallantois for CO₂. The present results suggest that the eggs must be turned periodically during incubation to prevent the distortion of normal oxygen exchange especially for the study of egg respiration".

Hagelin, et al (2013) studied that we know almost nothing about the chemosensory experiences of birds as they develop within an egg's fluid-filled environment. Given well-established literature on chemical detection of mammals in utero, we explored whether domestic chickens (*Gallus gallus domesticus*) exhibit a similar ability to detect or learn about chemical stimuli prior to breathing air. We incubated 18 eggs from embryonic day (E)9-18 in scented air containing Z-4-decenal and octanal, two key components of a citrusy-scented avian social odour (from Crested Auklets [*Aethia cristatella*]; Proc R Soc Lond 270:1323-1329, 2003). Control eggs were not exposed to scent. Behavioural responses of embryos were quantified by opening the shell and exposing embryos to three different test scents (Crested Auklet, wintergreen [novel scent], and water [unscented control]).

They conclude that embryos modified their behaviour after experiencing air-borne compounds that were transmitted into the egg's fluid environment".

Beattie (1964) studied that the measurements of the oxygen uptake and carbon dioxide output during the last 36 hr. of incubation show that the exchange of these two gases increases progressively from the start of pulmonary respiration until hatching. The evidence indicates that prior to the penetration of the shell by the beak of the embryo ("pipping") there is marked hypoxia and hypercapnia which are relieved when the embryo gains access to atmospheric air. At the time of hatching there is a very rapid release of carbon dioxide from the body surface and the internal surface of the allantoic membranes. There is no evidence to suggest that its release indicates either hypoxia or hypercapnia in the embryo immediately before hatching".

Burton & Tullett studied that this review is concerned with how bird embryos breathe. The first part discusses the ontogeny and mechanism of gaseous exchange, describing

briefly the development of the extra-embryonic membranes, the movement of oxygen across the shell and shell membranes into the blood and the elimination of carbon dioxide and the transition from respiration via the extra-embryonic membranes to pulmonary respiration. The discussion concerns mainly the domestic fowl, it can in general terms be applied to most birds. Egg weight, shell porosity and special circumstances, such as high altitude, unusual nesting or incubation regimes, have important effects on embryonic respiration. These are considered in the second part of the review".

Gabrielli & Accili (2010) studied that the chick chorio allantoic membrane is a very simple extraembryonic membrane which serves multiple functions during embryo development; it is the site of exchange of respiratory gases, calcium transport from the eggshell, acid-base homeostasis in the embryo, and ion and H₂O reabsorption from the allantoic fluid. All these functions are accomplished by its epithelia, the chorionic and the allantoic epithelium, by differentiation of a wide range of structural and molecular peculiarities which make them highly specialized, ion transporting epithelia".

Burton, et.al (1989) studied that the covering the whole shell or the shell over the chorioallantoic membrane with liquid paraffin resulted in the diffusion of oxygen through the shell over the air space before the onset of pulmonary respiration being reduced to a level insufficient to keep the embryo alive. During the parafoetal period respiratory exchange through the air space becomes progressively more important than that through the allantois with the result that the embryo can survive a blocking of the whole shell or allantoic shell during this period, provided that pulmonary respiration has been developed sufficiently and that the shell is pipped within a certain time limit. After pipping respiratory exchange can be effected by pulmonary respiration alone, without great danger to the embryo. It may therefore be concluded that the respiratory exchange through the allantoic shell is greater than through the air space shell up to the time of pipping. By measuring directly the gaseous exchange through the shell over the air space and allantoic shell it was shown that the amount of the diffusion through the shell over the air space was in proportion to the total diffusion, at least until the onset of pulmonary respiration. After that moment the absolute as well as the percentage exchange via the air space increased. The allantois did not begin to degenerate until pipping had taken place. Lung function then increased and soon took over completely the

function of the allantois. As soon as the allantoic respiration had been practically abolished hatching activities started with the result that hatching often followed within half an hour

MATERIALS AND METHODS

The present study was carried out with a view to analyse the breathing of hen's egg inside (fresh and cold egg) in different medium, ie; Water and Carbonated drink (soda).

1. Selection of egg:

Twenty eggs were taken, from same type.

- Ten are fresh eggs
- Ten are refrigerated for 24hrs.

2. Selection of medium:

Two different medium are taken, namely: water and carbonated drink (soda).

3. Preparation of medium:

3.1 Preparation of first medium

Take 3 ½ cup of water in a container box. Add (1 tsp) detergent and add (5 drops) red food colour to it. Mix well for sometimes without forming foam on water.

3.2 Preparation of second medium

Take 3 ½ cup of carbonated drink in a container box. Add (1tsp) detergent to it. Now, add food colour (5 drops) and mix well for sometimes.

4. Analysis of chick breath inside its shell:

Take two container boxes with water and carbonated drink on each. Pour 3 ½ cups of water in one container box and 3 ½ cups of carbonated drink in another container box. Add (1tsp) liquid dishwashing detergent and (1tsp) red food colour each to the container boxes.

Carefully set the fresh and cold eggs in each container box. Make sure that the eggs are submerged in the liquid, if any part of the egg is above the surface of the liquid, repeat the steps and add the extra liquid to the container box until the eggs are submerged. Note the starting time and soak the eggs for 5hrs. After the eggs have soaked in the liquid for 5hrs. carefully lift the eggs each from the two medium in every hour using a large spoon. Wipe it with a cotton towel. Now, crack the raw egg into a cup being carefully not to damage or crush the shell much. Set the empty egg shells on a plate and carefully inspect the inside of the shell.

OBSERVATION AND RESULT

The detergent is used to break the membranes of the egg so that the dye can make a concentrated, visible mark on the inside of the eggshell.

The present study was undertaken to analyse the breathing activity of embryonated eggs in water and carbonated medium for different hours.

Embryonated eggs in water medium

Analysing the breathing activity of the fresh embryonated egg in five hours it was observed that the first three hours the intake of oxygen is high whereas in fourth and fifth hour oxygen intake decreases gradually due to the intake of water from the medium. The embryonated egg faces difficulty to survive.

In the cold egg the breathing activity is absent due to the eggs treated 24 hours in refrigerator so that the embryonated eggs die.

Embryonated egg in carbonated medium

Analysing the breathing activity of the fresh embryonated egg in 5 hours it was observed that the first 4 hours the intake of oxygen is gradually increases due to the carbondioxide concentration in the carbonated medium decreases over time whereas in 5th hour the breathing activity of the embryonated eggs starts decreasing due to the intake of water from the medium. The embryonated eggs faces difficulty to survive.

As mentioned above, in the cold eggs the breathing activity is absent.

Colour grade chart

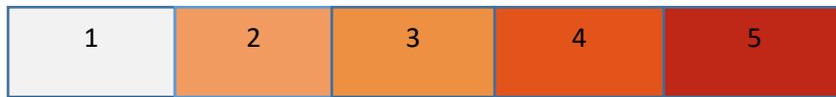
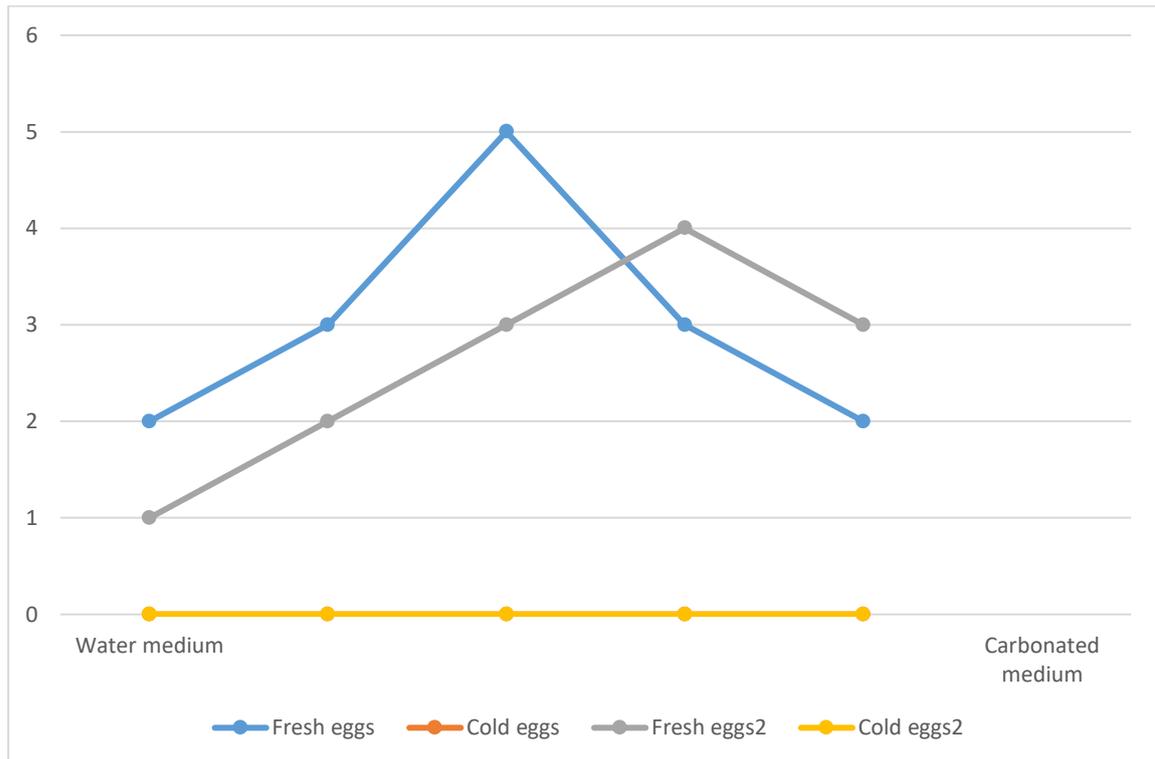


Table showing breathing activity of embryonated eggs in water and carbonated medium by colour gradient

Time	Water medium		Carbonated medium	
	Fresh eggs	Cold eggs	Fresh eggs	Cold eggs
1 hour	2	Nil	1	Nil
2 hour	3	Nil	2	Nil
3 hour	5	Nil	3	Nil
4 hour	3	Nil	4	Nil
5 hour	2	Nil	3	Nil

Graphical representation of breathing activity of embryonated eggs in water and carbonated medium by colour gradient



Water medium



Fresh egg in 1 hour



Fresh egg in 2 hours

Carbonated medium



Fresh egg in 1 hour



Fresh egg in 2 hours



Fresh egg in 3 hours



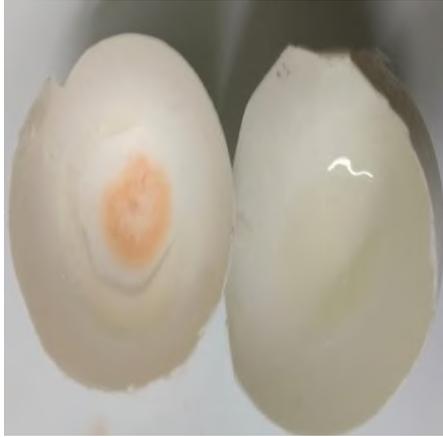
Fresh egg in 3 hours



Fresh egg in 4 hours



Fresh egg in 4 hours



Fresh egg in 5 hours



Fresh egg in 5 hours



In the cold egg the breathing activity is absent

DISCUSSION

Every animal including embryonated eggs needs oxygen to live.

Embryos developing inside the mother's uterus have an umbilical cord that takes in oxygen from their mother's bloodstream, and gets rid of carbon dioxide that is produced as waste but embryonated eggs that develop inside shells have no umbilical cord. Instead, they depend upon an air sac filled with oxygen that lies between two membranes which are directly under the hard shell of the egg. Oxygen enters the eggs through pores in the cuticle and passes through columns of crystals to the permeable shell membranes. Carbon dioxide and water vapour escape to the outside environment through these same pores.

The present study was undertaken to analyse the breathing activity of cold and fresh egg in water and carbonated medium. It also helpful in study the rate of oxygen and carbon dioxide in the medium which effect the breathing activity of embryonic chick. The gas moves through the pores of the egg shell by diffusion in the gas phase (Visschedijk, et.al (1980)).

According to the previous report it was demonstrated that small amounts of carbon dioxide in an atmosphere rich in oxygen initiated rhythmic respiration-like movements in incubating chick foetus several days before these movements occurred normally. Greater concentration of carbon dioxide or more complete anoxemia led to the establishment of stronger respiratory activity resembling the gasping of acute air-hunger. Near the end of 20th or beginning of 21st day of incubation the chick perforates its enclosing membranes and begins to breathe the air within the shell. Soon thereafter it chips a hole in the shell admitting atmospheric air. The respiratory allantois continues to function until hatching has been completed (Windle,et.al(1938)).

Kuo, Z. Y., & Shen, T. C(1937) reported that the development of respiration was studied by observing movements through transparent shell membranes and by recording them graphically in chicks taken out of the shell. The data indicate that development of respiration is a slow and continuous process. No abrupt changes are evidenced either before or after hatching. Drying of the skin and CO₂ tension are factors involved in stimulation of pulmonary respiration. Allantoic circulation remains for a time after breathing is established.

In the present work, water medium has hydrogen and oxygen. In the study embryonic chick in the water medium breath easily due to the presence of oxygen. But in carbonated medium the embryonic chick face difficulty in breathing due to the absence of oxygen.

On comparison of the breathing activity of fresh and cold embryonic chick in both water and carbonated medium the fresh egg in water medium shows high coloration inside the shell but no coloration in the cold egg. Where as in carbonated medium the fresh egg shows slight coloration inside the shell and no coloration in cold egg. The role of the detergent is to help break through the membranes of the egg so that the dye can make a concentrated, visible mark on the inside of the eggshell, rather than a light smear all over it. The detergent does not effect the eggshell.

CONCLUSION

Bird and reptile eggs have a hard shell. Shelled egg must get oxygen somehow for their growth and development. The breathing activity of chick inside its fresh and cold egg in different medium, ie; water and carbonated medium (soda).

The breathing activity of fresh embryonated egg in 5 hours was that the first 3 hours intake of oxygen is high were as in 4th and 5th hour oxygen intake decrease gradually. The embryonated eggs faces difficulty to survive.

In cold eggs breathing activity is absent.9

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ROLE OF TEMPERATURE IN THE BREEDING OF FISH - BLACK MOLLY (POECILIA SPHENOPS)



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Affiliated to Mahatma Gandhi University, Kottayam in Partial
Fulfillment of requirement for the degree of Bachelor of Science In
Zoology

2021-2022

CERTIFICATE

This is to certify that the project report entitled “**ROLE OF TEMPERATURE IN THE BREEDING OF FISH - BLACK MOLLY (POECILIA SPHENOPS)**”, submitted by **Ms. DAHLIA S PAUL** Reg. No. **AB19ZOO008** in partial fulfilment of the request of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under my guidance and supervision and to the best of my knowledge, this is her original effort.

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EXAMINERS

1)

2)

DECLARATION

I, hereby declare that this project work entitled “ROLE OF TEMPERATURE IN THE BREEDING OF FISH - BLACK MOLLY (POECILIA SPHENOPS)” is submitted to St. Teresa’s College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfillment of the requirements of Bachelor of Science degree in Zoology. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own.

Name: Dahlia S Paul
Reg.No: AB19ZOO008

Signature

ACKNOWLEDGEMENT

The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my project. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

I would like to thank God Almighty for the successful completion of my project.

I owe my deep gratitude to my project guide Dr. Reema Kuriakose, who took keen interest on my project work and guided me all along, till the completion of my project work by providing all necessary information.

I take this opportunity to express my profound gratitude to my parents and friends who helped me a lot in finishing the project within the limited time.

Dahlia S Paul

ROLE OF TEMPERATURE IN THE BREEDING
OF FISH - BLACK MOLLY (POECILIA
SPHENOPS)

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Abstract

Poecilia sphenops commonly known as black molly fish is one of the most popular aquarium fishes. In the present study, the effect of water temperature in fish breeding and mortality rate was investigated. Temperatures of 28°C, 30°C & 32°C were used and the study was conducted in aquarium tanks. It was observed that 28°C is the preferred temperature among the three. These findings will help to rear mollies in the suitable water temperatures for better production of offspring in small or large scale.

Introduction

Ornamental fishes are colourful and attractive species and are important part of aquaculture (Mandakranta et al., 2017). Ornamental fish keeping is one of the popular hobbies and it has resulted in increase of aquarium fish trade globally. This has paved way to financial openings and also enables relaxation of mind. (Sumithra et al.,2014) Hence it is essential to meet all requirements for the better development in this industry. Among the ornamental fishes, *Poecilia sphenops* (black mollies) is one of the commercially important fish which comes under Poeciliidae family. They mainly inhabit freshwater, but also found in brackish & saline habitats. They are viviparous and are easy to breed. Females can produce fry in large numbers in one live birth. (Divya,2018). There are many factors which affect the overall development of aquarium fishes. Temperature is a fundamental physical factor in the lives of fishes as they are poikilothermic. Dissolved oxygen concentration decreases with increase in temperature and in turn affects the rate of growth, reproductive processes and mortality in fishes. (Ned & Philip, 2011).

Scientific Classification	
Kingdom	Animalia
Phylum	Chordata
Class	Actinopterygii
Order	Cyprinodontiformes
Family	Poeciliidae
Genus	Poecilia
Species	sphenops

Review of Literature

Different studies were conducted on *Poecilia sphenops* (black molly fish) regarding the effect of temperature on growth, breeding & overall development. *Poecilia sphenops* is eurythermal and it can adapt to environmental variations. *Hernandez & Buckle (2002)*.

Benjamin et al., (2002) done a work to determine combined effect of pH/temperature on sex determination of *Poecilia sphenops*.

The thermal preference of black male molly fish was 25.5°C in summer and 29.6°C in winter whereas females preferred 29°C in both seasons (*Hernandez et al., 2002*). While *Wan Husna et al., (2014)* reported that black mollies were able to tolerate temperature ranged between 24-26.7°C.

It is also observed that the food intake by mollies was larger at the temperature 28 ±1°C than 22 ±1°C (*Nawras et al., 2007*).

Poecilia reticulata (guppy) also belongs to family Poeciliidae and different studies with respect to temperature were carried out in them. The highest survival of guppy was found at the temperatures of 26°C & 28°C (*Shah et al., 2017*).

Tarang Kumar Shah et al., (2017) conducted studies on guppies to find out the influence of water temperature on phenotypic sex ratio.

Methodology

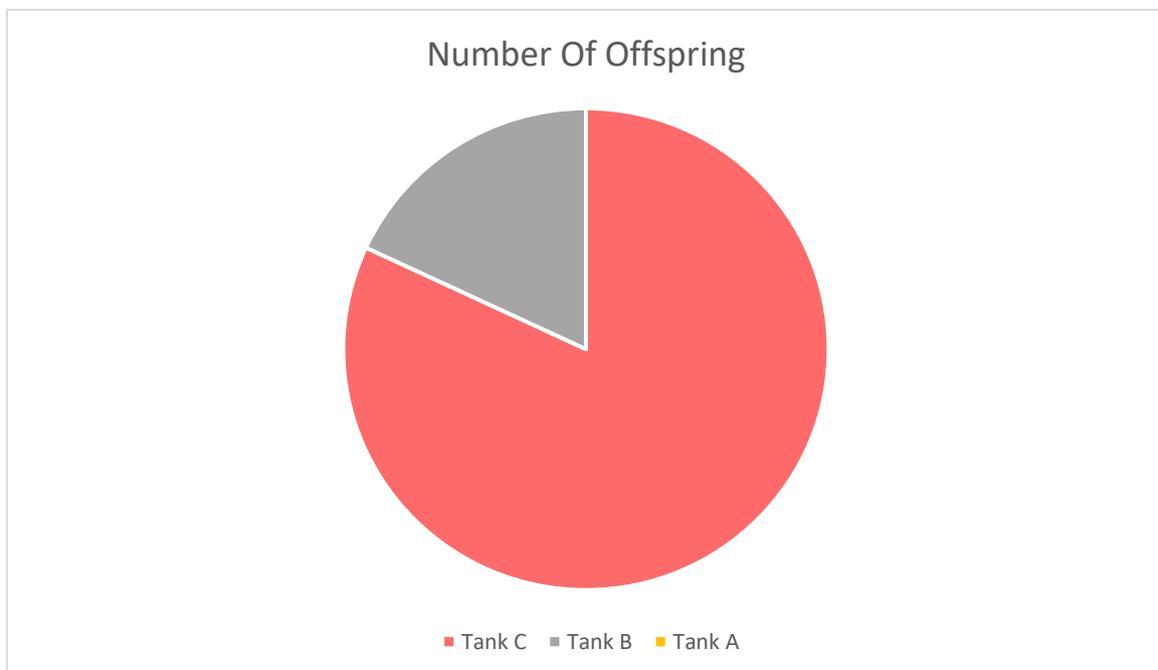
To detect the role of temperature in black molly fishes were selected for which three aquarium tanks were used which were labelled as A, B and C for the temperatures 32°C, 30°C and 28°C (normal water temperature) respectively. For Tank A and B, thermostat heaters were used for providing constant required temperature (32°C and 30°C respectively). The temperature of water in all the three tanks were monitored using a laboratory thermometer. The biofilters for all the three tanks were self – made using PVC pipes as they are eco-friendly and cost effective.

Six male and six female fishes were used for each tank. Before introducing the fishes into the tank, water in the tank was circulated for a day under normal conditions. The fishes were introduced into three tanks under normal conditions on next day so that the fishes get adapted to new surroundings. After 24 hours, the thermostat heaters in Tank A and B were switched on. Some amount of rock salt is added to all the three tanks to inhibit fungal infection. The number of offspring in each tank and mortality of the fishes were observed and the findings were noted. The experiment was carried out for one month.

Observation

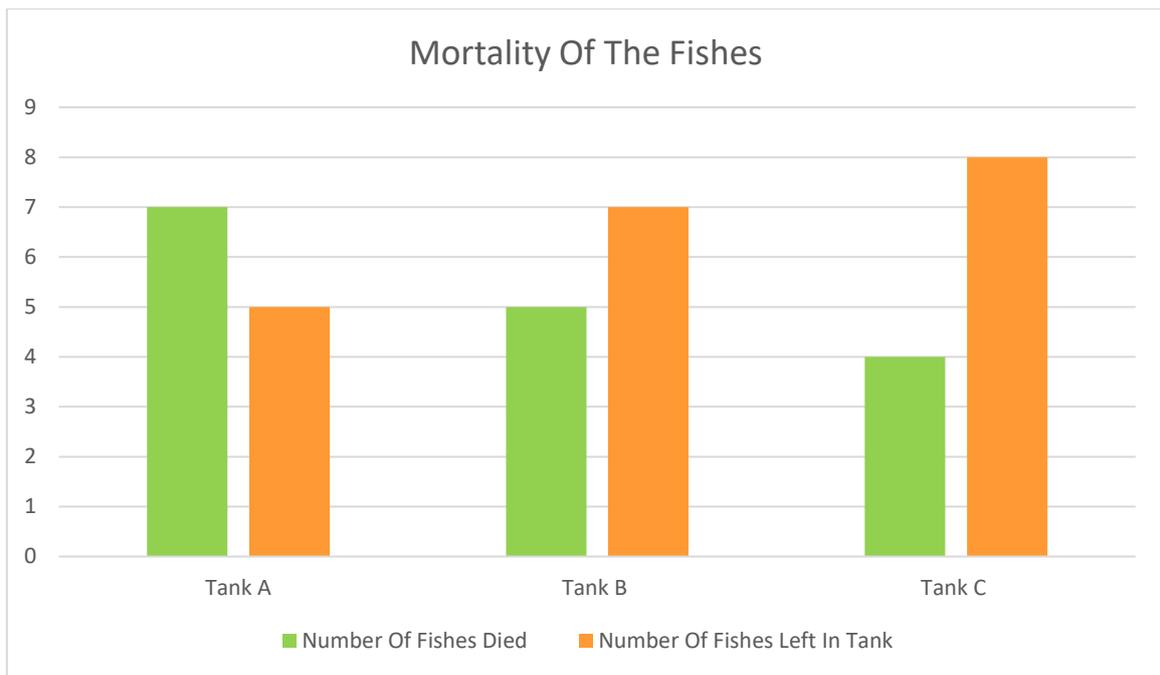
Number Of Offspring In Each Tank: -

	Tank A (32°C)	Tank B (30°C)	Tank C (28°C)
5 th Day	0	11	35
10 th Day	0	2	9
15 th Day	0	2	1
20 th Day	0	0	1
25 th Day	0	0	17
30 th Day	0	0	5
TOTAL	0	15	68



Mortality Of The Fishes: -

TANK	NUMBER OF FISHES DIED	NUMBER OF FISHES LEFT IN TANK
Tank A (32°C)	7	5
Tank B (30°C)	5	7
Tank C (28°C)	4	8



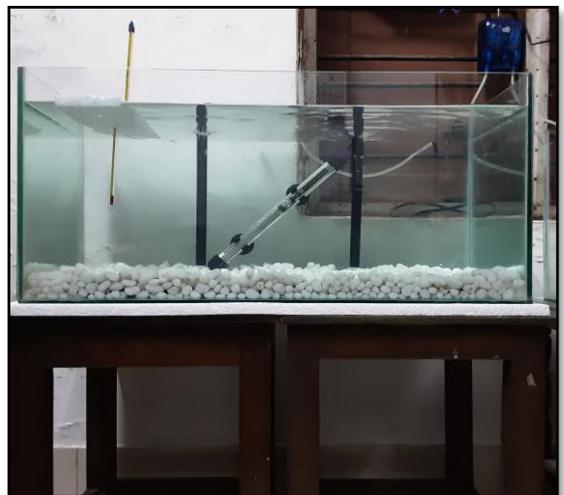


Fig 1: Setting up the Aquarium



Fig 2: Molly Fishes in the Aquarium



Fig 3: Fry of molly fish

Result

In the present study, the optimum temperature for molly fish breeding is 28°C (Tank C) followed by 30°C (Tank B). The fishes at 32°C (Tank A) did not produce any offspring. The mortality rate was maximum in 32°C, followed by 30°C. The fishes at 28°C had minimum mortality rate.

Discussion

It was reported that black mollies were able to tolerate temperature in the range between 24-26.7°C. *Wan Husna et al.*, (2014) and *Hernandez et al.*, (2002) reported that males preferred 25.5°C in summer and 29.6°C in winter whereas females preferred 29°C in both seasons.

But in the present study, it was found that mollies were able to tolerate a temperature of 28°C and produced 68 offspring than at 30°C which produced only 15 offspring, though there is only 2°C increase in temperature.

When the temperature was increased by 4°C (at 32°C), none of the offspring were produced by mollies. This shows that a small increase in temperature affects in the production of offspring. Out of these three temperatures, 28°C is found to be optimum for breeding black molly fishes. *Nawras et al.*, (2007) also observed that the food intake by mollies was larger at the temperature 28 ±1°C.

The mortality rate of the fishes was also recorded and it was found to be highest in 32°C, followed by 30°C and then at 28°C. This shows that water temperature is one of the principal environmental factors influencing the growth of ornamental fishes.

Conclusion

It can be concluded that from the experiment that *Poecilia sphenops* is found to be suitable in growing in the water having temperature at 28°C. They produced highest number of offspring and have least mortality rate. At 30°C, mollies produced few offspring. But the number was very less compared to those fishes at 28°C. Also, the mortality rate of the fishes at 30°C is found to be higher. At higher temperatures (32°C), the fishes will not produce offspring and they have highest mortality rate among the three.

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LARVICIDAL EFFECTS OF PLANT EXTRACTS ON MOSQUITO LARVAE



Project work by

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Affiliated to Mahatma Gandhi University, Kottayam in partial
Fulfilment of requirement for the degree of Bachelor of science
In Zoology
2019-2022

CERTIFICATE

This is to certify that the project report entitled “**LARVICIDAL EFFECTS OF PLANT EXTRACTS ON MOSQUITO LARVAE**”, submitted by **Ms. KANTIPUDI GOURI SREEPRIYA** Reg No: **AB19ZOO011** in partial fulfillment of the request of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under my guidance and supervision and to the best of my knowledge, this is her original effort.

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EXAMINERS

1)

2)

DECLARATION

I, hereby declare that this project work entitled, “**LARVICIDAL EFFECTS OF PLANT EXTRACTS ON MOSQUITO LARVAE**”, is submitted to St. Teresa’s College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science degree in Zoology. This work has not been undertaken nor submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own.

KANTIPUDI GOURI SREEPRIYA
Reg No: AB19ZOO011

Signature

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KANTIPUDI GOURI SREEPRIYA

ABSTRACT

Chemical control methods using synthetic insecticides had been favored for so long because of their speedy action and ease of application. However, it has now been realized that due to several reasons known, chemical insecticides and larvicides can no longer be used for vector control in the same scale as before from the point of view that environmental pollution has been taking place exponentially. Biologically active plant extracts are therefore used to be studied for their potential efficacy to minimize the extent of pollution and to reduce the cost.

Extracts derived from seven plant species, *Curcuma longa*, *Citrus lemon*, *Coleus barbatus*, *Azadirachta indica*, *Piper nigrum*, *Zingiber officinale*, *Allium sativum*, were evaluated for efficacy against larvae of mosquitoes using larvicidal bioassays. Plant extracts were extracted from fresh parts of plants and different concentrations of extract was prepared for each of the plant species. Insecticidal and repellent activity of extracts against larvae form of mosquitoes, collected from small pools located in and around Ernakulam were examined via bioassay method.

Results of current study on insecticidal and repellent activity of mentioned plant extracts against the larvae show considerable effects. Concentration of undiluted extract had the highest insecticidal and repellent activity against the larvae for all of the extracts and extracts of Neem and Pepper were seen more potent than the other plant extracts taken.

The highest larvicidal bioassay was established from *Azadirachta indica* (neem) and *Piper nigrum* (pepper).

The study affords some information regarding the action of different plant extracts and their potential in preventing the growth of mosquito larvae and therefore can be used in mosquito control. The study also shows that these plant extracts can be considered as good replacements for chemical pesticides but more experiments are needed for this purpose. Study on insecticidal activity of these plant extracts in the field condition can be considered as a subject for next experiments.

INTRODUCTION

Mosquitoes are members of a group of almost 3,600 species of small flies within the family Culicidae (from the Latin *culex* meaning “gnat”) The word “mosquito” (formed by *mosca* and diminutive *-ito*) is Spanish and Portuguese for “little fly”. Mosquitoes have a slender segmented body, one pair of wings, one pair of halteres, three pairs of long hair-like legs, and elongated mouthparts. The mosquito life cycle consists of egg, larva, pupa, and adult stages. Eggs are laid on the water surface; they hatch into motile larvae that feed on aquatic algae and organic material. These larvae are important food sources for many freshwater animals, such as dragonfly nymphs, many fish, and some birds such as ducks. The adult females of most species have tube-like mouthparts (called a proboscis) that can pierce the skin of a host and feed on blood, which contains protein and iron needed to produce eggs. Thousands of mosquito species feed on the blood of various hosts—vertebrates, including mammals, birds, reptiles, amphibians, and some fish; along with some invertebrates, primarily other arthropods. The mosquito’s saliva is transferred to the host during the bite, and can cause an itchy rash. In addition, many species can ingest pathogens while biting, and transmit them to future hosts. In this way, mosquitoes are important vectors of parasitic diseases such as malaria and filariasis, and arboviral diseases such as yellow fever, Chikungunya, West Nile, dengue fever, and Zika. By transmitting diseases, mosquitoes cause the deaths of more people than any other animal taxon: over 700,000 each year. It has been claimed that almost half of the people who have ever lived have died of mosquito-vector disease, but this claim is disputed, with more conservative estimates placing the death toll closer to 5% of all humans. Mosquitoes cannot live or function properly when the air temperature is below 10 degrees Celsius (50 degrees Fahrenheit). They are mostly active at 15–25 degrees Celsius (60–80 degrees Fahrenheit). Mosquito control manages the population of mosquitoes to reduce their damage to human health, economies, and enjoyment. Mosquito control is a vital public-health practice throughout the world and especially in the tropics because mosquitoes spread many diseases, such as malaria and the Zika virus. An obvious method for the control mosquito-borne diseases is the use of insecticides, and many synthetic agents has been developed and employed in the field with considerable success. However, one major drawback with the use of chemical insecticides is that they are non-selective and could be harmful to other organisms in the environment. It has also provoked undesirable effects, including toxicity to non-target organisms, and fostered

environmental and human health concerns. These problems have highlighted the need for the development of new strategies for selective mosquito larval control. Plant extracts or essential oils from plants may be alternative source of mosquito larval control agents, as they constitute a rich source of bioactive compounds that are biodegradable into nontoxic products and potentially suitable for use in control of mosquito larvae. Materials used for the experiment are lemon, ginger, garlic, pepper, neem, Tulsi, Mexican mint and lemon grass oil. The Citrus lemon is a species of small evergreen trees in the flowering plant family Rutaceae, native to Asia, primarily Northeast India, Northern Myanmar or China. It is a plant origin insecticide as an alternative to chemical insecticide, this study was undertaken to assess the larvicidal potential of citrus lemon. *Zingiber officinale* is a flowering plant whose rhizome, Ginger root or Ginger, is widely used as a spice and a folk medicine. Ginger has a greater larvicidal effect than *Allium sativum*, which is a species of bulbous flowering plant in the genus Allium. Garlic is a potent mosquito ovicide and larvicide. *Curcuma longa* is a flowering plant of the Ginger family, *Zingiberaceae*, the rhizome of which are used in cooking. Anti-parasitic effect of curcumin, which is one of the active compounds in *Curcuma longa*, has been observed to be dose dependent with greatest effects. *Piper nigrum* is a flowering vine in the family Piperaceae, cultivated for its fruit, known as a peppercorn, which is usually dried and used as a spice and seasoning. Black pepper shows potential as a larvicide for the control of certain malaria vector species. *Azadirachta indica*, is one of two species in the genus *Azadirachta*, and it is native to the Indian subcontinent and most of the countries in Africa. Neem is an effective larvicide against mosquito larvae. It is highly toxic to mosquito larvae and inhibits the development of pupae. *Coleus barbatus* is a semi-succulent perennial plant in the family *Lamiaceae* with a pungent oregano-like flavor and odor. It is an excellent option for keeping mosquitoes away. It has the greatest larvicidal effect.

Objective: To find eco-friendly larvicides to tackle the growing population of mosquitoes.

This has an added benefit of reducing the population in the environment.

REVIEW OF LITERATURE

Larvicidal activity of crude hexane, ethyl acetate, petroleum ether, acetone, and methanol extracts of the leaf of five species of cucurbitaceous plants, *Citrullus colocynthis*, *Coccinia indica*, *Cucumis sativus*, *Momordica charantia*, and *Trichosanthes anguina*, were tested against the early fourth instar larvae of *Aedes aegypti* L. and *Culex quinquefasciatus*. The larval mortality was observed after 24 h of exposure. All extracts showed moderate larvicidal effects; however, the highest larval mortality was found in petroleum ether extract of *Citrullus colocynthis*, methanol extracts of *Coccinia indica*, *Cucumis sativus*, *Momordica charantia*, and acetone extract of *Trichosanthes anguina* against the larvae of *Aedes aegypti* L (LC50 = 74.57, 309.46, 492.73, 199.14, and 554.20 ppm) and against *Culex quinquefasciatus* (LC50 = 88.24, 377.69, 623.80, 207.61, and 842.34 ppm), respectively. The petroleum ether extract of *Citrullus colocynthis* and methanol extract of *Momordica charantia* were more effective than the other extracts. This is an ideal eco-friendly approach for the control of the dengue vector, *Aedes aegypti*, and the lymphatic filariasis vector, *Culex quinquefasciatus*. (Rahuman & Venkatesan, 2008.)

The efficacy of methanol, benzene and ethyl it's solvent extract of leaf of *E.coronaria* and *C.pulcherrima* were tested against the early 3rd larvae of *Anopheles subpictus* and *Cx.tritaeniorhynchus*. the data where recorded and statistical data regarding the LC 50, LC90, Chi-square and 95% confidence limits were calculated, among three solvents tested the methanolic extract of *E. coronaria* and *C. pulcherrima* showed highest larvicidal activity against *Anopheles subpictus* and *Culex tritaeniorhynchus*. The LC 50 values were 86.47 (159.59) and 113.26 (207.73) ppm for *Anopheles subpictus* and 131.53 (245.37) and 165.28 (299.45) ppm for *Culex tritaeniorhynchus* respectively. No mortality was observed in control. The chi-square values were significant at $p < 0.05$ level. (Govindarajan et al., 2012)

Mosquito species of *Anopheles*, *Aedes* and *Culex* are major vectors for malaria, dengue, zika, chikungunya, filariasis and Japanese encephalitis diseases. *Bacillus thuringiensis subsp. israelensis* (Bti) is spore-forming bacterium having worldwide distribution produces proteins which are toxic to these mosquito larvae. The local isolate, Bti ISPC-12 showed high toxicity to mosquito larvae of *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*. The genes encoding insecticidal toxin proteins have been expressed in the E. coli expression system for molecular and structural studies. Sustained-release formulation using spore-crystal powder of Bti

ISPC-12 has been developed. The spore crystal powder (active ingredient) and formulation has been found to be safe to mammals. Field studies carried out in Anushaktinagar, Mumbai and RRCAT, Indore townships demonstrated the efficacy against mosquito larvae. Thus Bti ISPC-12 based formulation is a potent biopesticide for the management of mosquito population. (Kinkar & Makde, 2021)

Plant-based repellents have been used for generations in traditional practice as a personal protection measure against host-seeking mosquitoes. Knowledge on traditional repellent plants obtained through ethnobotanical studies is a valuable resource for the development of new natural products. Recently, commercial repellent products containing plant-based ingredients have gained increasing popularity among consumers, as these are commonly perceived as safe in comparison to long-established synthetic repellents although this is sometimes a misconception. To date insufficient studies have followed standard WHO Pesticide Evaluation Scheme guidelines for repellent testing. There is a need for further standardized studies in order to better evaluate repellent compounds and develop new products that offer high repellency as well as good consumer safety. This paper presents a summary of recent information on testing, efficacy and safety of plant-based repellents as well as promising new developments in the field. (Maia & Moore, 2011)

An insecticide containing Azadirachtina tree (*Azadirachta indica*) extract, was tested against *Culex pipiens* mosquito larvae and pupae in the east of the Republic of Algeria under laboratory conditions. First, after treatment of larval stage, LC50 and LC90 values for Azadirachtin were 0.35 and 1.28 mg/L in direct effect and 0.3-0.99 mg/l in indirect effect, respectively. Second, after treatment of the pupal stage, the LC50 and LC90 in direct effect were measured as 0.42 -1.24mg/l and in indirect effect was 0.39mg/l-1.14mg/l respectively. Mosquito adult fecundity were markedly decreased and sterility was increased by the Azadirachtin after treatment of the fourth instar and pupal stage. The treatment also prolonged the duration of the larval stage. The results show that the Azadirachtin is promising as a larvicidal agent against *Culex pipiens*, naturally occurring biopesticide could be an alternative for chemical pesticides. (Alouani, Rehimy & Soltani, 2009)

Mosquitoes transmit serious human diseases, causing millions of deaths every year and the development of resistance to chemical insecticides resulting in rebounding vectorial capacity. Plants may be alternative sources of mosquito control agents. This study assessed the role of

larvicidal activities of hexane, chloroform, ethyl acetate, acetone, and methanol dried leaf and bark extracts of *Annona squamosa* L., *Chrysanthemum indicum* L., and *Tridax procumbens* L. against the fourth instar larvae of malaria vector, *Anopheles subpictus* Grassi and Japanese encephalitis vector, *Culex tritaeniorhynchus* Giles. Larvicidal activities of three medicinal plant extracts were studied in the range of 4.69 to 1000 mg/l in the laboratory bioassays against early 4th instar larvae of *Anopheles subpictus* and *Culex tritaeniorhynchus*. The mortality data were subjected to probit analysis to determine the lethal concentrations (LC50 and LC90) to kill 50 and 90 percent of the treated larvae of the respective species. All plant extracts showed moderate effects after 24 h of exposure; however, the highest toxic effect of bark methanol extract of *Annona squamosa*, leaf ethyl acetate extract of *Chrysanthemum indicum* and leaf acetone extract of *Tridax procumbens* against the larvae of *Anopheles subpictus* (LC 50 = 93.80, 39.98 and 51.57 mg/l) and bark methanol extract of *squamosa*, leaf methanol extract of *Chrysanthemum indicum* and leaf ethyl acetate extract of *Tridax procumbens* against the larvae of *Culex tritaeniorhynchus* (LC50 = 104.94, 42.29 and 69.16 mg/l) respectively. This data suggests that the bark ethyl acetate and methanol extract of *Annona squamosa*, leaf ethyl acetate and methanol extract of *Chrysanthemum indicum*, acetone and ethyl acetate extract of *Tridax procumbens* have the potential to be used as an ecofriendly approach for the control of the *Anopheles subpictus*, and *Culex tritaeniorhynchus*. (Kamaraj et al.,2011)

Mosquitoes act as a vector for most of the life-threatening diseases like malaria, yellow fever, dengue fever, chikungunya fever, filariasis, encephalitis, West Nile Virus infection, etc. Under the Integrated Mosquito Management (IMM), emphasis was given on the application of alternative strategies in mosquito control. The continuous application of synthetic insecticides causes development of resistance in vector species, biological magnification of toxic substances through the food chain and adverse effects on environmental quality and non-target organisms including human health. Application of active toxic agents from plant extracts as an alternative mosquito control strategy was available from ancient times. These are non-toxic, easily available at affordable prices, biodegradable and show broad spectrum target-specific activities against different species of vector mosquitoes. In this study, the current state of knowledge on phytochemical sources and mosquitocidal activity, their mechanism of action on target population, variation of their larvicidal activity according to mosquito species, instar specificity, polarity of solvents used during extraction, nature of active ingredient and promising advances made in

biological control of mosquitoes by plant derived secondary metabolites have been reviewed. (Gosh, Chowdhury & Chandra, 2012).

In order to develop an environment-friendly botanical mosquito larvicide alternative to the chemical larvicides, extracts were made from the leaves of *Hyptis suaveolens*, *Lantana camara*, *Nerium oleander*, and *Tecoma stans* with three organic solvents such as methanol (ME), chloroform (CH), and petroleum ether (PE) using a Soxhlet extractor. The plant extracts were screened for larvicidal activity individually and in combination against the larvae of *Aedes aegypti* and *Culex quinquefasciatus* as per WHO protocol. Among the extracts, the maximum larvicidal activity was shown by the PE extract of *L. camara* (LC50 10.63 mg/L) followed by the PE extract of *Tecoma stans* (LC50 19.26 mg/L), ME extract of *Nerium oleander* (LC50 35.82 mg/L), and PE extract of *Hyptis suaveolens* (LC50 38.39 mg/L) against *Culex quinquefasciatus*. In the case of *Aedes aegypti*, the PE extract of *Tecoma stans* showed maximum activity with LC50 value of 55.41 mg/L followed by *Hyptis suaveolens* (LC50 64.49 mg/L), PE extract of *Lantana camara* (LC50 74.93 mg/L), and ME extract of *Nerium oleander* (LC50 84.09). A blend of these four extracts resulted in a combination with corresponding LC50 values of 4.32 and 7.19 mg/L against *Culex quinquefasciatus* and *Aedes aegypti*. The predator safety factors were 12.55 and 20.88 for *Gambusia affinis* with respect to *Aedes* and *Culex* larvae for the extract combination. Chemical constituents in extracts were also identified by FT-IR and GC-MS data. The present investigations suggest the possible use of this blend of botanical extracts as an ideal ecofriendly, larvicide against *Aedes aegypti* and *Culex quinquefasciatus* larvae (Hari & Mathew, 2018).

They examined the chemical composition of garlic and asafoetida essential oils and their individual and combined toxicity against larvae of *Culex pipiens Linnaeus* and *Culex restuans Theobald* (Diptera: *Culicidae*). The effect of the two essential oils on egg hatch was also examined. Ten and 12 compounds, respectively, were identified in garlic and asafoetida essential oils. Allyl disulfide (49.13%) and diallyl trisulfide (31.08%) were the most abundant compounds in garlic essential oil accounting for 80.2% of the total oil. In contrast, (E)-sec-butyl propenyl disulfide (30.03%), (Z)-sec-butyl propenyl disulfide (24.32%), and disulfide, methyl 1-(methylthio) propyl (21.87%) were the most abundant compounds in asafoetida essential oil. Allyl disulfide accounted for 7.38% of the total oil in asafoetida essential oil and was one of only three compounds found in both oils. For both mosquito species, garlic essential oil was more toxic than asafoetida essential oil with *Cx. restuans* (LC50: garlic = 2.7 ppm; asafoetida = 10.1 ppm) being more sensitive than

Culex pipiens (LC50: garlic = 7.5 ppm; asafoetida = 13.5 ppm). When combined, the two essential oils had antagonistic effects. The majority of *Culex* egg rafts exposed to garlic (73.1%) or asafoetida (55.8%) essential oils failed to hatch and larvae of the few that did hatch mostly died as first instars. Allyl disulfide exhibited strong ovicidal and larvicidal activity suggesting its important contribution to the overall toxicity of the two essential oils. Thus, garlic and asafoetida essential oils are potent mosquito ovicides and larvicides but if used jointly, they could undermine vector control programs. (Muturi, Ramirez & Rooney, 2018)

This study was conducted to determine the larvicidal effect of aqueous extract of garlic against the 4th instars of culex and anopheles mosquito larvae. Anopheles and culex mosquito larvae were obtained using a deeper from stagnant water in the fadama at kofar Kade along illela road, Sokoto and taken to the Physiology laboratory of biological sciences, Usmanu Danfodiyo University Sokoto for further analysis. Fresh samples of garlic were obtained from central market, Sokoto state and were taken to the physiology laboratory for processing. The concentration of the extract used was 0.5mg/ml, 2.0mg/ml and 3.0mg/ml were obtained by weighing 0.5mg, 2.0mg, and 3.0mg in 10ml of water. Mortality of *Culex* and *Anopheles* depends on the garlic extract and increase with time of exposure and concentration of the extract. 3.0mg/ml recorded the highest mortality rate of 3 hours of exposure for both *Culex* and *Anopheles* at a mean of 10.00 each while 0.5mg/ml recorded minimum mortality after 1 hour of exposure for both anopheles and culex with mean of 2.33 and 3.67 respectively. The study demonstrated the potency of garlic (*Allium sativum*) in managing the larvae and thus contributes as an affordable way to control *Anopheles* and *Culex* larvae of mosquito. (Kasim, Ann & Yahaya,2019)

METHODOLOGY

While mosquitoes spend most of their life in the air, newly born mosquitoes spend their time under water. They are known as mosquito larvae. Mosquito larvae are just one stage of a mosquito's life. In order to rear mosquito larvae, firstly a location was chosen and filled a bucket with water and placed it in open spaces. Mosquito larvae were also collected from wells and reared in plastic and enamel trays in tap water.

Plant materials such as *Curcuma longa*, *Citrus lemon*, *Coleus barbatus*, *Azadirachta indica*, *Piper nigrum*, *Zingiber officinale* and *Allium sativum* were collected from in and around Kochi.

Plant materials

Fresh leaves of *Azadirachta indica* (Neem), *Coleus barbatus* (Mexican mint), fruits of *Citrus limon* (Lemon), roots of *Zingiber officinale* (Ginger), *Curcuma longa* (Turmeric), bulbs of *Allium sativum* (Garlic), seeds of *Piper nigrum* (Pepper) were collected from different areas of Ernakulam. A larvicide is a type of insecticide used to control mosquitoes indoors and outdoors around your home. They work by killing mosquito larvae before they can grow into adults. Some formulations are activated when ingested by the mosquitoes, and some formulations work when they come into contact with the larvae.

Turmeric

Turmeric is a flowering plant, *Curcuma longa*, of the ginger family, *Zingiberaceae*, the rhizomes of which are used in cooking. Insect control properties of turmeric pertains to the powder, the plant extracts, the essential oil, and certain bioactive constituents of the plant. Its products have been found active as insect repellents and insecticidal agents. The presence of curcumin in turmeric is what makes it beneficial in so many ways. It helps in soothing the itching. The strong aroma of turmeric also acts as a mosquito repellent.

Pepper Black

Pepper is a flowering vine in the family *Piperaceae*, cultivated for its fruit, known as a peppercorn, which is usually dried and used as a spice and seasoning. The fruit is a drupe which is about 5 mm in diameter, dark red, and contains a stone which encloses a single pepper seed.

Garlic

Garlic is a species of bulbous flowering plant in the genus *Allium*. Its close relatives include the onion, shallot, leek, chive, Welsh onion and Chinese onion. Garlic makes a powerful natural insect repellent. Garlic can be used to repel a variety of crawling and flying insects, including mosquitoes,” according to Patrick Parker, SavATree Plant Health Care Program Director. One treatment with garlic is effective for 2 weeks and can repel insects for up to one month. The sulfurs contained within the garlic extract have been shown to be effective against a wide range of insects, including mosquitoes, and the lingering odor can deter mosquitoes from the area for weeks. It is thought that garlic may be an alternative mosquito repellent for humans as well.

Ginger

Ginger is a flowering plant whose rhizome, ginger root or ginger, is widely used as a spice and a folk medicine. It is a herbaceous perennial which grows annual pseudostems about one meter tall bearing narrow leaf blades. Ginger extracts have proven to be beneficial in offering medicinal, insecticidal and antioxidant values to humans, animals and plants. Research shows that ginger (*Zingiber officinale*, USDA zones 9-12) — especially its extracted oils — can repel certain mosquito species.

Neem

Azadirachta indica, commonly known as neem or Indian lilac, is a tree in the mahogany family *Meliaceae*. It is native to the Indian subcontinent. Neem leaf and its constituents have been demonstrated to exhibit immunomodulatory, anti-inflammatory, antihyperglycemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anticarcinogenic properties. Neem is of great importance and has promising larvicidal activity against important vectors of malaria, filaria, dengue, dengue haemorrhagic fever, yellow fever and chikungunya. The larvae of a number of mosquito species (including *Aedes* and *Anopheles*) are sensitive to neem.

They stop feeding and die within 24 hours after treatment. If neem derivatives are used alone, relatively high concentrations are required to obtain high mortality.

Mexican mint

Coleus amboinicus, synonym *Plectranthus amboinicus*, is a semi-succulent perennial plant in the family Lamiaceae with a pungent oregano-like flavor and odor. The pungent nature of mint deters bugs from making your home their home. Pests like ants, mosquitos, and mice will avoid mint plants whenever possible, and it can also help with other menaces like roaches, spiders, and flies.

Lemon

The lemon is a species of small evergreen trees in the flowering plant family *Rutaceae*, native to Asia, primarily Northeast India, Northern Myanmar or China. Citrus plants, as well as their crushed leaves and extracts made from them, naturally repel mosquitoes. Oranges, lemons, lavender, basil and catnip naturally produce oils that repel mosquitoes and are generally pleasant to the nose – unless you're of the feline persuasion.

Preparation of plant extracts

The leaves (100 g) of each plant (Neem, Mexican mint) and fruits (100g) of Lemon, Garlic, Ginger, Turmeric, Pepper were made into a paste mechanically using a commercial electrical stainless steel blender by mixing it with 100ml water each. From the paste formed, the liquid extract of each material was extracted using a strainer. For each plant extract, at first, 100 grams of the plant material was mixed with 100 ml of water and blended to make a paste. The solution thus formed was used as the stock solution (100g/100ml). Stock solution is the solution that is of the highest concentration. All the other solutions to perform the serial dilution was prepared from the stock solution.



Fig 1: extract of lemon



Fig 2: extract of garlic



Fig 3: extract of ginger

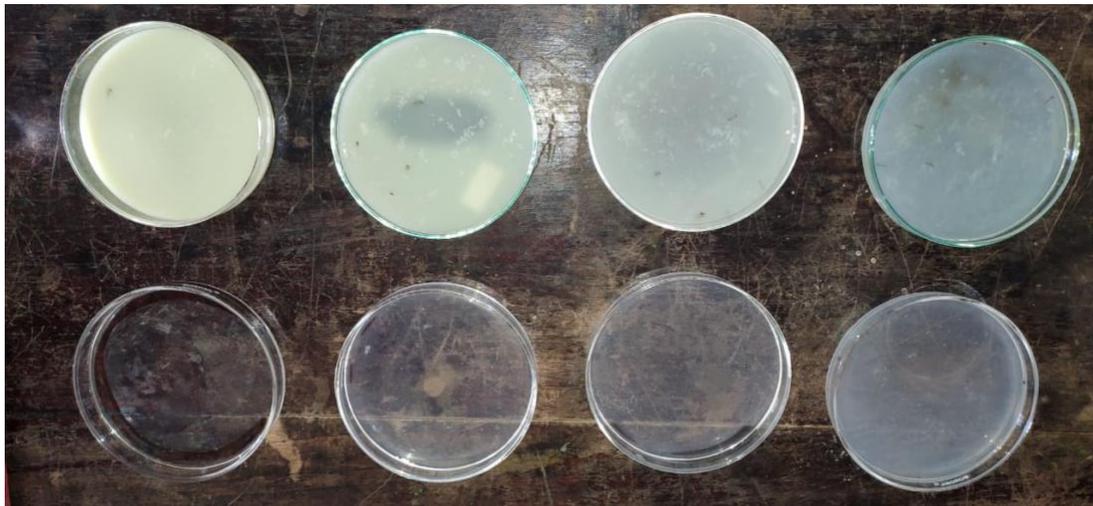


Fig 4: introduction of larvae in different concentration gradients of lemon extract



Fig 5: from top to bottom ginger, neem and pepper respectively

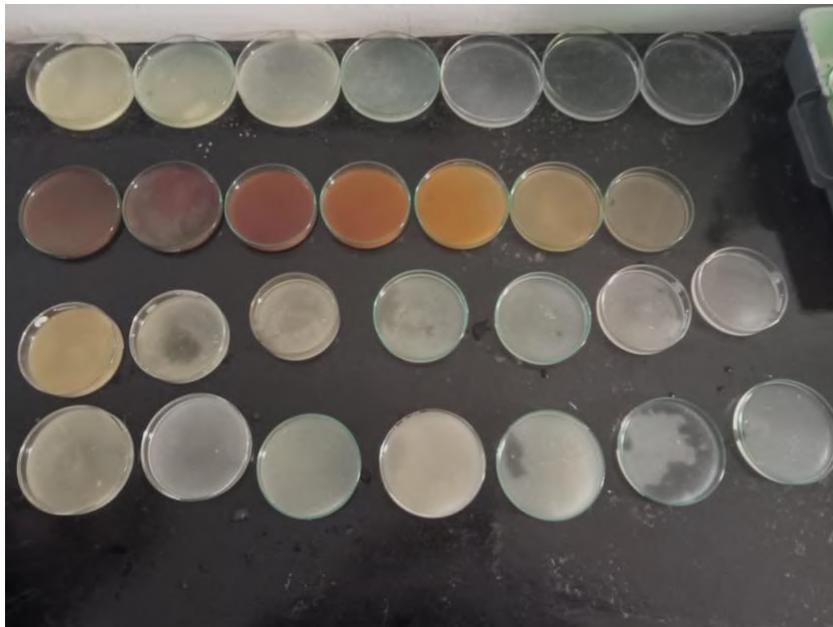


Fig: From top to bottom, garlic, turmeric, lemon and Mexican mint respectively

Larvicidal bioassay

A minimum of 5 larvae/ concentration were used for all the experiments. These experiments were repeated 3 times with 7 plant extracts in total. The percentage of mortality was calculated by using the formula:

Percentage of mortality = (No. of dead larvae/ No. of larvae introduced) x100

OBSERVATIONS AND RESULTS

Results are given in the table 1 & 2

Table 1: Percentage of Mortality of mosquito larvae after 17 hours of exposure

SL NO:	PLANT EXTRACTS	1gm/1ml percentage of mortality (%)	0.5gm/ml percentage of mortality (%)	0.25gm/ml percentage of mortality (%)	0.12gm/ml percentage of mortality (%)	0.06gm/ml percentage of mortality (%)	0.03gm/ml percentage of mortality (%)	0.01gm/ml percentage of mortality (%)
1	<i>Curcuma longa</i>	100	100	100	100	100	100	60
2	<i>Citrus lemon</i>	100	100	100	100	0	0	0
3	<i>Coleus barbatus</i>	100	100	100	100	0	0	0
4	<i>Azadirachta indica</i>	100	100	100	100	100	100	100
5	<i>Piper nigrum</i>	100	100	100	100	100	100	100
6	<i>Zingiber officinale</i>	100	100	100	100	100	80	60
7	<i>Allium sativum</i>	100	100	100	100	80	60	20

DISCUSSION

Mosquitoes are the major vector for many diseases. Mosquito vectored diseases include protozoan diseases, i.e., malaria, filarial diseases such as dog heartworm, and viruses such as dengue, encephalitis and yellow fever. In India, malaria caused due to the transmission of plasmodium by the female *Anopheles* mosquito is one of the most important causes of direct or indirect infant, child and adult mortality with approximately two to three million new cases arising every year. *Anopheles culicifacies* is the main vector of malaria, and India contributes 77 per cent of the total malaria in Southeast Asia. *Culex* is huge genus of mosquitoes that is a principle vector for transmission of diseases such as Japanese encephalitis, Lymphatic filariasis, West Nile fever etc. *Culex tritaeniorhynchus* and *Culex vishnui* are primary vectors of Japanese encephalitis (JE) virus, with a distribution throughout Southeast Asia and South. Seasonal outbreaks of acute encephalitis syndrome (AES) among children have been reported in the India causing high morbidity and mortality.

Though larvicides play a vital role in controlling mosquitoes in their breeding sites, these also show a negative impact in areas of beneficial and non-target organisms. In view of an increasing interest in developing plant origin insecticides as an alternative to chemical insecticide, this study was undertaken to assess the larvicidal potential of the extracts from the locally available plants against medically important species of malaria vector, *Anopheles* and Japanese encephalitis vector, *Culex*.

Our data suggest that the Pepper and Neem have the potential to be used as an eco-friendly approach for the control of the mosquito larvae.

The activity of plant extracts is often attributed to the complex mixture of active compounds. Seven different extracts were tested against mosquito larvae and 100 percent larval mortality was observed in extract of *Piper nigrum* and *Azadirachta indica* in all concentrations.

All plant extracts showed moderate toxic effect on *the larvae* after 24 h of exposure; however, the highest mortality was found in extract of *Piper nigrum* and *Azadirachta indica*.

CONCLUSION

Based on current study, it was concluded that the use of plant extracts is an alternative to synthetic insecticide as they are cheap, easily available and relatively safe to the natural enemies and other non-target species. Therefore, it is recommended to use different plant extracts for the control of mosquito larvae in open spaces.

From the table 1 & 2, it can be observed that extracts of seven plants had a significant mortality on the mosquito larvae. In all of the plant extracts, the highest effect occurred at a concentration of 1gm/ml while the smallest effect was at 0.15625gm/ml. The increased concentration led to increased larval mortality. The extract of Pepper and Neem showed the highest effect on the larvae. The smallest effect was shown by the extracts of Lemon and Mexican mint.

Repellent activity of Neem extract against mosquito larvae was different for different concentrations and its efficacy was more at the higher concentrations; and highest repellent activity was seen in undiluted extract and it gradually decreased with decrease in concentration (diluted extracts). Same results were observed for Pepper extracts and highest repellent activity was seen in undiluted extract.

Comparison between results of the seven plant species shows that in the same concentrations, repellent activity of Neem and Pepper was higher in all concentrations and this was common for all of the treatments. In the whole of concentrations, Neem and Pepper were more potent than other extracts.

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DNA BARCODING IN INDIAN MARINE FISHES

- *Pomadasys maculatus* and *Carangoides armatus*



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Affiliated to Mahatma Gandhi University, Kottayam in

Partial fulfilment of requirement for the

Degree of Bachelor of Science in Zoology

2021-2022

CERTIFICATE

This is to certify that the project report entitled “**DNA BARCODING IN INDIAN MARINE FISHES- *Pomadasys maculatus* and *Carangoides armatus***” submitted by **Ms. Namitha Vinod V**, Reg. No. **AB19ZO0014** in partial fulfilment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under my guidance and supervision and to my best knowledge, this is her original effort.

Dr. Meera Jan Abraham

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St. Teresa’s College (Autonomous)

Ernakulam

Ernakulam

EXAMINERS

1)

2)

DECLARATION

I, hereby declare that this project work entitled “**DNA BARCODING IN INDIAN MARINE FISHES- *Pomadasys maculatus* and *Carangoides armatus***” is submitted to St. Teresa’s College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science degree in Zoology. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report are entirely my own.

NAME: NAMITHA VINOD V

SIGNATURE

REGISTRATION NUMBER: AB19ZOO014

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NAMITHA VINOD V

DNA BARCODING IN INDIAN MARINE FISHES

- *Pomadasys maculatus* and *Carangoides armatus*

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ABSTRACT

The present study reflects DNA barcoding in two Indian Marine fishes. DNA barcoding involves sequencing a fragment of the mitochondrial cytochrome oxidase I (COI) gene, known as DNA barcodes, from taxonomically unknown specimens. The following study has been undertaken to research and analyse the barcode of Indian Marine fishes and their DNA sequences. DNA barcoding is a method of species identification using a short section of DNA from a specific gene. The present study focuses on barcoding the DNA of 2 genera of fishes collected from Beypore Fishing harbour, Kozhikode. The study was completed in 4 phases. The first two phases were collection and preservation of specimens for later analysis. Third phase consisted of DNA isolation from the collected samples and amplification of the sequence. Since DNA extraction is a sensitive process, buffers are added to stabilise the pH over cell lysis and isolation. The final phase consisted of PCR and barcoding the isolated DNA samples. From the 21 samples collected, fishes belonging to 8 genera were identified which include Pomadasys, Carangoides, Decapterus, Cynoglossus, Nemipterus, Selar, Mene, Leiognathus and Pericanthus out of which 2 genera were specifically analysed namely *Pomadasys maculatus* and *Carangoides armatus*.

INTRODUCTION

Identification of fishes contributes to the classification of each fish and the crucial function they play in the biological world, as well as the discovery of important food and medicinal ingredients from them. Fishes are the primary food source of humans, hence research on them has a significant impact on human life. Various approaches can be used to identify an unknown specimen. Identifying it with the use of morphology was a typical method employed previously, but morphology only provides external structural features, fishes have undergone multiple ontogenetic development, convergent and divergent evolution, identifying them only based on their morphological characteristics will be very challenging. As a result, morphological features alone are not sufficient for unknown specimens; in these cases, DNA barcoding is used.

DNA barcoding, or sequence-based specimen identification, was developed by Paul Hebert in 2003 to identify a broad range of taxa by sequencing a standardised short DNA fragment, the "DNA barcode". DNA Barcoding is a technique for identifying different species. It was first used in fishes by Ward in 2005. It functions by examining a specific DNA region and this specific area is known as the DNA barcode. For animal identification, the most broadly used barcode marker is mitochondrial Cytochrome C Oxidase subunit I (COI), which is highly conserved across species employing oxidative phosphorylation for metabolism. After that, the sequence of this DNA barcode is compared to a reference library that contains information on many different species and their barcodes. DNA Barcoding consists of the following steps: DNA Isolation, Amplifying the isolated DNA using Polymerase Chain Reaction, Gel Electrophoresis and Sequence Analysis. DNA isolation is a key step because, without high quality DNA, the PCR amplification will not be optimal. The following statements are some of the benefits of DNA barcoding over previous classification systems: DNA barcoding help in accurately distinguishing some species that are similar in morphology, cryptic species are indistinguishable biological groups that are incapable of interbreeding and cannot be distinguished using traditional methods of classification because traditional methods classify cryptic species as a single group, despite the fact that these species show genetic variation. (Lakra et al., 2011)

They can be easily distinguished through DNA Barcoding, Barcoding methods can often give information without causing harm to the animal studied. In cases of morphological ambiguity, such as with larval stages, DNA barcoding technology can help identify species. The applications of DNA barcoding includes ecological monitoring, early detection, control and removal of non-indigenous species, fisheries management, food safety and protection of endangered species. The results generated by DNA barcoding are limited or biased to the frequency of occurrence, and quantifying fish abundance from molecular data is another key problem for this approach.

There are several different types of DNA extraction methods. A few of them include Phenol-Chloroform Isoamyl Alcohol, Proteinase K, CTAB Method, Spin Column-based Methods and Magnetic Bead-based Technique. However the method implied on each specimen depends on sample type and purity and the yield of DNA that is to be obtained. DNA extraction is completed in 4 steps: lysis, separation, precipitation and purification. There are 2 main types of DNA sequencing methods. The classical method is known as Chain Termination method or Sanger Sequencing Method. Other modern methods that can process a large number of DNA molecules swiftly are collectively called High-Throughput sequencing (HTS) and Next-Generation sequencing methods (NGS). In the conducted study, DNA isolation was done using the DNeasy Blood & Tissue Kit and respective buffers namely Proteinase K, Buffer ATL, Ethanol, Buffer AL, Buffer AW1, Buffer AW2, Buffer AE and Buffer AP. Polymerase Chain Reaction (PCR) is a powerful method for amplifying particular segments of DNA, distinct from cloning and propagation within the host cell. Out of the various PCR techniques, amplification of the isolated sequence was done using normal Polymerase Chain Reaction. DNA sequencing was done to the amplified sequence using Sanger sequencing method. It is done to determine the nucleic acid sequence or order of nucleotides in DNA. Lastly, a phylogenetic tree was drawn with the help of the software MEGA 11 to identify the species of the specimen.

AIM AND OBJECTIVE

AIM

To identify two types of fishes using DNA barcoding.

OBJECTIVE

- To barcode the various types of Indian Marine Fishes.
- To provide the statistics, characteristics and presence of types of fishes in certain areas.
- To compare and classify the varieties of fishes in marine habitat.

REVIEW OF LITERATURE

DNA Barcoding is a molecular diagnostic method used for identification of species by using a standardised DNA sequence or genetic region which acts as the 'barcode'. 'DNA barcoding' is a new identification tool proposed by Hebert et al. (2003), and is a valuable addition to the taxonomic tool box. They advocated the use of short DNA sequences from the specified region of the genome termed as DNA barcode for biological identification. It implies sequencing of a standard DNA locus as a tool for identifying species. An ideal DNA barcode should be easily retrievable with a single primer pair, be amenable to bidirectional sequencing and effectively provide high discrimination among species.

According to Savoleinen et al., (2005), the scientific benefits of DNA barcoding include: (i) enabling species identification, including any life stage or fragment, (ii) facilitating species discoveries based on cluster analyses of gene sequences, (iii) promoting development of handheld DNA sequencing technology that can be applied in the field for biodiversity inventories and (iv) providing insight into the diversity of life.

Based on the works of Jeremy C. Andersen et al., (2019), the collection of DNA barcode sequences from unidentified specimens provides useful genomic data and at the same time DNA barcoding techniques are being used with increasing frequency to guide management decisions, particularly for the identification of alien invasive species (Dejean et al., 2012).

The present study utilises the standard Cytochrome C Oxidase subunit I (COI) which is found in most eukaryotes and highly conserved and so can be copied from unknown organisms. They also have less intraspecific (within species) variation than interspecific (between species) variation, known as the "Barcoding Gap". When fully developed, a COI identification system will provide a reliable, cost-effective and accessible solution to the current problem of species identification. Its assembly will also generate important new insights into the diversification of life and the rules of molecular evolution (Hebert et al., 2003). The mitochondrial genome of animals is a better target for analysis than the nuclear genome because of its lack of introns, its limited exposure to recombination and its haploid mode of inheritance (Saccone et al.,

1999). Robust primers also enable the routine recovery of specific segments of the mitochondrial genome (Folmer et al., 1994).

The major goal of DNA-barcoding efforts is to aid identification of specimens by matching sequences to a sequence library. The revolution introduced by DNA barcoding resides in the molecularisation, computerisation and standardisation of taxonomic approach. The identification and then the interpretation of molecular entities is the main goal of DNA barcoding that could be reached only by users with a sound theoretical background on what is identifiable by this technique (Casiraghi et al., 2010). Many authors have proposed DNA barcoding as an integrated approach with classical taxonomy for species identification and authentication. Modifications in extraction methods, primer sequences, use of an engineered polymerase and even the combining of barcodes from multiple loci has been used successfully to clear any issues related to DNA Barcoding in vertebrates.

In order to test the utility of DNA Barcoding in forensic vertebrate species identification, COI sequences from previously identified samples from humans and a variety of domestic and wild specimens of Brazilian mammals, birds, fishes were compared against the Barcode of Life Database (BOLD). BOLD provided a correct species-level identification for 12 out of the 20 queried sequences (60%) and presented the correct species as the best matched one for 17 out of 18 samples morphologically identified to this level (94%). (Carvalho, 2014)

Barcoding can be used as an alternative to traditional sampling methods in fish research. Barcoding procedures can often give information without causing harm to the animal being investigated. Hebert et al., (2003) proposed using DNA barcoding to help fish identification, which prompted the formation of Fish Barcode of Life (FISH-BOL), which aims to barcode all taxonomically documented fish species (Ward et al., 2009). The FISH-BOL project began in 2005, and roughly 8,000 of the 31,000 fish species recognised have been barcoded for the COI gene. According to the initial report, around 98 percent and 93 percent of marine and freshwater species may be distinguished using barcodes, respectively.

According to the work of Zhou et al., (2009), it is clear that the limited access to taxonomic expertise is an issue for large-scale biodiversity surveys. Their study shows that a

comprehensive DNA barcode library built on expertly identified specimens enables fast and accurate species identification. There will be easier ways of analysing bulk environmental samples which will become more widespread and less expensive over time, facilitating ecological and monitoring applications of the barcode library. Continued interaction with the taxonomic community during barcode-based biodiversity and monitoring studies, involving submitting specimens with novel sequences for determination or revision, will ensure the growth and maintenance of a high-quality database.

Pomadasys maculatus, also known as the saddle grunt or blotched javelin, is a species of marine ray-finned fish belonging to the family Haemulidae. It was discovered by Bloch in 1793. It is a harmless species native to the Indo-West Pacific region- throughout the Indian Ocean and the western Pacific, north to China, south to Australia. They are found in coastal waters over sand near reefs and feed on crustaceans and fishes. They are Oviparous and show distinct pairing during breeding. They have 12 dorsal spines and 13-14 dorsal soft rays, 3 anal spines and 7 anal soft rays. They are small-sized fish of moderately deep body. The chin with 2 pairs or small pores. Preferred temperature: 23.4 - 28.6, mean 27.4°C (Froese, R. & Pauly, D., 2022).

Carangoides armatus, also known as the longfin trevally, longfin kingfish, longfin cavalla or armed trevally, is a species of inshore marine fish in the family, Carangidae. It was discovered by Rüppell in 1830. Adults are found in coastal waters near corals and rocks, also in shallow lagoons. Solitary species on inshore reefs. Small groups frequently patrol the perimeter of reefs. Juveniles may enter estuaries. They have 9 dorsal spines and 19-22 dorsal soft rays, 3 anal spines and 16-18 anal soft rays (Froese, R. & Pauly, D., 2022).

METHODOLOGY

SPECIMEN COLLECTION

The fishes were collected from Beypore Fishing Harbour, Kozhikode. A total of 21 fish types were selected and labelled for analysis by DNA barcoding. The specimens were stored in the Museum at Kerala University of Fisheries and Ocean Studies, Panangad, Kerala.

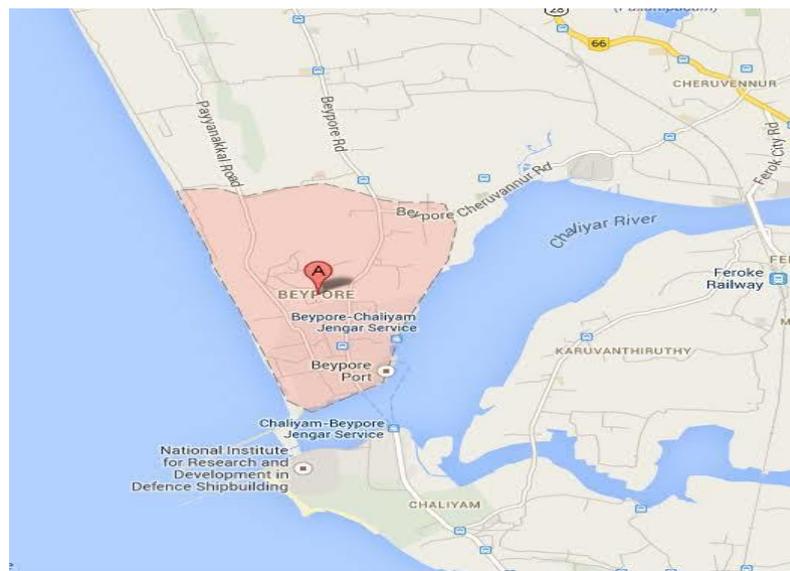


Fig. 1: Map showing Beypore Fishing Harbour, Kozhikode



Fig. 2: Fish samples collection

PROCEDURE

PREPARATION OF SAMPLE

The selected mature fish specimens were measured and mounted on a hard surface to observe the characteristic features. Muscle tissue samples were dissected using sterilised tools, preserved in 10% Formalin, properly labelled and stored in the refrigerator.

The protocol for DNA extraction, PCR amplification of CO1 gene, product purification and sequencing follow the study of Lakra et al. (2011).



Fig. 3: Preparation of fish samples

DNA ISOLATION

The preserved tissue samples were taken out of the vial and a portion (<25mg) of the flesh was transferred to a centrifuge tube and labelled. 200 μ L of Buffer ATL (Lysis Buffer) was added to the centrifuge tube followed by 20 μ L of Proteinase K. The mixture was vortexed to homogenise the contents and incubated in a thermomixer at 56°C for 2 hours until the whole tissue was completely digested. 200 μ L of Buffer AL (Lysis Buffer) was added to the centrifuge tube and incubated for another 10 minutes at 56°C. After removing the centrifuge tubes from the thermomixer, 200 μ L of 95% chilled Ethanol was added to it and then incubated at room

temperature for 5 minutes. The contents of the centrifuge tube were then transferred into labelled spin columns taken in 2ml collection tubes. The tubes were placed in a balanced configuration and centrifuged for 1 minute at 8000 rpm.

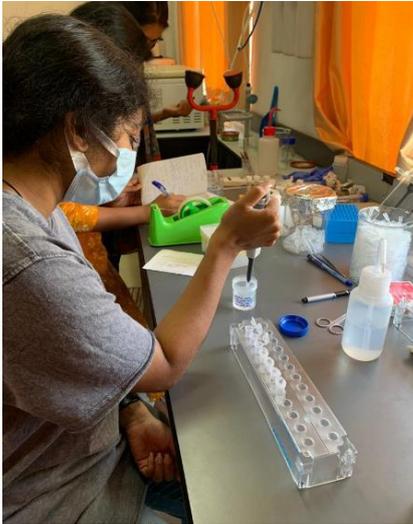


Fig. 4: Preparation of specimens for centrifugation

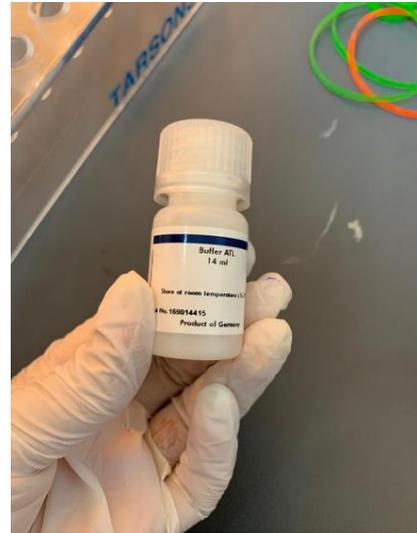


Fig. 5: ATL Buffer



Fig. 6: Thermomixer



Fig. 7: Centrifugation

The collection tubes were then replaced with new 2ml tubes. 500 μ L of Buffer AW1 (Wash Buffer) was added to the centrifuge tube and centrifuged for 3 minutes at 14000 rpm. The process was repeated with Buffer AW2 (Wash Buffer). The collection tubes were replaced with labelled centrifuge microtubes. 50 μ L of Buffer AE (Elution Buffer) was added to the centrifuge tube (incubated at room temperature for 1 minute) and then centrifuged for 1 minute at 8000 rpm. Another 50 μ L of Buffer AE was added to the centrifuge tube and incubated at room temperature for 1 minute and centrifuged at 1 minute at 8000 rpm. The

Buffer AE elutes the DNA from the spin column membrane into the centrifuge tube. The eluted DNA in the labelled centrifuge microtube was stored at -4°C.

PCR

Species-specific variations or polymorphisms in the DNA sequence that are spread randomly over the entire genome and result in characteristic DNA fingerprints have been exploited through use of polymerase chain reaction (PCR) and its variants. (Priyanka Mishra et al., 2015). The procedures are followed using INVITROGEN Genomic DNA Mini Kit.

24 µL Master Mix (312.5 µL Emerald Amp GT PCR, 31.25 µL Forward F1 Primer, 31.25 µL Reverse R1 Primer and 225 µL dH₂O) was added to new individual vials which were properly labelled. The primer pair LCO1490(59-GGTCACAAATCATAAAGATATTGG-39) and HCO2198(59-TAAACTTCAGGGTGACCAAAAATCA-39) was subsequently used to amplify a 658 bp fragment of the COI gene. The samples were taken out of storage and added to the Master Mix vials.

The next step in the process involved 35 cycles of PCR (involving Denaturation, Annealing and Extension followed by Final Extension) maintained at 4°C. The vortexed vials were kept in the PCR Machine until a temperature of 105°C was attained after which the process started and took around two and a half hours to complete.

- DENATURATION
Done at 95°C for 5 minutes

- ANNEALING
Done at 58°C (but may vary depending on Primers used)

- EXTENSION
Done at 72°C



Fig. 8: Genomic DNA Mini Kit

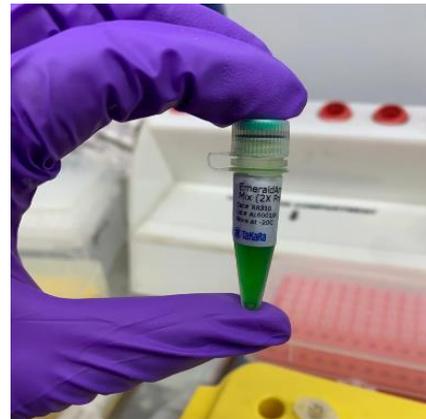


Fig. 9: Master Mix



Fig. 10: PCR Machine

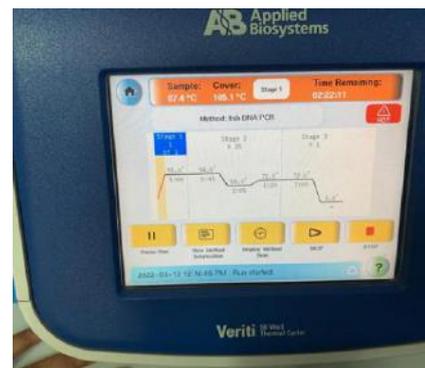


Fig. 11: Progress of PCR

PREPARATION OF GEL

0.4g Agarose Special Powder was added to 100mL Borosil and mixed well. This was transferred to a gel tray with wells/pits.

GEL ELECTROPHORESIS

Anode and Cathode electrodes were placed in a container and TE Buffer was added until the electrodes were completely immersed. The amplified sample mixtures were poured into the wells/pits. The Electrophoresis Machine was set to 90-91 Volts and run for about 20 minutes. The gel is then transferred onto a Bio Rad Imager for analysis of results of DNA imaging on the computer. The isolated DNA samples were given for sequencing.

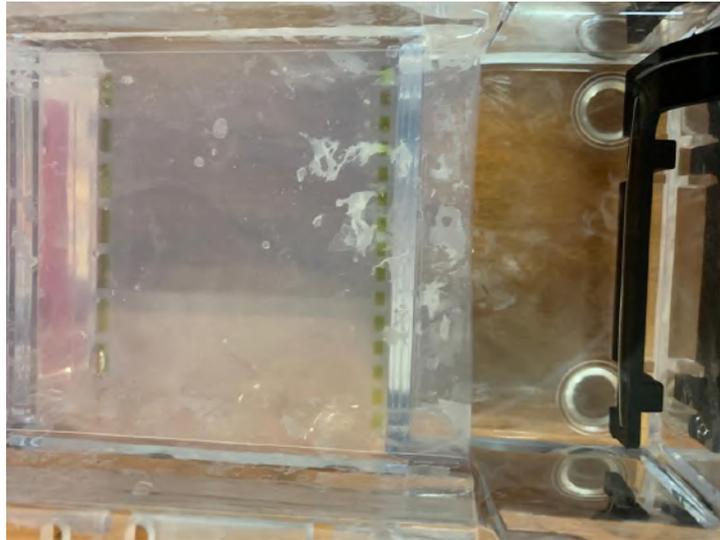


Fig. 12: DNA Bands separation after Gel electrophoresis



Fig. 13: Analysis of DNA Bands

PHYLOGENETIC TREE

A phylogenetic tree is made and analysed using various softwares. In the field of genome analysis, biologists seek to identify important genes or chromosome regions by comparing phylogenetic trees and analysing the mutation at which locus might affect phenotypic traits (Ge et al., 2020).

The dataset of 637 basepairs of *Pomadasys maculatus* and 643 basepairs of *Carangoides armatus* are acquired from the ICBN nucleotide sequences of COI. These sequences were then

aligned using the MUSCLE (Edgar, 2004) sequence algorithm implemented in MEGA 11. From the aligned sequences a phylogenetic tree was constructed by using the Maximum Likelihood (ML) method. The most common way to estimate the reliability of a phylogenetic tree is by the bootstrap method (Hall, 2013).

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc Len	Accession
<input checked="" type="checkbox"/> Pomadasys maculatus voucher st. 1 AN3 cytochrome c oxidase subunit I (COX1) gene, partial cds, mitochondrial	Pomadasys mac...	1177	1177	93%	0.0	100.00%	637	ON142495.1
<input checked="" type="checkbox"/> Pomadasys maculatus voucher ZSI/SRC F-19B cytochrome c oxidase subunit I (COI) gene, partial cds, mitoch...	Pomadasys mac...	1164	1164	93%	0.0	99.69%	668	MK331957.1
<input checked="" type="checkbox"/> Pomadasys maculatus voucher ZSI/SRC F-19 cytochrome c oxidase subunit I (COI) gene, partial cds, mitoch...	Pomadasys mac...	1164	1164	93%	0.0	99.69%	668	MK331956.1
<input checked="" type="checkbox"/> Pomadasys maculatus isolate BSS cytochrome c oxidase subunit I (COX1) gene, partial cds, mitochondrial	Pomadasys mac...	1157	1157	93%	0.0	99.22%	663	ON183260.1
<input checked="" type="checkbox"/> Pomadasys maculatus voucher DUZM_MF_245.2 cytochrome oxidase subunit I (COI) gene, partial cds, mitochon...	Pomadasys mac...	1131	1131	90%	0.0	99.68%	655	MH4230986.1
<input checked="" type="checkbox"/> Pomadasys maculatus voucher DUZM_MF_245.4 cytochrome oxidase subunit I (COI) gene, partial cds, mitoc...	Pomadasys mac...	1129	1129	91%	0.0	99.20%	650	MK989526.1
<input checked="" type="checkbox"/> Pomadasys maculatus voucher DUZM_MF_245.2 cytochrome oxidase subunit I (COI) gene, partial cds, mitoc...	Pomadasys mac...	1125	1125	90%	0.0	99.51%	630	MH429346.1
<input checked="" type="checkbox"/> Pomadasys maculatus isolate FCC1901SB-14 cytochrome c oxidase subunit I (COI) gene, partial cds	Pomadasys mac...	1120	1120	92%	0.0	98.73%	642	MN458364.1
<input checked="" type="checkbox"/> Pomadasys maculatus isolate F1798SM-11 from Bangladesh cytochrome oxidase subunit I (COI) gene, partial...	Pomadasys mac...	1101	1101	89%	0.0	99.18%	611	MK346693.1
<input checked="" type="checkbox"/> Pomadasys maculatus isolate 12058 cytochrome c oxidase subunit I (COX1) gene, partial cds, mitochondrial	Pomadasys mac...	1101	1101	88%	0.0	99.67%	626	MW509667.1
<input checked="" type="checkbox"/> Pomadasys maculatus isolate VSKP_HBR_AU_78 cytochrome c oxidase subunit I gene, partial cds, mitochon...	Pomadasys mac...	1058	1058	88%	0.0	99.50%	609	MN747959.2
<input checked="" type="checkbox"/> Pomadasys maculatus isolate F1798SM-10 from Bangladesh cytochrome oxidase subunit I (COI) gene, partial...	Pomadasys mac...	1096	1096	89%	0.0	99.02%	611	MK346692.1
<input checked="" type="checkbox"/> Pomadasys maculatus voucher F1803SM-44 cytochrome c oxidase subunit I (COX1) gene, partial cds, mitoch...	Pomadasys mac...	1083	1083	88%	0.0	99.01%	604	MT374170.1
<input checked="" type="checkbox"/> Pomadasys maculatus voucher DUZM_MF_245.5 cytochrome oxidase subunit I (COI) gene, partial cds, mitoc...	Pomadasys mac...	1077	1077	87%	0.0	99.00%	601	MK995076.1
<input checked="" type="checkbox"/> Pomadasys maculatus voucher PGN171 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Pomadasys mac...	1029	1029	94%	0.0	95.50%	707	KF714996.1
<input checked="" type="checkbox"/> Pomadasys maculatus voucher FBGN-SAU-Dhaka F1602sb-38-2 cytochrome oxidase subunit I (COI) gene, p...	Pomadasys mac...	1005	1005	81%	0.0	99.28%	556	MF580665.1

Fig. 14: BLAST Results of *Pomadasys maculatus*

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc Len	Accession
<input checked="" type="checkbox"/> Carangoides armatus voucher CEW0144 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	Carangoides ar...	1195	1195	96%	0.0	99.25%	686	KU317894.1
<input checked="" type="checkbox"/> Carangoides armatus voucher NBFGR.CHN.102A1 cytochrome c oxidase subunit I (COI) gene, partial cds, mit...	Carangoides ar...	1192	1192	96%	0.0	99.24%	684	MK348217.1
<input checked="" type="checkbox"/> Carangoides armatus voucher st0_s3 cytochrome c oxidase subunit I (COX1) gene, partial cds, mitochondrial	Carangoides ar...	1188	1188	93%	0.0	100.00%	643	ON150841.1
<input checked="" type="checkbox"/> Atropus atropos voucher CEW0046 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	Atropus atropos	1175	1175	96%	0.0	98.64%	700	KU179061.1
<input checked="" type="checkbox"/> Atropus atropos voucher ABG1 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Atropus atropos	1164	1164	91%	0.0	100.00%	631	KY849506.1
<input checked="" type="checkbox"/> Carangoides armatus voucher BW-A10391 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Carangoides ar...	1158	1158	93%	0.0	99.22%	652	JN312991.1
<input checked="" type="checkbox"/> Ulua mentalis voucher USNM 444126 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Ulua mentalis	1157	1157	93%	0.0	99.22%	651	MH235727.1
<input checked="" type="checkbox"/> Carangoides armatus FRLM.43863 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds	Carangoides ar...	1155	1155	91%	0.0	100.00%	625	LC646692.1
<input checked="" type="checkbox"/> Carangoides armatus voucher EADF_161 cytochrome c oxidase subunit I (COX1) gene, partial cds, mitochon...	Carangoides ar...	1149	1149	94%	0.0	98.62%	663	MT076549.1
<input checked="" type="checkbox"/> Carangoides armatus FRLM.43862 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds	Carangoides ar...	1144	1144	91%	0.0	99.68%	625	LC646691.1
<input checked="" type="checkbox"/> Carangoides armatus voucher KAU14-553 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	Carangoides ar...	1142	1142	93%	0.0	98.76%	652	MF375317.1
<input checked="" type="checkbox"/> Carangoides armatus voucher KAU14-347 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	Carangoides ar...	1142	1142	93%	0.0	98.76%	652	MF375316.1
<input checked="" type="checkbox"/> Carangoides armatus FRLM.43850 mitochondrial COX1 gene for cytochrome c oxidase subunit 1, partial cds	Carangoides ar...	1138	1138	91%	0.0	99.52%	625	LC646690.1
<input checked="" type="checkbox"/> Carangoides armatus voucher KAU14-346 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	Carangoides ar...	1136	1136	93%	0.0	98.60%	652	MF375311.1
<input checked="" type="checkbox"/> Ulua mentalis isolate F1612Sh-83 cytochrome oxidase subunit I (COI) gene, partial cds	Ulua mentalis	1109	1109	90%	0.0	98.72%	624	MK024416.1
<input checked="" type="checkbox"/> Carangoides armatus voucher UMTF 9364 cytochrome c oxidase subunit I (COX1) gene, partial cds, mitochon...	Carangoides ar...	1079	1079	85%	0.0	100.00%	588	MT646208.1

Fig. 15: BLAST Results of *Carangoides armatus*

Carangoides armatus



Fig. 18: *Carangoides armatus* (Courtesy of Wikipedia)

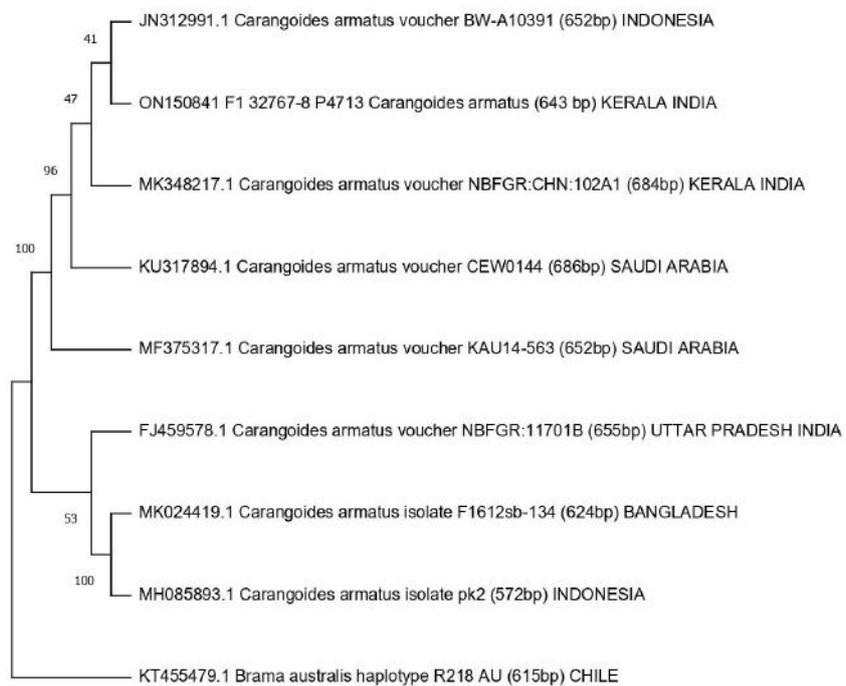


Fig. 19: Phylogenetic Tree of *Carangoides armatus*

Voucher Specimen- ON150841

Outgroup- KT455479

DISCUSSION

The analysis of morphological traits has been the most traditional method used for species identification and taxonomy. However, when morphological identification is compromised, genetic identification can be used to associate sequences from unknown samples to a sequence from a reference sample. Based on a standard region of 650 base pairs of the subunit I of Cytochrome C Oxidase mitochondrial gene (COI) and using a validated reference database, the DNA Barcoding system for cataloguing and identifying animal species has been proposed.

Present study suggests that DNA barcoding has been successful in identifying and discriminating the vast majority of marine ichthyofauna (fishes). The DNA barcoding method is an effective tool for species identification, particularly with specimens that are damaged, incomplete, unknown or consisting of several morphologically distinct stages.

The objective of DNA barcoding analyses is simple- to assign each unknown sequence to a set of referenced (tagged-specimen) sequences extracted, for instance, from databases like BOLD (Casiraghi, 2010). Many different bioinformatics approaches are available to reach this aim. Although it has many advantages, it also has its limitations. In some cases, related species may present identical sequences making DNA barcodes useless for species discrimination.

DNA Barcoding is already effective for species identification in many cases and, although presenting some limitations, the use of the tool must be improved and widespread in forensic casework. DNA sequences are increasingly being used for the rapid quantification of biodiversity and for some taxonomic groups, which may provide a more accurate overview of species diversity than traditional (i.e., morphological) methods. While these approaches can be used to highlight biogeographic regions that are risks due to anthropogenic disturbances and have great potential for use by ecologists, evolutionary, and conservation biologists, frequently the utility of these methods are constrained by the presence of incomplete reference libraries. Here, we have presented a technique that can be utilised by any community DNA sequencing project to rapidly categorise unidentified specimens or

specimens from species whose biogeographic status is unknown as representing either likely native or non-native species.

Pomadasys maculatus (saddle grunt fish) is native to Indo - west pacific region. They are found in coastal waters over sand near reefs and feed on crustacean & fishes. They are a group of migratory fishes that migrate from sea to fresh water and vice versa for the purpose of breeding. They have oblong body, compressed, dorsal and ventral profiles are equally convex. The body is silvery grey with a series of dark vertical bands dorso-laterally. A large black spot on/in dorsal fin between third and sixth or seventh spines are also seen.

Carangoides armatus are marine fishes living in brackish waters. The distribution is seen along Indo-west Pacific and West coast of Madagascar, to Southern India and Sri Lanka. They have a deep body with a steep head and scutes on the caudal peduncle. They possess 19-22 dorsal fin soft rays and 1 gill arch. The body is blue-grey in colour with a blackish dorsal fin. They feed on small fishes. (FishIDER, 2022).

A phylogenetic tree is an estimate of the relationships among taxa (or sequences) and their hypothetical common ancestors. Today most phylogenetic trees are built from molecular data: DNA or protein sequences which are orthologous to infer the evolutionary relationship among organisms.

Originally, the purpose of most molecular phylogenetic trees was to estimate the relationships among the species represented by those sequences, but today the purposes have expanded to include understanding the relationships among the sequences themselves without regard to the host species, inferring the functions of genes that have not been studied experimentally, and elucidating mechanisms that lead to microbial outbreaks among many others.

The most basic assumption of phylogenetic analysis is that all the sequences on a tree are homologous, that is, descended from a common ancestor. Alignment programs will align sequences, homologous or not. All tree-building programs will make a tree from that alignment. However, if the sequences are not actually descended from a common ancestor,

the tree will be meaningless and may quite well be misleading. The most reliable way to identify sequences that are homologous to the sequence of interest is to do a Basic Local Alignment Search Tool (BLAST) search using the sequence of interest as a query.

Building a phylogenetic tree requires four distinct steps namely, identification, alignment estimation and presentation. The program MEGA was used to implement the above steps in a phylogenetic tree construction. The same method was used for the construction of the phylogenetic trees of the fishes identified (Hall, 2013).

Bootstrap values are used to distinguish how many times out of 100 the same branch was observed when repeating the phylogenetic reconstruction on a re-sampled set of data. Interpretation of Bootstrap value is controversial. Felsenstein (1985) proposed that Bootstrap value of 95% or greater be considered statistically significant and indicate support for a clade: alternative nodes can be rejected if they occur in less than 5% of the Bootstrap estimate. There are generally accepted thresholds for "good" and "moderate" support, e.g. $BS > 70/80$ is considered good, and $50/60 < BS < 70/80$ is considered moderate.

The Bootstrap proportion has variously been interpreted as:

1. A measure of reliability, telling us what would be expected to happen if we repeated our experiment;
2. A measure of accuracy, telling us about the probability of our experimental result being true; and
3. A measure of confidence, interpreted as a conditional probability similar to those in standard statistical hypothesis tests (i.e. measuring Type I errors or false positives).

(Morrison, 2014)

The phylogenetic tree of fishes *Pomadasys maculatus* and *Carangoides armatus* were specifically analysed in the conducted study.

In the phylogenetic tree of *Pomadasys maculatus*, the voucher specimen was named as ON142495 of 637 base pairs. The native place of *Pomadasys maculatus* is China, making the

region its type locality. The voucher specimen for study was collected from Beypore, Kerala. The dendrogram was analysed based on the similarities and differences between different branches, clades and Bootstrap values. Here, species of similar interests or species with most similarities are shown in a clade. The ones with least similarities are shown in different branches. The type locality voucher is named as KY371990 and is obtained from China. The sequences for alignment are obtained from GenBank. The final dataset of 10 sequences were aligned using MUSCLE (Edgar, 2004) in MEGA 11. An outgroup is chosen to highlight the difference between the specimen under study and species. Here, the outgroup chosen is *Sphyraena barracuda* and it has no similarities to the genus *Pomadasys*. It can be observed that the Bootstrap value varies a lot between the type locality specimen and voucher specimen. There is a chance that the voucher specimen comes under different species since they don't align with the type locality specimen. This could be because they are cryptic species or due to taxonomical ambiguities. Even though they are taxonomically identified as *Pomadasys maculatus*, the dendrogram drawn with the sequence obtained from GenBank shows that they are different. Sequences generated in the present study have been submitted to GenBank under accession number ON150841.

In the dendrogram of *Carangoides armatus*, the specimen obtained is named as ON150841 of 643 base pairs. The type locality is Indonesia. The tree is analysed based on the similarities and differences between clades and Bootstrap values. Once the sequences are MUSCLE aligned and a dendrogram is drawn, the species are shown in branches or clades in accordance with their similarities. Species of similar interests or species with most similarities are shown in a clade. The ones with least similarities are shown in different branches. Here, the type locality specimen is named JN312991 found in Indonesia. The specimen under study is obtained from Kerala. Both the voucher specimen and type locality specimen are in a single clade indicating that they have very little differences. The outgroup chosen to highlight the difference is *Barma australis* which shows zero similarities to *Carangoides*. Since the similarities between the type locality voucher and voucher specimen is above 40, it can be said that they belong to the same specimen of fishes. The variations are vague and similarities are more prominent making it a less divergent species of Carangidae. Sequences generated in the present study have been submitted to GenBank under accession number ON150841.

By analysing the phylogenetic tree or dendrogram, it is observed that the voucher specimen of *Pomadasys maculatus* having accession number ON150841 may be a cryptic species whereas the voucher specimen of *Carangoides armatus* having accession number ON150841 may belong to the same specimen of fishes.

Through this study, a reliable DNA barcode reference library for the marine fish in south India has been established, which could be used to assign fish species by screening sequences against it in the future. This could contribute to achieving better monitoring, conservation, and management of fisheries in this overexploited region.

CONCLUSION

DNA barcoding is a very useful tool for identifying unknown specimens. Identification of a fish can be done by taking a small sample from the specimen without causing much damage to them. Even with a small sample, it will be adequate to find the sequence and thus the unknown specimens can be identified. The DNA Barcoding method of identifying unknown species is extremely accurate. DNA barcoding uses genetic-level identification of species to get insight into their evolutionary relationships. Genetic material is the only thing that is transferred down to the next generation. So, as compared to traditional methods such as identifying fish using morphology, which can be misleading and has a higher possibility of error, analysis involving genetic material is significantly better. A phylogenetic tree might be used to interpret the results. A single glance reveals the clade or group to which the unknown species belongs. Phylogenetic trees are the most basic and easiest approach to understanding the results of DNA Barcoding.

The discriminatory power of COI barcodes is emphasized and their application to cases requiring species level resolution starting from unknown sequences. There are high results of reliability to DNA barcodes from public reference libraries, to identify species from different geographical origins. The ability to assign species with high precision from DNA samples of disparate quality and origin has major utility in several fields, from forensics, fisheries and conservation programs to control of fish products authenticity.

It is well known that no identification method (morphological, biochemical, genetic or whatsoever based) can truly identify species, because species are entities in continuous evolution and it is theoretically impossible to statically define such dynamic matter.

The results of the present study of DNA Barcoding will facilitate other lines of research in biodiversity assessments of unknown fishes.

The present study has also demonstrated that DNA barcoding holds great promise as a tool for rapid biodiversity assessment in unknown fishes.

From the dendrogram for *Pomadasys maculatus*, it can be observed that there is variation between the Bootstrap values of the type locality specimen and voucher specimen. There is a possibility that the voucher specimen comes under different species since they don't align with the type locality specimen. This could be because they are cryptic species or due to taxonomical ambiguities. Even though they are taxonomically identified as *Pomadasys maculatus*, the dendrogram drawn with the sequence obtained from GenBank shows that they are different. But in the case of *Carangoides armatus*, since the similarities between the type locality voucher and voucher specimen is above 40, it can be said that they belong to the same specimen of fishes. The variations are vague and similarities are more prominent making it a less divergent species of Carangidae. In the present study, one species is similar but the other, even though they are taxonomically identified within the same genera *Carangoides*, their similarities are very vague.

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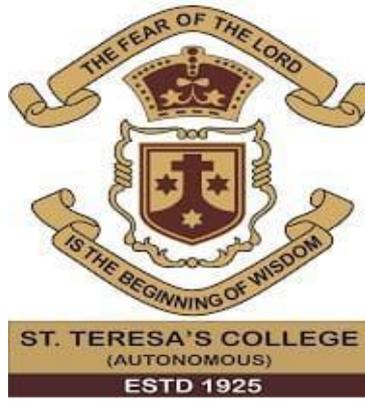
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DNA BARCODING IN INDIAN MARINE FISHES

- *Mene maculata* and *Leiognathus equulus*.



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Partial fulfilment of requirement for the

Degree of Bachelor of Science in Zoology

2021-2022

CERTIFICATE

This is to certify that the project report entitled “DNA BARCODING IN INDIAN MARINE FISHES- *Mene maculata* and *Leiognathus equulus*” submitted by Ms. SARA MARIA SHAJI, Reg. No: AB19ZOO016 in partial fulfilment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under my guidance and supervision and to my best knowledge, this is her original effort.

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Ernakulam

EXAMINERS

1)

2)

DECLARATION

I, hereby declare that this project work entitled “**DNA BARCODING IN INDIAN MARINE FISHES- *Mene maculata* and *Leiognathus equulus***” is submitted to St. Teresa’s College (Autonomous), Ernakulum affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science degree in Zoology. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report are entirely my own.

NAME: SARA MARIA SHAJI

SIGNATURE

REGISTRATION NUMBER: AB19ZOO016

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SARA MARIA SHAJI

DNA BARCODING IN INDIAN MARINE FISHES-

Mene maculata and Leiognathus equulus.

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REFERENCE	24

ABSTRACT

The present study reflects DNA barcoding in two Indian Marine fishes. DNA barcoding involves sequencing a fragment of the mitochondrial cytochrome oxidase I (COI) gene, known as DNA barcodes, from taxonomically unknown specimens. The following study has been undertaken to research and analyse the barcode of Indian Marine fishes and their DNA sequences. DNA barcoding is a method of species identification using a short section of DNA from a specific gene. The present study focuses on barcoding the DNA of 2 genera of fishes collected from Beypore Fishing harbour, Kozhikode. The study was completed in 4 phases. The first two phases were collection and preservation of specimens for later analysis. Third phase consisted of DNA isolation from the collected samples and amplification of the sequence. Since DNA extraction is a sensitive process, buffers are added to stabilise the pH over cell lysis and isolation. The final phase consisted of PCR and barcoding the isolated DNA samples. From the 21 samples collected, fishes belonging to 8 genera were identified which include Pomadasys, Carangoides, Decapterus, Cynoglossus, Nemipterus, Selar, Mene, Leiognathus and Pericanthus out of which 2 genera were specifically analysed namely *Mene maculata* and *Leiognathus equulus*.

INTRODUCTION

Identification of fishes contributes to the classification of each fish and the crucial function they play in the biological world, as well as the discovery of important food and medicinal ingredients from them. Fishes are the primary food source of humans, hence research on them has a significant impact on human life. Various approaches can be used to identify an unknown specimen. Identifying it with the use of morphology was a typical method employed previously, but morphology only provides external structural features, fishes have undergone multiple ontogenetic development, convergent and divergent evolution, identifying them only based on their morphological characteristics will be very challenging. As a result, morphological features alone are not sufficient for unknown specimens; in these cases, DNA barcoding is used.

DNA barcoding, or sequence-based specimen identification, was developed by Paul Hebert in 2003 to identify a broad range of taxa by sequencing a standardised short DNA fragment, the "DNA barcode". DNA Barcoding is a technique for identifying different species. It was first used in fishes by Ward in 2005. It functions by examining a specific DNA region and this specific area is known as the DNA barcode. For animal identification, the most broadly used barcode marker is mitochondrial Cytochrome C Oxidase subunit I (COI), which is highly conserved across species employing oxidative phosphorylation for metabolism. After that, the sequence of this DNA barcode is compared to a reference library that contains information on many different species and their barcodes. DNA Barcoding consists of the following steps: DNA Isolation, Amplifying the isolated DNA using Polymerase Chain Reaction, Gel Electrophoresis and Sequence Analysis. DNA isolation is a key step because, without high quality DNA, the PCR amplification will not be optimal. The following statements are some of the benefits of DNA barcoding over previous classification systems: DNA barcoding help in accurately distinguishing some species that are similar in morphology, cryptic species are indistinguishable biological groups that are incapable of interbreeding and cannot be distinguished using traditional methods of classification because traditional methods classify cryptic species as a single group, despite the fact that these species show genetic variation. (Lakra et al., 2011)

They can be easily distinguished through DNA Barcoding, Barcoding methods can often give information without causing harm to the animal studied. In cases of morphological ambiguity, such as with larval stages, DNA barcoding technology can help identify species. The applications of DNA barcoding includes ecological monitoring, early detection, control and removal of non-indigenous species, fisheries management, food safety and protection of endangered species. The results generated by DNA barcoding are limited or biased to the frequency of occurrence, and quantifying fish abundance from molecular data is another key problem for this approach.

There are several different types of DNA extraction methods. A few of them include Phenol-Chloroform Isoamyl Alcohol, Proteinase K, CTAB Method, Spin Column-based Methods and Magnetic Bead-based Technique. However the method implied on each specimen depends on sample type and purity and the yield of DNA that is to be obtained. DNA extraction is completed in 4 steps: lysis, separation, precipitation and purification. There are 2 main types of DNA sequencing methods. The classical method is known as Chain Termination method or Sanger Sequencing Method. Other modern methods that can process a large number of DNA molecules swiftly are collectively called High-Throughput sequencing (HTS) and Next-Generation sequencing methods (NGS). In the conducted study, DNA isolation was done using the DNeasy Blood & Tissue Kit and respective buffers namely Proteinase K, Buffer ATL, Ethanol, Buffer AL, Buffer AW1, Buffer AW2, Buffer AE and Buffer AP. Polymerase Chain Reaction (PCR) is a powerful method for amplifying particular segments of DNA, distinct from cloning and propagation within the host cell. Out of the various PCR techniques, amplification of the isolated sequence was done using normal Polymerase Chain Reaction. DNA sequencing was done to the amplified sequence using Sanger sequencing method. It is done to determine the nucleic acid sequence or order of nucleotides in DNA. Lastly, a phylogenetic tree was drawn with the help of the software MEGA 11 to identify the species of the specimen.

AIM AND OBJECTIVES

AIM

To identify two types of fishes by using DNA barcoding.

OBJECTIVE

- To barcode the various types of Indian Marine Fishes.
- To provide the statistics, characteristics and presence of types of fishes in certain areas.
- To compare and classify the varieties of fishes in marine habitat.

REVIEW OF LITERATURE

DNA Barcoding is a molecular diagnostic method used for identification of species by using a standardised DNA sequence or genetic region which acts as the 'barcode'. 'DNA barcoding' is a new identification tool proposed by Hebert et al. (2003), and is a valuable addition to the taxonomic tool box. They advocated the use of short DNA sequences from the specified region of the genome termed as DNA barcode for biological identification. It implies sequencing of a standard DNA locus as a tool for identifying species. An ideal DNA barcode should be easily retrievable with a single primer pair, be amenable to bidirectional sequencing and effectively provide high discrimination among species.

According to Savoleinen et al., (2005), the scientific benefits of DNA barcoding include: (i) enabling species identification, including any life stage or fragment, (ii) facilitating species discoveries based on cluster analyses of gene sequences, (iii) promoting development of handheld DNA sequencing technology that can be applied in the field for biodiversity inventories and (iv) providing insight into the diversity of life.

Based on the works of Jeremy C. Andersen et al., (2019), the collection of DNA barcode sequences from unidentified specimens provides useful genomic data and at the same time DNA barcoding techniques are being used with increasing frequency to guide management decisions, particularly for the identification of alien invasive species (Dejean et al., 2012).

This study utilises the standard Cytochrome C Oxidase subunit I (COI) which is found in most eukaryotes and highly conserved and so can be copied from unknown organisms. They also have less intraspecific (within species) variation than interspecific (between species) variation, known as the "Barcoding Gap". When fully developed, a COI identification system will provide a reliable, cost-effective and accessible solution to the current problem of species identification. Its assembly will also generate important new insights into the diversification of life and the rules of molecular evolution (Hebert et al., 2003). The mitochondrial genome of animals is a better target for analysis than the nuclear genome because of its lack of introns, its limited exposure to recombination and its haploid mode of

inheritance (Saccone et al., 1999). Robust primers also enable the routine recovery of specific segments of the mitochondrial genome (Folmer et al., 1994).

The major goal of DNA-barcoding efforts is to aid identification of specimens by matching sequences to a sequence library. The revolution introduced by DNA barcoding resides in the molecularisation, computerization and standardisation of taxonomic approach. The identification and then the interpretation of molecular entities is the main goal of DNA barcoding that could be reached only by users with a sound theoretical background on what is identifiable by this technique (Casiraghi et al., 2010). Many authors have proposed DNA barcoding as an integrated approach with classical taxonomy for species identification and authentication. Modifications in extraction methods, primer sequences, use of an engineered polymerase and even the combining of barcodes from multiple loci has been used successfully to clear any issues related to DNA Barcoding in vertebrates.

In order to test the utility of DNA Barcoding in forensic vertebrate species identification, COI sequences from previously identified samples from humans and a variety of domestic and wild specimens of Brazilian mammals, birds, fishes were compared against the Barcode of Life Database (BOLD). BOLD provided a correct species-level identification for 12 out of the 20 queried sequences (60%) and presented the correct species as the best matched one for 17 out of 18 samples morphologically identified to this level (94%). (Carvalho, 2014)

Barcoding can be used as an alternative to traditional sampling methods in fish research. Barcoding procedures can often give information without causing harm to the animal being investigated. Hebert et al., (2003) proposed using DNA barcoding to help fish identification, which prompted the formation of Fish Barcode of Life (FISH-BOL), which aims to barcode all taxonomically documented fish species (Ward et al., 2009). The FISH-BOL project began in 2005, and roughly 8,000 of the 31,000 fish species recognised have been barcoded for the COI gene. According to the initial report, around 98 percent and 93 percent of marine and freshwater species may be distinguished using barcodes, respectively.

According to the work of Zhou et al., (2009), it is clear that the limited access to taxonomic expertise is an issue for large-scale biodiversity surveys. Their study shows that a

comprehensive DNA barcode library built on expertly identified specimens enables fast and accurate species identification. There will be easier ways of analysing bulk environmental samples which will become more widespread and less expensive over time, facilitating ecological and monitoring applications of the barcode library. Continued interaction with the taxonomic community during barcode-based biodiversity and monitoring studies, involving submitting specimens with novel sequences for determination or revision, will ensure the growth and maintenance of a high-quality database.

Mene maculata or moon fish, a fish native to the Indian ocean, is found throughout the Indo-Pacific ranging from the eastern coast of Africa, India, Philippines, Northern Australia and Japan (Du et al., 2012). It inhabits deeper coastal waters especially around coral reefs (Carpenter et al., 1997). They feed on copepods, shrimp and fish larvae. *Mene maculata* can be identified by a laterally compressed disc-like body, distinctly shaped maxillae and long ascending processes of pre-maxilla (Matt et al., 2005). The complete mitogenome has 16,733 base pairs (53.8% A + T content) and is made up of a total 37 genes and a putative non-coding control region (Shengping et al.,). They have 3 dorsal spines and 40-45 dorsal soft rays. The colour of the body is metallic blue on the dorsal side and silvery on the ventral side.

Leiognathus equulus also known as common ponyfish is found in river mouths and muddy inshore areas. Allen (G.R., 1991) often in mangrove areas (Kuitert R.H. et al., 2001). Adults are coastal inhabitants found on soft bottoms, usually between depths of 10-70 metres. (Allen, G.R. et al., 2002). They're strictly carnivorous fish feeding on blue-green algae, green algae, diatoms, rotifers, gastropods, nematodes etc. (Lankadhikara L.M.C.V. et al., 2004). They're deep bodied with short rounded snout and large eyes, strongly arched back, naked head with nuchal spines and protracted mouth (Frosskal, 1775). The gas bladder has the purpose of reflecting bioluminescent light from circumesophageal light organ (Margaret, J.M. 1983). They have 8 dorsal spines and 15-16 dorsal soft rays and 3 anal spines 14-15 anal soft rays. The body is black greyish colour with silver belly.

METHODOLOGY

SPECIMEN COLLECTION

The fishes were collected from Beypore Fishing Harbour, Kozhikode. A total of 21 fish types were selected and labelled for analysis by DNA barcoding. The specimens were stored in the Museum at Kerala University of Fisheries and Ocean Studies, Panangad, Kerala.

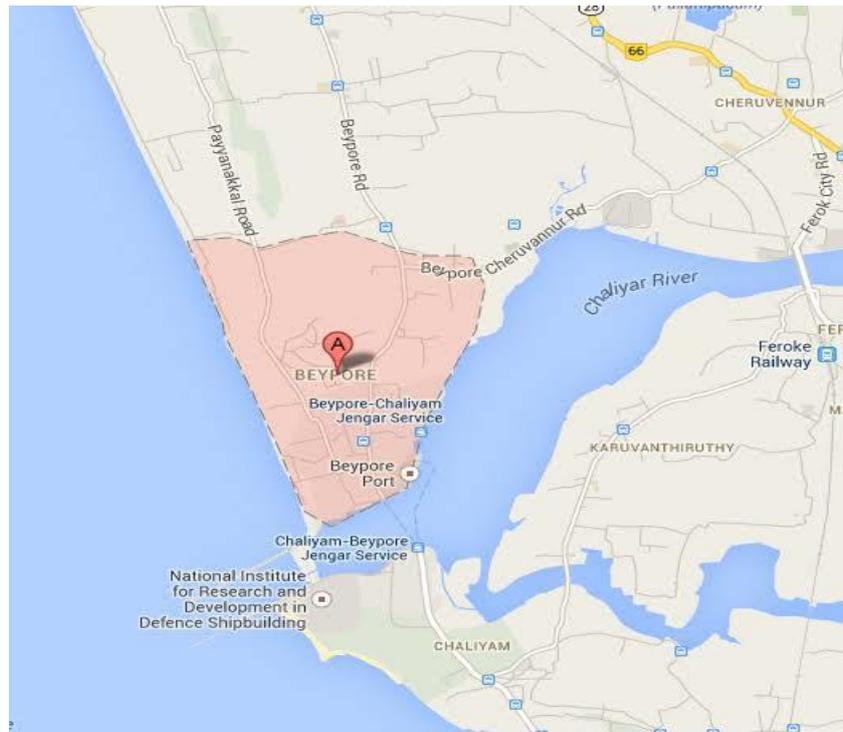


Fig. 1: Map showing Beypore Fishing Harbour, Kozhikode



Fig. 2: Fish samples collected

PROCEDURE

PREPARATION OF SAMPLE

The selected mature fish specimens were measured and mounted on a hard surface to observe the characteristic features. Muscle tissue samples were dissected using sterilised tools, preserved in 10% Formalin, properly labelled and stored in the refrigerator.

The protocol for DNA extraction, PCR amplification of CO1 gene, product purification and sequencing follow the study of Lakra et al. (2011).



Fig. 3: Preparation of fish samples

DNA ISOLATION

The preserved tissue samples were taken out of the vial and a portion (<25mg) of the flesh was transferred to a centrifuge tube and labelled. 200 μ L of Buffer ATL (Lysis Buffer) was added to the centrifuge tube followed by 20 μ L of Proteinase K. The mixture was vortexed to homogenise the contents and incubated in a thermomixer at 56°C for 2 hours until the whole tissue was completely digested. 200 μ L of Buffer AL (Lysis Buffer) was added to the centrifuge tube and incubated for another 10 minutes at 56°C. After removing the centrifuge tubes from

the thermomixer, 200 μ L of 95% chilled Ethanol was added to it and then incubated at room temperature for 5 minutes. The contents of the centrifuge tube were then transferred into labelled spin columns taken in 2ml collection tubes. The tubes were placed in a balanced configuration and centrifuged for 1 minute at 8000 rpm.



Fig. 4: Vortexing the microtubes

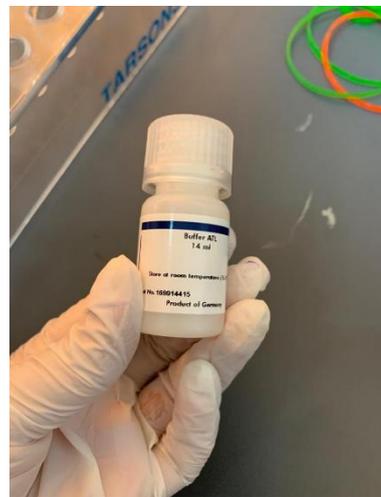


Fig. 5: ATL Buffer



Fig. 6: Thermomixer



Fig. 7: Centrifugation

The collection tubes were then replaced with new 2ml tubes. 500 μ L of Buffer AW1 (Wash Buffer) was added to the centrifuge tube and centrifuged for 3 minutes at 14000 rpm. The process was repeated with Buffer AW2 (Wash Buffer). The collection tubes were replaced with labelled centrifuge microtubes. 50 μ L of Buffer AE (Elution Buffer) was added to the centrifuge tube (incubated at room temperature for 1 minute) and then centrifuged for 1 minute at 8000 rpm. Another 50 μ L of Buffer AE was added to the centrifuge tube and incubated at room temperature for 1 minute and centrifuged at 1 minute at 8000 rpm. The

Buffer AE elutes the DNA from the spin column membrane into the centrifuge tube. The eluted DNA in the labelled centrifuge microtube was stored at -4°C.

PCR

Species-specific variations or polymorphisms in the DNA sequence that are spread randomly over the entire genome and result in characteristic DNA fingerprints have been exploited through use of polymerase chain reaction (PCR) and its variants. (Priyanka Mishra et al., 2015). The procedures are followed using INVITROGEN Genomic DNA Mini Kit.

24 µL Master Mix (312.5 µL Emerald Amp GT PCR, 31.25 µL Forward F1 Primer, 31.25 µL Reverse R1 Primer and 225 µL dH₂O) was added to new individual vials which were properly labelled. The primer pair LCO1490(59-GGTCAACAAATCATAAAGATATTGG-39) and HCO2198(59-TAAACTTCAGGGTGACCAAAAAATCA-39) was subsequently used to amplify a 658 bp fragment of the COI gene. The samples were taken out of storage and added to the Master Mix vials.

The next step in the process involved 35 cycles of PCR (involving Denaturation, Annealing and Extension followed by Final Extension) maintained at 4°C. The vortexed vials were kept in the PCR Machine until a temperature of 105°C was attained after which the process started and took around two and a half hours to complete.

- DENATURATION
Done at 95°C for 5 minutes

- ANNEALING
Done at 58°C (but may vary depending on Primers used)

- EXTENSION
Done at 72°C



Fig. 8: Genomic DNA Mini Kit



Fig. 9: Master Mix



Fig. 10: PCR Machine

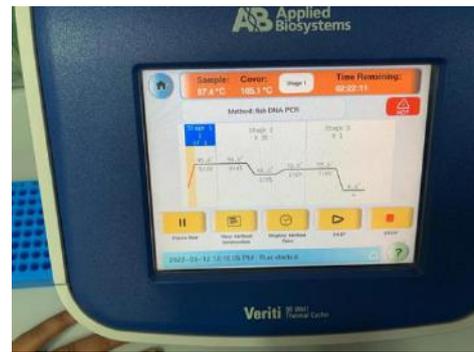


Fig. 11: Progress of PCR

PREPARATION OF GEL

0.4g Agarose Special Powder was added to 100mL Borosil and mixed well. This was transferred to a gel tray with wells/pits.

GEL ELECTROPHORESIS

Anode and Cathode electrodes were placed in a container and TE Buffer was added until the electrodes were completely immersed. The amplified sample mixtures were poured into the wells/pits. The Electrophoresis Machine was set to 90-91 Volts and run for about 20 minutes. The gel is then transferred onto a Bio Rad Imager for analysis of results of DNA imaging on the computer. The isolated DNA samples were given for sequencing.

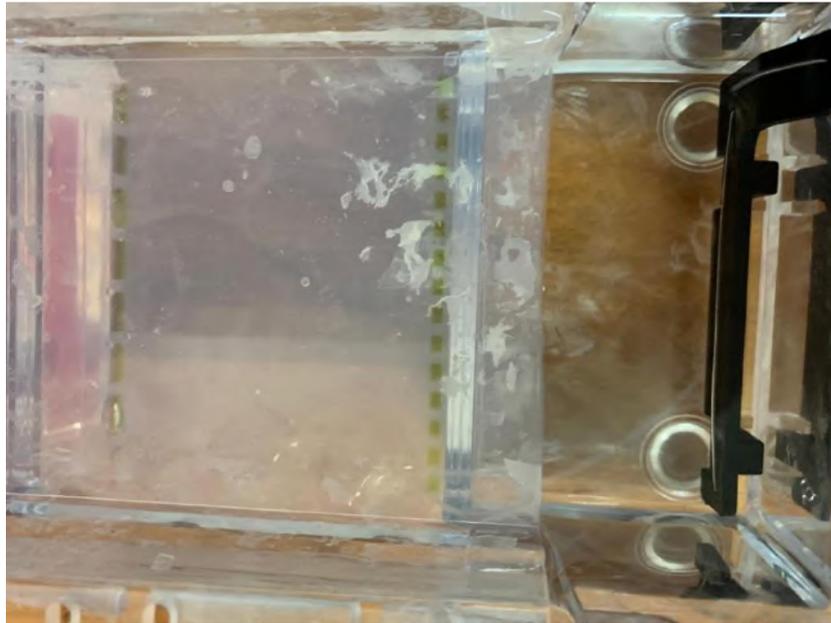


Fig. 12: DNA Bands separation after Gel electrophoresis



Fig. 13: Analysis of DNA Bands

PHYLOGENETIC TREE

A phylogenetic tree is made and analysed using various softwares. In the field of genome analysis, biologists seek to identify important genes or chromosome regions by comparing phylogenetic trees and analysing the mutation at which locus might affect phenotypic traits (Ge et al., 2020).

The dataset of 637 basepairs of *Mene maculata* and 643 basepairs of *Leiognathus equulus* are acquired from the ICBN nucleotide sequences of COI. These sequences were then aligned using the MUSCLE (Edgar, 2004) sequence algorithm implemented in MEGA 11. From the aligned sequences a phylogenetic tree was constructed by using the Maximum Likelihood (ML) method. The most common way to estimate the reliability of a phylogenetic tree is by the bootstrap method (Hall, 2013).

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc Len	Accession
<input checked="" type="checkbox"/> Mene maculata mitochondrial complete genome	Mene maculata	1179	1179	94%	0.0	96.69%	16733	NC_037226.1
<input checked="" type="checkbox"/> Mene maculata voucher 917 s1 cytochrome c oxidase subunit I (COXI) gene, partial cds, mitochondrial	Mene maculata	1177	1177	93%	0.0	100.00%	637	ON150753.1
<input checked="" type="checkbox"/> Mene maculata voucher FBGN:SAU-Dhaka F15125b-02 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1173	1173	94%	0.0	99.53%	715	ME580546.1
<input checked="" type="checkbox"/> Mene maculata mitochondrial DNA complete genome except for D-loop	Mene maculata	1168	1168	94%	0.0	99.38%	16292	AB355909.1
<input checked="" type="checkbox"/> Mene maculata voucher F14 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1157	1157	91%	0.0	99.84%	691	KJ202178.1
<input checked="" type="checkbox"/> Mene maculata voucher NBFGR:2901A cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1147	1147	90%	0.0	100.00%	655	FJ342937.1
<input checked="" type="checkbox"/> Mene maculata voucher MBCSC Fish BH11226138 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1142	1142	90%	0.0	100.00%	652	JN242545.1
<input checked="" type="checkbox"/> Mene maculata isolate FSC5002-86 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1142	1142	90%	0.0	100.00%	652	EF607444.1
<input checked="" type="checkbox"/> Mene maculata voucher IO92 cytochrome c oxidase subunit I (COXI) gene, partial cds, mitochondrial	Mene maculata	1140	1140	90%	0.0	99.84%	620	MT774159.1
<input checked="" type="checkbox"/> Mene maculata isolate SP61 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1136	1136	90%	0.0	100.00%	650	MK843727.1
<input checked="" type="checkbox"/> Mene maculata voucher GDC1872 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1136	1136	90%	0.0	100.00%	634	KY371755.1
<input checked="" type="checkbox"/> Mene maculata voucher GDC1875 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1136	1136	90%	0.0	100.00%	634	KY371754.1
<input checked="" type="checkbox"/> Mene maculata voucher SY121 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1136	1136	90%	0.0	100.00%	634	KY371753.1
<input checked="" type="checkbox"/> Mene maculata voucher SY122 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1136	1136	90%	0.0	100.00%	634	KY371752.1
<input checked="" type="checkbox"/> Mene maculata voucher SY123 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1136	1136	90%	0.0	100.00%	634	KY371751.1
<input checked="" type="checkbox"/> Mene maculata voucher SY124 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Mene maculata	1136	1136	90%	0.0	100.00%	634	KY371750.1

Fig. 14: BLAST Results of *Mene maculata*

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc Len	Accession
<input checked="" type="checkbox"/> Leiognathus equulus voucher PGN195 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1182	1182	96%	0.0	98.80%	702	KF714951.1
<input checked="" type="checkbox"/> Leiognathus equulus cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1173	1173	96%	0.0	98.50%	708	KF309403.1
<input checked="" type="checkbox"/> Leiognathus equulus voucher PGN5 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1173	1173	95%	0.0	98.79%	688	KF714953.1
<input checked="" type="checkbox"/> Leiognathus equulus isolate A1 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1160	1160	96%	0.0	98.20%	1236	EU381032.1
<input checked="" type="checkbox"/> Leiognathus equulus voucher PGN15 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1153	1153	95%	0.0	98.10%	689	KF714955.1
<input checked="" type="checkbox"/> Leiognathus equulus voucher WL-M208 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1146	1146	92%	0.0	99.06%	655	EF509537.1
<input checked="" type="checkbox"/> Leiognathus equulus LIE-fish4K mitochondrial cox1 gene for cytochrome c oxidase subunit I, partial cds	Leiognathus equ...	1134	1134	92%	0.0	98.75%	655	LC558788.1
<input checked="" type="checkbox"/> Leiognathus equulus voucher WL-M209 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1134	1134	92%	0.0	98.76%	655	EF509538.1
<input checked="" type="checkbox"/> Leiognathus equulus voucher WL-M310 cytochrome c oxidase I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1131	1131	92%	0.0	98.74%	655	EU352205.1
<input checked="" type="checkbox"/> Leiognathus equulus voucher BW-A9055 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1129	1129	92%	0.0	98.74%	652	HQ564498.1
<input checked="" type="checkbox"/> Leiognathus equulus voucher BW-A7733 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1129	1129	92%	0.0	98.74%	652	GU673710.1
<input checked="" type="checkbox"/> Leiognathus equulus voucher EBRC/ZSIF:10966 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1127	1127	91%	0.0	98.89%	1166	MK689371.1
<input checked="" type="checkbox"/> Leiognathus equulus cytochrome oxidase subunit I gene, partial cds, mitochondrial	Leiognathus equ...	1127	1127	89%	0.0	99.52%	624	KJ949385.1
<input checked="" type="checkbox"/> Leiognathus equulus voucher NS1310 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1125	1125	91%	0.0	98.74%	634	KY371657.1
<input checked="" type="checkbox"/> Leiognathus equulus voucher NBFGR 35309B cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1125	1125	92%	0.0	98.58%	655	FJ367966.1
<input checked="" type="checkbox"/> Leiognathus equulus voucher SP-130-1 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	Leiognathus equ...	1123	1123	92%	0.0	98.43%	654	MW488672.1

Fig. 15: BLAST Results of *Leiognathus equulus*

OBSERVATION AND RESULT

The amplified Cytochrome Oxidase I (COI) sequences were identified using BLAST. The fishes identified based on matches of $\geq 97\%$ similarity to a published sequence in the NCBI GenBank database were *Mene maculata* and *Leiognathus equulus*.

Mene maculata



Fig. 16: *Mene maculata*

Mene maculata Dendrogram

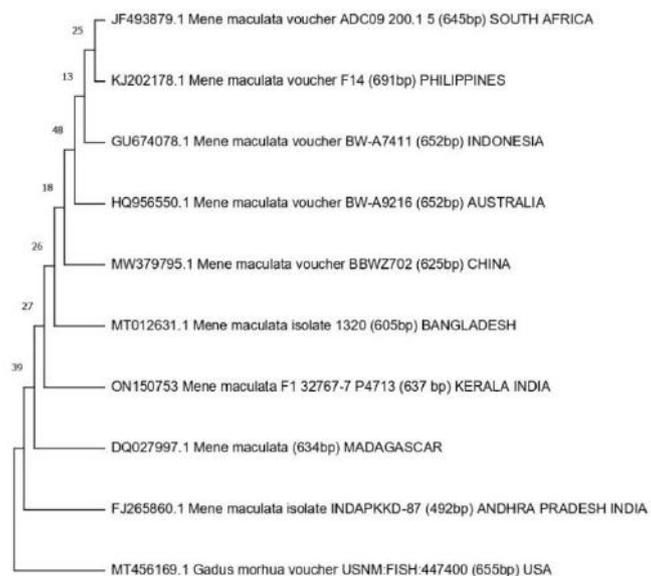


Fig. 17: Phylogenetic Tree of *Mene maculata*

Voucher Specimen- ON150753

Outgroup- MT456169

DISCUSSION

The analysis of morphological traits has been the most traditional method used for species identification and taxonomy. However, when morphological identification is compromised, genetic identification can be used to associate sequences from unknown samples to a sequence from a reference sample. Based on a standard region of 650 base pairs of the subunit I of Cytochrome C Oxidase mitochondrial gene (COI) and using a validated reference database, the DNA Barcoding system for cataloguing and identifying animal species has been proposed.

Present study suggests that DNA barcoding has been successful in identifying and discriminating the vast majority of marine ichthyofauna (fishes). The DNA barcoding method is an effective tool for species identification, particularly with specimens that are damaged, incomplete, unknown or consisting of several morphologically distinct stages.

The objective of DNA barcoding analyses is simple- to assign each unknown sequence to a set of referenced (tagged-specimen) sequences extracted, for instance, from databases like BOLD (Casiraghi, 2010). Many different bioinformatics approaches are available to reach this aim. Although it has many advantages, it also has its limitations. In some cases, related species may present identical sequences making DNA barcodes useless for species discrimination.

DNA Barcoding is already effective for species identification in many cases and, although presenting some limitations, the use of the tool must be improved and widespread in forensic casework. DNA sequences are increasingly being used for the rapid quantification of biodiversity and for some taxonomic groups, which may provide a more accurate overview of species diversity than traditional (i.e., morphological) methods. While these approaches can be used to highlight biogeographic regions that are risks due to anthropogenic disturbances and have great potential for use by ecologists, evolutionary, and conservation biologists, frequently the utility of these methods are constrained by the presence of incomplete reference libraries. Here, we have presented a technique that can be utilised by any

community DNA sequencing project to rapidly categorise unidentified specimens or specimens from species whose biogeographic status is unknown as representing either likely native or non-native species.

Mene maculata are marine fishes living in brackish waters. They are distributed along the coasts of East Africa, South Africa to southern Japan. They have a deep almost triangular body. They inhabit deeper coastal waters near the bottom, on both the continental shelves and around major island groups. Found in schools and feeds on benthic invertebrates (Froese,R.,& Pauly,.D. 2022).

Leiognathus equulus are marine fishes living in brackish waters. They are distributed along Red sea, Persian Gulf and East Africa. Body is deep, compressed with a strongly humped back. Found in river mouths and muddy inshore areas often in mangrove areas. Adults move in schools. They feed on ploychates, small crustaceans, small fishes and worms (Froese,R.,& Pauly,.D. 2022).

A phylogenetic tree is an estimate of the relationships among taxa (or sequences) and their hypothetical common ancestors. Today most phylogenetic trees are built from molecular data: DNA or protein sequences which are orthologous to infer the evolutionary relationship among organisms.

Originally, the purpose of most molecular phylogenetic trees was to estimate the relationships among the species represented by those sequences, but today the purposes have expanded to include understanding the relationships among the sequences themselves without regard to the host species, inferring the functions of genes that have not been studied experimentally, and elucidating mechanisms that lead to microbial outbreaks among many others.

The most basic assumption of phylogenetic analysis is that all the sequences on a tree are homologous, that is, descended from a common ancestor. Alignment programs will align sequences, homologous or not. All tree-building programs will make a tree from that alignment. However, if the sequences are not actually descended from a common ancestor,

the tree will be meaningless and may quite well be misleading. The most reliable way to identify sequences that are homologous to the sequence of interest is to do a Basic Local Alignment Search Tool (BLAST) search using the sequence of interest as a query.

Building a phylogenetic tree requires four distinct steps namely, identification, alignment estimation and presentation. The program MEGA was used to implement the above steps in a phylogenetic tree construction. The same method was used for the construction of the phylogenetic trees of the fishes identified (Hall, 2013).

Bootstrap values are used to distinguish how many times out of 100 the same branch was observed when repeating the phylogenetic reconstruction on a re-sampled set of data. Interpretation of Bootstrap value is controversial. Felsenstein (1985) proposed that Bootstrap value of 95% or greater be considered statistically significant and indicate support for a clade: alternative nodes can be rejected if they occur in less than 5% of the Bootstrap estimate. There are generally accepted thresholds for "good" and "moderate" support, e.g. $BS > 70/80$ is considered good, and $50/60 < BS < 70/80$ is considered moderate.

The Bootstrap proportion has variously been interpreted as:

1. A measure of reliability, telling us what would be expected to happen if we repeated our experiment;
2. A measure of accuracy, telling us about the probability of our experimental result being true; and
3. A measure of confidence, interpreted as a conditional probability similar to those in standard statistical hypothesis tests (i.e. measuring Type I errors or false positives).

(Morrison, 2014)

The phylogenetic tree of fishes *Mene maculata* and *Leiognathus equulus* were specifically analysed in the conducted study.

In the phylogenetic tree of *Mene maculata*, the specimen obtained is named as ON150753. The native place of *Mene maculata* is Southern/Eastern Africa making the place it's the type

locality. The dendrogram is analysed on the basis of type locality, bootstrap value and its similarities. Once the sequences are muscle aligned and a dendrogram is drawn, the species are shown in branches or clades in accordance with their similarities. Here the type locality specimen is named JF493879 and is found in East Africa. And the specimen for current study is obtained from Kerala. The clades are on different branches and Bootstrap value shows vast differentiations. Even if the Bootstrap values are not defined, the outgroup shows mild similarities to the voucher specimen. It can be observed that the outgroup specimen and the specimen under study are more similar than the voucher specimen obtained from the type locality. There is a chance that the voucher specimen comes under different species since they don't align with the type locality specimen. This could be because they are cryptic species or due to taxonomical ambiguities. Even though they are taxonomically identified as *Mene maculata*, the dendrogram drawn with the sequence obtained from GenBank shows that they are different.

In the phylogenetic tree of *Leiognathus equulus*, the voucher specimen is named as st5 Kerala India. The native place of *Leiognathus equulus* is Tranquebar, Tamil Nadu, making the region its type of locality. The voucher specimen is collected from Beypore Kerala. The dendrogram is analysed based on the similarities and differences between clades and Bootstrap values. Here species of similar interests or species with most similarities are shown in a clade. The ones with least similarities are shown in different branches. The type locality voucher is named as KX 147301 and is found in Tamil Nadu. If the difference in Bootstrap value is less than 40, one could say that the similarities between two species are very vague or they belong to two different species. Here an outgroup is chosen to show the significant difference between the two genus of fishes. The outgroup chosen is *Pellona ditchela* and it has null similarities to the genus *Leiognathus*. Since the similarities between the type locality voucher and voucher specimen is below 40, it can be said that even though they belong to the same genus, they are dissimilar. The similarities are vague and differences are more prominent in the dendrogram making it less divergent species of *Leiognathus*.

Both the specimen selected for study are generically identified as *Mene* and *Leiognathus*. But they are variant species of *Mene maculata* and *Leiognathus equulus* respectively due to taxonomical ambiguities. They can be classified as cryptic species.

Through this study, a reliable DNA barcode reference library for the marine fish in south India has been established, which could be used to assign fish species by screening sequences against it in the future. This could contribute to achieving better monitoring, conservation, and management of fisheries in this overexploited region.

CONCLUSION

DNA barcoding is a very useful tool for identifying unknown specimens. Identification of a fish can be done by taking a small sample from the specimen without causing much damage to them. Even with a small sample, it will be adequate to find the sequence and thus the unknown specimens can be identified. The DNA Barcoding method of identifying unknown species is extremely accurate. DNA barcoding uses genetic-level identification of species to get insight into their evolutionary relationships. Genetic material is the only thing that is transferred down to the next generation. So, as compared to traditional methods such as identifying fish using morphology, which can be misleading and has a higher possibility of error, analysis involving genetic material is significantly better. A phylogenetic tree might be used to interpret the results. A single glance reveals the clade or group to which the unknown species belongs. Phylogenetic trees are the most basic and easiest approach to understanding the results of DNA Barcoding.

The discriminatory power of COI barcodes is emphasized and their application to cases requiring species level resolution starting from unknown sequences. There are high results of reliability to DNA barcodes from public reference libraries, to identify species from different geographical origins. The ability to assign species with high precision from DNA samples of disparate quality and origin has major utility in several fields, from forensics, fisheries and conservation programs to control of fish products authenticity.

It is well known that no identification method (morphological, biochemical, genetic or whatsoever based) can truly identify species, because species are entities in continuous evolution and it is theoretically impossible to statically define such dynamic matter.

The results of the present study of DNA Barcoding will facilitate other lines of research in biodiversity assessments of unknown fishes.

The present study has also demonstrated that DNA barcoding holds great promise as a tool for rapid biodiversity assessment in unknown fishes.

From the dendrogram of *Mene maculata* and *Leiognathus equulus*, it can be observed that there is variation between the Bootstrap values of the type locality specimen and voucher specimen. There is a possibility that the voucher specimen comes under different species since they don't align with the type locality specimen. This could be because they are cryptic species or due to taxonomical ambiguities. Even though they are taxonomically identified as *Mene maculata* and *Leiognathus equulus*, the dendrogram drawn with the sequence obtained from GenBank shows that they are different. In the present study, both species are different from the other, even though they are taxonomically identified within the same genera Mene and Leiognatha, their similarities are very vague.

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COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICK AND DUCK EGGS



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partial fulfilment of requirement for the
degree of Bachelor of Science in Zoology

2021-2022

CERTIFICATE

This is to certify that the project report entitled "**COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICK AND DUCK EGGS**" submitted by Ms. DAYANA MARY M.M Reg. No. AB19ZOO021 in partial fulfilment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Soja Louis and this is her original effort.

Dr. Soja Louis

Assistant Professor & Head of Department

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Ernakulam

EXAMINERS

1).....

2).....

PLACE: ERNAKULAM

DATE: 09/05/2022

DECLARATION

I, Ms. Dayana Mary M.M, hereby declare that this project work entitled "**COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICK AND DUCK EGGS**" is submitted to St. Teresa's College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science Degree in Zoology. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own

Name: DAYANA MARY M.M

Signature

Reg.No: AB19ZOO021

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DAYANA MARY M.M

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ABSTRACT

COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICKEN AND DUCK EGGS

This experiment was conducted to study first 5 stages of embryonic development in three different birds. Chick, Duck, and Quail were used in the study. These birds have different hatching time. Chicken is one of the most common and widespread domesticated animal. One of the greatest miracles of nature is the transformation of egg into chicken. Egg laying is stimulated by long stretches of day light that occur during the warmer months. The time between Ovulation and Egg laying is approximately 23 to 26 hours. Fertilized embryos develop quickly, and chicks hatch approximately 21 days later. Ducks are called waterfowl. A duck have water-proof feathers. Duck eggs are notable because they are almost 50% larger than a large sized hen's egg. Duck shell have tiny holes that allow it to breathe. Respiratory gases as well as water vapour travel through these pores. Duck eggs have wide range of vitamins and minerals. Quails are very small birds. They have streak and buffed feathers in either blue, black, brown, cream or white color. Quail eggs are main source of protein.

The results shows that early stages of embryonic development in these birds are somewhat similar. Embryonic development of chick in first five days shows the following features; fully formed primitive streak, appearance of optic cup, brain and spinal cord, beginning of heart beat, development of all organs and separation of three toes. In embryonic development of duck, the appearance of primitive streak which extends to center of area pellucida, neural fold development, distinct head and trunk, formation of amniotic cavity etc were observed. Enlarged blastoderm, appearance of vitelline membrane, beginning

of blood circulation, appearance of eyes and C shaped embryo were the characters observed in first five days of quail embryonic development.

INTRODUCTION

Embryonic development in birds has been widely researched. The hatching time of birds' eggs is very diverse, ranging from 11 to 100 Days, which is mainly thought to be due to differences in embryonic development. Aim of the experiment was to compare the embryonic development in three different eggs of Quail, chicken and duck, which were easily available. An egg is the organic vessel containing the zygote in which an embryo develops until it can survive on its own, at which point the animal hatches.

QUAIL

Scientific name: *Coturnix coturnix*

Kingdom: Animalia

Phylum: Chordata

Class: Aves

Order: Galliformes

Superfamily: Phasianoidea

Quail is a collective name for several genera of mid-sized birds generally placed in the order Galliforms. Quail eggs are little thicker than normal eggs because they have an extra layer of thickness where the colour sites. The egg shell was soft, with colour between white to light sand colour, and a smooth texture which allows good deposition of colour spots, with different colour levels from black to brown spots. The egg size was smaller compared to chicken and duck eggs. It was about 1 ¼ inch long and 1 inch wide and its mass is 9gm. Generally Quail takes about 18 days of incubation, but they can hatch as early as day 16 or as late as 20days.

Quail eggs are considered a delicacy in many parts of the world, including Asia, Europe, and North America.

CHICKEN

Scientific name: *Gallus gallus domesticus*

Kingdom: Animalia

Phylum: Chordata

Class: Aves

Order: Galliformes

Family: Phasianidae

Chicken is a domesticated bird. Usually white hens lay white eggs, and brown hens lay brown eggs. Eggs that are not white have pigments deposited on them as the eggs travel through the hen's oviduct. Blue and other egg colours are formed as the egg develops and the colour appears on both the inside and outside of the shell. Eggs usually become fertile about four days after the rooster has been introduced to the hens.

Chicken eggs contain within a calcium carbonate-based hard shell, which is about 11% of the weight, the egg whites (albumen) and the egg yolk, separated by membranes. Eggs are nutritionally valuable due to their content of high-value proteins, fat, lecithin, vitamins and minerals. The structural components of the egg include the shell and shell membranes the albumen or white including the thick albumen, the outer thin albumen, the inner thin albumen, and the chalazae; and the yolk. The average egg length was 34.4mm, width 24.7mm and volume 10.6cm and its mass is 50gm. Egg takes about 21 days to hatch.

DUCK

Scientific name: *Anas platyrhynchos*

Kingdom: Animalia

Phylum: Chordata

Class: Aves

Order: Anseriformes

Superfamily: Anatoidea

Family: Anatidae

Duck is the common name for numerous species of waterfowl in the family Anatidae. Ducks are generally smaller and shorter-necked than swans and geese, which are members of the same family.

Duck eggs are notable because they're almost 50% larger than a large-sized hen's egg. They have a large, golden, creamy yolk, and many people love them for their rich, extra-egg flavour. Their shells are also a treat for the eyes. The colour depends on the breed of the duck, though the shell colour sometimes varies even within the same breed. Duck egg yolks get their orange-yellow colour from natural pigments called carotenoids. Size of a duck egg is about 5-7 inches and its mass is 70gm. It takes about 28 days for hatching.

Duck eggs are an excellent source of selenium, providing almost half of the daily value in one egg. Duck eggs also provide vitamin D, the "sunshine vitamin." Low levels of vitamin D are associated with depression and seasonal affective disorder.

All these three eggs had varied features and the hatching time was also different. Quail eggs hatch early compared to hen and duck eggs. The study focuses on the different developmental stages of quail, chicken and duck eggs and comparing it.

AIM

To compare the embryonic development in Chick, Duck and Quail using incubator.

OBJECTIVE

- To compare the embryonic development in chick, duck, quail using homemade incubator.
- To check the hatching rate of eggs belong to different strain.
- To demonstrate fertility using candling method and floating test.
- To observe the variation in hatching days.

REVIEW OF LITERATURE

Anna *et al.* (2012) Article explains how to practice floating test for detecting egg viability.

Doty (2011) her study explains about the Hamburger-Hamilton stages which are sequence of images depicting 46 chronological stages in chick development. In this study the images begin with a fertilized egg and end with a fully developed chick. The stages were determined by the number of somites and each stage was at an interval of three somites. Somites or segmented blocks of mesoderm, bud off sequentially during vertebrate development and can therefore be used as a timing landmark. The embryos used for the photographs were from different varieties of chickens: white leghorns, barred Plymouth Rock, and Rhode Island reds.

Jacob (2022) explained the parts of eggs in his article and mentioned that everything that an embryo needs to develop, grow and hatch must be provided in the egg when the egg is laid. If a hen receives sub-optimal feed, there may be a lot of developmental problems with the embryos when the eggs are incubated.

Warin (2009) explained in his article about embryonic development of chick egg, from day 1 to 21 using images of embryonic stages. It also explains the distinguishing features of infertile and fertile egg.

Smith (2004) explained in his article about the following topics that improve the producer's success. They are: Selection of hatching eggs, egg care and storage, incubators, incubating conditions, sanitation trouble shooting failures.

Incubation and Hatching techniques of eggs was explained by Archer *et al.* For successful hatch one must store and incubate eggs carefully. Incubation and hatching of eggs are influenced by factors such as environmental conditions, handling, sanitation and record keeping.

Ramteke *et al.* (2013) Article helps to study the embryonic development of Japanese quail and to identify the period of incubation where the Japanese quail embryo gives the faster ontogeny than chick embryo.

Mormio explains the embryonic development using the photo presentation from the Purdue Research Institute along with the authors own photos of candling eggs throughout the 21 chicken egg incubation period.

The Article is about candling Duck eggs. Candling is an old term that means the application of bright light to an egg to see what is inside. Originally it was done by using candles in a dark room. Now a day flashlight or an actual candling light are used for candling. Article includes pictures of developing duck eggs, from Day 1 to Day 25 and also the pictures of early dead embryo.

Ghali *et al* (2010) they presented a novel way of adapting the wellknown EC culture of whole chick embryos to time lapse imaging and to functional molecular studies using blocking agents. The novelty of their method stems from the ability to apply blocking agents *ex ovo* as well as *in ovo*. They were able to study the function of a set of molecules by culturing developing embryos *ex ovo* in tissue culture media containing these molecules or by injecting them underneath the live embryo *in ovo*. The *in ovo* preparation is particularly valuable since it extends the period of time during which the developmental function of the molecule can be studied and it provides an easy,

reproducible method for screening a batch of molecules. These new techniques will prove very helpful in visualizing and understanding the role of specific molecules during embryonic morphogenesis, including blood vessel formation. They attained two objectives in this study. First, they devised a flexible ex-ovo method that combines the ability to image the live, whole embryo with the application of blocking agents. Second, they devised a simple method for studying the function of secreted factors through the application of blocking agents.

One dilemma in comparing embryo development between poultry species is the absence of a common reference of sequential stages of morphogenetic development. To counter this problem, Sellier *et al* (2006) in his paper the normal table of embryogenesis was devised to accurately assess embryo development during the oviductal and incubation phases of embryogenesis. This paper is not only important for research on the morphogenetic development of the avian embryo, but it is also useful for investigators in the poultry industry attempting to uncover the basis of fertility and hatchability problems.

Givisiez *et al* (2020) their study addresses the main changes in metabolism and intestinal development throughout incubation, and also addresses scientific advances, limitations and future perspectives associated with the use of in ovo feeding that has been regarded as an important technology by the poultry industry.

ShanshanLi *et al* (2019) their study aimed to establish a comparison of complete morphological development staging for ducks (*Anas platyrhynchos*) and geese (*Anser cygnoides*) with the embryonic staging system by Hamburger and Hamilton (HH) for the chicken (*Gallus gallus*). Their results

show that morphological development in the chicken, duck, and goose are similar in the early stages. The major differences occurred after stage 27 of embryonic development, where the beak shape in ducks and geese was wider and longer than in chickens. In addition, the second and third interdigital webs of the hind limb of the chicken were found to be degraded from stage 31, and eventually vanished at stage 35; however, they were retained in ducks and geese. They established embryonic developmental staging systems for ducks and geese, from fertilization to hatching, which can be used in comparative studies with these 3 species.

Ainsworth *et al.* The results of this study demonstrate that there are only minimal differences identified in the rate of quail embryonic development when compared with chick embryos up to 5.5 days of incubation. Therefore, up to this period, chick and quail embryos can be directly compared using either incubation times or descriptive details and the stages used are identical (stages 4–28). After 5.5 days of incubation there is a slight increase in the developmental rate of the quail embryos and hence attributing equivalent stages to both chick and quail embryos based on incubation times is no longer possible. The overall descriptions for each stage (stages 29–35) are still similar, however, so comparisons are still possible. From 8.5 days of incubation the rate of development for the quail accelerates in comparison to the rate for the chick, so there are significant anatomical differences between chick and quail embryos at these time-points. Therefore, at these later stages of development (stages 36–46) the H.H Stage series is no longer comparable to the quail series in terms of either incubation times or morphological descriptions, making the quail series completely distinct for these stages. So in this paper they developed a definitive developmental stage series for Japanese quail so that differences are fully characterized, misconceptions or

assumptions are avoided, and the results of comparative studies are not distorted.

Guryeva *et al.* Their results showed that the hatching rate of quail in the flight group made up 38 % from the laboratory and 44 % from the synchronous control. Embryonic death during the incubation period both in the control and in the flight groups was caused by their position within the egg. Flight group embryos and hatched birds did not differ from the control groups in body size either. Body length of the flight group embryos at all stages of development was slightly less than in the control groups, but within the limits of norm. In the growth dynamics of extremities, the differences between the flight and control groups exceed the diapason of individual fluctuations in earth conditions.

Saraswati *et al.* This experiment was conducted to determine the development of Japanese quail embryo (*Coturnix coturnix japonica*), through observation and measurement of embryo organ development from the age of one day until hatching. The study used 15 female quails and 5 male quails. 15 female quails were divided into 5 cages, each cage containing 3 quails females and 1 male quail. Eggs which are inserted into an egg incubator is produced when the quail began the age of 3 months. Descriptive observation has been made towards the development of organs in the embryo. Based on the results of the study, the growth and development of quail embryo organs occur in stages until hatching occurred during the 16 days.

Dupuy *et al.* In this study, an objective means to stage development of the duck embryo is lacking, Such a staging procedure, is described there .The staging scheme presented there provides objective morphological criteria describing the embryonic development of the duck.

METHODOLOGY

MATERIALS REQUIRED

Styrofoam box, bulb, holder, wire, adapter, connector, switch board, 3 inch fan, 20 chicken eggs, 20 duck eggs, 20 quail eggs, lens, water, bowl, saw dust, nylon cable ties, Thermostat.

INCUBATOR

Incubator was set in a Styrofoam box. Entire top of the box was removed and saw dust was spread on the floor of the box. A hole was cut on one side of Styrofoam box. The hole contain the light bulb and socket. Then a socket with 12 volt bulb was inserted. Socket was fixed using nylon cable wire. Then a digital thermostat was added, and it was placed on the side of the box. Then sensor of the thermostat was inserted inside the box through a hole, on the side that is opposite to the bulb. A bulb, thermostat and adaptor was connected to the circuit. Since the main function of incubator was to keep the temperature and humidity inside at an optimum level a thermostat with high rate of accuracy was selected for the experiment. Thermostat was set in such a way that the bulb turns off, when the temperature inside the box exceeds 37.6C in the case of all the 3 type of eggs. A bowl of water was placed. It was the humidity source. Light was turned on and regular inspections of incubator was done during the study to monitor the humidity and temperature.

PROCEDURE

Fertile eggs can be hatched by using an egg incubator. After setting incubator, eggs were placed inside the incubator. Here 20 chicken, Duck, and Quail eggs were used. After setting the eggs, incubation process begins. An important part of it is turning or rotating the eggs. Eggs will need to be turned a minimum of 3 times per day. Mark one side of the egg with pencil that will help us to keep track of which eggs had been turned. Proper sanitation should be followed while touching the eggs. Incubator was closed.

DATA COLLECTION

Eggs are cracked at the top and a small portion of the shell is carefully removed to observe the embryonic development under a lens. Chick, duck and quill eggs are separately observed under the lens. Clear pictures of respective eggs are taken for comparison.

Then the analysis was done using candling method from day 6. Candling of eggs was done towards the middle of the incubation period from day 7-10, eggs can be candled to determine if the embryos are growing properly. It is done by simply shining a light through an egg. White or light colored shells are easy to candle while darker shells require brighter light.

Interior is clear for infertile eggs and they are removed.

A ring of red is visible within the egg, which contains a dead embryo. It was removed.

If there is blood vessels within the egg there is a live embryo inside. If broken eggs are see, it should be removed from the incubator. Stop turning the chicken, duck and quail eggs at day 18, 25, 14 of incubation respectively.

Chicken eggs required a hatching period of 21 days, whereas duck eggs require 28 days and Quail eggs require 18 days.

OBSERVATION AND RESULT

QUAIL EGG

20 eggs were used for incubation. On first day, a small portion of egg shell was cracked and observed that the blastodisc was located on the top of the yolk and appeared irregular in shape. It was an infertile egg. Another egg was taken and when observed it was found to be a fertile egg because, the germinal disc was at the blastodermal stage. The blastoderm was enlarged and the segmentation cavity under area pellucida attained a shape of dark ring and the area pellucida and area opaca were visible. On the second day, the egg yolk had more development. The blastoderm enlarged in size; the vitelline membrane was visible and the blastoderm had a donut shaped structure. On day 3, three eggs were cracked because, the first two eggs were infertile. In the third egg embryo was lying on its left side. The beginning of blood circulation in embryo can be observed; vitelline membrane spreads over the yolk surface. Head and trunk were distinct. Cardiac structure appeared, which begins to beat.

On day 4 development of amniotic cavity, which surround the embryo, can be observed. It was filled with amniotic fluid. It protects the embryo and allows it to move. Allantoic vesicle also appeared. Eyes were visible. On fifth day of development, the embryo attained a sensible increase in size; embryo formed a C shape; the head moved closer to the tail and limbs extended. From day 8-10 the development of embryo inside the egg was observed using candling method. On day 8, when candling was done, four eggs were found to be infertile. The next egg observed was fertile. The veins were clearly visible; the whole embryo was well defined and the beak appeared. The movement of embryo can be observed.

On ninth day, the toes are completely separated from each other. The beak and feet began to keratinize and the amniotic fluid decreased in quantity. On tenth day, more veining can be seen; but it was difficult to get a clear image. On the 12th day of incubation, floating test is done for all remaining eggs to determine that the embryo was live or dead. In that test, 3 eggs were removed because they were dead and settled under water. All others float on water and were wriggling. Those eggs contained a live embryo. On 14th day, the rotation of egg was stopped. Eggs were kept for lockdown period. On 18th day three eggs were hatched and on 19th 2 eggs were hatched. Thus a total of 5 eggs were hatched at the end of experiment.

Embryonic development in first 5 days of quail embryo:



A



B



C



D



E

Plate 1: A- DAY 1; B- DAY 2; C- DAY 3; D- DAY 4; E- DAY 5

CHICK EGG

20 eggs were placed in the incubator for hatching. On 1st day, when the top portion of the shell was cracked, egg yolk was visible and it was observed that, the chick embryo was oval in shape; The primitive streak was fully formed; area opaca and area pellucida were visible; area opaca further modified into area vasculosa and area vitellina. On day two, the completion of vitelline (extra embryonic) circulatory system and formation of two pairs of aortic arches occurred. Twisting of heart and formation of chambers was also observed. Vitelline vessels were visible; formation of optic cup, brain, and spinal chord; commencement of blood circulation; presence of amnion; tail and leg buds were visible. On day 3, one egg was found to be infertile. So it was removed from the incubator. Another fertile egg was taken and in that egg, heart began to beat and appearance of blood vessels can be observed. Amniotic tail fold was developed. It extended opposite to head fold. Eyes, lungs, and liver we're clearly visible. Atrium, vitelline vein, nerve chord, cranial flexure, mesenchyme and diencephalon can be observed.

On day 4, amniotic cavity was developed, which surround the embryo and it was filled with amniotic fluid. It protects the embryo and allowed it to move. Appearance of amniotic vesicle, plays a major role in calcium reabsorption, respiration, and waste storage. By the end of fourth day of incubation, the embryo had all the organs needed to sustain life after hatching. Most of the embryo parts can be identified in this stage. On 5th day optic vesicle became distinct. In the wings demarcation of elbow and knee joint was observed and the first 3 toes were separated. From 6th day onwards, candling was done.

On day 9, candling experiment was conducted, and observed the following features; air cells can be seen, the eye of embryo can be seen as dark round

spots, blood vessels were visible, embryo appeared like a dark mass and the movement of embryo inside the shell can be seen clearly. On 10th day of incubation, the embryo resembled a solid mass. The body Differentiation can be seen; Appearance of egg-tooth; toes and beak can be identified. The embryonal movements are clearly visible. On 12th day, the air cells grow as incubation progresses. The toes were fully formed and beak was keratinized. Body was lightly covered with feathers. Now, the embryo had the aspect of a chick. On 15th day of incubation, floating experiment was done and in that experiment 4 eggs settled down. That means the eggs were decayed, infertile, or the chick formed inside was dead. Those eggs were removed. Rotation of eggs was stopped at 18th day. On 20th day 4 eggs were hatched and on 21st day 6 eggs were hatched. A total of 10 eggs were hatched at the end of experiment.

Embryonic development in the first five days of chick embryo:



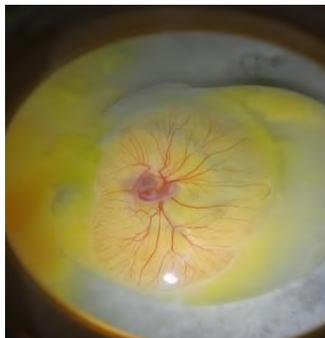
A



B



C



D



E

PLATE 2: A- DAY 1; B- DAY 2; C- DAY 3; D- DAY 4; E- DAY 5

DUCK EGG

20 eggs were used for observation. On day one, when the top portion of egg was cracked, it was observed, that the germinal disc was at the blastodermal stage. An embryonic shield was formed; primitive streak appeared and it extend to the center of pellucida. Area opaca and area pellucida can be distinguished very clearly. Area opaca further modified into area vasculosa and area vitellina. On day 2, area vasculosa and area vitellina become distinct. Primitive streak began to regress and neural fold starts to develop. On day three, first egg taken was infertile. In the next fertile egg taken, vitelline membrane spreads over the yolk surface; Embryo was lying on its left side. There was beginning of heart formation; the first indication of heart formation was defined by a paired primordia, along with primary optic vesicles. Blood circulation had started; cephalic bud, wing and leg buds were visible, neural tube developed and the head and trunk can be discerned.

On day 4, allantois appears and it became vesicular. Vitelline arteries were well developed and amniotic cavity was formed. On 5th day elbow and knee joints became distinct. On 9th, 10th, and 11th day candling experiment was done and in that it was observed that 2 eggs were infertile. So those eggs were removed. On 9th day it can be seen that the embryo became a dark mass. The eyes can be seen as a dark spot. Blood vessels spread inside the egg; and the air cell can be seen. The movement of embryo was visible, and the movement was very fast.

On 10th day, it was observed that, the embryo had development; its body was well developed. Appearance of beak and foot can be found. On day11, small feathers were formed on the body; the air cells had grown, beak and

foot growth progressed and the toes were differentiated. The movement of embryo slows down. Floating experiment was conducted on day 15 and in that experiment 3 embryos were found to be dead and they were removed. On 24th day, 3 egg had crack on shell. On 25th day floating test was again conducted and in that, 2 eggs were removed because the embryo was already dead. On 26th day of incubation, 4 eggs hatched and on 27th day 3 more eggs were hatched. A total of 7 eggs were hatched at the end of experiment.

Embryonic development in first 5 days of duck embryo:



A



B



C



D



E

PLATE 3: A- DAY 1; B- DAY 2; C- DAY 3; D- DAY 4; E- DAY 5

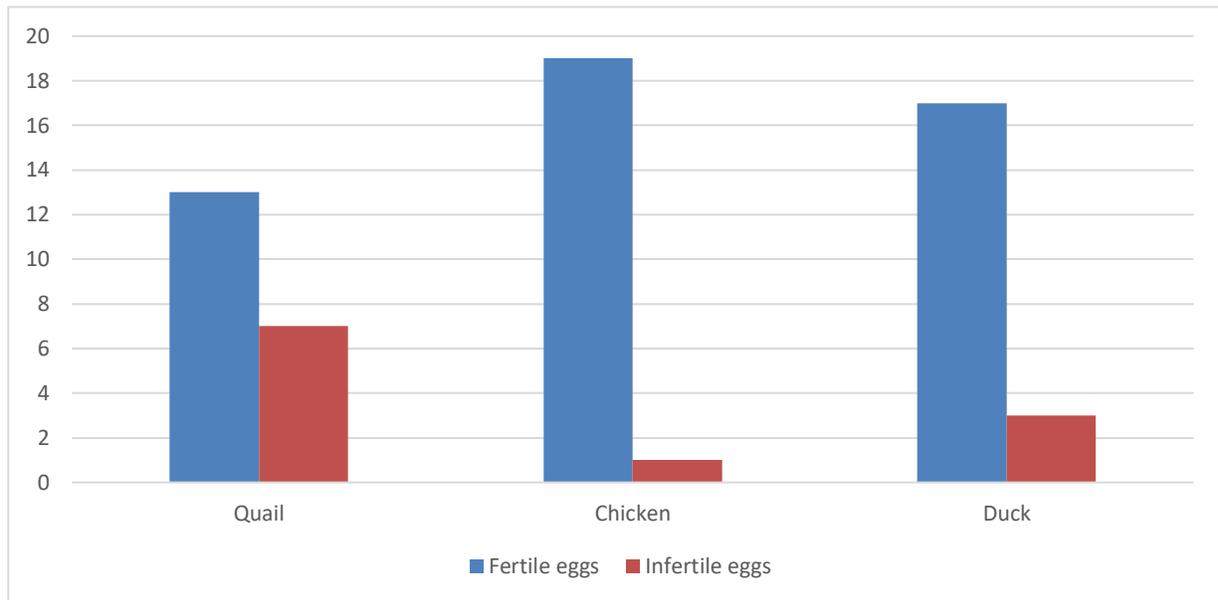
DAY	Strain		
	Quail	Chicken	Duck
Day 1	<ul style="list-style-type: none"> • Blastoderm is enlarged. • Segmentation cavity attains the shape of a dark ring. • Area opaca and area pellucida were visible. 	<ul style="list-style-type: none"> • Embryo is oval in shape • Primitive streak was fully formed • Area opaca and area pellucida were visible • Area opaca further modifies into area vasculosa and area vitellina. 	<ul style="list-style-type: none"> • Germinal disc at blastodermal stage. • Embryonic shield is formed • Primitive streak appears and extend to centre of area pellucida • Area opaca and area pellucida can be distinguished • Area opaca further modifies into area vasculosa and area vitellina
Day 2	<ul style="list-style-type: none"> • Blastoderm enlarges and attains a donut shape. • Vitelline membrane appears 	<ul style="list-style-type: none"> • Completion of vitelline circulatory system. • Formation of two pair of aortic arches. • Twisting of heart and formation of chambers • Visible Vitelline vessels • Formation of optic cup, brain, spinal chord • Commencement of blood circulation • Presence of amnion • Tail and leg bud visible 	<ul style="list-style-type: none"> • Area vasculosa and area vitellina are clearly distinguishable • Primitive streak began to regress • Neural fold starts to develop.

Day 3	<ul style="list-style-type: none"> • Embryo lying towards left • Beginning of blood circulation • Vitelline membrane spreads over yolk • Head and trunk distinguished • Cardiac structures began to beat 	<ul style="list-style-type: none"> • Starting of heart beat • Visible blood vessel • Amniotic tail fold developed • Clearly visible Eyes, lungs, and liver. • Atrium, Vitelline vein, nerve chord, cranial flexure, mesenchyme and diencephalon are visible. 	<ul style="list-style-type: none"> • Embryo lying towards left • Vitelline membrane spreads over yolk • Beginning of heart formation • Blood circulation start • Cephalic, wing and leg buds are visible. • Neural tube develops • Head and trunk are distinguishable
Day 4	<ul style="list-style-type: none"> • Amniotic cavity develops and gets filled with amniotic fluid • Allantoic vesicle appears • Eyes are visible 	<ul style="list-style-type: none"> • Amniotic cavity develops and gets filled with amniotic fluid. • Amniotic vesicle appears • Development of all organs needed to sustain life after hatching 	<ul style="list-style-type: none"> • Allantois appears and become vesicular • Amniotic cavity is formed • Vitelline arteries are well developed.
Day 5	<ul style="list-style-type: none"> • Embryo greatly increases in size • Embryo forms a c shape • Head moves closer to tail • Limbs extend 	<ul style="list-style-type: none"> • Distinct optic vesicle • Demarcation of elbow and knee joint is observable in wings • Separation of three toes 	<ul style="list-style-type: none"> • Elbow and knee joint become distinct.

Table 1: Showing embryonic development of quail, chicken, and duck during first 5 days of incubation.

SPECIES	FERTILE EGGS	INFERTILE EGGS
Quail	13	7
Chicken	19	1
Duck	17	3

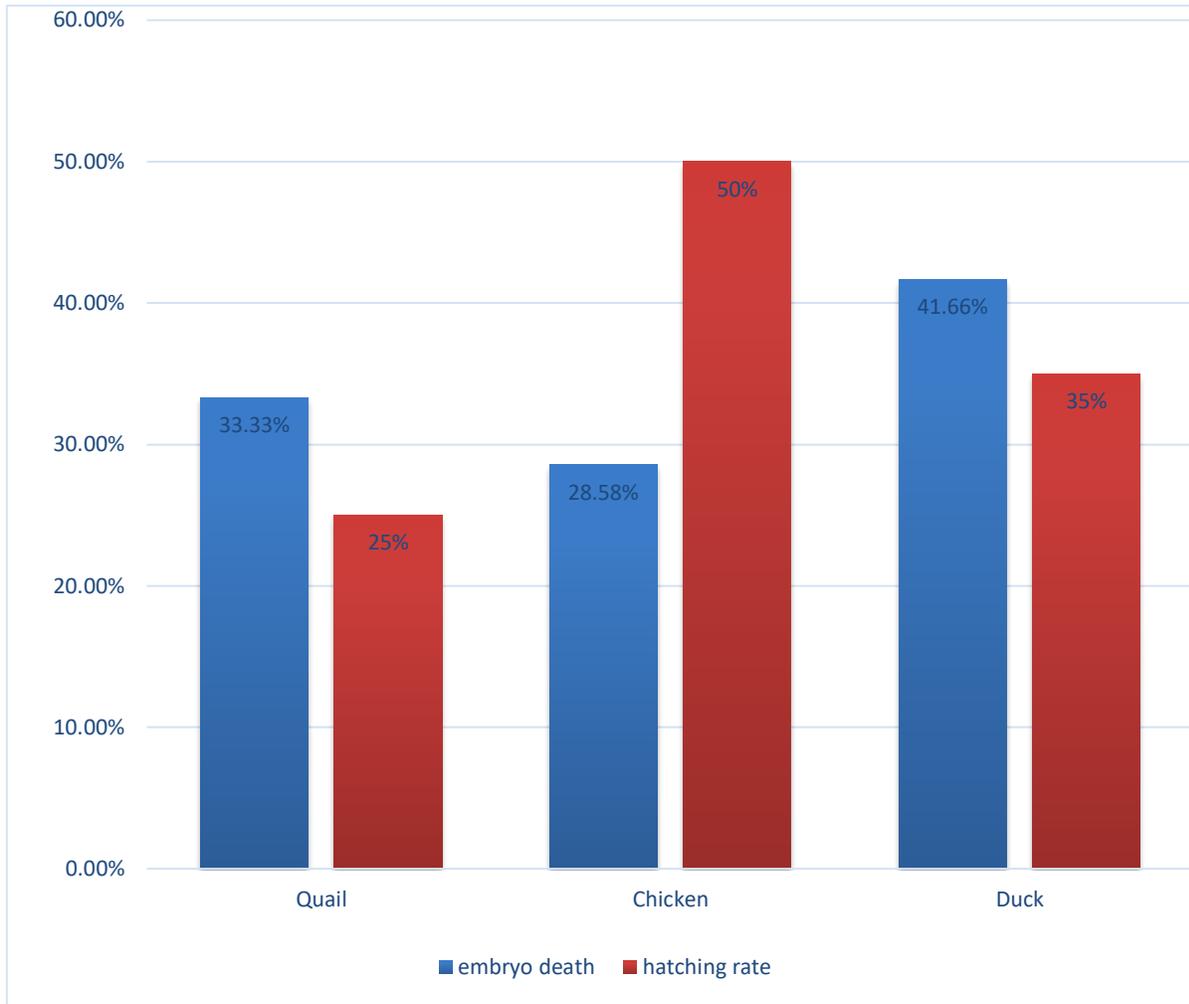
Table 2: Showing fertile and infertile eggs in each species



Graph 1: Showing the fertility and infertility of eggs

SPECIES	EMBRYO DEATH	HATCHING RATE
Quail	33.33%	25%
Chicken	28.6%	50%
Duck	41.7%	35%

Table 3: Showing embryo death and hatch rate of each species



Graph 2: Showing the embryo death and hatch rate of each species

DISCUSSION

Nearly 60 eggs were used in the development of Quail, Chicken and Duck using incubator working at same temperature. On the first day of embryonic development area opaca and area pellucida are clearly distinguishable in the three eggs. On day two vitelline membranes became visible. Commencement of blood circulation was observed on the second day in chick egg, but in quail and duck egg it was observed on day 3. Amniotic cavity develops and gets filled with amniotic fluid on day 4. On day 5 embryo forms a C shape, and head moves closer to tail in case of quail egg, whereas elbow and knee joints became distinct in chick and duck.

Similar kind of research were conducted by several scientists. The research conducted by ShanshanLi, ShibinBai, XiaQin, Junpeng Zhang, Irwin, Zhan, Zhewang (2019), aim to establish a comparison of complete morphological development staging for ducks and geese with the embryonic staging system by Hamburger and Hamilton for the chicken. The morphological development in chicken duck and goose are similar in the early stages.

According to the studies by Saraswati, Tana, faculty of science and mathematics, Diponegoro University Semarang, Indonesia, (2015)

Based on result the growth and development of quail embryo organs occur in stages until hatching occurred during the 16 days. Formation of blastoderm, differentiation of primitive streak mesoderm, circulatory system begins to develop, development of C shaped embryo were visible in the early days.

Ainsworth, Stanley and Evans, (2010) The results of this study demonstrate that there are only minimal differences Identified in the rate of quail embryonic

development When compared with chick embryos up to 5.5 days of incubation. Therefore, up to this period, chick and quail embryos can be directly compared using either incubation times or descriptive details and the stages used are identical.

Jacob explained about the parts of egg. Everything that an embryo needs to develop, grow and hatch must be provided in the egg when the egg is laid. Archer and Cartwright they explained about the important factors of incubating and hatching eggs like storage of eggs, environmental conditions, handling etc.

Warin explains about embryonic development of chick egg, from day 1 to 21 using images of embryonic stages. Also through this, we can distinguish an unfertile egg and a fertile egg.

Ramteke, charde, zade and Gabhane(2013) their article helps to study the embryonic development of Japanese Quail and to identify the period of incubation where the Japanese quail embryo gives the faster ontogeny than chick embryo.

CONCLUSION

The present work concludes by studying the specific developmental features of quail, duck and chicken embryos. Compared with the previous studies using chick, duck and quail, this study is mainly focussed on the embryonic development of these strains in first five days. The organogenetic sequence of quail, chicken and duck embryos are uniform; nevertheless, the incubation period of three species are different. Incubation period of chicken egg was 21 days whereas quail required 18 days and for duck egg it was 28 days. Fertility rate was high for chicken eggs when compared to quail and duck eggs. Out of 20 eggs 19, 17, 13 eggs were fertile for chick, duck and quail eggs respectively. Embryo death was mostly observed in late stages in duck eggs, with the reason being insufficient evaporation of water and the presence of comparatively thick shell. Hatching rate was found to be maximum in chicken eggs with 50% hatchability whereas, it is only 35% and 25% for duck and quail eggs respectively. Embryonic development in these three species is similar at early stages and difference occur only in late stages. Observation on the development of chick, duck, and, quail embryos can be used for the maintenance and management of these species.

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COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICK AND DUCK EGGS



Project work by
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Submitted to St. Teresa's college (Autonomous) Ernakulam
Affiliated to Mahatma Gandhi University, Kottayam
in partial fulfilment of requirement for the
degree of Bachelor of Science in Zoology

2021-2022

CERTIFICATE

This is to certify that the project report entitled "**COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICK AND DUCK EGGS**" submitted by Ms. Delwia Mary Reeta Reg. No. AB19ZOO022 in partial fulfilment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Soja Louis and this is her original effort.

Dr. Soja Louis
Assistant Professor & Head of Department
Department of Zoology
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Ernakulam

EXAMINERS

1).....

2).....

PLACE: ERNAKULAM

DATE: 09/05/2022

DECLARATION

I, Ms. Delwia Mary Reeta, hereby declare that this project work entitled **“COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICK AND DUCK EGGS”** is submitted to St. Teresa’s College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science Degree in Zoology. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own

Name: DELWIA MARY REETA

Signature

Reg.No: AB19ZOO022

ACKNOWLEDGEMENT

The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my project. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

I owe my deep gratitude to my project guide Dr. Soja Louis, who took keen interest on my project work and guided me all along, till the completion of my project work by providing all necessary information for developing a good system.

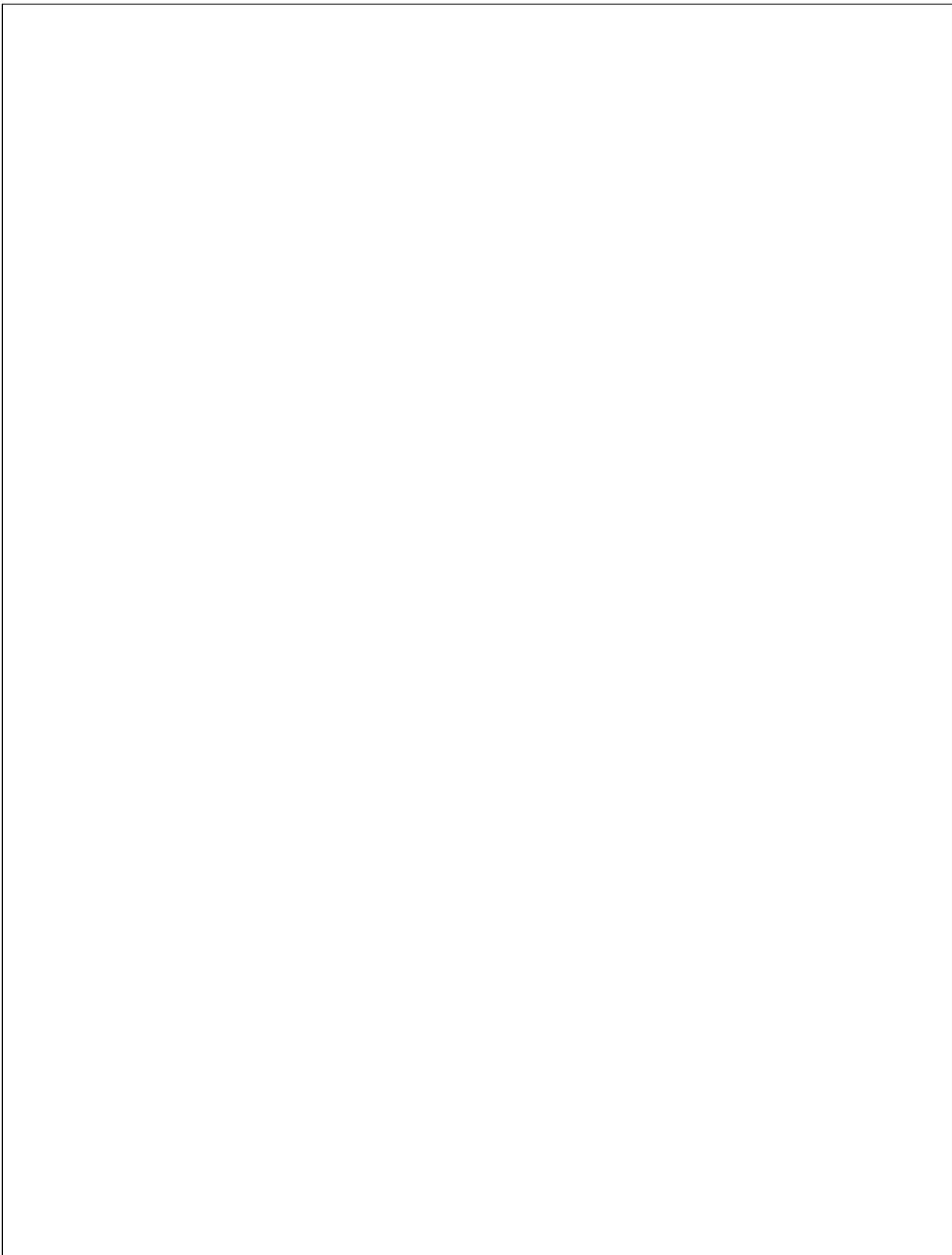
I take this opportunity to express my profound gratitude to my parents and friends who helped me a lot in finishing the project within the limited time. Also, I would like to extend my sincere esteems to all staff in laboratory for their timely support.

Last but not least I would like to thank God Almighty for the successful completion of my project.

DELWIA MARY REETA

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ABSTRACT

COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICKEN AND DUCK EGGS

This experiment was conducted to study first 5 stages of embryonic development in three different birds. Chick, Duck, and Quail were used in the study. These birds have different hatching time. Chicken is one of the most common and widespread domesticated animal. One of the greatest miracles of nature is the transformation of egg into chicken. Egg laying is stimulated by long stretches of day light that occur during the warmer months. The time between Ovulation and Egg laying is approximately 23 to 26 hours. Fertilized embryos develop quickly, and chicks hatch approximately 21 days later. Ducks are called waterfowl. A duck have water-proof feathers. Duck eggs are notable because they are almost 50% larger than a large sized hen's egg. Duck shell have tiny holes that allow it to breathe. Respiratory gases as well as water vapour travel through these pores. Duck eggs have wide range of vitamins and minerals. Quails are very small birds. They have streak and buffed feathers in either blue, black, brown, cream or white color. Quail eggs are main source of protein.

The results shows that early stages of embryonic development in these birds are somewhat similar. Embryonic development of chick in first five days shows the following features; fully formed primitive streak, appearance of optic cup, brain and spinal cord, beginning of heart beat, development of all organs and separation of three toes. In embryonic development of duck, the appearance of primitive streak which extends to center of area pellucida, neural fold development, distinct head and trunk, formation of amniotic cavity etc were observed. Enlarged blastoderm, appearance of vitelline membrane, beginning

of blood circulation, appearance of eyes and C shaped embryo were the characters observed in first five days of quail embryonic development.

INTRODUCTION

Embryonic development in birds has been widely researched. The hatching time of birds' eggs is very diverse, ranging from 11 to 100 Days, which is mainly thought to be due to differences in embryonic development. Aim of the experiment was to compare the embryonic development in three different eggs of Quail, chicken and duck, which were easily available. An egg is the organic vessel containing the zygote in which an embryo develops until it can survive on its own, at which point the animal hatches.

QUAIL

Scientific name: *Coturnix coturnix*

Kingdom: Animalia

Phylum: Chordata

Class: Aves

Order: Galliformes

Superfamily: Phasianoidea

Quail is a collective name for several genera of mid-sized birds generally placed in the order Galliforms. Quail eggs are little thicker than normal eggs because they have an extra layer of thickness where the colour sites. The egg shell was soft, with colour between white to light sand colour, and a smooth texture which allows good deposition of colour spots, with different colour levels from black to brown spots. The egg size was smaller compared to chicken and duck eggs. It was about 1 ¼ inch long and 1 inch wide and its mass is 9gm. Generally Quail takes about 18 days of incubation, but they can hatch as early as day 16 or as late as 20days.

Quail eggs are considered a delicacy in many parts of the world, including Asia, Europe, and North America.

CHICKEN

Scientific name: *Gallus gallus domesticus*

Kingdom: Animalia

Phylum: Chordata

Class: Aves

Order: Galliformes

Family: Phasianidae

Chicken is a domesticated bird. Usually white hens lay white eggs, and brown hens lay brown eggs. Eggs that are not white have pigments deposited on them as the eggs travel through the hen's oviduct. Blue and other egg colours are formed as the egg develops and the colour appears on both the inside and outside of the shell. Eggs usually become fertile about four days after the rooster has been introduced to the hens.

Chicken eggs contain within a calcium carbonate-based hard shell, which is about 11% of the weight, the egg whites (albumen) and the egg yolk, separated by membranes. Eggs are nutritionally valuable due to their content of high-value proteins, fat, lecithin, vitamins and minerals. The structural components of the egg include the shell and shell membranes the albumen or white including the thick albumen, the outer thin albumen, the inner thin albumen, and the chalazae; and the yolk. The average egg length was 34.4mm, width 24.7mm and volume 10.6cm and its mass is 50gm. Egg takes about 21 days to hatch.

DUCK

Scientific name: *Anas platyrhynchos*

Kingdom: Animalia

Phylum: Chordata

Class: Aves

Order: Anseriformes

Superfamily: Anatoidea

Family: Anatidae

Duck is the common name for numerous species of waterfowl in the family Anatidae. Ducks are generally smaller and shorter-necked than swans and geese, which are members of the same family.

Duck eggs are notable because they're almost 50% larger than a large-sized hen's egg. They have a large, golden, creamy yolk, and many people love them for their rich, extra-egg flavour. Their shells are also a treat for the eyes. The colour depends on the breed of the duck, though the shell colour sometimes varies even within the same breed. Duck egg yolks get their orange-yellow colour from natural pigments called carotenoids. Size of a duck egg is about 5-7 inches and its mass is 70gm. It takes about 28 days for hatching.

Duck eggs are an excellent source of selenium, providing almost half of the daily value in one egg. Duck eggs also provide vitamin D, the "sunshine vitamin." Low levels of vitamin D are associated with depression and seasonal affective disorder.

All these three eggs had varied features and the hatching time was also different. Quail eggs hatch early compared to hen and duck eggs. The study focuses on the different developmental stages of quail, chicken and duck eggs and comparing it.

AIM

To compare the embryonic development in Chick, Duck and Quail using incubator.

OBJECTIVE

- To compare the embryonic development in chick, duck, quail using homemade incubator.
- To check the hatching rate of eggs belong to different strain.
- To demonstrate fertility using candling method and floating test.
- To observe the variation in hatching days.

REVIEW OF LITERATURE

Anna *et al.* (2012) Article explains how to practice floating test for detecting egg viability.

Doty (2011) her study explains about the Hamburger-Hamilton stages which are sequence of images depicting 46 chronological stages in chick development. In this study the images begin with a fertilized egg and end with a fully developed chick. The stages were determined by the number of somites and each stage was at an interval of three somites. Somites or segmented blocks of mesoderm, bud off sequentially during vertebrate development and can therefore be used as a timing landmark. The embryos used for the photographs were from different varieties of chickens: white leghorns, barred Plymouth Rock, and Rhode Island reds.

Jacob (2022) explained the parts of eggs in his article and mentioned that everything that an embryo needs to develop, grow and hatch must be provided in the egg when the egg is laid. If a hen receives sub-optimal feed, there may be a lot of developmental problems with the embryos when the eggs are incubated.

Warin (2009) explained in his article about embryonic development of chick egg, from day 1 to 21 using images of embryonic stages. It also explains the distinguishing features of infertile and fertile egg.

Smith (2004) explained in his article about the following topics that improve the producer's success. They are: Selection of hatching eggs, egg care and storage, incubators, incubating conditions, sanitation trouble shooting failures.

Incubation and Hatching techniques of eggs was explained by Archer *et al.* For successful hatch one must store and incubate eggs carefully. Incubation and hatching of eggs are influenced by factors such as environmental conditions, handling, sanitation and record keeping.

Ramteke *et al.* (2013) Article helps to study the embryonic development of Japanese quail and to identify the period of incubation where the Japanese quail embryo gives the faster ontogeny than chick embryo.

Mormio explains the embryonic development using the photo presentation from the Purdue Research Institute along with the authors own photos of candling eggs throughout the 21 chicken egg incubation period.

The Article is about candling Duck eggs. Candling is an old term that means the application of bright light to an egg to see what is inside. Originally it was done by using candles in a dark room. Now a day flashlight or an actual candling light are used for candling. Article includes pictures of developing duck eggs, from Day 1 to Day 25 and also the pictures of early dead embryo.

Ghali *et al* (2010) they presented a novel way of adapting the wellknown EC culture of whole chick embryos to time lapse imaging and to functional molecular studies using blocking agents. The novelty of their method stems from the ability to apply blocking agents *ex ovo* as well as *in ovo*. They were able to study the function of a set of molecules by culturing developing embryos *ex ovo* in tissue culture media containing these molecules or by injecting them underneath the live embryo *in ovo*. The *in ovo* preparation is particularly valuable since it extends the period of time during which the developmental function of the molecule can be studied and it provides an easy,

reproducible method for screening a batch of molecules. These new techniques will prove very helpful in visualizing and understanding the role of specific molecules during embryonic morphogenesis, including blood vessel formation. They attained two objectives in this study. First, they devised a flexible ex-ovo method that combines the ability to image the live, whole embryo with the application of blocking agents. Second, they devised a simple method for studying the function of secreted factors through the application of blocking agents.

One dilemma in comparing embryo development between poultry species is the absence of a common reference of sequential stages of morphogenetic development. To counter this problem, Sellier *et al* (2006) in his paper the normal table of embryogenesis was devised to accurately assess embryo development during the oviductal and incubation phases of embryogenesis. This paper is not only important for research on the morphogenetic development of the avian embryo, but it is also useful for investigators in the poultry industry attempting to uncover the basis of fertility and hatchability problems.

Givisiez *et al* (2020) their study addresses the main changes in metabolism and intestinal development throughout incubation, and also addresses scientific advances, limitations and future perspectives associated with the use of in ovo feeding that has been regarded as an important technology by the poultry industry.

ShanshanLi *et al* (2019) their study aimed to establish a comparison of complete morphological development staging for ducks (*Anas platyrhynchos*) and geese (*Anser cygnoides*) with the embryonic staging system by Hamburger and Hamilton (HH) for the chicken (*Gallus gallus*). Their results

show that morphological development in the chicken, duck, and goose are similar in the early stages. The major differences occurred after stage 27 of embryonic development, where the beak shape in ducks and geese was wider and longer than in chickens. In addition, the second and third interdigital webs of the hind limb of the chicken were found to be degraded from stage 31, and eventually vanished at stage 35; however, they were retained in ducks and geese. They established embryonic developmental staging systems for ducks and geese, from fertilization to hatching, which can be used in comparative studies with these 3 species.

Ainsworth *et al.* The results of this study demonstrate that there are only minimal differences identified in the rate of quail embryonic development when compared with chick embryos up to 5.5 days of incubation. Therefore, up to this period, chick and quail embryos can be directly compared using either incubation times or descriptive details and the stages used are identical (stages 4–28). After 5.5 days of incubation there is a slight increase in the developmental rate of the quail embryos and hence attributing equivalent stages to both chick and quail embryos based on incubation times is no longer possible. The overall descriptions for each stage (stages 29–35) are still similar, however, so comparisons are still possible. From 8.5 days of incubation the rate of development for the quail accelerates in comparison to the rate for the chick, so there are significant anatomical differences between chick and quail embryos at these time-points. Therefore, at these later stages of development (stages 36–46) the H.H Stage series is no longer comparable to the quail series in terms of either incubation times or morphological descriptions, making the quail series completely distinct for these stages. So in this paper they developed a definitive developmental stage series for Japanese quail so that differences are fully characterized, misconceptions or

assumptions are avoided, and the results of comparative studies are not distorted.

Guryeva *et al.* Their results showed that the hatching rate of quail in the flight group made up 38 % from the laboratory and 44 % from the synchronous control. Embryonic death during the incubation period both in the control and in the flight groups was caused by their position within the egg. Flight group embryos and hatched birds did not differ from the control groups in body size either. Body length of the flight group embryos at all stages of development was slightly less than in the control groups, but within the limits of norm. In the growth dynamics of extremities, the differences between the flight and control groups exceed the diapason of individual fluctuations in earth conditions.

Saraswati *et al.* This experiment was conducted to determine the development of Japanese quail embryo (*Coturnix coturnix japonica*), through observation and measurement of embryo organ development from the age of one day until hatching. The study used 15 female quails and 5 male quails. 15 female quails were divided into 5 cages, each cage containing 3 quails females and 1 male quail. Eggs which are inserted into an egg incubator is produced when the quail began the age of 3 months. Descriptive observation has been made towards the development of organs in the embryo. Based on the results of the study, the growth and development of quail embryo organs occur in stages until hatching occurred during the 16 days.

Dupuy *et al.* In this study, an objective means to stage development of the duck embryo is lacking, Such a staging procedure, is described there .The staging scheme presented there provides objective morphological criteria describing the embryonic development of the duck.

METHODOLOGY

MATERIALS REQUIRED

Styrofoam box, bulb, holder, wire, adapter, connector, switch board, 3 inch fan, 20 chicken eggs, 20 duck eggs, 20 quail eggs, lens, water, bowl, saw dust, nylon cable ties, Thermostat.

INCUBATOR

Incubator was set in a Styrofoam box. Entire top of the box was removed and saw dust was spread on the floor of the box. A hole was cut on one side of Styrofoam box. The hole contain the light bulb and socket. Then a socket with 12 volt bulb was inserted. Socket was fixed using nylon cable wire. Then a digital thermostat was added, and it was placed on the side of the box. Then sensor of the thermostat was inserted inside the box through a hole, on the side that is opposite to the bulb. A bulb, thermostat and adaptor was connected to the circuit. Since the main function of incubator was to keep the temperature and humidity inside at an optimum level a thermostat with high rate of accuracy was selected for the experiment. Thermostat was set in such a way that the bulb turns off, when the temperature inside the box exceeds 37.6C in the case of all the 3 type of eggs. A bowl of water was placed. It was the humidity source. Light was turned on and regular inspections of incubator was done during the study to monitor the humidity and temperature.

PROCEDURE

Fertile eggs can be hatched by using an egg incubator. After setting incubator, eggs were placed inside the incubator. Here 20 chicken, Duck, and Quail eggs were used. After setting the eggs, incubation process begins. An important part of it is turning or rotating the eggs. Eggs will need to be turned a minimum of 3 times per day. Mark one side of the egg with pencil that will help us to keep track of which eggs had been turned. Proper sanitation should be followed while touching the eggs. Incubator was closed.

DATA COLLECTION

Eggs are cracked at the top and a small portion of the shell is carefully removed to observe the embryonic development under a lens. Chick, duck and quill eggs are separately observed under the lens. Clear pictures of respective eggs are taken for comparison.

Then the analysis was done using candling method from day 6. Candling of eggs was done towards the middle of the incubation period from day 7-10, eggs can be candled to determine if the embryos are growing properly. It is done by simply shining a light through an egg. White or light colored shells are easy to candle while darker shells require brighter light.

Interior is clear for infertile eggs and they are removed.

A ring of red is visible within the egg, which contains a dead embryo. It was removed.

If there is blood vessels within the egg there is a live embryo inside. If broken eggs are see, it should be removed from the incubator. Stop turning the chicken, duck and quail eggs at day 18, 25, 14 of incubation respectively.

Chicken eggs required a hatching period of 21 days, whereas duck eggs require 28 days and Quail eggs require 18 days.

OBSERVATION AND RESULT

QUAIL EGG

20 eggs were used for incubation. On first day, a small portion of egg shell was cracked and observed that the blastodisc was located on the top of the yolk and appeared irregular in shape. It was an infertile egg. Another egg was taken and when observed it was found to be a fertile egg because, the germinal disc was at the blastodermal stage. The blastoderm was enlarged and the segmentation cavity under area pellucida attained a shape of dark ring and the area pellucida and area opaca were visible. On the second day, the egg yolk had more development. The blastoderm enlarged in size; the vitelline membrane was visible and the blastoderm had a donut shaped structure. On day 3, three eggs were cracked because, the first two eggs were infertile. In the third egg embryo was lying on its left side. The beginning of blood circulation in embryo can be observed; vitelline membrane spreads over the yolk surface. Head and trunk were distinct. Cardiac structure appeared, which begins to beat.

On day 4 development of amniotic cavity, which surround the embryo, can be observed. It was filled with amniotic fluid. It protects the embryo and allows it to move. Allantoic vesicle also appeared. Eyes were visible. On fifth day of development, the embryo attained a sensible increase in size; embryo formed a C shape; the head moved closer to the tail and limbs extended. From day 8-10 the development of embryo inside the egg was observed using candling method. On day 8, when candling was done, four eggs were found to be infertile. The next egg observed was fertile. The veins were clearly visible; the whole embryo was well defined and the beak appeared. The movement of embryo can be observed.

On ninth day, the toes are completely separated from each other. The beak and feet began to keratinize and the amniotic fluid decreased in quantity. On tenth day, more veining can be seen; but it was difficult to get a clear image. On the 12th day of incubation, floating test is done for all remaining eggs to determine that the embryo was live or dead. In that test, 3 eggs were removed because they were dead and settled under water. All others float on water and were wriggling. Those eggs contained a live embryo. On 14th day, the rotation of egg was stopped. Eggs were kept for lockdown period. On 18th day three eggs were hatched and on 19th 2 eggs were hatched. Thus a total of 5 eggs were hatched at the end of experiment.

Embryonic development in first 5 days of quail embryo:



A



B



C



D



E

Plate 1: A- DAY 1; B- DAY 2; C- DAY 3; D- DAY 4; E- DAY 5

CHICK EGG

20 eggs were placed in the incubator for hatching. On 1st day, when the top portion of the shell was cracked, egg yolk was visible and it was observed that, the chick embryo was oval in shape; The primitive streak was fully formed; area opaca and area pellucida were visible; area opaca further modified into area vasculosa and area vitellina. On day two, the completion of vitelline (extra embryonic) circulatory system and formation of two pairs of aortic arches occurred. Twisting of heart and formation of chambers was also observed. Vitelline vessels were visible; formation of optic cup, brain, and spinal chord; commencement of blood circulation; presence of amnion; tail and leg buds were visible. On day 3, one egg was found to be infertile. So it was removed from the incubator. Another fertile egg was taken and in that egg, heart began to beat and appearance of blood vessels can be observed. Amniotic tail fold was developed. It extended opposite to head fold. Eyes, lungs, and liver we're clearly visible. Atrium, vitelline vein, nerve chord, cranial flexure, mesenchyme and diencephalon can be observed.

On day 4, amniotic cavity was developed, which surround the embryo and it was filled with amniotic fluid. It protects the embryo and allowed it to move. Appearance of amniotic vesicle, plays a major role in calcium reabsorption, respiration, and waste storage. By the end of fourth day of incubation, the embryo had all the organs needed to sustain life after hatching. Most of the embryo parts can be identified in this stage. On 5th day optic vesicle became distinct. In the wings demarcation of elbow and knee joint was observed and the first 3 toes were separated. From 6th day onwards, candling was done.

On day 9, candling experiment was conducted, and observed the following features; air cells can be seen, the eye of embryo can be seen as dark round

spots, blood vessels were visible, embryo appeared like a dark mass and the movement of embryo inside the shell can be seen clearly. On 10th day of incubation, the embryo resembled a solid mass. The body Differentiation can be seen; Appearance of egg-tooth; toes and beak can be identified. The embryonal movements are clearly visible. On 12th day, the air cells grow as incubation progresses. The toes were fully formed and beak was keratinized. Body was lightly covered with feathers. Now, the embryo had the aspect of a chick. On 15th day of incubation, floating experiment was done and in that experiment 4 eggs settled down. That means the eggs were decayed, infertile, or the chick formed inside was dead. Those eggs were removed. Rotation of eggs was stopped at 18th day. On 20th day 4 eggs were hatched and on 21st day 6 eggs were hatched. A total of 10 eggs were hatched at the end of experiment.

Embryonic development in the first five days of chick embryo:



A



B



C



D



E

PLATE 2: A- DAY 1; B- DAY 2; C- DAY 3; D- DAY 4; E- DAY 5

DUCK EGG

20 eggs were used for observation. On day one, when the top portion of egg was cracked, it was observed, that the germinal disc was at the blastodermal stage. An embryonic shield was formed; primitive streak appeared and it extend to the center of pellucida. Area opaca and area pellucida can be distinguished very clearly. Area opaca further modified into area vasculosa and area vitellina. On day 2, area vasculosa and area vitellina become distinct. Primitive streak began to regress and neural fold starts to develop. On day three, first egg taken was infertile. In the next fertile egg taken, vitelline membrane spreads over the yolk surface; Embryo was lying on its left side. There was beginning of heart formation; the first indication of heart formation was defined by a paired primordia, along with primary optic vesicles. Blood circulation had started; cephalic bud, wing and leg buds were visible, neural tube developed and the head and trunk can be discerned.

On day 4, allantois appears and it became vesicular. Vitelline arteries were well developed and amniotic cavity was formed. On 5th day elbow and knee joints became distinct. On 9th, 10th, and 11th day candling experiment was done and in that it was observed that 2 eggs were infertile. So those eggs were removed. On 9th day it can be seen that the embryo became a dark mass. The eyes can be seen as a dark spot. Blood vessels spread inside the egg; and the air cell can be seen. The movement of embryo was visible, and the movement was very fast.

On 10th day, it was observed that, the embryo had development; its body was well developed. Appearance of beak and foot can be found. On day11, small feathers were formed on the body; the air cells had grown, beak and

foot growth progressed and the toes were differentiated. The movement of embryo slows down. Floating experiment was conducted on day 15 and in that experiment 3 embryos were found to be dead and they were removed. On 24th day, 3 egg had crack on shell. On 25th day floating test was again conducted and in that, 2 eggs were removed because the embryo was already dead. On 26th day of incubation, 4 eggs hatched and on 27th day 3 more eggs were hatched. A total of 7 eggs were hatched at the end of experiment.

Embryonic development in first 5 days of duck embryo:



A



B



C



D



E

PLATE 3: A- DAY 1; B- DAY 2; C- DAY 3; D- DAY 4; E- DAY 5

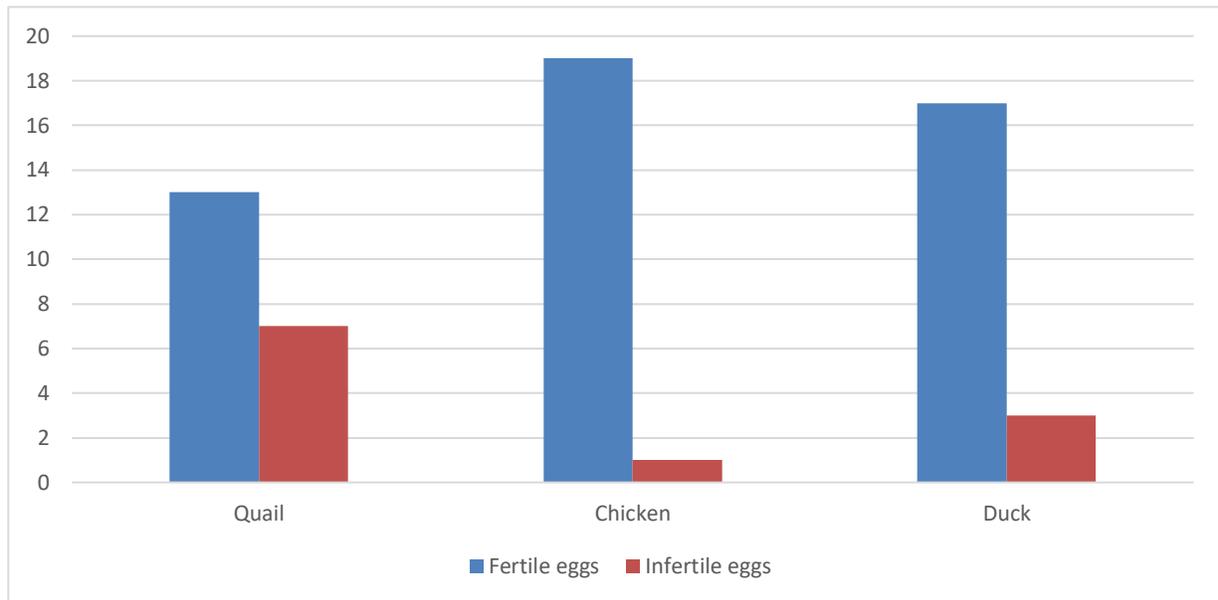
DAY	Strain		
	Quail	Chicken	Duck
Day 1	<ul style="list-style-type: none"> • Blastoderm is enlarged. • Segmentation cavity attains the shape of a dark ring. • Area opaca and area pellucida were visible. 	<ul style="list-style-type: none"> • Embryo is oval in shape • Primitive streak was fully formed • Area opaca and area pellucida were visible • Area opaca further modifies into area vasculosa and area vitellina. 	<ul style="list-style-type: none"> • Germinal disc at blastodermal stage. • Embryonic shield is formed • Primitive streak appears and extend to centre of area pellucida • Area opaca and area pellucida can be distinguished • Area opaca further modifies into area vasculosa and area vitellina
Day 2	<ul style="list-style-type: none"> • Blastoderm enlarges and attains a donut shape. • Vitelline membrane appears 	<ul style="list-style-type: none"> • Completion of vitelline circulatory system. • Formation of two pair of aortic arches. • Twisting of heart and formation of chambers • Visible Vitelline vessels • Formation of optic cup, brain, spinal chord • Commencement of blood circulation • Presence of amnion • Tail and leg bud visible 	<ul style="list-style-type: none"> • Area vasculosa and area vitellina are clearly distinguishable • Primitive streak began to regress • Neural fold starts to develop.

Day 3	<ul style="list-style-type: none"> • Embryo lying towards left • Beginning of blood circulation • Vitelline membrane spreads over yolk • Head and trunk distinguished • Cardiac structures began to beat 	<ul style="list-style-type: none"> • Starting of heart beat • Visible blood vessel • Amniotic tail fold developed • Clearly visible Eyes, lungs, and liver. • Atrium, Vitelline vein, nerve chord, cranial flexure, mesenchyme and diencephalon are visible. 	<ul style="list-style-type: none"> • Embryo lying towards left • Vitelline membrane spreads over yolk • Beginning of heart formation • Blood circulation start • Cephalic, wing and leg buds are visible. • Neural tube develops • Head and trunk are distinguishable
Day 4	<ul style="list-style-type: none"> • Amniotic cavity develops and gets filled with amniotic fluid • Allantoic vesicle appears • Eyes are visible 	<ul style="list-style-type: none"> • Amniotic cavity develops and gets filled with amniotic fluid. • Amniotic vesicle appears • Development of all organs needed to sustain life after hatching 	<ul style="list-style-type: none"> • Allantois appears and become vesicular • Amniotic cavity is formed • Vitelline arteries are well developed.
Day 5	<ul style="list-style-type: none"> • Embryo greatly increases in size • Embryo forms a c shape • Head moves closer to tail • Limbs extend 	<ul style="list-style-type: none"> • Distinct optic vesicle • Demarcation of elbow and knee joint is observable in wings • Separation of three toes 	<ul style="list-style-type: none"> • Elbow and knee joint become distinct.

Table 1: Showing embryonic development of quail, chicken, and duck during first 5 days of incubation.

SPECIES	FERTILE EGGS	INFERTILE EGGS
Quail	13	7
Chicken	19	1
Duck	17	3

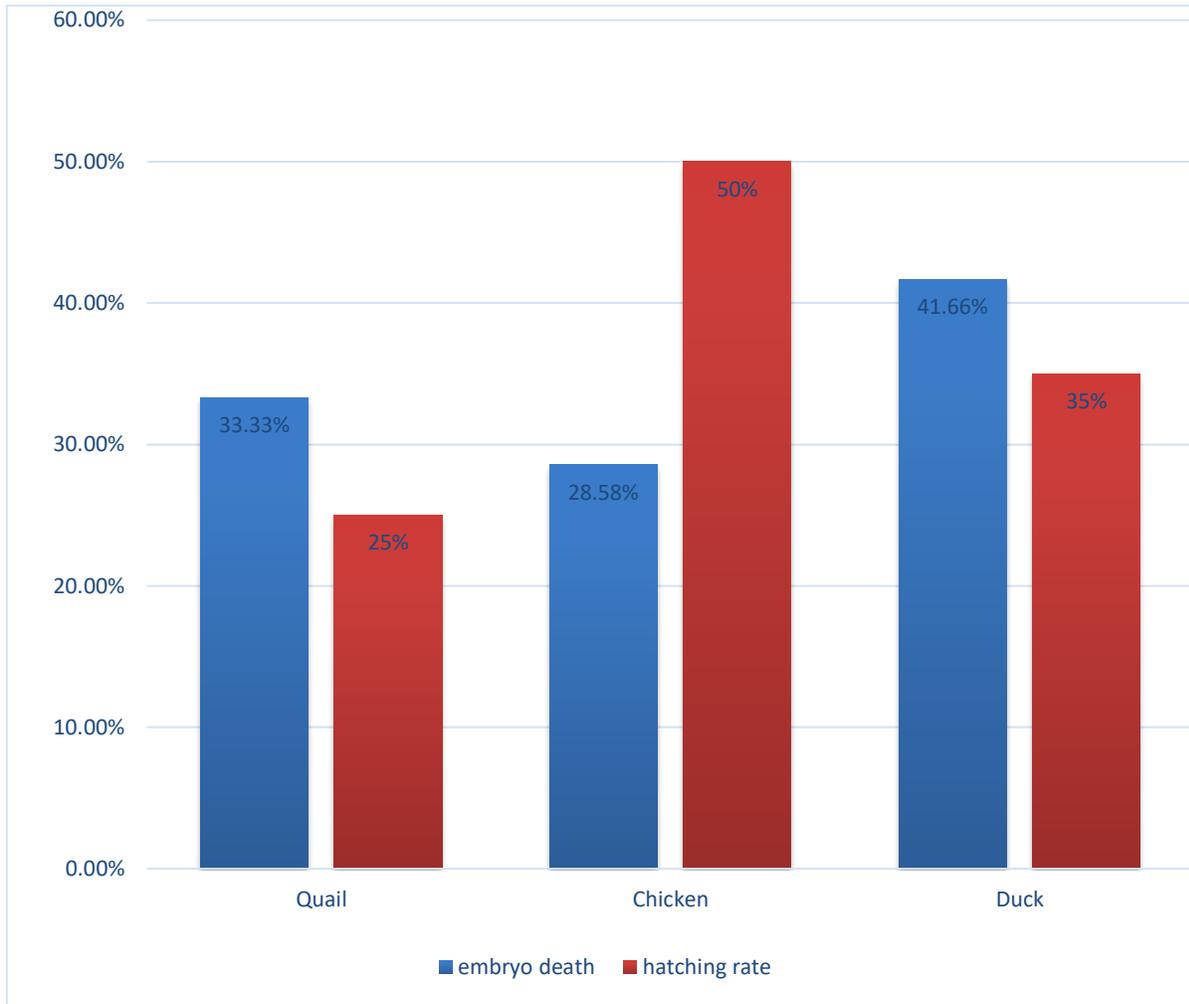
Table 2: Showing fertile and infertile eggs in each species



Graph 1: Showing the fertility and infertility of eggs

SPECIES	EMBRYO DEATH	HATCHING RATE
Quail	33.33%	25%
Chicken	28.6%	50%
Duck	41.7%	35%

Table 3: Showing embryo death and hatch rate of each species



Graph 2: Showing the embryo death and hatch rate of each species

DISCUSSION

Nearly 60 eggs were used in the development of Quail, Chicken and Duck using incubator working at same temperature. On the first day of embryonic development area opaca and area pellucida are clearly distinguishable in the three eggs. On day two vitelline membranes became visible. Commencement of blood circulation was observed on the second day in chick egg, but in quail and duck egg it was observed on day 3. Amniotic cavity develops and gets filled with amniotic fluid on day 4. On day 5 embryo forms a C shape, and head moves closer to tail in case of quail egg, whereas elbow and knee joints became distinct in chick and duck.

Similar kind of research were conducted by several scientists. The research conducted by ShanshanLi, ShibinBai, XiaQin, Junpeng Zhang, Irwin, Zhan, Zhewang (2019), aim to establish a comparison of complete morphological development staging for ducks and geese with the embryonic staging system by Hamburger and Hamilton for the chicken. The morphological development in chicken duck and goose are similar in the early stages.

According to the studies by Saraswati, Tana, faculty of science and mathematics, Diponegoro University Semarang, Indonesia, (2015)

Based on result the growth and development of quail embryo organs occur in stages until hatching occurred during the 16 days. Formation of blastoderm, differentiation of primitive streak mesoderm, circulatory system begins to develop, development of C shaped embryo were visible in the early days.

Ainsworth, Stanley and Evans, (2010) The results of this study demonstrate that there are only minimal differences Identified in the rate of quail embryonic

development When compared with chick embryos up to 5.5 days of incubation. Therefore, up to this period, chick and quail embryos can be directly compared using either incubation times or descriptive details and the stages used are identical.

Jacob explained about the parts of egg. Everything that an embryo needs to develop, grow and hatch must be provided in the egg when the egg is laid. Archer and Cartwright they explained about the important factors of incubating and hatching eggs like storage of eggs, environmental conditions, handling etc.

Warin explains about embryonic development of chick egg, from day 1 to 21 using images of embryonic stages. Also through this, we can distinguish an unfertile egg and a fertile egg.

Ramteke, charde, zade and Gabhane(2013) their article helps to study the embryonic development of Japanese Quail and to identify the period of incubation where the Japanese quail embryo gives the faster ontogeny than chick embryo.

CONCLUSION

The present work concludes by studying the specific developmental features of quail, duck and chicken embryos. Compared with the previous studies using chick, duck and quail, this study is mainly focussed on the embryonic development of these strains in first five days. The organogenetic sequence of quail, chicken and duck embryos are uniform; nevertheless, the incubation period of three species are different. Incubation period of chicken egg was 21 days whereas quail required 18 days and for duck egg it was 28 days. Fertility rate was high for chicken eggs when compared to quail and duck eggs. Out of 20 eggs 19, 17, 13 eggs were fertile for chick, duck and quail eggs respectively. Embryo death was mostly observed in late stages in duck eggs, with the reason being insufficient evaporation of water and the presence of comparatively thick shell. Hatching rate was found to be maximum in chicken eggs with 50% hatchability whereas, it is only 35% and 25% for duck and quail eggs respectively. Embryonic development in these three species is similar at early stages and difference occur only in late stages. Observation on the development of chick, duck, and, quail embryos can be used for the maintenance and management of these species.

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AGE DETERMINATION AND MOBILITY
STUDIES IN FISHES USING SCALES



Project Work by

DIYA SABU

REG.NO: AB19ZOO023

UNDER THE GUIDANCE OF

Dr. SOJA LOUIS

DEPARTMENT OF ZOOLOGY

ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM

Submitted to St. Teresa's college (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfillment of the requirements of Bachelor of Science degree in Zoology.

2019 - 2022

CERTIFICATE

This is to certify that the project report entitled “**AGE DETERMINATION AND MOBILITY STUDIES IN FISHES USING SCALES**” submitted by **DIYA SABU**, Reg. No. **AB19ZOO023** in partial fulfillment of the requirements of

Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Soja Louis and this is her original effort.

Dr. Soja Louis
Assistant Professor & Head of the Department
Department of Zoology
St Teresa's College (Autonomous)
St. Teresa's college
Ernakulam

EXAMINERS

1)

2)

DECLARATION

I, Ms. DIYA SABU, hereby declare that this project report entitled “**AGE DETERMINATION AND MOBILITY STUDIES IN FISHES USING SCALES**” is a bonafide record of work done by me during the academic year 2021-2022 in partial fulfilment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam.

This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

AB19ZOO023

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ABSTRACT

Ocean biome is a largest of all, covering almost three-quarters of the planets surface. It includes a wide variety of marine habitats. Fishes are vertebrates that have always lived underwater since the evolution of the earliest ancestors around 530 million years ago. Throughout earth's history one constant challenge for all animals is been attaining protection from the elements, predators and microorganisms. In ancestral animals of fishes and reptiles a robust preserve form of integuments, scales etc arose. It has a variety of functions including protection and locomotory assistance. Most fishes have scales that protect their delicate skin. Each scale is separate but the scales overlap like tiles on a roof to allow movement. Scales originated within ostracoderms, the ancestor to all fishes today. Cycloid scales of salmon and carp, ctenoid scales of perch, ganoid scales of surgeons and gars, placoid scales of sharks and rays are examples. Fish scales primarily serves two purpose: protection and locomotion. These slippery scales present in the body, protect their body from the environment, parasites, and predators. Scales develops as external growth of the skin on the epidermis. The scales reduce the friction with the water. What distinguishes the scales from each other is both their composition and how they balance those two functions. The more evolved or derived scales have more balance functionality between protection and locomotion. Cosmoid and ganoid scales are the most ancestral type of scales while ctenoid and cycloid scales are most type of scales.

Experiment and studies were conducted on various fishes with cycloid and ctenoid types of scales. Scales were collected from different fishes for this purpose, and mounted. Structural components and other features were observed under the microscope. Both scales had structural diffrences. The studies helped to understand pivotal role played by scales in the aquatic life of fishes. Scales are the most widely

used aging structure in North America because of their non-lethal ease of collection. Counting the number of annuli (rings) on a scale provides the fish age and the spacing between rings is proportional to the growth of the fish. Knowledge of fish age characteristics is necessary for stock assessments, and to develop management or conservation plans.

INTRODUCTION

The evolution of fishes began about 530 million years ago during the Cambrian explosion. The first fish belongs to Agnatha, or jawless fish, found in the early Paleozoic (Ordovician) 505 mya. They were small (few inches to a foot long initially), freshwater bottom-feeding animals. The circular mouth lacks jaws. Use their gills as both straining devices and as respiratory structures. There is no internal bony skeleton instead thick bony plates and scales that cover the body. Fishes make up more than half of all vertebrate species. They are especially important in the study of vertebrate evolution because several important vertebrate traits evolved in fish. Fish show great diversity in body size. They range in length from about 8 millimeters (0.3 inches) to 16 meters (about 53 feet). Most are ectothermic and covered with scales.

Scales are small plate-shaped dermal or epidermal structures that are found in the outer skeletons of fish, reptiles, or some mammals. The skeletons of many vertebrates are covered by two types of scales, namely epidermal and dermal. The epidermis contains numerous mucus cells. These cells secrete mucus or slime, which prevents parasites, fungi, pathogens, etc. from entering the skin easily. Most fish bear scales. While some agnatha and catfishes have no scales. Some fishes, especially paddle fish (*Polyodon*), mirror carp (*Cyprinus carpio*) have partial scales. Other fish such as trout and freshwater eel have very small scales. The scales contain a variety of pigments that give the fish a variety of colors. When the fish hatches from the egg, its body is covered by small scales. As the fish grow so does the scales. However, the number of scales remains the same throughout life but the lost scales can be restored at some point. A small circular growth ring is formed in the scales and this ring is called circuli or circulus (in singular). Circulus formed in summer are quite wide whereas circulus formed in winter are intertwined. In that densely enclosed region a black circle is formed which is known as the annulus. The age of the fish is determined by counting the number of annulus in scales. Scales cover most of the body and protect the skin from injury. The scales form a lateral line in the body of the fish along the side of the body and play an important role in detecting vibrations in the water as it acts as a sensory receptor. Fish scales can be divided into four based on shape, namely: Placoid, Cycloid, Ganoid, Ctenoid.

Type of scales found in most living bony fish (Osteichthyes) are of two types, namely: Cycloid and Ctenoid scales. Fishes like tank goby (*Glossogobius giuris*), thilapia (*Oreochromis mossambicus*), sardine (*Sardina longiceps*) etc possess cycloid type of scales and fishes like mullet (*Mugil cephalus*), karimeen (*Etroplus suratensis*), pink perch (*Nemipterus japonicus*) etc have ctenoid type of scale. Detailed structure of the fish scale can be helpful in the identification of fishes up to

major groups and species levels, phylogeny, sexual dimorphism, age determination, past environment experienced by fish, discriminating between hatchery reared and wild populations, migration, pathology of fish scale due to water pollution of the water body and for the growth studies. Shape, size and number of scale are suitable tools in fish taxonomy and using it dates back to first half of 19th century when Agassiz (1833-1843) used it in fish taxonomy for the first time. He classified the fishes into 4 groups based on the scale morphology (Jawad and Al-Jufaili, 2007). During the late 19th century and the first half of the 20th century and with the great advancements in the field of light microscopy, the importance of scale morphology in systematics increased significantly. The importance of scale morphology used in classification was strengthened with the introduction and development of Scanning Electron Microscopy (SEM), so that scales of many different fish species have been studied using SEM (Jawad and All-Jufaili, 2007).

With regard to the importance of scale morphology in this research ultrastructures of scale. The ease of collection of this aging structure is not without its tradeoffs, as the major bias of scales used as an age estimation structure is their tendency to underestimate the age of older fish. The most commonly used techniques involve counting natural growth rings on the scales, otoliths, vertebrae, fin spines, eye lenses, teeth, or bones of the jaw, pectoral girdle, and opercular series. Fish ages are often examined along with measurements of length and weight which combined can provide information on stock composition, age at maturity, life span, mortality, and production.

This topic was selected because, performing age structure analysis are important in growth analysis, population dynamics estimates and resource management. Data from the study can delineate individuals into specific age classes. Exploited species

often have the older, larger individuals removed from the population because they are the first removed by fishers leaving the younger smaller individuals. The effect may have serious consequences for that population. By performing age analysis studies we can identify these types of effects as well their implications to the status of the population. The project, enabled to understand more about the structural peculiarities of cycloid and ctenoid scales.

CYCLOID SCALE FISHES

TANK GOPY - *Glossogobius giuris*

Glossogobius giuris, the tank goby, is a species of goby native to fresh, marine and brackish waters from the Red Sea and East Africa through South Asia and the Indian Ocean to China, Australia and the islands of the Pacific Ocean. This species can also be found in the aquarium trade. It is also known as the bar-eyed goby, flat-headed goby and the Gangetic tank goby. The head is depressed with a protruding lower jaw while the body takes on a compressed appearance towards to caudal fin. Normally brown or light brown with various darker brown spots and flecks along the sides. Ranges in size from 40 to 50 cm maximum (16-20 inches).

TILAPIA - *Oreochromis mossambicus*

Tilapia is the common name for nearly a hundred species of cichlid fish from the coelotilapine, coptodonine, heterotilapine, oreochromine, pelmatolapiine, and tilapiine tribes with the economically most important species placed in the Coptodonini and Oreochromini. Tilapia are mainly freshwater fish inhabiting shallow streams, ponds, rivers, and lakes, and less commonly found living in

brackish water. Tilapia typically have laterally compressed, deep bodies. Like other cichlids, their lower pharyngeal bones are fused into a single tooth-bearing structure. A complex set of muscles allows the upper and lower pharyngeal bones to be used as a second set of jaws for processing food, allowing a division of labor between the "true jaws" (mandibles) and the "pharyngeal jaws". This means they are efficient feeders that can capture and process a wide variety of food items. Their mouths are protrusible, usually bordered with wide and often swollen lips. The jaws have conical teeth. Some Nile tilapia can grow as long as 2.0 ft.

SARDINE - Sardina longiceps

Sardines have a flat body which is covered with large, reflective, silvery scales. In the middle of their belly, they have a set of specialized scales, known as scutes, which are jagged and point backwards. Having very small teeth or no teeth at all, sardines eat plankton, which they filter from the water through their gills. While numerous species of sardines live off the coasts of India, China, Indonesia, and Japan, single sardine species dominate in areas like the English Channel and the California coast. Sardines are basically a warm-water fish, but occur as far north as Norway.

CTENOID SCALE FISHES

MULLET - Mugil cephalus

The flathead grey mullet (*Mugil cephalus*) is an important food fish species in the mullet family Mugilidae. It is found in coastal tropical and subtropical waters worldwide. Its length is typically 30 to 75 centimetres (12 to 30 in). It is known with

numerous English names, including the flathead mullet, striped mullet (US, American Fisheries Society name), black mullet, bully mullet, common mullet, grey mullet, sea mullet and mullet, among others. The flathead grey mullet is a mainly diurnal coastal species that often enters estuaries and rivers. It usually schools over sand mud bottoms, feeding on zooplankton. The adult fish normally feed on algae in fresh water. It occupies fresh, brackish and marine habitats in depths ranging between 0-120 metres (0-394 ft) and with temperatures between 8-24 °C (46-75 °F).

PEARL SPOT - *Etroplus suratensis*

The green chromide (*Etroplus suratensis*) is a species of cichlid fish is native to fresh and brackish water habitats in some parts in India such as Kerala, Goa, Chilika Lake in Odisha and Sri Lanka. The species was first described by Marcus Elieser Bloch in 1790. Other common name include pearlspot cichlid, banded pearlspot, and striped chromide. In Kerala, it is known locally as the karimeen. In Tamilnadu, it is known locally as the 'pappan or pappa. In Goa the fish is known as kalundar. The green chromide lives in brackish water habitat types, such as river deltas. It eats mainly aquatic plants, including filamentous algae and diatoms, but it consumes the occasional molluscs and other animal matter. This species engages in attentive parental care in which several adults care for each brood.

PINK PERCH - *Nemipterus japonicus*

Pink Perch or Rani is a common freshwater fish in India. Pink in colour and small in size, this fish has a mild taste when cooked. Due to just 4- 5% of body fat, this fish is not oily and hence it is called a lean fish. This is your go to go meat if you are planning to lose weight. Pink Perch gets its name due to the pink hue and yellow strips with scales on its skin. It has a mild flavour with a delicate, soft texture that makes it ideal for curries, fried or grilled preparations.

AIM

This project work aims to analyse the size variations of scales in different parts of fishes, determine age of fish by counting the anuli of scale.

OBJECTIVES

The objective of the present study was 1) to evaluate the size variation of scales in different parts of fishes by measuring the scales 2) determining the age of fishes by counting anuli by mounting the scales.

REVIEW OF LITERATURE

One of the unique features in a fish is the presence of the scales (except fishes of order siluriformes) on the outer surface of the body. A fish scale can tell us a great deal about the fish's life story, including where it has been since it hatched. These scales are magic tool for studying a life cycle as a whole. Various types of scales are found in fishes eg. placoid, cycloid, ganoid, cosmoid or ctenoid. Placoid scales are found in cartilaginous fishes and rest all are found in bony fishes. These are derived from the connective tissue of the dermis and form the exoskeleton. Scales of fish are used for classification, identification and growth studies of different fishes, pollution indicators etc.

The scales of bony fishes are derived entirely from the dermal layer of the skin and overlap one another like the tiles. The overlapping (imbrications) of the scale is important in the sense that it imparts mechanical support. Each scale is shaped roughly like a human finger nail whose front end is inserted deep into the dermal layer, while the hinder end is free of exposed and bears the pigment cells or chromatophores on it. These chromatophores provide specific colour to the fish body. These scales have a soft anterior part and hard posterior part. The dorsal surface of the scales is rough as it bears the lines of growth, whereas the ventral surface that touches skin in shining. The cycloid scale has concentric rings around the focus and these rings/lines of growth as sclerites. Cycloid and ctenoid scales increase in size, growth rings called circuli become visible. These rings look a little like the growth rings in the trunk of a tree. During the cooler months of the year the scale (and otoliths) grows more slowly and the circuli are closer together leaving a band called an annulus. By counting the annuli it is possible estimate the age of the fish. This technique is extensively used by fisheries biologists. Only the anterior and

lateral sides of scale have these circuli. These are the marks of periodical growth of fish. Any sudden changes in fish's environment is recorded on the scales in the form of alteration in the circuli shape, pattern or altered elemental deposition thus making these hard structures a testimony to life history of the fish. These revealing marks may be annual marks, winter marks or the larval marks.

Scales at the shoulder of the fish between the head and the dorsal fin is best suited for age determination. Scales are almost two-dimensional structures. The anterior part is formed of a series of sclerites which should extend in a regular pattern from the centre of the scale. The structural discontinuities used for age determination result from irregularities in the pattern of the sclerites; they may be slightly distorted or they may be slightly closely spaced than the majority of the sclerites; usually the discontinuities are narrow and they are usually called 'rings'. Scales are thin structures they need no preparation before viewing; the scales should be cleaned before they are stored. For reading, the slide with mounted scales is placed on the stage of a low-power microscope. The magnification used depends upon the size of the scale; in general, the lowest possible magnification is the best because it enables the whole scale pattern to be seen.

The use of age information is an integral part of fisheries today. Hilborn and Walters (1992) state that "the most valuable information obtained from sampled catch, at least for temperate waters, is age." The development and acceptance of methods for age determination in fishes represents a critical early stage in fisheries science, and at times was fraught with more controversy than today's wide usage of the methods would suggest. In 2006, the Fisheries Management Section of the American Fisheries Society formed the ad hoc Assessment of Fish Aging Techniques Committee to assess the status of aging of freshwater fishes in North America (see

Maceina *et al.* this issue). As part of the committee's goals, an historical survey of the earliest references to aging of fishes and their initial application to fisheries studies was undertaken.

Antoni van Leeuwenhoek of Holland, who used his experience counting threads in cloth at a dry goods store with magnifying glasses to develop improved lenses that he used to construct microscopes, became one of the leading microscopists of the 1600s. Leeuwenhoek possessed a wide-ranging curiosity that included issues surrounding demographics of animal populations (Egerton 1968). Curiously, Leeuwenhoek's studies of fish scales appear to have been at least in part inspired by Biblical strictures against eating fish without scales. His earliest writings on fish scales appeared in a letter to the Royal Society of London, and focused on the European eel (*Anguilla anguilla*) and the burbot (*Lota lota*), which he was drawn to as a result of their reported lack of scales "which two sorts of fish, the Jews will not eat, as forbidden by the Law of Moses." (Leeuwenhoek 1685).

Petersen is most often credited with first proposing length-based methods for age determination (Allen 1917; Ricker 1975). His work appears to have been preceded by Joseph T. Cunningham. Cunningham, working at the Marine Biological Association's Plymouth Lab, attempted to use lengths from known-aged fish he reared in aquaria to assign ages to wild-caught fish, focusing on flatfish and cod (Cunningham 1891, 1892). Cunningham's efforts were not rewarded by clear-cut results: "It is evident there is considerable variation in the rate of growth in nature, from the difficulty of distinguishing in a large number of fish those of one year's, two years', and three years' growth" (Cunningham 1891).

Hederström examined the vertebrae of pike and concluded that the rings that could be discerned on them were growth rings that could be used to determine the fish's age. His reasoning revealed a thoroughly scientific approach, and included verification that (1) both sides of a vertebra had the same number of rings, (2) all vertebrae in an individual possessed the same number of rings, (3) larger fish had more rings on their vertebrae than smaller fish, and (4) the number of rings matched the age of fish "known either from experience or from other circumstances" (Hederström 1759). Hederström went on to present length-at-age data for pike that agree well with modern estimates and also reported that he had confirmed the applicability of using rings on vertebrae for determining the age of a variety of other species, including European perch (*Perca fluviatilis*), roach (*Rutilus rutilus*), bream (*Abramis brama*) etc.

Detailed structure of the fish scale, helpful in the identification of fish up to major groups and species levels can be obtained from: (Abraham *et al.* 1966; Bartulović *et al.* 2011). Detailed structure of the fish scale, helpful in the identification of fish up to major groups and species levels can be obtained from: (Abraham *et al.* 1966; Bartulović *et al.* 2011).

Thompson's (1910) statistical concerns by presenting comparisons of a normal curve to their age-frequency curve, concluding that "the dissimilarity of the two curves is, in fact, so great as to exclude any idea of the age-curve following the usual law of biological variation" and that "it seems to us impossible to explain the observed facts as a result of common variation, even if the help of a mathematical statistician were enlisted."

Huntsman of the Fisheries Research Board of Canada was keeping abreast of the developments in Europe, however, and in 1918 presented a paper to the Royal Society of Canada on its potential applications, soon followed by a similar presentation to the American Fisheries Society (Huntsman 1918, 1919). Carlander (1987) credits Huntsman's papers with bringing aging methods to the attention of North American workers.

Borodin (1924) used scales to assess American shad (*Alosa sapidissima*) in the Connecticut River, and a study of the use of otoliths in the same system followed soon after (Barney 1924). A search of articles in the Transactions reveals only one other application of aging to fish studies in the 1920s, but an increase to 84 in the 1930s, 74 during the 1940s, 112 during the 1950s, followed by rapid increases to 231 in the 1960s and 370 during the decade of the 1970s.

Petersen's work using lengths to assign ages to blenny (*Zoarces viviparus*) received more notice than Cunningham's, but was characterized by the same difficulties (Petersen 1892, summarized by Ricker 1975). Petersen constructed what are now known as length-frequency graphs, proposing that the peaks, or modes, that were evident across the range of smaller to larger size classes represented progressively older age-classes of fish.

By correlating the marginal growth of scales (the amount of growth between the last ring or annulus and the margin of the scale) with the season of the year in which the scales were collected, Walford and Mosher (1943a: 9 and 1943b) have shown for the Pacific pilchard or sardine, *Sardinops caerulea* (Girard) 1854, that these rings are formed annually and consequently can be used for age determination as well as for back-calculation of the length of the fish at a given earlier age.

Age determination based on the analysis of the growth marks of calcified structures is the specific aim of the Sclerochronology Laboratory of the INSTM (SLI), which was created in 2000. The methods used in SLI to identify and count growth marks on mineralized structures in fishes and to interpret the corresponding data. Is monitored till now, the species of interest to the SLI are small pelagic fishes. Age determination of *Mullus surmuletus* has been performed either by counting scale annuli (Gharbi, 1980) or otolith (in toto) growth marks (Jabeur, 1999).

In 1859, Robert Bell reported that one could use these growth rings to reliably determine the age of all fish after examination of sucker (*Catostomus sp.*) vertebrae and yellow perch (*Perca flavescens*) scales that he raised in a pond for two years showed “two rings or circles.”

Stuart Thomson, with encouragement and support from Walter Garstang and Allen at the Plymouth Laboratory of the Marine Biological Association of the United Kingdom, extended Hoffbauer’s work with freshwater fishes to important commercial marine species. His detailed work with pollack (*Pollachius pollachius*), poor cod (*Trisopterus minutus*), whiting (*Merlangius merlangius*), haddock (*Melanogrammus aeglefinus*), and cod convinced him that Hoffbauer’s findings could be applied to marine species (Thomson 1902, 1904).

METHODOLOGY

3 different types of fishes were selected each for cycloid and ctenoid scales. Tank gopy, thilapia, sardine for cycloid scale and pearl spot, mullet, pink perch for ctenoid scale. These fishes were selected from various markets. Specimens were collected

during March, 2022. Care was taken during collection of specimen to avoid fishes with damaged scales. First step was cleaning of the specimens, it was washed with clean water. The next step was de-scaling. Scales were taken from the body carefully, using forceps and washed the scales with water again. Measurements of the scale were noted. Measurements were taken using white threads, length and breadth of the scales were measured. With the help of a ruler right measurements of length and breadth were obtained.

Colour of the scales of all the fishes were observed. The shape and other morphological differences between two type of scales were also noted. Scales from different regions like cephalic, caudal, dorsal, pelvic and pectoral regions were collected. The final step of the experiment was the comparative study of scales of ctenoid and cycloid scales. The scales were mounted for further morphological observations.

Mounting of Scales

The materials required:

1. Forceps
2. Watch glass-2
3. 10 % KOH solution
4. Spirit lamp
5. Match box
6. Distilled water
7. Fine brush
8. clean grease free microscopic slides
9. Leishmann's stain



Fig. 1 – Materials required for mounting the scales

Procedure:

1. Fishes with cycloid and ctenoid scales are de-scaled (scales from pectoral, pelvic, caudal, dorsal, head regions were collected, this can be done by careful scrapping of fish. Care should be taken not to damage the scales, they are then transferred to 10% KOH solution taken in a test tube.
2. Heat the fish scales in 10% of KOH solution for 10-20 seconds.
(Note: Heating is done to dissolve the covering epithelium over heating will cause curling of the scales.)
3. Wash the fish scales in distilled water (2 times), for removing the KOH solution.
4. Take few drops of Leishmann's stain in a watch glass. Transfer the scales into that.
5. Wait for 3 minutes. Wash off the excess stain.
6. Transfer the scales to a glass slide.
7. Observe the slide under dissecting microscope and under the low power (4X) of simple microscope.
8. Repeat the same for other fishes with ctenoid scale.

9. Compare all the slides and note down the differences and morphologies of the scales.

Age Determination

Procedure: Scales are prepared for study by mounting the whole scales on glass slides or, more commonly, by pressing imprints of the scale circuli into transparent plastic. These scales or scale impressions are examined under a low-power microscope or by use of a microprojector, and the rings or annuli are counted.

Applications of Scale Method: Fish of temperate regions shows clear rings, which are true marks. This is because there is a sharp difference between the temperatures two seasons—summer—the period of faster growth, and winter—the period of slow growth or no growth. Therefore, the calculation of the age of fish by annuli is most reliable in temperate fish. This method is more reliably applicable in case of salmons, carps, cod and herrings, established a method of estimating age of fish based on scales.



Fig. 1 – Tank Gopy



Fig. 2 – Tilapia



Fig. 3 – Sardine



Fig. 4 – Mullet



Fig. 5 – PEARL SPOT

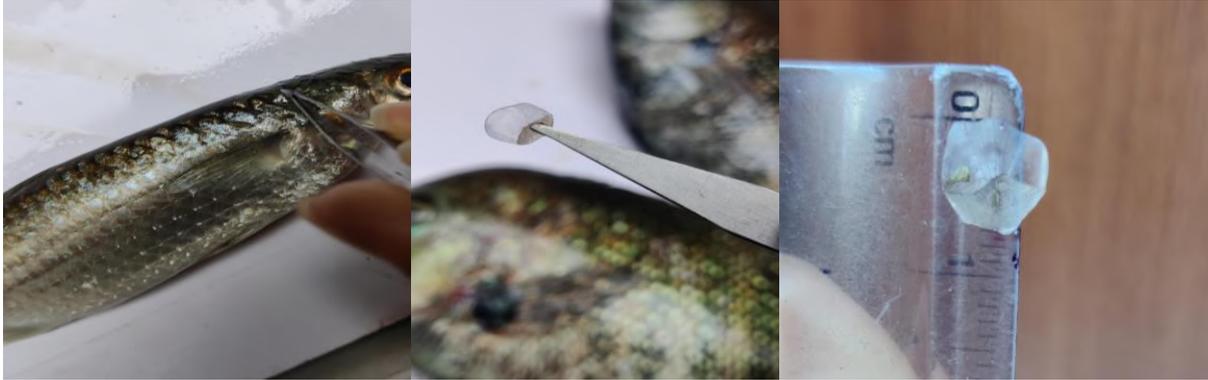


Fig. 6 – Pink Perch



(a)

(b)



(c)

(d)

(e)

Pleat 1 – Procedure for measuring the size of scale

- a) Measuring the length of the fish
- b) Measuring the width of the fish
- c) De-scaling the fish
- d) Scale of fish
- e) Measuring the scale

OBSERVATION

	FISH 1		FISH 2		FISH 3		FISH 4		FISH 5	
	L	B	L	B	L	B	L	B	L	B
TANKGOPY	13.1cm	4cm	10.2cm	3.7cm	11.6cm	4cm	9.2cm	3cm	8.9cm	3cm
TILAPIA	19cm	7cm	20cm	10cm	21cm	8cm	22cm	8cm	19cm	7cm
SARDINE	14.6cm	3.5cm	16.1cm	4.3cm	16.5cm	4.2cm	16.3cm	4.2cm	15.1cm	3.6cm
MULLET	18.5cm	5cm	16.5cm	4.3cm	15cm	4.3cm	14.5cm	3cm	13cm	3cm

PEARL SPOT	18.5cm	10cm	17cm	8.5cm	15cm	9cm	14.5cm	7cm	14cm	7cm
PINK PERCH	18.7cm	5.9cm	18.7cm	5.8cm	17.8cm	5.8cm	16.8cm	6.7cm	19.1cm	5.7cm

Table 1 – Measurement of fish size

	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.2cm	0.2cm	0.4cm	0.4cm	0.6cm	0.6cm	0.5cm	0.5cm	0.6cm	0.4cm
Fish2	0.2cm	0.2cm	0.4cm	0.5cm	0.5cm	0.5cm	0.5cm	0.4cm	0.5cm	0.4cm
Fish3	0.2cm	0.2cm	0.4cm	0.3cm	0.5cm	0.5cm	0.5cm	0.6cm	0.5cm	0.4cm
Fish4	0.3cm	0.3cm	0.4cm	0.4cm	0.4cm	0.5cm	0.5cm	0.5cm	0.5cm	0.4cm
Fish5	0.3cm	0.3cm	0.4cm	0.4cm	0.6cm	0.6cm	0.4cm	0.4cm	0.5cm	0.4cm

Table 2 – Measurement of scales in different parts of tank gopy

	HEAD REGION		PELVIC REGION		PECTORAL REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.4cm	0.6cm	0.6cm	0.7cm	0.3cm	0.4cm	0.5cm	0.6cm	0.5cm	0.5cm
Fish2	0.8cm	0.6cm	0.6cm	0.8cm	0.4cm	0.3cm	0.5cm	0.5cm	0.5cm	0.5cm
Fish3	0.7cm	0.7cm	0.6cm	0.8cm	0.4cm	0.3cm	0.4cm	0.4cm	0.4cm	0.5cm
Fish4	0.6cm	0.8cm	0.6cm	0.6cm	0.5cm	0.3cm	0.6cm	0.5cm	0.6cm	0.5cm
Fish5	0.5cm	0.6cm	0.5cm	0.6cm	0.4cm	0.2cm	0.5cm	0.5cm	0.5cm	0.4cm

Table 3 – Measurement of scales in different parts of tilapia

	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.3cm	0.3	0.5cm	0.4cm	0.4cm	0.4cm	0.4cm	0.2cm	0.5cm	0.4cm

Fish2	0.3cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm	0.4cm	0.4cm	0.3cm	0.3cm
Fish3	0.5cm	0.3cm	0.7cm	0.6cm	0.5cm	0.5cm	0.5cm	0.4cm	0.3cm	0.3cm
Fish4	0.5cm	0.5cm	0.6cm	0.6cm	0.5cm	0.5cm	0.5cm	0.5cm	0.3cm	0.3cm
Fish5	0.5cm	0.5cm	0.6cm	0.4cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm	0.4cm

Table 4 – Measurement of scales in different parts of sardine

	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.8cm	0.8cm	1cm	0.9cm	0.9cm	0.6cm	1.1cm	0.9cm	0.7cm	0.5cm
Fish2	0.8cm	0.8cm	0.9cm	0.8cm	0.7cm	0.6cm	0.9cm	0.8cm	0.7cm	0.5cm
Fish3	0.5cm	0.4cm	0.5cm	0.4cm	0.7cm	0.5cm	0.6cm	0.6cm	0.6cm	0.5cm
Fish4	0.5cm	0.5cm	0.6cm	0.6cm	0.5cm	0.4cm	0.6cm	0.6cm	0.4cm	0.4cm
Fish5	0.7cm	0.6cm	0.8cm	0.7cm	0.5cm	0.3cm	0.7cm	0.6cm	0.6cm	0.4cm

Table 5 – Measurement of scales in different parts of mullet fish

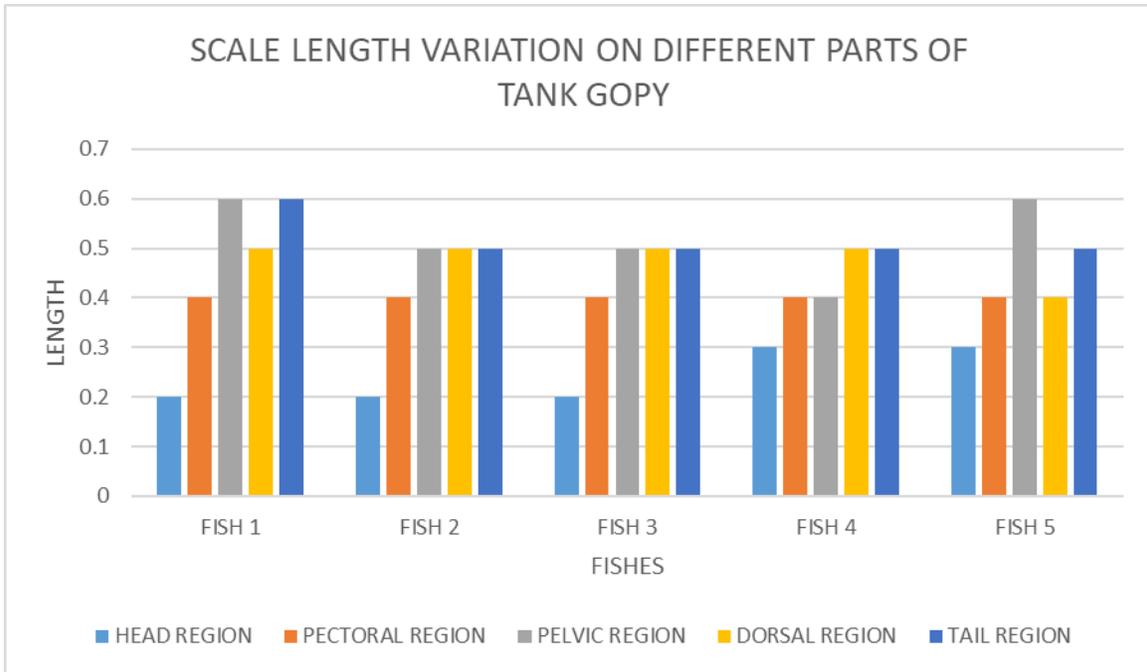
	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish 1	0.7cm	0.5cm	0.7cm	0.7cm	0.6cm	0.4cm	0.7cm	0.5cm	0.6cm	0.5cm
Fish 2	0.5cm	0.7cm	0.5cm	0.6cm	0.5cm	0.5cm	0.6cm	0.6cm	0.5cm	0.5cm
Fish 3	0.5cm	0.6cm	0.6cm	0.6cm	0.6cm	0.5cm	0.6cm	0.5cm	0.5cm	0.4cm
Fish 4	0.5cm	0.4cm	0.5cm	0.5cm	0.5cm	0.5cm	0.6cm	0.5cm	0.5cm	0.5cm
Fish 5	0.4cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm

Table 6– Measurement of scales in different parts of pearl spot

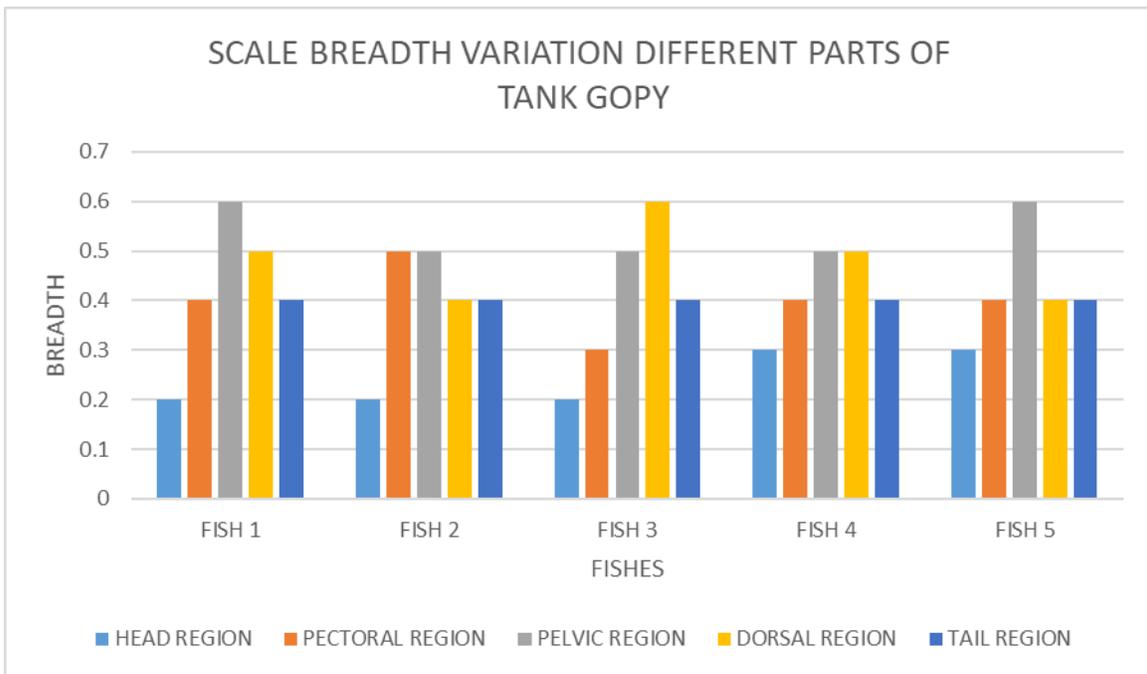
	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.6cm	0.6cm	0.6cm	0.9cm	0.6cm	0.6cm	0.5cm	0.4cm	0.4cm	0.4cm
Fish2	0.4cm	0.4cm	0.6cm	0.7cm	0.6cm	0.7cm	0.6cm	0.5cm	0.5cm	0.5cm
Fish3	0.5cm	0.4cm	0.6cm	0.8cm	0.6cm	0.6cm	0.6cm	0.5cm	0.4cm	0.4cm
Fish4	0.6cm	0.6cm	0.5cm	0.9cm	0.5cm	0.5cm	0.5cm	0.5cm	0.3cm	0.3cm
Fish5	0.6cm	0.5cm	0.6cm	0.9cm	0.6cm	0.7cm	0.6cm	0.5cm	0.4cm	0.4cm

Table 7 – Measurement of scales in different parts of pink perch

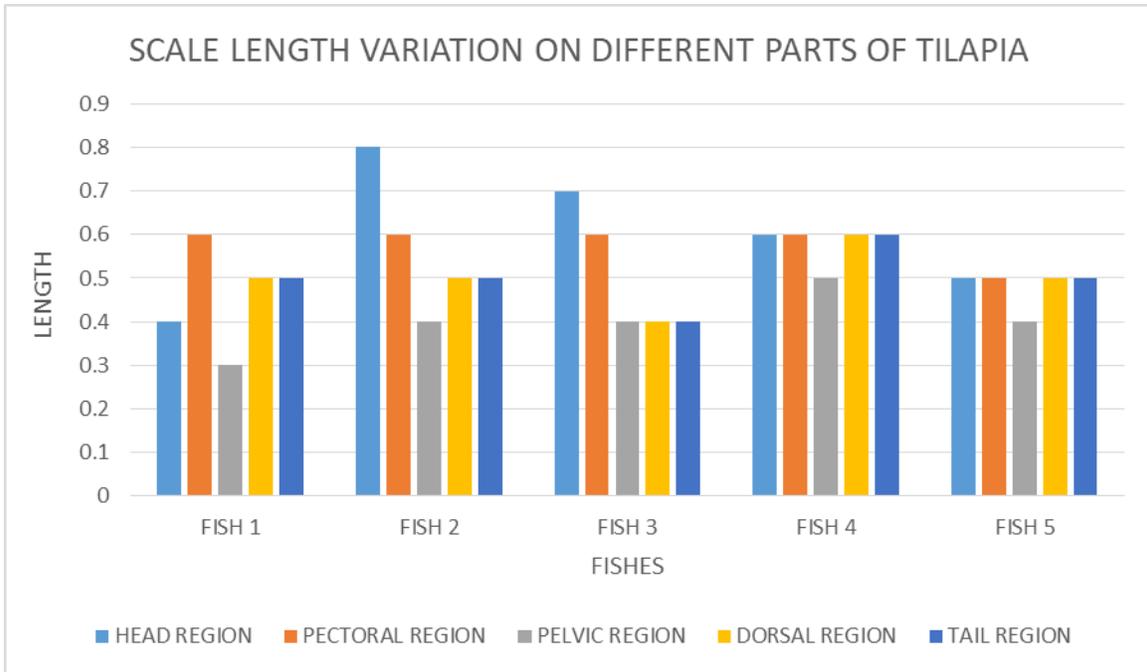
RESULT



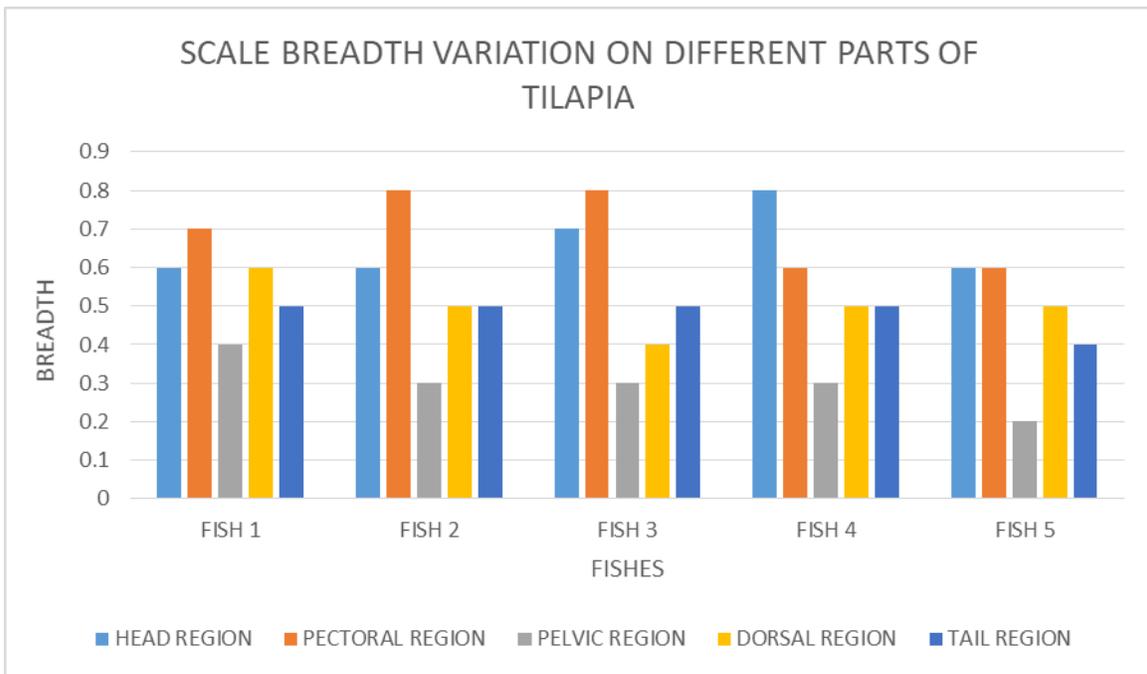
Graph 1:



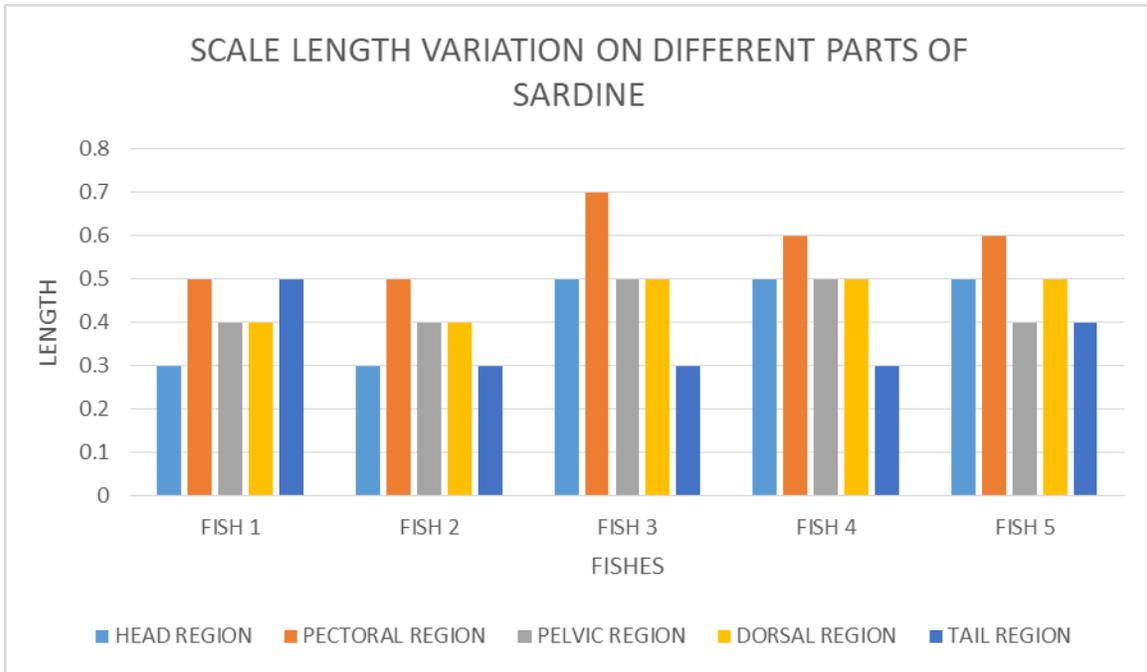
Graph 2:



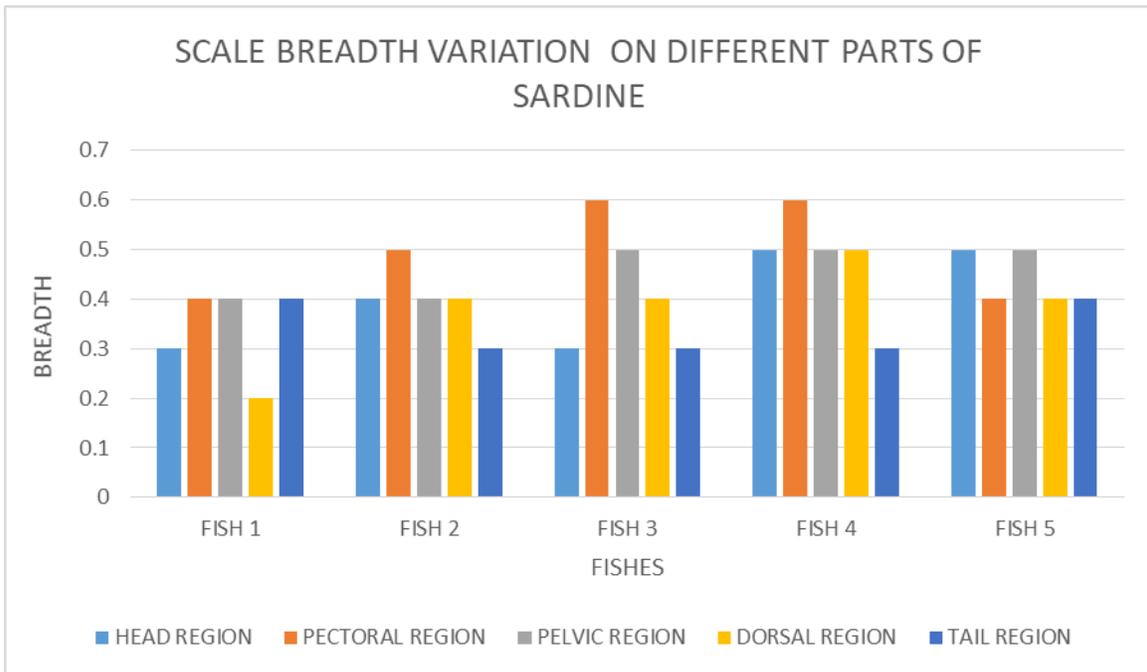
Graph 3



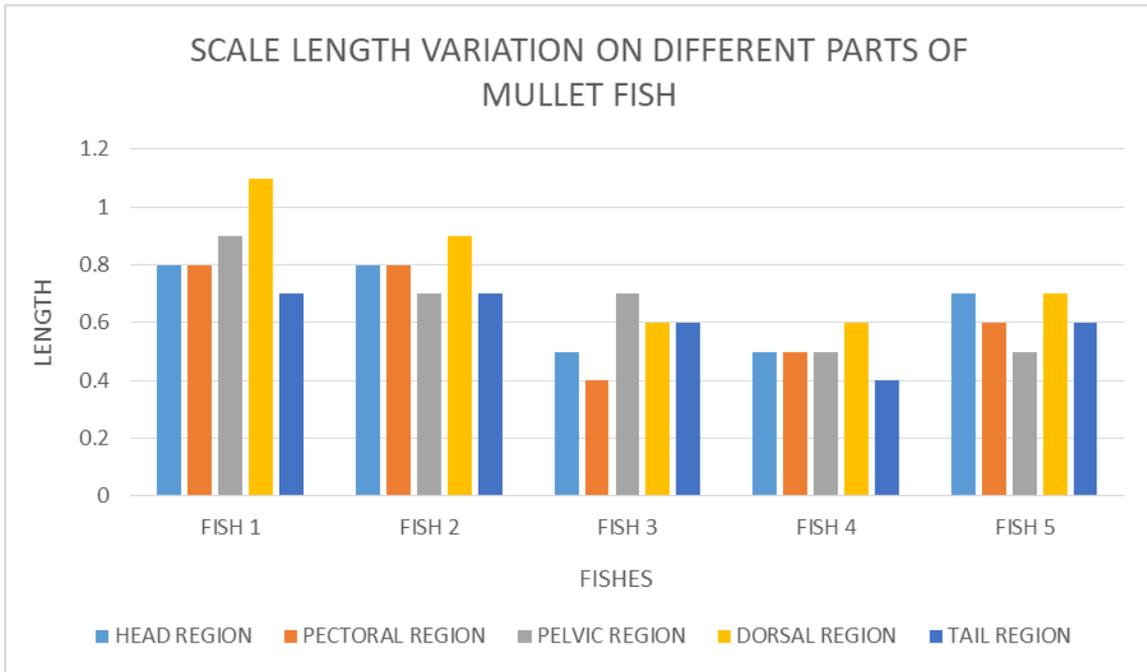
Graph 4:



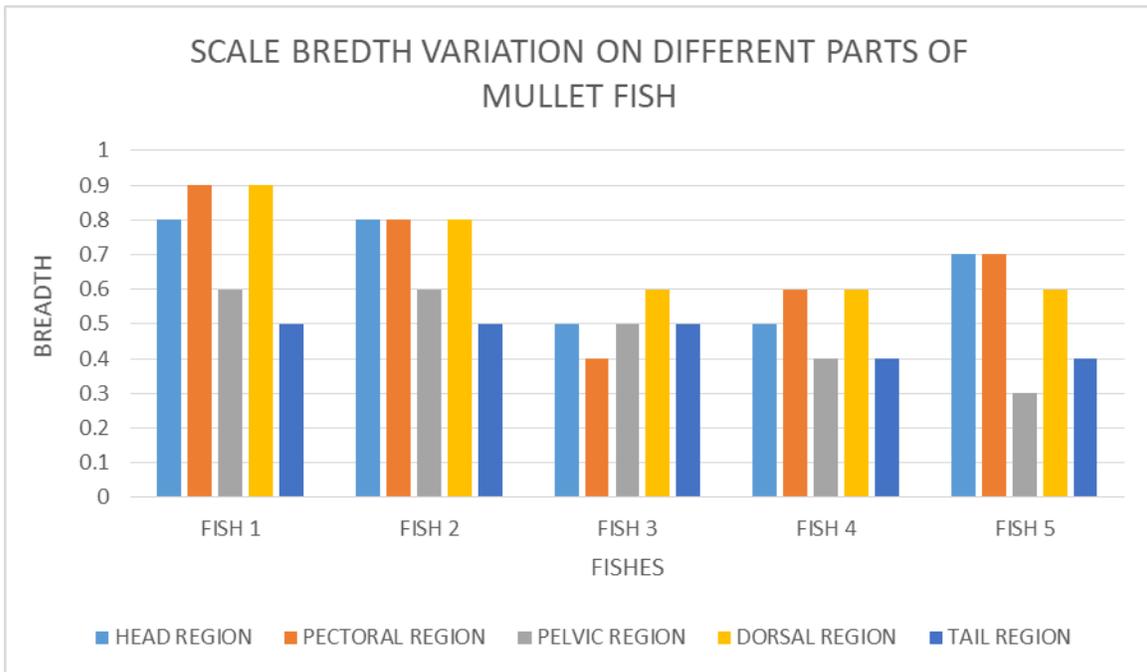
Graph 5:



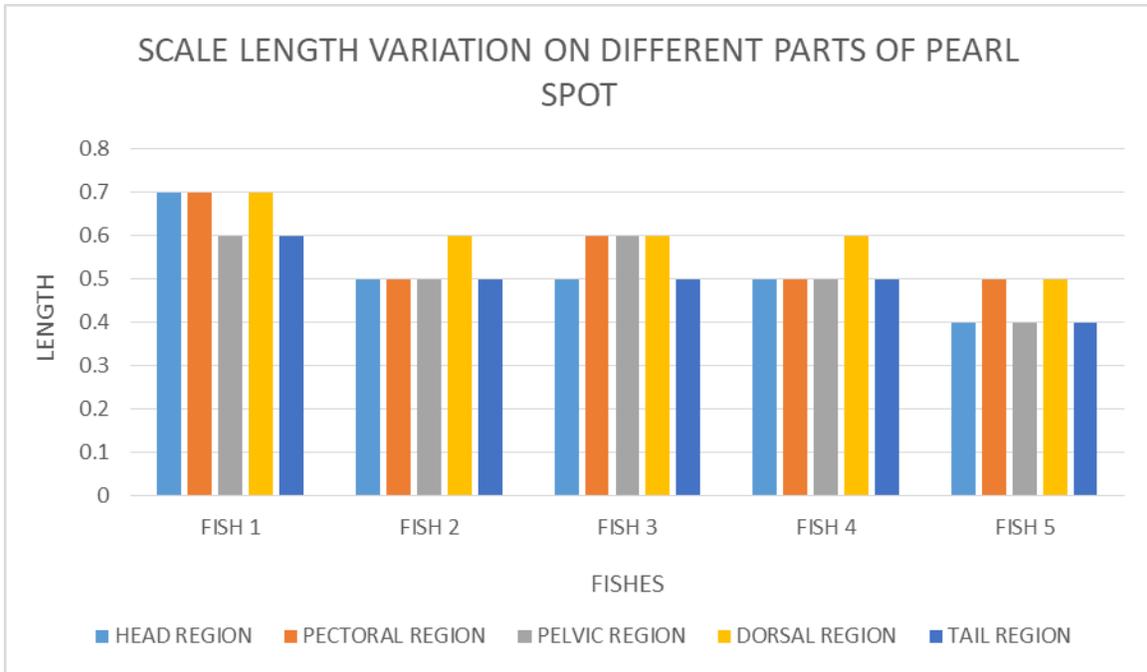
Graph 6:



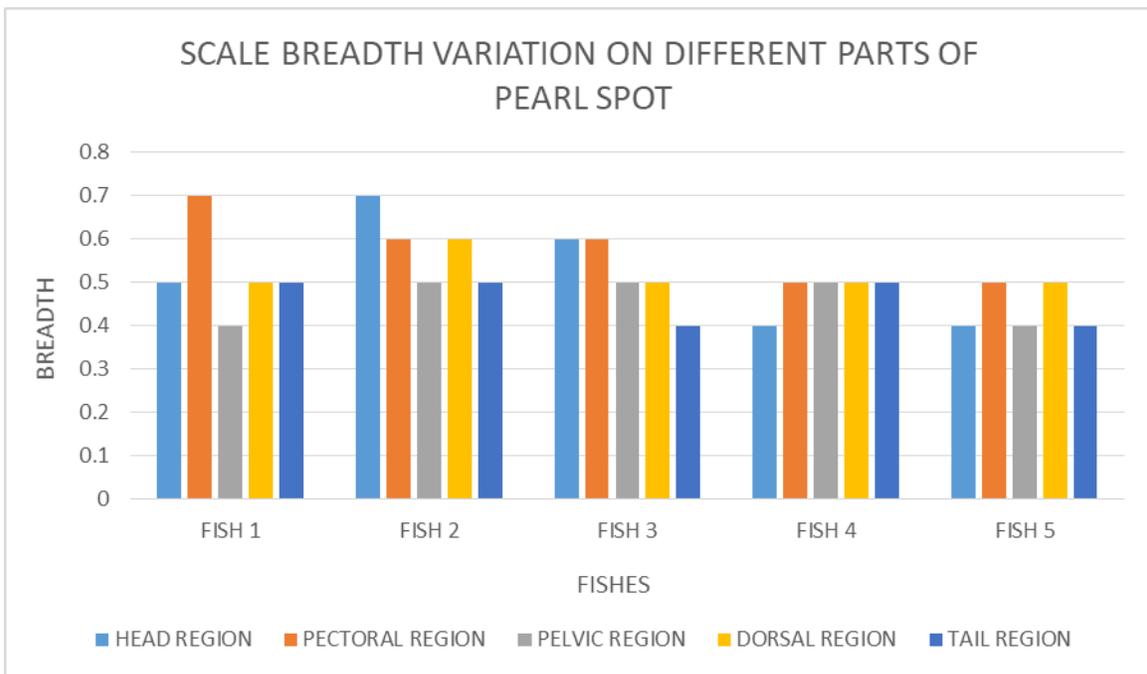
Graph 7:



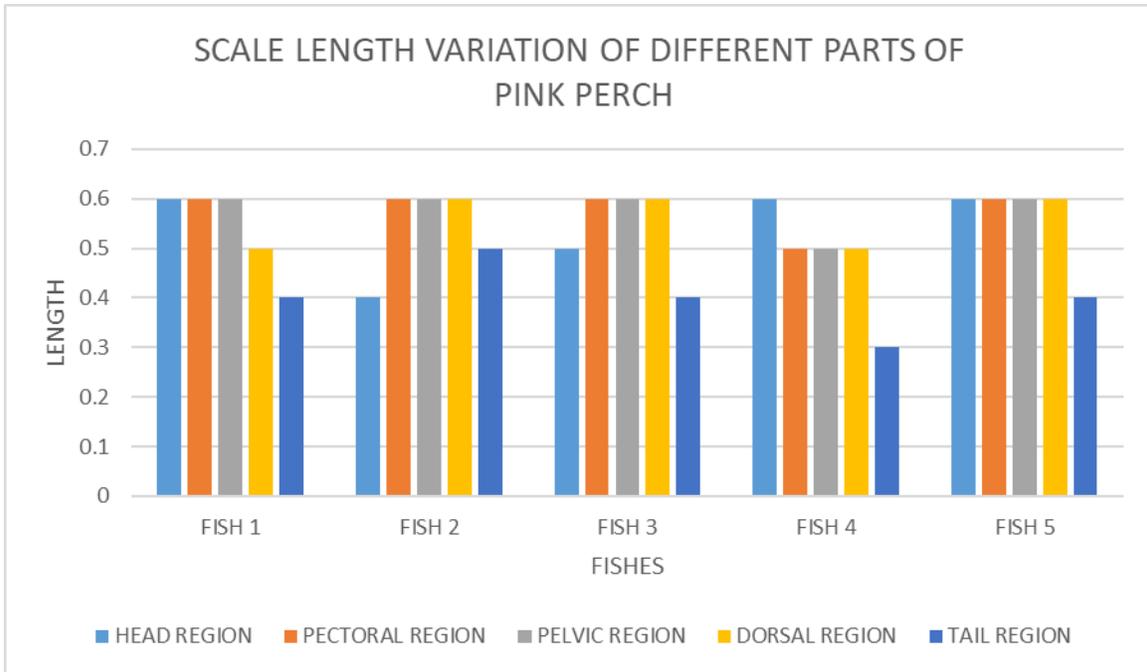
Graph 8:



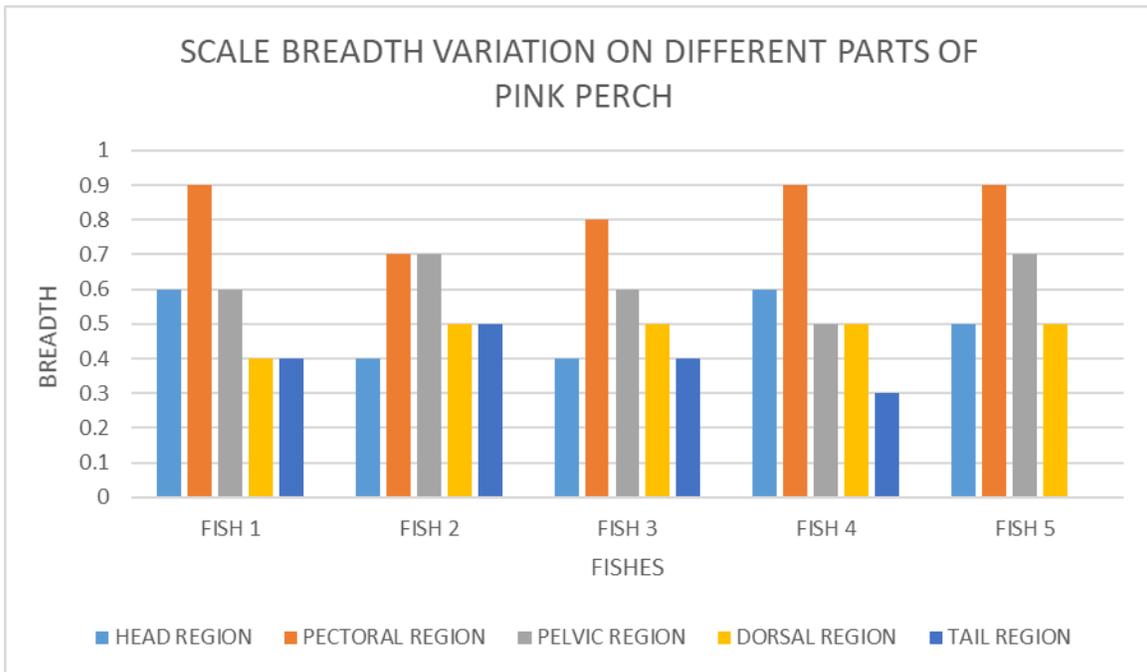
Graph 9:



Graph 10:



Graph 11:



Graph 12:

TANK GOPY		
FISHES	NO. OF ANULI	AGE OF FISH
FISH 1	1	1 YEAR
FISH 2	2	2 YEARS
FISH 3	1	1 YEAR
FISH 4	0	
FISH 5	0	
TILAPILA		
FISH 1	1	1 YEAR
FISH 2	0	
FISH 3	0	
FISH 4	<u>1</u>	1 YEAR
FISH 5	<u>1</u>	1 YEAR
SARDINE		
FISH 1	2	2 YEARS
FISH 2	3	3 YEARS
FISH 3	4	4 YEARS
FISH 4	3	3 YEARS
FISH 5	2	2 YEARS
MULLET FISH		
FISH 1	2	2 YEARS
FISH 2	2	2 YEARS
FISH 3	2	2 YEARS
FISH 4	1	1 YEAR
FISH 5	1	1 YEAR
PEARL SPOT		

FISH 1	4	4 YEARS
FISH 2	3	3 YEARS
FISH 3	2	2 YEARS
FISH 4	2	2 YEARS
FISH 5	1	1 YEAR
PINK PERCH		
FISH 1	3	3 YEARS
FISH 2	1	1 YEAR
FISH 3	4	4 YEARS
FISH 4	3	3 YEARS
FISH 5	3	3 YEARS

DISCUSSION

The body of an ideal fish is covered by thin scales. The scales develop as external growths of the epidermis or skin. The epidermis contains numerous mucus cells. These cells secrete mucus or slime, which prevents parasites, fungi, pathogens, etc. from entering the skin easily. Agnatha is an infra phylum of jawless fish in the phylum Chordata, subphylum Vertebrata, consisting of both present (cyclostomes) and extinct (conodonts and ostracoderms) species. Among recent animals, cyclostomes are sister to all vertebrates with jaws, known as gnathostomes. In modern agnathans, the body is covered in skin, with neither dermal nor epidermal scales. The skin of hagfish has copious slime glands, the slime constituting their defense mechanism. The slime can sometimes clog up enemy fishes' gills, causing them to die. In direct contrast, many extinct agnathans sported extensive exoskeletons composed of either massive, heavy dermal armour or small

mineralized scales. Most fish bear scales. The scales contain a variety of pigments that give the fish a variety of colors. The scales form a lateral line in the body of the fish along the side of the body and play an important role in detecting vibrations in the water as it acts as a sensory receptor. Cycloid and ctenoid scales are found in the majority of bony fishes (the Teleostei).

In this project two different types of scales were studied namely cycloid and ctenoid. Fishes having cycloid and ctenoid scales were collected, descaled, stained and observed. It was noticed that both the scales differed in shape and structure. Ctenoid scales have a variously developed spiny posterior margin whereas cycloid scales have a smooth posterior margin lacking ctenii. The number and distribution of scales were not same either in all the six fishes. In fishes like sardine, tank goby, pink perch it was observed that the size of the scale in the tail region was comparatively smaller than scales in other regions of the body. These fishes are active swimmers, their rapid tail movement is what allows them to swim smoothly against the friction offered by the water. The small size of scales in the tail region is an adaptation for their rapid tail movement. Scales in other regions like pectoral, dorsal and head were bigger than the ones in tail region as they are not much involved in movement. Coloration was another feature that was observed, the margin of the scale in pink perch was pink in colour.

The coloration is an adaptation for various purposes like camouflage, mating etc. Scales are of value for age determination in many of bony fishes, a broad grouping which includes most fishes of importance for food. Scales are formed when newly hatched fish complete their larval stages, and soon cover the entire body, with the exception of head and fins. In most species they lie in an overlapping pattern much like shingles on a roof and serve as a protective coat. Scale growth begins with the formation of the scale center or focus and growth is outward from this focus, though it is greatest toward the forward margin of the scale. Fine ridges called circuli are

laid down in a circular pattern around the focus as growth proceeds. Many circuli are added to the scale each year. Fish growth is reflected in scale growth. Circuli are widely spaced in warm seasons when fish growth is rapid, and closely spaced in cold seasons when it is slow. Fish growth stops in winter. The growth of a fish during one year is shown on its scales as a series of widely spaced spring and summer circuli followed by a series of closely spaced fall and winter circuli. Since fishes continue to grow throughout their lives, this pattern is repeated each year. The outer edge of a series of closely spaced circuli is generally taken to be the end of growth for that year and this point is referred to as the year mark or annulus. The age of a fish is determined by counting the number of annuli or year marks.

CONCLUSION

The scales comprise a non-growing "crown" composed of dentine, with a sometimes-ornamented enameloid upper surface and an aspidine base. Its growing base is made of cell-free bone, which sometimes developed anchorage structures to fix it in the side of the fish. They are small, thin, cornified, calcareous or bony plates which fit closely together or overlap. When the arrangement of scales on fish body is concerned, they are most often imbricated and thus, overlap like shingles on the roof, with their free margins directed towards the tail, so as to minimise the friction of water. Two different types of scales observed in the project were cycloid and

ctenoid scales. Cycloid scales are somewhat circular in appearance, in addition to being thin and translucent. The center of these scales is thicker, and can observe several concentric lines of growth. Another interesting fact about these lines of growth is that they indicate the age of the fish when counted. The greater the number of concentric rings, the older the fish. Ctenoid scales have specific comb-like projections at the back. In form, structure and arrangement, they are very similar to cycloid scales. However, ctenoid scales attach themselves more firmly to the skin. The rear parts of these scales don't overlap and they have several comb-like teeth. Some scales possess colour as in pink perch, these colouration help in mating and camouflage it also make them attractive. Scales exhibit variations in size in different regions of the body. Scales found near the tail and head regions are comparatively small than the scales in the, pectoral and dorsal regions. Size of the scales contributes much to the locomotion of the fish. Small scales in the tail region enable rapid movement of tail and move smoothly in water against the friction offered by it. More detailed studies can be conducted on fish scales, considering the structural and mechanical properties.

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AGE DETERMINATION AND MOBILITY
STUDIES IN FISHES USING SCALES



Project Work by

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REG.NO: AB19ZOO024

UNDER THE GUIDANCE OF

Dr. SOJA LOUIS

DEPARTMENT OF ZOOLOGY

ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM

Submitted to St. Teresa's college (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfillment of the requirements of Bachelor of Science degree in Zoology.

2019 - 2022

CERTIFICATE

This is to certify that the project report entitled "**AGE DETERMINATION AND MOBILITY STUDIES IN FISHES USING SCALES**" submitted by LAVANYA M, Reg. No. AB19ZOO024 in partial fulfillment of the requirements of Bachelor of

Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Soja Louis and this is her original effort.

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EXAMINERS

1)

2)

DECLARATION

I, Ms. LAVANYA M, hereby declare that this project report entitled “**AGE DETERMINATION AND MOBILITY STUDIES IN FISHES USING SCALES**” is a bonafide record of work done by me during the academic year 2021-2022 in partial fulfilment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam.

This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

AB19ZOO024

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ABSTRACT

Ocean biome is a largest of all, covering almost three-quarters of the planets surface. It includes a wide variety of marine habitats. Fishes are vertebrates that have always lived underwater since the evolution of the earliest ancestors around 530 million years ago. Throughout earth's history one constant challenge for all animals is been attaining protection from the elements, predators and microorganisms. In ancestral animals of fishes and reptiles a robust preserve form of integuments, scales etc arose. It has a variety of functions including protection and locomotory assistance. Most fishes have scales that protect their delicate skin. Each scale is separate but the scales overlap like tiles on a roof to allow movement. Scales originated within ostracoderms, the ancestor to all fishes today. Cycloid scales of salmon and carp, ctenoid scales of perch, ganoid scales of surgeons and gars, placoid scales of sharks and rays are examples. Fish scales primarily serves two purpose: protection and locomotion. These slippery scales present in the body, protect their body from the environment, parasites, and predators. Scales develops as external growth of the skin on the epidermis. The scales reduce the friction with the water. What distinguishes the scales from each other is both their composition and how they balance those two functions. The more evolved or derived scales have more balance functionality between protection and locomotion. Cosmoid and ganoid scales are the most ancestral type of scales while ctenoid and cycloid scales are most type of scales.

Experiment and studies were conducted on various fishes with cycloid and ctenoid types of scales. Scales were collected from different fishes for this purpose, and mounted. Structural components and other features were observed under the microscope. Both scales had structural differences. The studies helped to understand pivotal role played by scales in the aquatic life of fishes. Scales are the most widely used aging structure in North America because of their non-lethal ease of collection. Counting the number of annuli (rings) on a scale provides the fish age and the

spacing between rings is proportional to the growth of the fish. Knowledge of fish age characteristics is necessary for stock assessments, and to develop management or conservation plans.

INTRODUCTION

The evolution of fishes began about 530 million years ago during the Cambrian explosion. The first fish belongs to Agnatha, or jawless fish, found in the early Paleozoic (Ordovician) 505 mya. They were small (few inches to a foot long initially), freshwater bottom-feeding animals. The circular mouth lacks jaws. Use their gills as both straining devices and as respiratory structures. There is no internal bony skeleton instead thick bony plates and scales that cover the body. Fishes make up more than half of all vertebrate species. They are especially important in the study of vertebrate evolution because several important vertebrate traits evolved in fish. Fish show great diversity in body size. They range in length from about 8 millimeters (0.3 inches) to 16 meters (about 53 feet). Most are ectothermic and covered with scales.

Scales are small plate-shaped dermal or epidermal structures that are found in the outer skeletons of fish, reptiles, or some mammals. The skeletons of many

vertebrates are covered by two types of scales, namely epidermal and dermal. The epidermis contains numerous mucus cells. These cells secrete mucus or slime, which prevents parasites, fungi, pathogens, etc. from entering the skin easily. Most fish bear scales. While some agnatha and catfishes have no scales. Some fishes, especially paddle fish (*Polyodon*), mirror carp (*Cyprinus carpio*) have partial scales. Other fish such as trout and freshwater eel have very small scales. The scales contain a variety of pigments that give the fish a variety of colors. When the fish hatches from the egg, its body is covered by small scales. As the fish grow so does the scales. However, the number of scales remains the same throughout life but the lost scales can be restored at some point. A small circular growth ring is formed in the scales and this ring is called circuli or circulus (in singular). Circulus formed in summer are quite wide whereas circulus formed in winter are intertwined. In that densely enclosed region a black circle is formed which is known as the annulus. The age of the fish is determined by counting the number of annulus in scales. Scales cover most of the body and protect the skin from injury. The scales form a lateral line in the body of the fish along the side of the body and play an important role in detecting vibrations in the water as it acts as a sensory receptor. Fish scales can be divided into four based on shape, namely: Placoid, Cycloid, Ganoid, Ctenoid.

Type of scales found in most living bony fish (Osteichthyes) are of two types, namely: Cycloid and Ctenoid scales. Fishes like tank goby (*Glossogobius giuris*), tilapia (*Oreochromis mossambicus*), sardine (*Sardina longiceps*) etc possess cycloid type of scales and fishes like mullet (*Mugil cephalus*), karimeen (*Etroplus suratensis*), pink perch (*Nemipterus japonicus*) etc have ctenoid type of scale. Detailed structure of the fish scale can be helpful in the identification of fishes up to major groups and species levels, phylogeny, sexual dimorphism, age determination, past environment experienced by fish, discriminating between hatchery reared and

wild populations, migration, pathology of fish scale due to water pollution of the water body and for the growth studies. Shape, size and number of scale are suitable tools in fish taxonomy and using it dates back to first half of 19th century when Agassiz (1833-1843) used it in fish taxonomy for the first time. He classified the fishes into 4 groups based on the scale morphology (Jawad and Al-Jufaili, 2007). During the late 19th century and the first half of the 20th century and with the great advancements in the field of light microscopy, the importance of scale morphology in systematics increased significantly. The importance of scale morphology used in classification was strengthened with the introduction and development of Scanning Electron Microscopy (SEM), so that scales of many different fish species have been studied using SEM (Jawad and All-Jufaili, 2007).

With regard to the importance of scale morphology in this research ultrastructures of scale. The ease of collection of this aging structure is not without its tradeoffs, as the major bias of scales used as an age estimation structure is their tendency to underestimate the age of older fish. The most commonly used techniques involve counting natural growth rings on the scales, otoliths, vertebrae, fin spines, eye lenses, teeth, or bones of the jaw, pectoral girdle, and opercular series. Fish ages are often examined along with measurements of length and weight which combined can provide information on stock composition, age at maturity, life span, mortality, and production.

This topic was selected because, performing age structure analysis are important in growth analysis, population dynamics estimates and resource management. Data from the study can delineate individuals into specific age classes. Exploited species often have the older, larger individuals removed from the population because they are the first removed by fishers leaving the younger smaller individuals. The effect

may have serious consequences for that population. By performing age analysis studies we can identify these types of effects as well their implications to the status of the population. The project, enabled to understand more about the structural peculiarities of cycloid and ctenoid scales.

CYCLOID SCALE FISHES

TANK GOPY - *Glossogobius giuris*

Glossogobius giuris, the tank goby, is a species of goby native to fresh, marine and brackish waters from the Red Sea and East Africa through South Asia and the Indian Ocean to China, Australia and the islands of the Pacific Ocean. This species can also be found in the aquarium trade. It is also known as the bar-eyed goby, flat-headed goby and the Gangetic tank goby. The head is depressed with a protruding lower jaw while the body takes on a compressed appearance towards to caudal fin. Normally brown or light brown with various darker brown spots and flecks along the sides. Ranges in size from 40 to 50 cm maximum (16-20 inches).

TILAPIA - *Oreochromis mossambicus*

Tilapia is the common name for nearly a hundred species of cichlid fish from the coelotilapine, coptodonine, heterotilapine, oreochromine, pelmatolapiine, and tilapiine tribes with the economically most important species placed in the Coptodonini and Oreochromini. Tilapia are mainly freshwater fish inhabiting shallow streams, ponds, rivers, and lakes, and less commonly found living in brackish water. Tilapia typically have laterally compressed, deep bodies. Like other cichlids, their lower pharyngeal bones are fused into a single tooth-bearing structure.

A complex set of muscles allows the upper and lower pharyngeal bones to be used as a second set of jaws for processing food, allowing a division of labor between the "true jaws" (mandibles) and the "pharyngeal jaws". This means they are efficient feeders that can capture and process a wide variety of food items. Their mouths are protrusible, usually bordered with wide and often swollen lips. The jaws have conical teeth. Some Nile tilapia can grow as long as 2.0 ft.

SARDINE - *Sardina longiceps*

Sardines have a flat body which is covered with large, reflective, silvery scales. In the middle of their belly, they have a set of specialized scales, known as scutes, which are jagged and point backwards. Having very small teeth or no teeth at all, sardines eat plankton, which they filter from the water through their gills. While numerous species of sardines live off the coasts of India, China, Indonesia, and Japan, single sardine species dominate in areas like the English Channel and the California coast. Sardines are basically a warm-water fish, but occur as far north as Norway.

CTENOID SCALE FISHES

MULLET - *Mugil cephalus*

The flathead grey mullet (*Mugil cephalus*) is an important food fish species in the mullet family Mugilidae. It is found in coastal tropical and subtropical waters worldwide. Its length is typically 30 to 75 centimetres (12 to 30 in). It is known with numerous English names, including the flathead mullet, striped mullet (US, American Fisheries Society name), black mullet, bully mullet, common mullet, grey

mullet, sea mullet and mullet, among others. The flathead grey mullet is a mainly diurnal coastal species that often enters estuaries and rivers. It usually schools over sand mud bottoms, feeding on zooplankton. The adult fish normally feed on algae in fresh water. It occupies fresh, brackish and marine habitats in depths ranging between 0-120 metres (0-394 ft) and with temperatures between 8-24 °C (46-75 °F).

PEARL SPOT - *Eetroplus suratensis*

The green chromide (*Eetroplus suratensis*) is a species of cichlid fish is native to fresh and brackish water habitats in some parts in India such as Kerala, Goa, Chilika Lake in Odisha and Sri Lanka. The species was first described by Marcus Elieser Bloch in 1790. Other common name include pearlspot cichlid, banded pearlspot, and striped chromide. In Kerala, it is known locally as the karimeen. In Tamilnadu, it is known locally as the 'pappan or pappa. In Goa the fish is known as kalundar. The green chromide lives in brackish water habitat types, such as river deltas. It eats mainly aquatic plants, including filamentous algae and diatoms, but it consumes the occasional molluscs and other animal matter. This species engages in attentive parental care in which several adults care for each brood.

PINK PERCH - *Nemipterus japonicus*

Pink Perch or Rani is a common freshwater fish in India. Pink in colour and small in size, this fish has a mild taste when cooked. Due to just 4- 5% of body fat, this fish is not oily and hence it is called a lean fish. This is your go to go meat if you are planning to lose weight. Pink Perch gets its name due to the pink hue and yellow strips with scales on its skin. It has a mild flavour with a delicate, soft texture that makes it ideal for curries, fried or grilled preparations.

AIM

This project work aims to analyse the size variations of scales in different parts of fishes, determine age of fish by counting the anuli of scale.

OBJECTIVES

The objective of the present study was 1) to evaluate the size variation of scales in different parts of fishes by measuring the scales 2) determining the age of fishes by counting anuli by mounting the scales.

REVIEW OF LITERATURE

One of the unique features in a fish is the presence of the scales (except fishes of order siluriformes) on the outer surface of the body. A fish scale can tell us a great deal about the fish's life story, including where it has been since it hatched. These scales are magic tool for studying a life cycle as a whole. Various types of scales are found in fishes eg. placoid, cycloid, ganoid, cosmoid or ctenoid. Placoid scales are found in cartilaginous fishes and rest all are found in bony fishes. These are derived from the connective tissue of the dermis and form the exoskeleton. Scales of fish are used for classification, identification and growth studies of different fishes, pollution indicators etc.

The scales of bony fishes are derived entirely from the dermal layer of the skin and overlap one another like the tiles. The overlapping (imbrications) of the scale is important in the sense that it imparts mechanical support. Each scale is shaped roughly like a human finger nail whose front end is inserted deep into the dermal layer, while the hinder end is free of exposed and bears the pigment cells or chromatophores on it. These chromatophores provide specific colour to the fish body. These scales have a soft anterior part and hard posterior part. The dorsal surface of the scales is rough as it bears the lines of growth, whereas the ventral surface that touches skin in shining. The cycloid scale has concentric rings around the focus and these rings/lines of growth as sclerites. Cycloid and ctenoid scales increase in size, growth rings called circuli become visible. These rings look a little like the growth rings in the trunk of a tree. During the cooler months of the year the scale (and otoliths) grows more slowly and the circuli are closer together leaving a band called an annulus. By counting the annuli it is possible estimate the age of the fish. This technique is extensively used by fisheries biologists. Only the anterior and lateral sides of scale have these circuli. These are the marks of periodical growth of fish. Any sudden changes in fish's environment is recorded on the scales in the form

of alteration in the circuli shape, pattern or altered elemental deposition thus making these hard structures a testimony to life history of the fish. These revealing marks may be annual marks, winter marks or the larval marks.

Scales at the shoulder of the fish between the head and the dorsal fin is best suited for age determination. Scales are almost two-dimensional structures. The anterior part is formed of a series of sclerites which should extend in a regular pattern from the centre of the scale. The structural discontinuities used for age determination result from irregularities in the pattern of the sclerites; they may be slightly distorted or they may be slightly closely spaced than the majority of the sclerites; usually the discontinuities are narrow and they are usually called 'rings'. Scales are thin structures they need no preparation before viewing; the scales should be cleaned before they are stored. For reading, the slide with mounted scales is placed on the stage of a low-power microscope. The magnification used depends upon the size of the scale; in general, the lowest possible magnification is the best because it enables the whole scale pattern to be seen.

The use of age information is an integral part of fisheries today. Hilborn and Walters (1992) state that "the most valuable information obtained from sampled catch, at least for temperate waters, is age." The development and acceptance of methods for age determination in fishes represents a critical early stage in fisheries science, and at times was fraught with more controversy than today's wide usage of the methods would suggest. In 2006, the Fisheries Management Section of the American Fisheries Society formed the ad hoc Assessment of Fish Aging Techniques Committee to assess the status of aging of freshwater fishes in North America (see Maceina *et al.* this issue). As part of the committee's goals, an historical survey of

the earliest references to aging of fishes and their initial application to fisheries studies was undertaken.

Antoni van Leeuwenhoek of Holland, who used his experience counting threads in cloth at a dry goods store with magnifying glasses to develop improved lenses that he used to construct microscopes, became one of the leading microscopists of the 1600s. Leeuwenhoek possessed a wide-ranging curiosity that included issues surrounding demographics of animal populations (Egerton 1968). Curiously, Leeuwenhoek's studies of fish scales appear to have been at least in part inspired by Biblical strictures against eating fish without scales. His earliest writings on fish scales appeared in a letter to the Royal Society of London, and focused on the European eel (*Anguilla anguilla*) and the burbot (*Lota lota*), which he was drawn to as a result of their reported lack of scales "which two sorts of fish, the Jews will not eat, as forbidden by the Law of Moses." (Leeuwenhoek 1685).

Petersen is most often credited with first proposing length-based methods for age determination (Allen 1917; Ricker 1975). His work appears to have been preceded by Joseph T. Cunningham. Cunningham, working at the Marine Biological Association's Plymouth Lab, attempted to use lengths from known-aged fish he reared in aquaria to assign ages to wild-caught fish, focusing on flatfish and cod (Cunningham 1891, 1892). Cunningham's efforts were not rewarded by clear-cut results: "It is evident there is considerable variation in the rate of growth in nature, from the difficulty of distinguishing in a large number of fish those of one year's, two years', and three years' growth" (Cunningham 1891).

Hederström examined the vertebrae of pike and concluded that the rings that could be discerned on them were growth rings that could be used to determine the fish's

age. His reasoning revealed a thoroughly scientific approach, and included verification that (1) both sides of a vertebra had the same number of rings, (2) all vertebrae in an individual possessed the same number of rings, (3) larger fish had more rings on their vertebrae than smaller fish, and (4) the number of rings matched the age of fish “known either from experience or from other circumstances” (Hederström 1759). Hederström went on to present length-at-age data for pike that agree well with modern estimates and also reported that he had confirmed the applicability of using rings on vertebrae for determining the age of a variety of other species, including European perch (*Perca fluviatilis*), roach (*Rutilus rutilus*), bream (*Abramis brama*) etc.

Detailed structure of the fish scale, helpful in the identification of fish up to major groups and species levels can be obtained from: (Abraham *et al.* 1966; Bartulović *et al.* 2011). Detailed structure of the fish scale, helpful in the identification of fish up to major groups and species levels can be obtained from: (Abraham *et al.* 1966; Bartulović *et al.* 2011).

Thompson’s (1910) statistical concerns by presenting comparisons of a normal curve to their age-frequency curve, concluding that “the dissimilarity of the two curves is, in fact, so great as to exclude any idea of the age-curve following the usual law of biological variation” and that “it seems to us impossible to explain the observed facts as a result of common variation, even if the help of a mathematical statistician were enlisted.”

Huntsman of the Fisheries Research Board of Canada was keeping abreast of the developments in Europe, however, and in 1918 presented a paper to the Royal Society of Canada on its potential applications, soon followed by a similar

presentation to the American Fisheries Society (Huntsman 1918, 1919). Carlander (1987) credits Huntsman's papers with bringing aging methods to the attention of North American workers.

Borodin (1924) used scales to assess American shad (*Alosa sapidissima*) in the Connecticut River, and a study of the use of otoliths in the same system followed soon after (Barney 1924). A search of articles in the Transactions reveals only one other application of aging to fish studies in the 1920s, but an increase to 84 in the 1930s, 74 during the 1940s, 112 during the 1950s, followed by rapid increases to 231 in the 1960s and 370 during the decade of the 1970s.

Petersen's work using lengths to assign ages to blenny (*Zoarces viviparus*) received more notice than Cunningham's, but was characterized by the same difficulties (Petersen 1892, summarized by Ricker 1975). Petersen constructed what are now known as length-frequency graphs, proposing that the peaks, or modes, that were evident across the range of smaller to larger size classes represented progressively older age-classes of fish.

By correlating the marginal growth of scales (the amount of growth between the last ring or annulus and the margin of the scale) with the season of the year in which the scales were collected, Walford and Mosher (1943a: 9 and 1943b) have shown for the Pacific pilchard or sardine, *Sardinops caerulea* (Girard) 1854, that these rings are formed annually and consequently can be used for age determination as well as for back-calculation of the length of the fish at a given earlier age.

Age determination based on the analysis of the growth marks of calcified structures is the specific aim of the Sclerochronology Laboratory of the INSTM (SLI), which was created in 2000. The methods used in SLI to identify and count growth marks

on mineralized structures in fishes and to interpret the corresponding data. Is monitored till now, the species of interest to the SLI are small pelagic fishes. Age determination of *Mullus surmuletus* has been performed either by counting scale annuli (Gharbi, 1980) or otolith (in toto) growth marks (Jabeur, 1999).

In 1859, Robert Bell reported that one could use these growth rings to reliably determine the age of all fish after examination of sucker (*Catostomus sp.*) vertebrae and yellow perch (*Perca flavescens*) scales that he raised in a pond for two years showed “two rings or circles.”

Stuart Thomson, with encouragement and support from Walter Garstang and Allen at the Plymouth Laboratory of the Marine Biological Association of the United Kingdom, extended Hoffbauer’s work with freshwater fishes to important commercial marine species. His detailed work with pollack (*Pollachius pollachius*), poor cod (*Trisopterus minutus*), whiting (*Merlangius merlangius*), haddock (*Melanogrammus aeglefinus*), and cod convinced him that Hoffbauer’s findings could be applied to marine species (Thomson 1902, 1904).

METHODOLOGY

3 different types of fishes were selected each for cycloid and ctenoid scales. Tank goby, thilapia, sardine for cycloid scale and pearl spot, mullet, pink perch for ctenoid scale. These fishes were selected from various markets. Specimens were collected during March, 2022. Care was taken during collection of specimen to avoid fishes with damaged scales. First step was cleaning of the specimens, it was washed with clean water. The next step was de-scaling. Scales were taken from the body

carefully, using forceps and washed the scales with water again. Measurements of the scale were noted. Measurements were taken using white threads, length and breadth of the scales were measured. With the help of a ruler right measurements of length and breadth were obtained.

Colour of the scales of all the fishes were observed. The shape and other morphological differences between two type of scales were also noted. Scales from different regions like cephalic, caudal, dorsal, pelvic and pectoral regions were collected. The final step of the experiment was the comparative study of scales of ctenoid and cycloid scales. The scales were mounted for further morphological observations.

Mounting of Scales

The materials required:

1. Forceps
2. Watch glass-2
3. 10 % KOH solution
4. Spirit lamp
5. Match box
6. Distilled water
7. Fine brush
8. clean grease free microscopic slides
9. Leishmann's stain



Fig. 1 – Materials required for mounting the scales

Procedure:

1. Fishes with cycloid and ctenoid scales are de-scaled (scales from pectoral, pelvic, caudal, dorsal, head regions were collected, this can be done by careful scrapping of fish. Care should be taken not to damage the scales, they are then transferred to 10% KOH solution taken in a test tube.
2. Heat the fish scales in 10% of KOH solution for 10-20 seconds.
(Note: Heating is done to dissolve the covering epithelium over heating will cause curling of the scales.)
3. Wash the fish scales in distilled water (2 times), for removing the KOH solution.
4. Take few drops of Leishmann's stain in a watch glass. Transfer the scales into that.
5. Wait for 3 minutes. Wash off the excess stain.
6. Transfer the scales to a glass slide.
7. Observe the slide under dissecting microscope and under the low power (4X) of simple microscope.
8. Repeat the same for other fishes with ctenoid scale.

9. Compare all the slides and note down the differences and morphologies of the scales.

Age Determination

Procedure: Scales are prepared for study by mounting the whole scales on glass slides or, more commonly, by pressing imprints of the scale circuli into transparent plastic. These scales or scale impressions are examined under a low-power microscope or by use of a microprojector, and the rings or annuli are counted.

Applications of Scale Method: Fish of temperate regions shows clear rings, which are true marks. This is because there is a sharp difference between the temperatures two seasons—summer—the period of faster growth, and winter—the period of slow growth or no growth. Therefore, the calculation of the age of fish by annuli is most reliable in temperate fish. This method is more reliably applicable in case of salmons, carps, cod and herrings, established a method of estimating age of fish based on scales.



Fig. 1 – Tank Gopy



Fig. 2 – Tilapia



Fig. 3 – Sardine



Fig. 4 – Mullet



Fig. 5 – PEARL SPOT

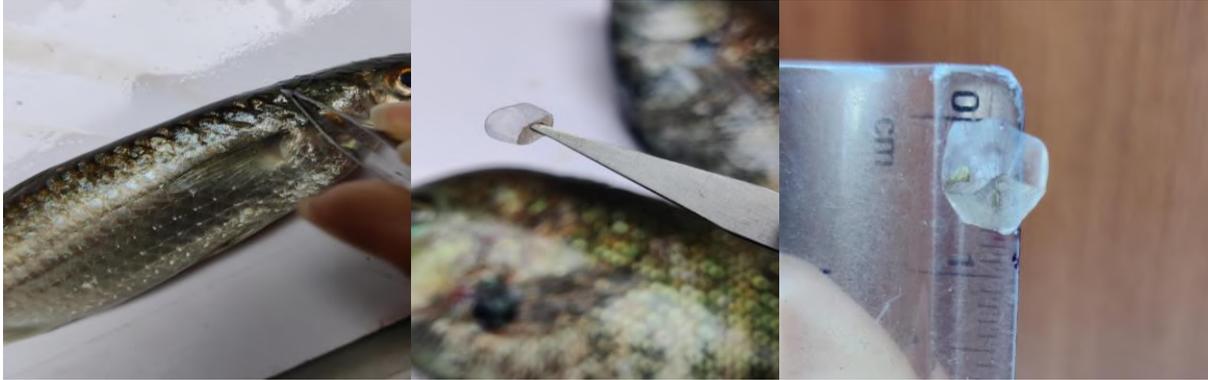


Fig. 6 – Pink Perch



(a)

(b)



(c)

(d)

(e)

Pleat 1 – Procedure for measuring the size of scale

- a) Measuring the length of the fish
- b) Measuring the width of the fish
- c) De-scaling the fish
- d) Scale of fish
- e) Measuring the scale

OBSERVATION

	FISH 1		FISH 2		FISH 3		FISH 4		FISH 5	
	L	B	L	B	L	B	L	B	L	B
TANGOPY	13.1cm	4cm	10.2cm	3.7cm	11.6cm	4cm	9.2cm	3cm	8.9cm	3cm
TILAPIA	19cm	7cm	20cm	10cm	21cm	8cm	22cm	8cm	19cm	7cm
SARDINE	14.6cm	3.5cm	16.1cm	4.3cm	16.5cm	4.2cm	16.3cm	4.2cm	15.1cm	3.6cm
MULLET	18.5cm	5cm	16.5cm	4.3cm	15cm	4.3cm	14.5cm	3cm	13cm	3cm

PEARL SPOT	18.5cm	10cm	17cm	8.5cm	15cm	9cm	14.5cm	7cm	14cm	7cm
PINK PERCH	18.7cm	5.9cm	18.7cm	5.8cm	17.8cm	5.8cm	16.8cm	6.7cm	19.1cm	5.7cm

Table 1 – Measurement of fish size

	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.2cm	0.2cm	0.4cm	0.4cm	0.6cm	0.6cm	0.5cm	0.5cm	0.6cm	0.4cm
Fish2	0.2cm	0.2cm	0.4cm	0.5cm	0.5cm	0.5cm	0.5cm	0.4cm	0.5cm	0.4cm
Fish3	0.2cm	0.2cm	0.4cm	0.3cm	0.5cm	0.5cm	0.5cm	0.6cm	0.5cm	0.4cm
Fish4	0.3cm	0.3cm	0.4cm	0.4cm	0.4cm	0.5cm	0.5cm	0.5cm	0.5cm	0.4cm
Fish5	0.3cm	0.3cm	0.4cm	0.4cm	0.6cm	0.6cm	0.4cm	0.4cm	0.5cm	0.4cm

Table 2 – Measurement of scales in different parts of tank gopy

	HEAD REGION		PELVIC REGION		PECTORAL REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.4cm	0.6cm	0.6cm	0.7cm	0.3cm	0.4cm	0.5cm	0.6cm	0.5cm	0.5cm
Fish2	0.8cm	0.6cm	0.6cm	0.8cm	0.4cm	0.3cm	0.5cm	0.5cm	0.5cm	0.5cm
Fish3	0.7cm	0.7cm	0.6cm	0.8cm	0.4cm	0.3cm	0.4cm	0.4cm	0.4cm	0.5cm
Fish4	0.6cm	0.8cm	0.6cm	0.6cm	0.5cm	0.3cm	0.6cm	0.5cm	0.6cm	0.5cm
Fish5	0.5cm	0.6cm	0.5cm	0.6cm	0.4cm	0.2cm	0.5cm	0.5cm	0.5cm	0.4cm

Table 3 – Measurement of scales in different parts of tilapia

	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.3cm	0.3	0.5cm	0.4cm	0.4cm	0.4cm	0.4cm	0.2cm	0.5cm	0.4cm

Fish2	0.3cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm	0.4cm	0.4cm	0.3cm	0.3cm
Fish3	0.5cm	0.3cm	0.7cm	0.6cm	0.5cm	0.5cm	0.5cm	0.4cm	0.3cm	0.3cm
Fish4	0.5cm	0.5cm	0.6cm	0.6cm	0.5cm	0.5cm	0.5cm	0.5cm	0.3cm	0.3cm
Fish5	0.5cm	0.5cm	0.6cm	0.4cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm	0.4cm

Table 4 – Measurement of scales in different parts of sardine

	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.8cm	0.8cm	1cm	0.9cm	0.9cm	0.6cm	1.1cm	0.9cm	0.7cm	0.5cm
Fish2	0.8cm	0.8cm	0.9cm	0.8cm	0.7cm	0.6cm	0.9cm	0.8cm	0.7cm	0.5cm
Fish3	0.5cm	0.4cm	0.5cm	0.4cm	0.7cm	0.5cm	0.6cm	0.6cm	0.6cm	0.5cm
Fish4	0.5cm	0.5cm	0.6cm	0.6cm	0.5cm	0.4cm	0.6cm	0.6cm	0.4cm	0.4cm
Fish5	0.7cm	0.6cm	0.8cm	0.7cm	0.5cm	0.3cm	0.7cm	0.6cm	0.6cm	0.4cm

Table 5 – Measurement of scales in different parts of mullet fish

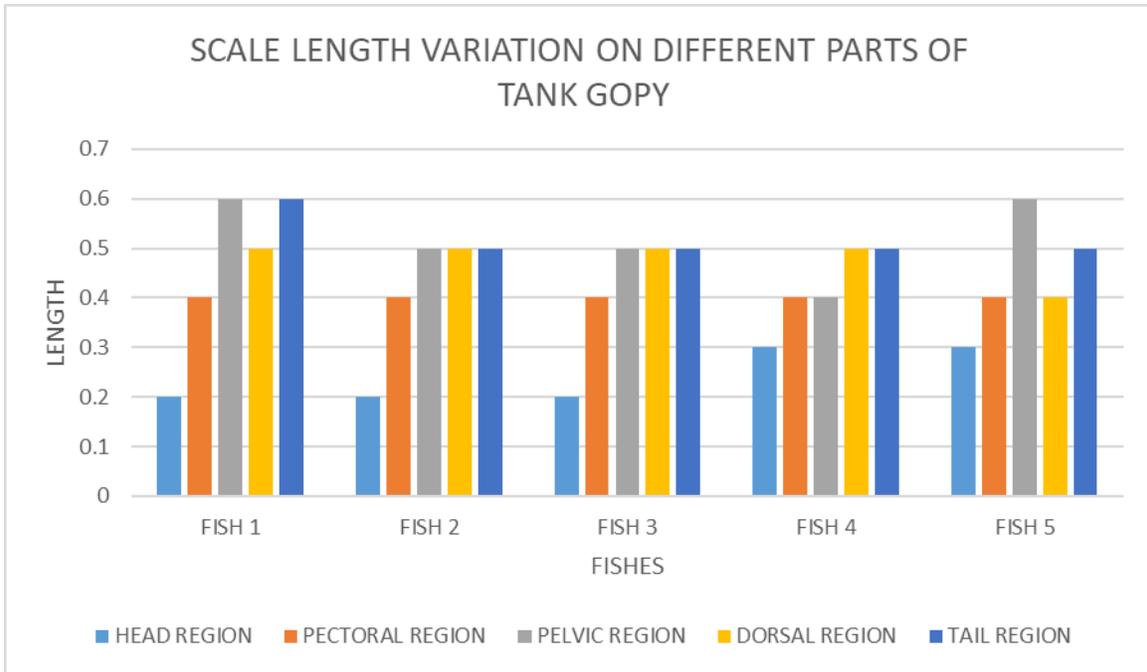
	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish 1	0.7cm	0.5cm	0.7cm	0.7cm	0.6cm	0.4cm	0.7cm	0.5cm	0.6cm	0.5cm
Fish 2	0.5cm	0.7cm	0.5cm	0.6cm	0.5cm	0.5cm	0.6cm	0.6cm	0.5cm	0.5cm
Fish 3	0.5cm	0.6cm	0.6cm	0.6cm	0.6cm	0.5cm	0.6cm	0.5cm	0.5cm	0.4cm
Fish 4	0.5cm	0.4cm	0.5cm	0.5cm	0.5cm	0.5cm	0.6cm	0.5cm	0.5cm	0.5cm
Fish 5	0.4cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm

Table 6– Measurement of scales in different parts of pearl spot

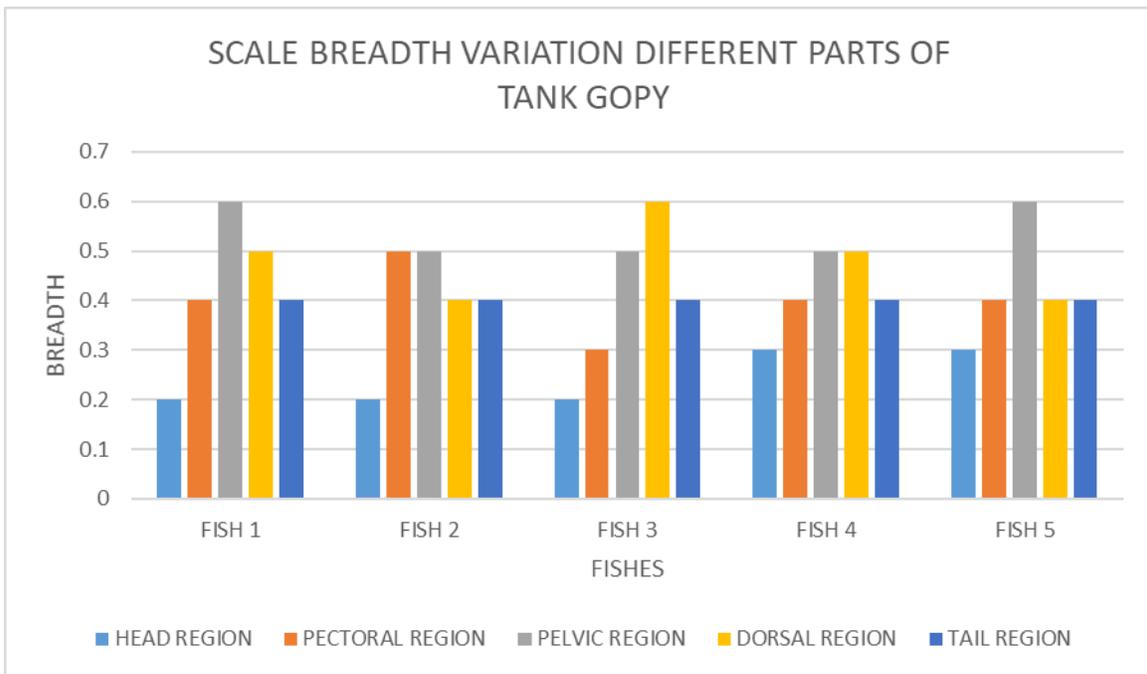
	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.6cm	0.6cm	0.6cm	0.9cm	0.6cm	0.6cm	0.5cm	0.4cm	0.4cm	0.4cm
Fish2	0.4cm	0.4cm	0.6cm	0.7cm	0.6cm	0.7cm	0.6cm	0.5cm	0.5cm	0.5cm
Fish3	0.5cm	0.4cm	0.6cm	0.8cm	0.6cm	0.6cm	0.6cm	0.5cm	0.4cm	0.4cm
Fish4	0.6cm	0.6cm	0.5cm	0.9cm	0.5cm	0.5cm	0.5cm	0.5cm	0.3cm	0.3cm
Fish5	0.6cm	0.5cm	0.6cm	0.9cm	0.6cm	0.7cm	0.6cm	0.5cm	0.4cm	0.4cm

Table 7 – Measurement of scales in different parts of pink perch

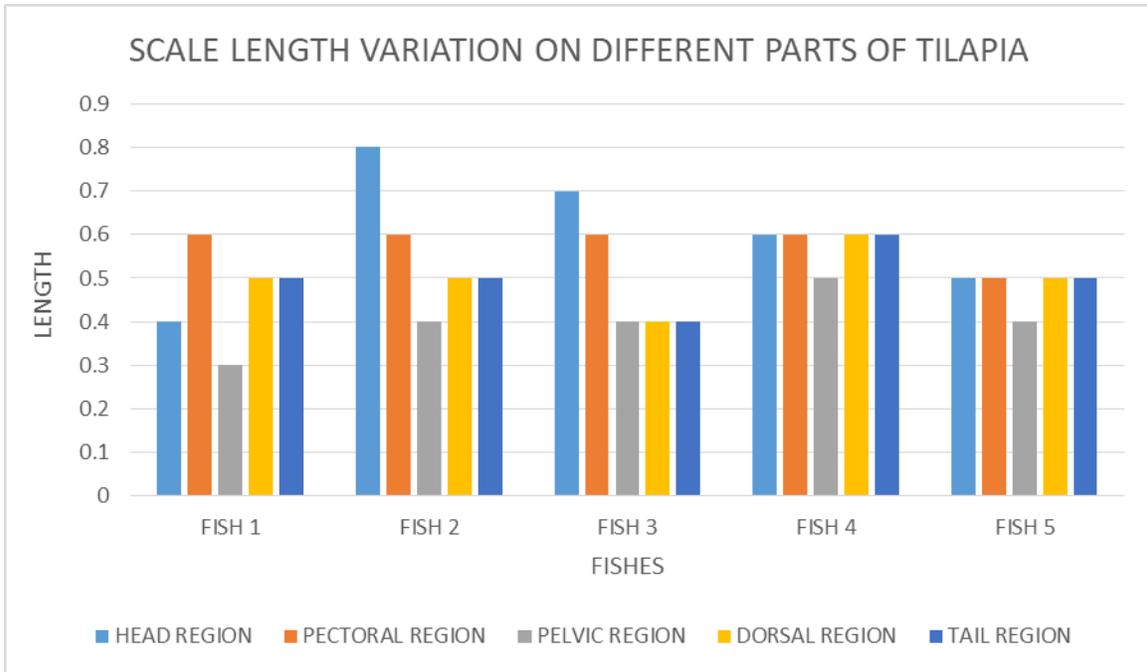
RESULT



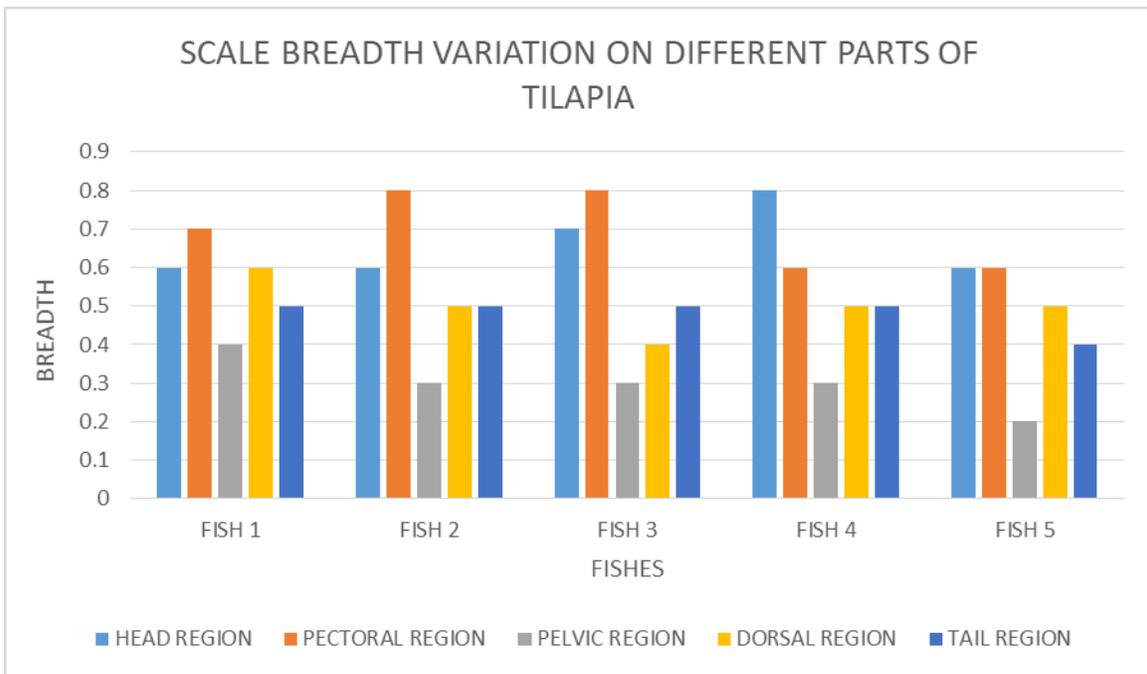
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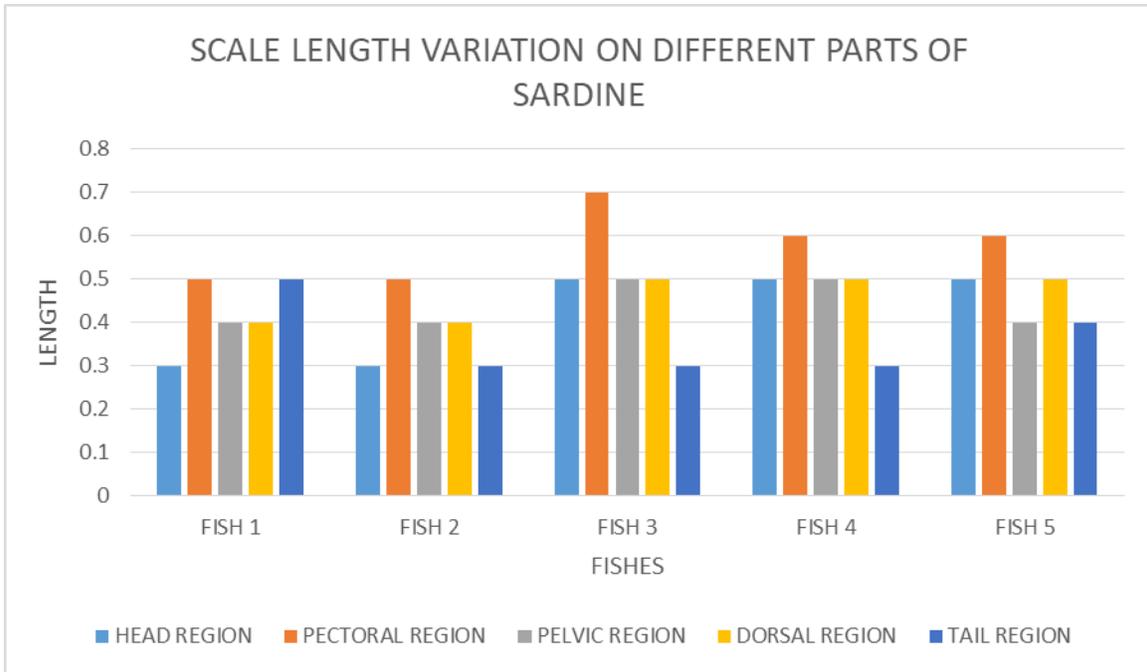
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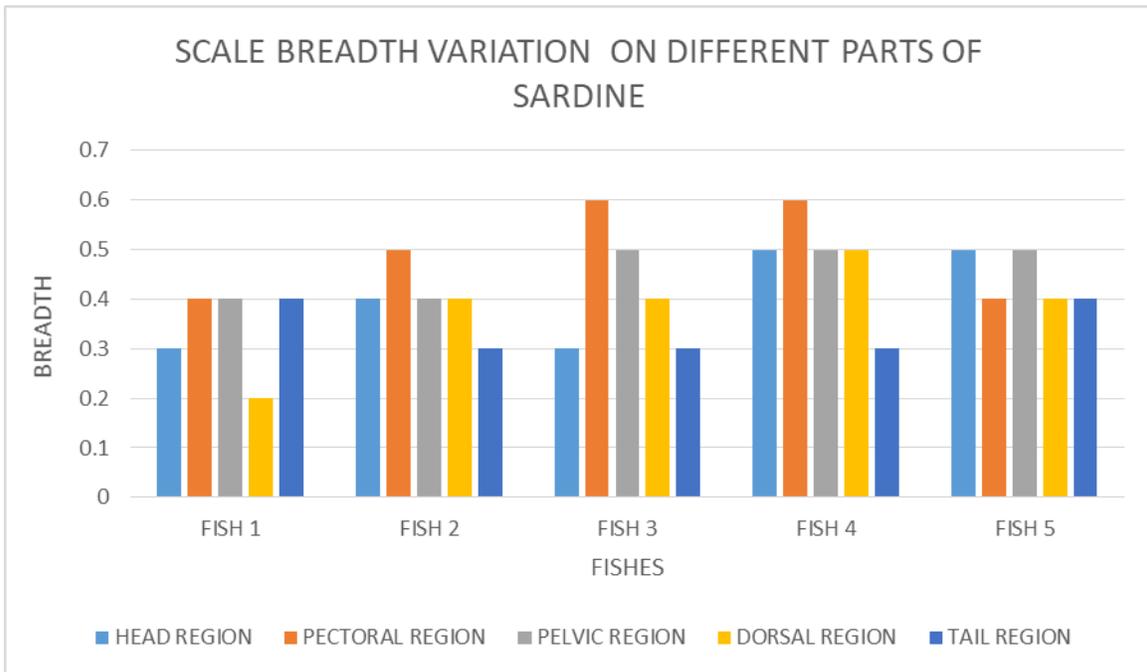
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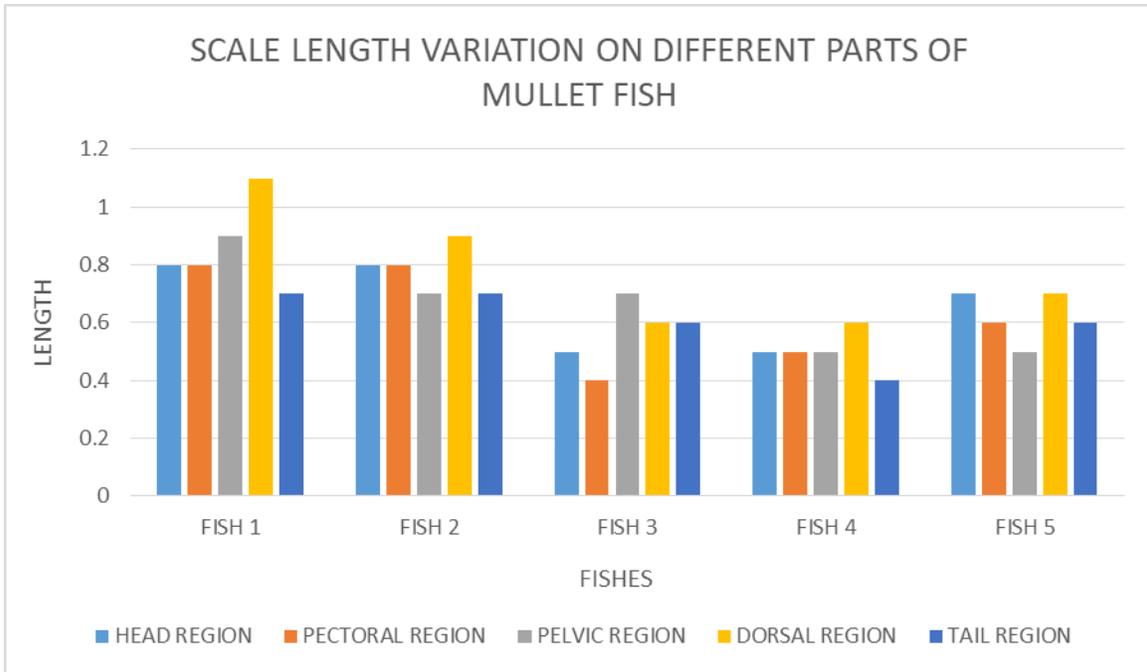
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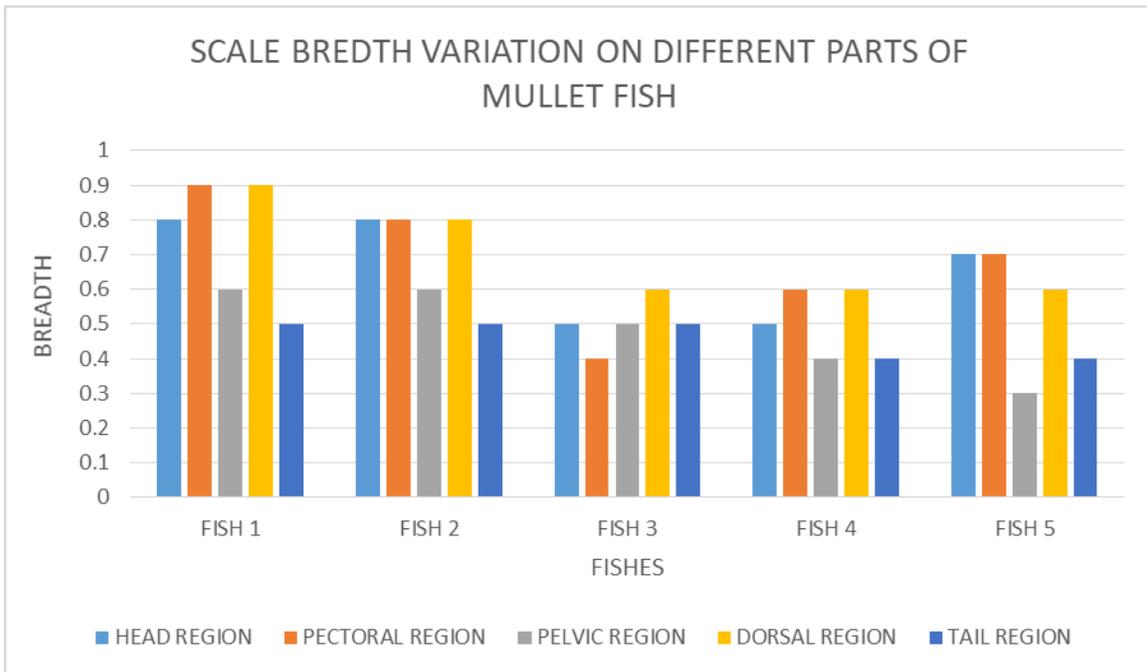
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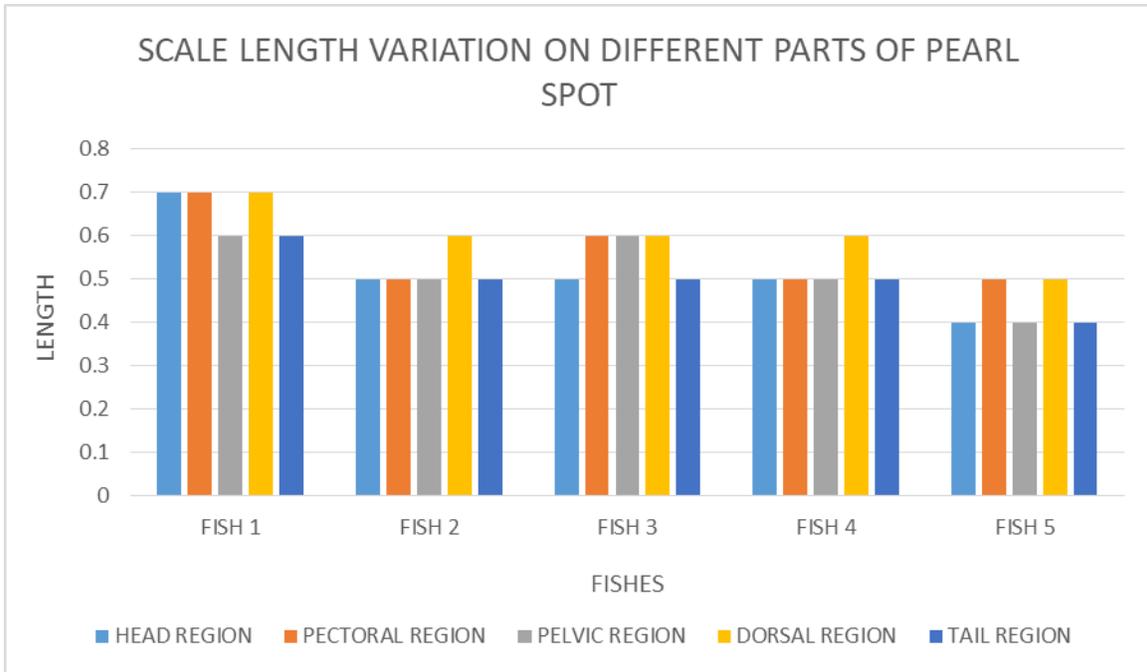
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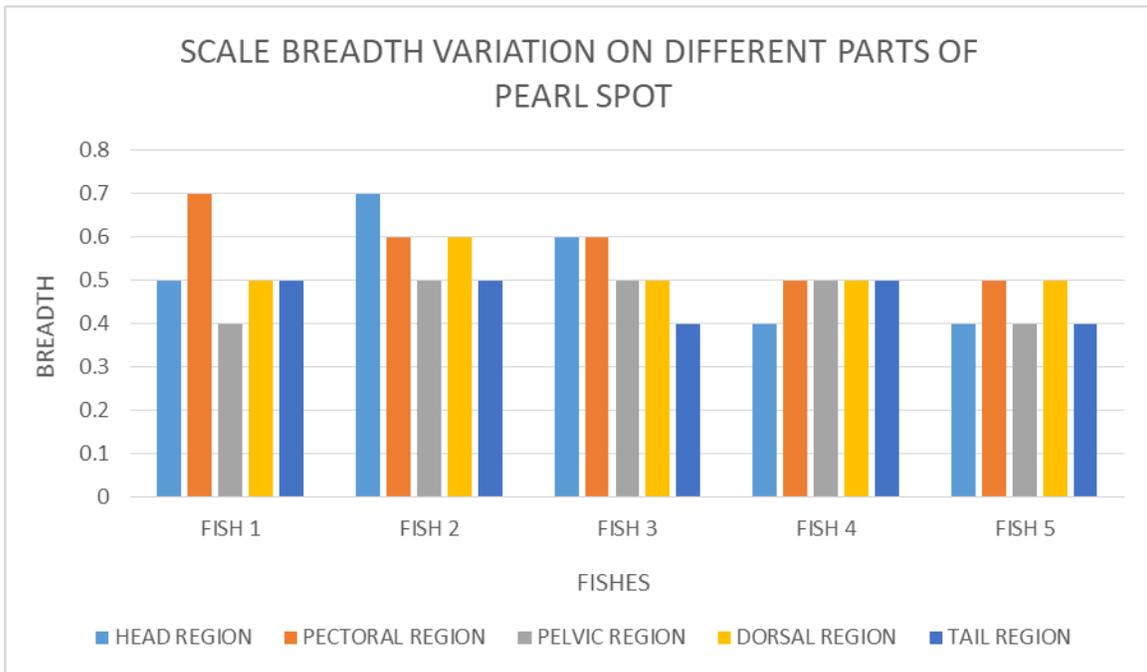
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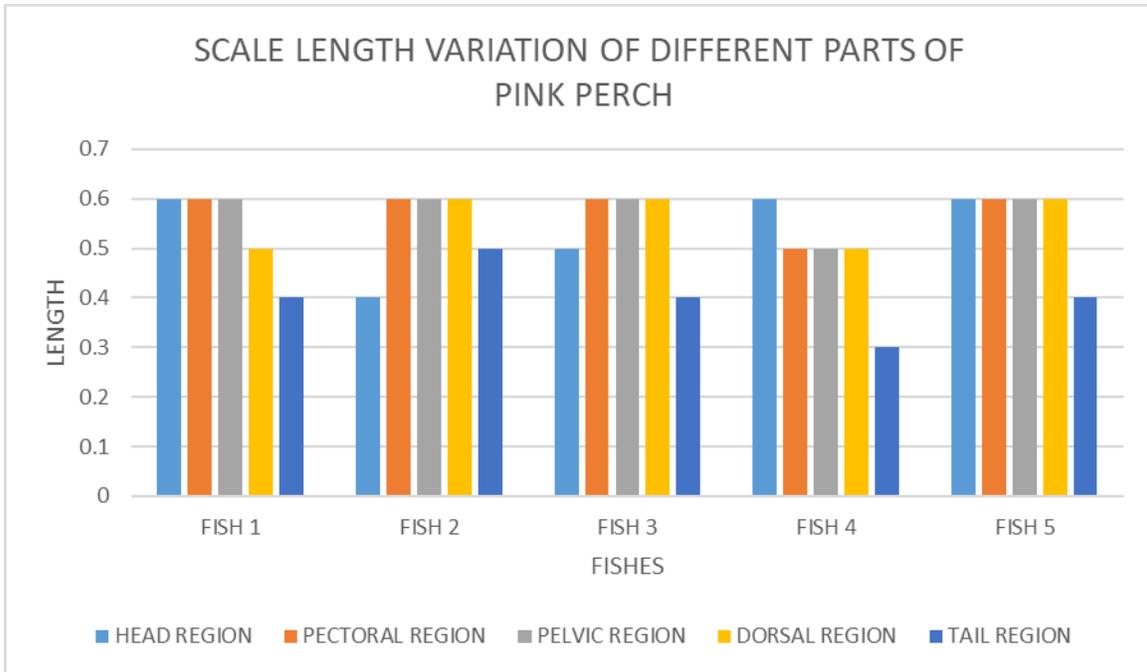
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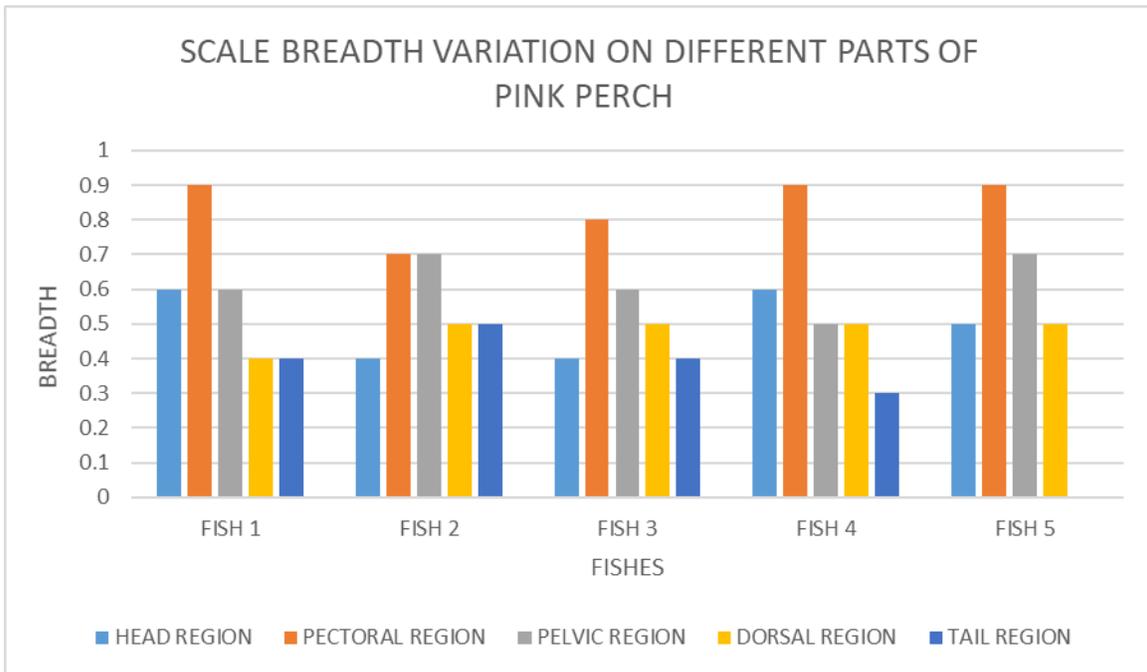
Graph 9:



Graph 10:



Graph 11:



Graph 12:

TANK GOPY		
FISHES	NO. OF ANULI	AGE OF FISH
FISH 1	1	1 YEAR
FISH 2	2	2 YEARS
FISH 3	1	1 YEAR
FISH 4	0	
FISH 5	0	
TILAPILA		
FISH 1	1	1 YEAR
FISH 2	0	
FISH 3	0	
FISH 4	<u>1</u>	1 YEAR
FISH 5	<u>1</u>	1 YEAR
SARDINE		
FISH 1	2	2 YEARS
FISH 2	3	3 YEARS
FISH 3	4	4 YEARS
FISH 4	3	3 YEARS
FISH 5	2	2 YEARS
MULLET FISH		
FISH 1	2	2 YEARS
FISH 2	2	2 YEARS
FISH 3	2	2 YEARS
FISH 4	1	1 YEAR
FISH 5	1	1 YEAR
PEARL SPOT		

FISH 1	4	4 YEARS
FISH 2	3	3 YEARS
FISH 3	2	2 YEARS
FISH 4	2	2 YEARS
FISH 5	1	1 YEAR
PINK PERCH		
FISH 1	3	3 YEARS
FISH 2	1	1 YEAR
FISH 3	4	4 YEARS
FISH 4	3	3 YEARS
FISH 5	3	3 YEARS

DISCUSSION

The body of an ideal fish is covered by thin scales. The scales develop as external growths of the epidermis or skin. The epidermis contains numerous mucus cells. These cells secrete mucus or slime, which prevents parasites, fungi, pathogens, etc. from entering the skin easily. Agnatha is an infra phylum of jawless fish in the phylum Chordata, subphylum Vertebrata, consisting of both present (cyclostomes) and extinct (conodonts and ostracoderms) species. Among recent animals, cyclostomes are sister to all vertebrates with jaws, known as gnathostomes. In modern agnathans, the body is covered in skin, with neither dermal nor epidermal scales. The skin of hagfish has copious slime glands, the slime constituting their defense mechanism. The slime can sometimes clog up enemy fishes' gills, causing them to die. In direct contrast, many extinct agnathans sported extensive exoskeletons composed of either massive, heavy dermal armour or small

mineralized scales. Most fish bear scales. The scales contain a variety of pigments that give the fish a variety of colors. The scales form a lateral line in the body of the fish along the side of the body and play an important role in detecting vibrations in the water as it acts as a sensory receptor. Cycloid and ctenoid scales are found in the majority of bony fishes (the Teleostei).

In this project two different types of scales were studied namely cycloid and ctenoid. Fishes having cycloid and ctenoid scales were collected, descaled, stained and observed. It was noticed that both the scales differed in shape and structure. Ctenoid scales have a variously developed spiny posterior margin whereas cycloid scales have a smooth posterior margin lacking ctenii. The number and distribution of scales were not same either in all the six fishes. In fishes like sardine, tank goby, pink perch it was observed that the size of the scale in the tail region was comparatively smaller than scales in other regions of the body. These fishes are active swimmers, their rapid tail movement is what allows them to swim smoothly against the friction offered by the water. The small size of scales in the tail region is an adaptation for their rapid tail movement. Scales in other regions like pectoral, dorsal and head were bigger than the ones in tail region as they are not much involved in movement. Coloration was another feature that was observed, the margin of the scale in pink perch was pink in colour.

The coloration is an adaptation for various purposes like camouflage, mating etc. Scales are of value for age determination in many of bony fishes, a broad grouping which includes most fishes of importance for food. Scales are formed when newly hatched fish complete their larval stages, and soon cover the entire body, with the exception of head and fins. In most species they lie in an overlapping pattern much like shingles on a roof and serve as a protective coat. Scale growth begins with the formation of the scale center or focus and growth is outward from this focus, though it is greatest toward the forward margin of the scale. Fine ridges called circuli are

laid down in a circular pattern around the focus as growth proceeds. Many circuli are added to the scale each year. Fish growth is reflected in scale growth. Circuli are widely spaced in warm seasons when fish growth is rapid, and closely spaced in cold seasons when it is slow. Fish growth stops in winter. The growth of a fish during one year is shown on its scales as a series of widely spaced spring and summer circuli followed by a series of closely spaced fall and winter circuli. Since fishes continue to grow throughout their lives, this pattern is repeated each year. The outer edge of a series of closely spaced circuli is generally taken to be the end of growth for that year and this point is referred to as the year mark or annulus. The age of a fish is determined by counting the number of annuli or year marks.

CONCLUSION

The scales comprise a non-growing "crown" composed of dentine, with a sometimes-ornamented enameloid upper surface and an aspidine base. Its growing base is made of cell-free bone, which sometimes developed anchorage structures to fix it in the side of the fish. They are small, thin, cornified, calcareous or bony plates which fit closely together or overlap. When the arrangement of scales on fish body is concerned, they are most often imbricated and thus, overlap like shingles on the roof, with their free margins directed towards the tail, so as to minimise the friction of water. Two different types of scales observed in the project were cycloid and

ctenoid scales. Cycloid scales are somewhat circular in appearance, in addition to being thin and translucent. The center of these scales is thicker, and can observe several concentric lines of growth. Another interesting fact about these lines of growth is that they indicate the age of the fish when counted. The greater the number of concentric rings, the older the fish. Ctenoid scales have specific comb-like projections at the back. In form, structure and arrangement, they are very similar to cycloid scales. However, ctenoid scales attach themselves more firmly to the skin. The rear parts of these scales don't overlap and they have several comb-like teeth. Some scales possess colour as in pink perch, these colouration help in mating and camouflage it also make them attractive. Scales exhibit variations in size in different regions of the body. Scales found near the tail and head regions are comparatively small than the scales in the, pectoral and dorsal regions. Size of the scales contributes much to the locomotion of the fish. Small scales in the tail region enable rapid movement of tail and move smoothly in water against the friction offered by it. More detailed studies can be conducted on fish scales, considering the structural and mechanical properties.

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AGE DETERMINATION AND MOBILITY
STUDIES IN FISHES USING SCALES



Project Work by

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UNDER THE GUIDANCE OF

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DEPARTMENT OF ZOOLOGY

ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM

Submitted to St. Teresa's college (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfillment of the requirements of Bachelor of Science degree in Zoology.

2019 - 2022

CERTIFICATE

This is to certify that the project report entitled “**AGE DETERMINATION AND MOBILITY STUDIES IN FISHES USING SCALES**” submitted by Margrett Aneeta Simenthy, Reg. No. AB19ZOO025 in partial fulfillment of the requirements

of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Soja Louis and this is her original effort.

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EXAMINERS

1)

2)

DECLARATION

I, Ms. Margrett Aneeta Simenthy, hereby declare that this project report entitled “**AGE DETERMINATION AND MOBILITY STUDIES IN FISHES USING SCALES**” is a bonafide record of work done by me during the academic year 2021-2022 in partial fulfilment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam.

This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

Margrett Aneeta
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ABSTRACT

Ocean biome is a largest of all, covering almost three-quarters of the planets surface. It includes a wide variety of marine habitats. Fishes are vertebrates that have always lived underwater since the evolution of the earliest ancestors around 530 million years ago. Throughout earth's history one constant challenge for all animals is been attaining protection from the elements, predators and microorganisms. In ancestral animals of fishes and reptiles a robust preserve form of integuments, scales etc arose. It has a variety of functions including protection and locomotory assistance. Most fishes have scales that protect their delicate skin. Each scale is separate but the scales overlap like tiles on a roof to allow movement. Scales originated within ostracoderms, the ancestor to all fishes today. Cycloid scales of salmon and carp, ctenoid scales of perch, ganoid scales of surgeons and gars, placoid scales of sharks and rays are examples. Fish scales primarily serves two purpose: protection and locomotion. These slippery scales present in the body, protect their body from the environment, parasites, and predators. Scales develops as external growth of the skin on the epidermis. The scales reduce the friction with the water. What distinguishes the scales from each other is both their composition and how they balance those two functions. The more evolved or derived scales have more balance functionality between protection and locomotion. Cosmoid and ganoid scales are the most ancestral type of scales while ctenoid and cycloid scales are most type of scales.

Experiment and studies were conducted on various fishes with cycloid and ctenoid types of scales. Scales were collected from different fishes for this purpose, and mounted. Structural components and other features were observed under the microscope. Both scales had structural diffrences. The studies helped to understand pivotal role played by scales in the aquatic life of fishes. Scales are the most widely

used aging structure in North America because of their non-lethal ease of collection. Counting the number of annuli (rings) on a scale provides the fish age and the spacing between rings is proportional to the growth of the fish. Knowledge of fish age characteristics is necessary for stock assessments, and to develop management or conservation plans.

INTRODUCTION

The evolution of fishes began about 530 million years ago during the Cambrian explosion. The first fish belongs to Agnatha, or jawless fish, found in the early Paleozoic (Ordovician) 505 mya. They were small (few inches to a foot long initially), freshwater bottom-feeding animals. The circular mouth lacks jaws. Use their gills as both straining devices and as respiratory structures. There is no internal bony skeleton instead thick bony plates and scales that cover the body. Fishes make up more than half of all vertebrate species. They are especially important in the study of vertebrate evolution because several important vertebrate traits evolved in fish. Fish show great diversity in body size. They range in length from about 8 millimeters (0.3 inches) to 16 meters (about 53 feet). Most are ectothermic and covered with scales.

Scales are small plate-shaped dermal or epidermal structures that are found in the outer skeletons of fish, reptiles, or some mammals. The skeletons of many vertebrates are covered by two types of scales, namely epidermal and dermal. The epidermis contains numerous mucus cells. These cells secrete mucus or slime, which prevents parasites, fungi, pathogens, etc. from entering the skin easily. Most fish bear scales. While some agnatha and catfishes have no scales. Some fishes, especially paddle fish (*Polyodon*), mirror carp (*Cyprinus carpio*) have partial scales. Other fish such as trout and freshwater eel have very small scales. The scales contain a variety of pigments that give the fish a variety of colors. When the fish hatches from the egg, its body is covered by small scales. As the fish grow so does the scales. However, the number of scales remains the same throughout life but the lost scales can be restored at some point. A small circular growth ring is formed in the scales and this ring is called circuli or circulus (in singular). Circulus formed in summer are quite wide whereas circulus formed in winter are intertwined. In that densely enclosed region a black circle is formed which is known as the annulus. The age of the fish is determined by counting the number of annulus in scales. Scales cover most of the body and protect the skin from injury. The scales form a lateral line in the body of the fish along the side of the body and play an important role in detecting vibrations in the water as it acts as a sensory receptor. Fish scales can be divided into four based on shape, namely: Placoid, Cycloid, Ganoid, Ctenoid.

Type of scales found in most living bony fish (*Osteichthyes*) are of two types, namely: Cycloid and Ctenoid scales. Fishes like tank goby (*Glossogobius giuris*), thilapia (*Oreochromis mossambicus*), sardine (*Sardina longiceps*) etc possess cycloid type of scales and fishes like mullet (*Mugil cephalus*), karimeen (*Etroplus suratensis*), pink perch (*Nemipterus japonicus*) etc have ctenoid type of scale. Detailed structure of the fish scale can be helpful in the identification of fishes up to

major groups and species levels, phylogeny, sexual dimorphism, age determination, past environment experienced by fish, discriminating between hatchery reared and wild populations, migration, pathology of fish scale due to water pollution of the water body and for the growth studies. Shape, size and number of scale are suitable tools in fish taxonomy and using it dates back to first half of 19th century when Agassiz (1833-1843) used it in fish taxonomy for the first time. He classified the fishes into 4 groups based on the scale morphology (Jawad and Al-Jufaili, 2007). During the late 19th century and the first half of the 20th century and with the great advancements in the field of light microscopy, the importance of scale morphology in systematics increased significantly. The importance of scale morphology used in classification was strengthened with the introduction and development of Scanning Electron Microscopy (SEM), so that scales of many different fish species have been studied using SEM (Jawad and All-Jufaili, 2007).

With regard to the importance of scale morphology in this research ultrastructures of scale. The ease of collection of this aging structure is not without its tradeoffs, as the major bias of scales used as an age estimation structure is their tendency to underestimate the age of older fish. The most commonly used techniques involve counting natural growth rings on the scales, otoliths, vertebrae, fin spines, eye lenses, teeth, or bones of the jaw, pectoral girdle, and opercular series. Fish ages are often examined along with measurements of length and weight which combined can provide information on stock composition, age at maturity, life span, mortality, and production.

This topic was selected because, performing age structure analysis are important in growth analysis, population dynamics estimates and resource management. Data from the study can delineate individuals into specific age classes. Exploited species

often have the older, larger individuals removed from the population because they are the first removed by fishers leaving the younger smaller individuals. The effect may have serious consequences for that population. By performing age analysis studies we can identify these types of effects as well their implications to the status of the population. The project, enabled to understand more about the structural peculiarities of cycloid and ctenoid scales.

CYCLOID SCALE FISHES

TANK GOPY - *Glossogobius giuris*

Glossogobius giuris, the tank goby, is a species of goby native to fresh, marine and brackish waters from the Red Sea and East Africa through South Asia and the Indian Ocean to China, Australia and the islands of the Pacific Ocean. This species can also be found in the aquarium trade. It is also known as the bar-eyed goby, flat-headed goby and the Gangetic tank goby. The head is depressed with a protruding lower jaw while the body takes on a compressed appearance towards to caudal fin. Normally brown or light brown with various darker brown spots and flecks along the sides. Ranges in size from 40 to 50 cm maximum (16-20 inches).

TILAPIA - *Oreochromis mossambicus*

Tilapia is the common name for nearly a hundred species of cichlid fish from the coelotilapine, coptodonine, heterotilapine, oreochromine, pelmatolapiine, and tilapiine tribes with the economically most important species placed in the Coptodonini and Oreochromini. Tilapia are mainly freshwater fish inhabiting shallow streams, ponds, rivers, and lakes, and less commonly found living in

brackish water. Tilapia typically have laterally compressed, deep bodies. Like other cichlids, their lower pharyngeal bones are fused into a single tooth-bearing structure. A complex set of muscles allows the upper and lower pharyngeal bones to be used as a second set of jaws for processing food, allowing a division of labor between the "true jaws" (mandibles) and the "pharyngeal jaws". This means they are efficient feeders that can capture and process a wide variety of food items. Their mouths are protrusible, usually bordered with wide and often swollen lips. The jaws have conical teeth. Some Nile tilapia can grow as long as 2.0 ft.

SARDINE - Sardina longiceps

Sardines have a flat body which is covered with large, reflective, silvery scales. In the middle of their belly, they have a set of specialized scales, known as scutes, which are jagged and point backwards. Having very small teeth or no teeth at all, sardines eat plankton, which they filter from the water through their gills. While numerous species of sardines live off the coasts of India, China, Indonesia, and Japan, single sardine species dominate in areas like the English Channel and the California coast. Sardines are basically a warm-water fish, but occur as far north as Norway.

CTENOID SCALE FISHES

MULLET - Mugil cephalus

The flathead grey mullet (*Mugil cephalus*) is an important food fish species in the mullet family Mugilidae. It is found in coastal tropical and subtropical waters worldwide. Its length is typically 30 to 75 centimetres (12 to 30 in). It is known with

numerous English names, including the flathead mullet, striped mullet (US, American Fisheries Society name), black mullet, bully mullet, common mullet, grey mullet, sea mullet and mullet, among others. The flathead grey mullet is a mainly diurnal coastal species that often enters estuaries and rivers. It usually schools over sand mud bottoms, feeding on zooplankton. The adult fish normally feed on algae in fresh water. It occupies fresh, brackish and marine habitats in depths ranging between 0-120 metres (0-394 ft) and with temperatures between 8-24 °C (46-75 °F).

PEARL SPOT - *Etroplus suratensis*

The green chromide (*Etroplus suratensis*) is a species of cichlid fish is native to fresh and brackish water habitats in some parts in India such as Kerala, Goa, Chilika Lake in Odisha and Sri Lanka. The species was first described by Marcus Elieser Bloch in 1790. Other common name include pearlspot cichlid, banded pearlspot, and striped chromide. In Kerala, it is known locally as the karimeen. In Tamilnadu, it is known locally as the 'pappan or pappa. In Goa the fish is known as kalundar. The green chromide lives in brackish water habitat types, such as river deltas. It eats mainly aquatic plants, including filamentous algae and diatoms, but it consumes the occasional molluscs and other animal matter. This species engages in attentive parental care in which several adults care for each brood.

PINK PERCH - *Nemipterus japonicus*

Pink Perch or Rani is a common freshwater fish in India. Pink in colour and small in size, this fish has a mild taste when cooked. Due to just 4- 5% of body fat, this fish is not oily and hence it is called a lean fish. This is your go to go meat if you are planning to lose weight. Pink Perch gets its name due to the pink hue and yellow strips with scales on its skin. It has a mild flavour with a delicate, soft texture that makes it ideal for curries, fried or grilled preparations.

AIM

This project work aims to analyse the size variations of scales in different parts of fishes, determine age of fish by counting the anuli of scale.

OBJECTIVES

The objective of the present study was 1) to evaluate the size variation of scales in different parts of fishes by measuring the scales 2) determining the age of fishes by counting anuli by mounting the scales.

REVIEW OF LITERATURE

One of the unique features in a fish is the presence of the scales (except fishes of order siluriformes) on the outer surface of the body. A fish scale can tell us a great deal about the fish's life story, including where it has been since it hatched. These scales are magic tool for studying a life cycle as a whole. Various types of scales are found in fishes eg. placoid, cycloid, ganoid, cosmoid or ctenoid. Placoid scales are found in cartilaginous fishes and rest all are found in bony fishes. These are derived from the connective tissue of the dermis and form the exoskeleton. Scales of fish are used for classification, identification and growth studies of different fishes, pollution indicators etc.

The scales of bony fishes are derived entirely from the dermal layer of the skin and overlap one another like the tiles. The overlapping (imbrications) of the scale is important in the sense that it imparts mechanical support. Each scale is shaped roughly like a human finger nail whose front end is inserted deep into the dermal layer, while the hinder end is free of exposed and bears the pigment cells or chromatophores on it. These chromatophores provide specific colour to the fish body. These scales have a soft anterior part and hard posterior part. The dorsal surface of the scales is rough as it bears the lines of growth, whereas the ventral surface that touches skin in shining. The cycloid scale has concentric rings around the focus and these rings/lines of growth as sclerites. Cycloid and ctenoid scales increase in size, growth rings called circuli become visible. These rings look a little like the growth rings in the trunk of a tree. During the cooler months of the year the scale (and otoliths) grows more slowly and the circuli are closer together leaving a band called an annulus. By counting the annuli it is possible estimate the age of the fish. This technique is extensively used by fisheries biologists. Only the anterior and

lateral sides of scale have these circuli. These are the marks of periodical growth of fish. Any sudden changes in fish's environment is recorded on the scales in the form of alteration in the circuli shape, pattern or altered elemental deposition thus making these hard structures a testimony to life history of the fish. These revealing marks may be annual marks, winter marks or the larval marks.

Scales at the shoulder of the fish between the head and the dorsal fin is best suited for age determination. Scales are almost two-dimensional structures. The anterior part is formed of a series of sclerites which should extend in a regular pattern from the centre of the scale. The structural discontinuities used for age determination result from irregularities in the pattern of the sclerites; they may be slightly distorted or they may be slightly closely spaced than the majority of the sclerites; usually the discontinuities are narrow and they are usually called 'rings'. Scales are thin structures they need no preparation before viewing; the scales should be cleaned before they are stored. For reading, the slide with mounted scales is placed on the stage of a low-power microscope. The magnification used depends upon the size of the scale; in general, the lowest possible magnification is the best because it enables the whole scale pattern to be seen.

The use of age information is an integral part of fisheries today. Hilborn and Walters (1992) state that "the most valuable information obtained from sampled catch, at least for temperate waters, is age." The development and acceptance of methods for age determination in fishes represents a critical early stage in fisheries science, and at times was fraught with more controversy than today's wide usage of the methods would suggest. In 2006, the Fisheries Management Section of the American Fisheries Society formed the ad hoc Assessment of Fish Aging Techniques Committee to assess the status of aging of freshwater fishes in North America (see

Maceina *et al.* this issue). As part of the committee's goals, an historical survey of the earliest references to aging of fishes and their initial application to fisheries studies was undertaken.

Antoni van Leeuwenhoek of Holland, who used his experience counting threads in cloth at a dry goods store with magnifying glasses to develop improved lenses that he used to construct microscopes, became one of the leading microscopists of the 1600s. Leeuwenhoek possessed a wide-ranging curiosity that included issues surrounding demographics of animal populations (Egerton 1968). Curiously, Leeuwenhoek's studies of fish scales appear to have been at least in part inspired by Biblical strictures against eating fish without scales. His earliest writings on fish scales appeared in a letter to the Royal Society of London, and focused on the European eel (*Anguilla anguilla*) and the burbot (*Lota lota*), which he was drawn to as a result of their reported lack of scales "which two sorts of fish, the Jews will not eat, as forbidden by the Law of Moses." (Leeuwenhoek 1685).

Petersen is most often credited with first proposing length-based methods for age determination (Allen 1917; Ricker 1975). His work appears to have been preceded by Joseph T. Cunningham. Cunningham, working at the Marine Biological Association's Plymouth Lab, attempted to use lengths from known-aged fish he reared in aquaria to assign ages to wild-caught fish, focusing on flatfish and cod (Cunningham 1891, 1892). Cunningham's efforts were not rewarded by clear-cut results: "It is evident there is considerable variation in the rate of growth in nature, from the difficulty of distinguishing in a large number of fish those of one year's, two years', and three years' growth" (Cunningham 1891).

Hederström examined the vertebrae of pike and concluded that the rings that could be discerned on them were growth rings that could be used to determine the fish's age. His reasoning revealed a thoroughly scientific approach, and included verification that (1) both sides of a vertebra had the same number of rings, (2) all vertebrae in an individual possessed the same number of rings, (3) larger fish had more rings on their vertebrae than smaller fish, and (4) the number of rings matched the age of fish "known either from experience or from other circumstances" (Hederström 1759). Hederström went on to present length-at-age data for pike that agree well with modern estimates and also reported that he had confirmed the applicability of using rings on vertebrae for determining the age of a variety of other species, including European perch (*Perca fluviatilis*), roach (*Rutilus rutilus*), bream (*Abramis brama*) etc.

Detailed structure of the fish scale, helpful in the identification of fish up to major groups and species levels can be obtained from: (Abraham *et al.* 1966; Bartulović *et al.* 2011). Detailed structure of the fish scale, helpful in the identification of fish up to major groups and species levels can be obtained from: (Abraham *et al.* 1966; Bartulović *et al.* 2011).

Thompson's (1910) statistical concerns by presenting comparisons of a normal curve to their age-frequency curve, concluding that "the dissimilarity of the two curves is, in fact, so great as to exclude any idea of the age-curve following the usual law of biological variation" and that "it seems to us impossible to explain the observed facts as a result of common variation, even if the help of a mathematical statistician were enlisted."

Huntsman of the Fisheries Research Board of Canada was keeping abreast of the developments in Europe, however, and in 1918 presented a paper to the Royal Society of Canada on its potential applications, soon followed by a similar presentation to the American Fisheries Society (Huntsman 1918, 1919). Carlander (1987) credits Huntsman's papers with bringing aging methods to the attention of North American workers.

Borodin (1924) used scales to assess American shad (*Alosa sapidissima*) in the Connecticut River, and a study of the use of otoliths in the same system followed soon after (Barney 1924). A search of articles in the Transactions reveals only one other application of aging to fish studies in the 1920s, but an increase to 84 in the 1930s, 74 during the 1940s, 112 during the 1950s, followed by rapid increases to 231 in the 1960s and 370 during the decade of the 1970s.

Petersen's work using lengths to assign ages to blenny (*Zoarces viviparus*) received more notice than Cunningham's, but was characterized by the same difficulties (Petersen 1892, summarized by Ricker 1975). Petersen constructed what are now known as length-frequency graphs, proposing that the peaks, or modes, that were evident across the range of smaller to larger size classes represented progressively older age-classes of fish.

By correlating the marginal growth of scales (the amount of growth between the last ring or annulus and the margin of the scale) with the season of the year in which the scales were collected, Walford and Mosher (1943a: 9 and 1943b) have shown for the Pacific pilchard or sardine, *Sardinops caerulea* (Girard) 1854, that these rings are formed annually and consequently can be used for age determination as well as for back-calculation of the length of the fish at a given earlier age.

Age determination based on the analysis of the growth marks of calcified structures is the specific aim of the Sclerochronology Laboratory of the INSTM (SLI), which was created in 2000. The methods used in SLI to identify and count growth marks on mineralized structures in fishes and to interpret the corresponding data. Is monitored till now, the species of interest to the SLI are small pelagic fishes. Age determination of *Mullus surmuletus* has been performed either by counting scale annuli (Gharbi, 1980) or otolith (in toto) growth marks (Jabeur, 1999).

In 1859, Robert Bell reported that one could use these growth rings to reliably determine the age of all fish after examination of sucker (*Catostomus sp.*) vertebrae and yellow perch (*Perca flavescens*) scales that he raised in a pond for two years showed “two rings or circles.”

Stuart Thomson, with encouragement and support from Walter Garstang and Allen at the Plymouth Laboratory of the Marine Biological Association of the United Kingdom, extended Hoffbauer’s work with freshwater fishes to important commercial marine species. His detailed work with pollack (*Pollachius pollachius*), poor cod (*Trisopterus minutus*), whiting (*Merlangius merlangius*), haddock (*Melanogrammus aeglefinus*), and cod convinced him that Hoffbauer’s findings could be applied to marine species (Thomson 1902, 1904).

METHODOLOGY

3 different types of fishes were selected each for cycloid and ctenoid scales. Tank gopy, thilapia, sardine for cycloid scale and pearl spot, mullet, pink perch for ctenoid scale. These fishes were selected from various markets. Specimens were collected

during March, 2022. Care was taken during collection of specimen to avoid fishes with damaged scales. First step was cleaning of the specimens, it was washed with clean water. The next step was de-scaling. Scales were taken from the body carefully, using forceps and washed the scales with water again. Measurements of the scale were noted. Measurements were taken using white threads, length and breadth of the scales were measured. With the help of a ruler right measurements of length and breadth were obtained.

Colour of the scales of all the fishes were observed. The shape and other morphological differences between two type of scales were also noted. Scales from different regions like cephalic, caudal, dorsal, pelvic and pectoral regions were collected. The final step of the experiment was the comparative study of scales of ctenoid and cycloid scales. The scales were mounted for further morphological observations.

Mounting of Scales

The materials required:

1. Forceps
2. Watch glass-2
3. 10 % KOH solution
4. Spirit lamp
5. Match box
6. Distilled water
7. Fine brush
8. clean grease free microscopic slides
9. Leishmann's stain



Fig. 1 – Materials required for mounting the scales

Procedure:

1. Fishes with cycloid and ctenoid scales are de-scaled (scales from pectoral, pelvic, caudal, dorsal, head regions were collected, this can be done by careful scrapping of fish. Care should be taken not to damage the scales, they are then transferred to 10% KOH solution taken in a test tube.
2. Heat the fish scales in 10% of KOH solution for 10-20 seconds.
(Note: Heating is done to dissolve the covering epithelium over heating will cause curling of the scales.)
3. Wash the fish scales in distilled water (2 times), for removing the KOH solution.
4. Take few drops of Leishmann's stain in a watch glass. Transfer the scales into that.
5. Wait for 3 minutes. Wash off the excess stain.
6. Transfer the scales to a glass slide.
7. Observe the slide under dissecting microscope and under the low power (4X) of simple microscope.
8. Repeat the same for other fishes with ctenoid scale.

9. Compare all the slides and note down the differences and morphologies of the scales.

Age Determination

Procedure: Scales are prepared for study by mounting the whole scales on glass slides or, more commonly, by pressing imprints of the scale circuli into transparent plastic. These scales or scale impressions are examined under a low-power microscope or by use of a microprojector, and the rings or annuli are counted.

Applications of Scale Method: Fish of temperate regions shows clear rings, which are true marks. This is because there is a sharp difference between the temperatures two seasons—summer—the period of faster growth, and winter—the period of slow growth or no growth. Therefore, the calculation of the age of fish by annuli is most reliable in temperate fish. This method is more reliably applicable in case of salmons, carps, cod and herrings, established a method of estimating age of fish based on scales.



Fig. 1 – Tank Gopy



Fig. 2 – Tilapia



Fig. 3 – Sardine



Fig. 4 – Mullet



Fig. 5 – PEARL SPOT

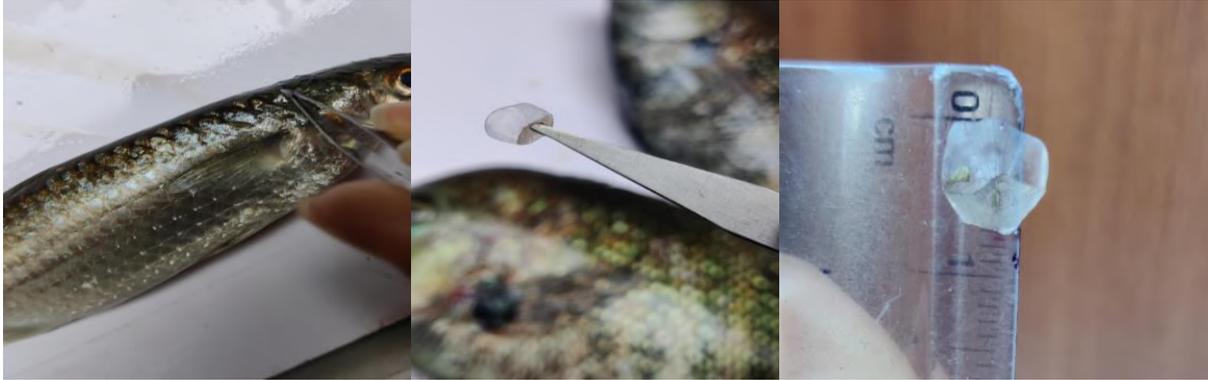


Fig. 6 – Pink Perch



(a)

(b)



(c)

(d)

(e)

Pleat 1 – Procedure for measuring the size of scale

- a) Measuring the length of the fish
- b) Measuring the width of the fish
- c) De-scaling the fish
- d) Scale of fish
- e) Measuring the scale

OBSERVATION

	FISH 1		FISH 2		FISH 3		FISH 4		FISH 5	
	L	B	L	B	L	B	L	B	L	B
TANKGOPY	13.1cm	4cm	10.2cm	3.7cm	11.6cm	4cm	9.2cm	3cm	8.9cm	3cm
TILAPIA	19cm	7cm	20cm	10cm	21cm	8cm	22cm	8cm	19cm	7cm
SARDINE	14.6cm	3.5cm	16.1cm	4.3cm	16.5cm	4.2cm	16.3cm	4.2cm	15.1cm	3.6cm
MULLET	18.5cm	5cm	16.5cm	4.3cm	15cm	4.3cm	14.5cm	3cm	13cm	3cm

PEARL SPOT	18.5cm	10cm	17cm	8.5cm	15cm	9cm	14.5cm	7cm	14cm	7cm
PINK PERCH	18.7cm	5.9cm	18.7cm	5.8cm	17.8cm	5.8cm	16.8cm	6.7cm	19.1cm	5.7cm

Table 1 – Measurement of fish size

	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.2cm	0.2cm	0.4cm	0.4cm	0.6cm	0.6cm	0.5cm	0.5cm	0.6cm	0.4cm
Fish2	0.2cm	0.2cm	0.4cm	0.5cm	0.5cm	0.5cm	0.5cm	0.4cm	0.5cm	0.4cm
Fish3	0.2cm	0.2cm	0.4cm	0.3cm	0.5cm	0.5cm	0.5cm	0.6cm	0.5cm	0.4cm
Fish4	0.3cm	0.3cm	0.4cm	0.4cm	0.4cm	0.5cm	0.5cm	0.5cm	0.5cm	0.4cm
Fish5	0.3cm	0.3cm	0.4cm	0.4cm	0.6cm	0.6cm	0.4cm	0.4cm	0.5cm	0.4cm

Table 2 – Measurement of scales in different parts of tank gopy

	HEAD REGION		PELVIC REGION		PECTORAL REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.4cm	0.6cm	0.6cm	0.7cm	0.3cm	0.4cm	0.5cm	0.6cm	0.5cm	0.5cm
Fish2	0.8cm	0.6cm	0.6cm	0.8cm	0.4cm	0.3cm	0.5cm	0.5cm	0.5cm	0.5cm
Fish3	0.7cm	0.7cm	0.6cm	0.8cm	0.4cm	0.3cm	0.4cm	0.4cm	0.4cm	0.5cm
Fish4	0.6cm	0.8cm	0.6cm	0.6cm	0.5cm	0.3cm	0.6cm	0.5cm	0.6cm	0.5cm
Fish5	0.5cm	0.6cm	0.5cm	0.6cm	0.4cm	0.2cm	0.5cm	0.5cm	0.5cm	0.4cm

Table 3 – Measurement of scales in different parts of tilapia

	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.3cm	0.3	0.5cm	0.4cm	0.4cm	0.4cm	0.4cm	0.2cm	0.5cm	0.4cm

Fish2	0.3cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm	0.4cm	0.4cm	0.3cm	0.3cm
Fish3	0.5cm	0.3cm	0.7cm	0.6cm	0.5cm	0.5cm	0.5cm	0.4cm	0.3cm	0.3cm
Fish4	0.5cm	0.5cm	0.6cm	0.6cm	0.5cm	0.5cm	0.5cm	0.5cm	0.3cm	0.3cm
Fish5	0.5cm	0.5cm	0.6cm	0.4cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm	0.4cm

Table 4 – Measurement of scales in different parts of sardine

	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.8cm	0.8cm	1cm	0.9cm	0.9cm	0.6cm	1.1cm	0.9cm	0.7cm	0.5cm
Fish2	0.8cm	0.8cm	0.9cm	0.8cm	0.7cm	0.6cm	0.9cm	0.8cm	0.7cm	0.5cm
Fish3	0.5cm	0.4cm	0.5cm	0.4cm	0.7cm	0.5cm	0.6cm	0.6cm	0.6cm	0.5cm
Fish4	0.5cm	0.5cm	0.6cm	0.6cm	0.5cm	0.4cm	0.6cm	0.6cm	0.4cm	0.4cm
Fish5	0.7cm	0.6cm	0.8cm	0.7cm	0.5cm	0.3cm	0.7cm	0.6cm	0.6cm	0.4cm

Table 5 – Measurement of scales in different parts of mullet fish

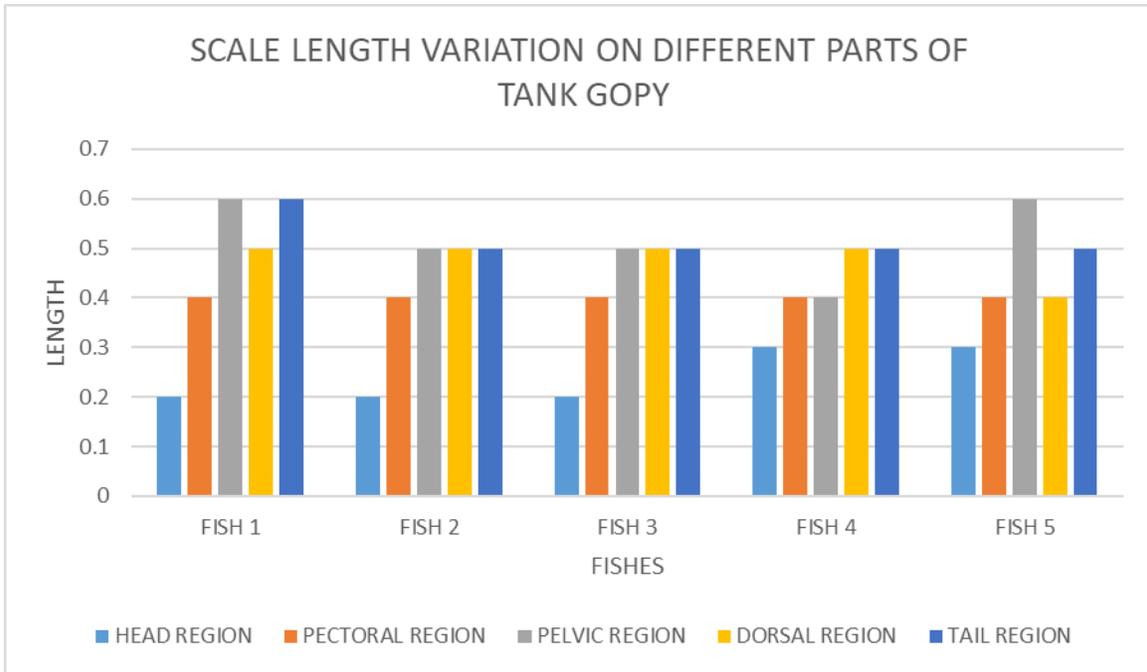
	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish 1	0.7cm	0.5cm	0.7cm	0.7cm	0.6cm	0.4cm	0.7cm	0.5cm	0.6cm	0.5cm
Fish 2	0.5cm	0.7cm	0.5cm	0.6cm	0.5cm	0.5cm	0.6cm	0.6cm	0.5cm	0.5cm
Fish 3	0.5cm	0.6cm	0.6cm	0.6cm	0.6cm	0.5cm	0.6cm	0.5cm	0.5cm	0.4cm
Fish 4	0.5cm	0.4cm	0.5cm	0.5cm	0.5cm	0.5cm	0.6cm	0.5cm	0.5cm	0.5cm
Fish 5	0.4cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm	0.5cm	0.5cm	0.4cm	0.4cm

Table 6– Measurement of scales in different parts of pearl spot

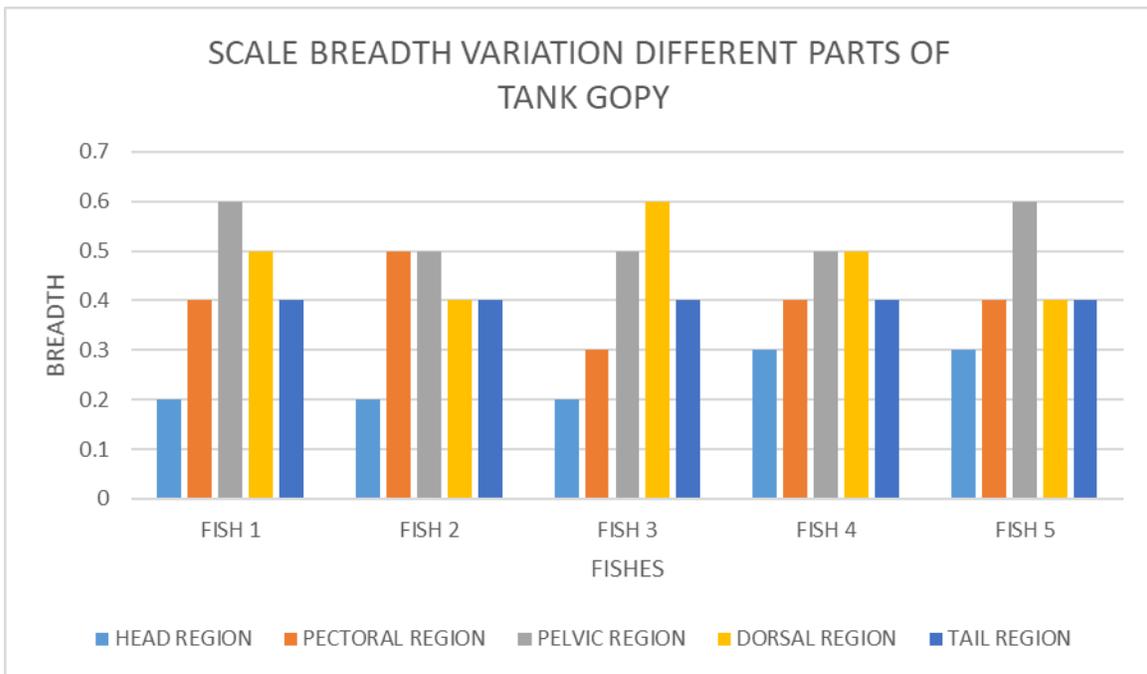
	HEAD REGION		PECTORAL REGION		PELVIC REGION		DORSAL REGION		CUADAL REGION	
	L	B	L	B	L	B	L	B	L	B
Fish1	0.6cm	0.6cm	0.6cm	0.9cm	0.6cm	0.6cm	0.5cm	0.4cm	0.4cm	0.4cm
Fish2	0.4cm	0.4cm	0.6cm	0.7cm	0.6cm	0.7cm	0.6cm	0.5cm	0.5cm	0.5cm
Fish3	0.5cm	0.4cm	0.6cm	0.8cm	0.6cm	0.6cm	0.6cm	0.5cm	0.4cm	0.4cm
Fish4	0.6cm	0.6cm	0.5cm	0.9cm	0.5cm	0.5cm	0.5cm	0.5cm	0.3cm	0.3cm
Fish5	0.6cm	0.5cm	0.6cm	0.9cm	0.6cm	0.7cm	0.6cm	0.5cm	0.4cm	0.4cm

Table 7 – Measurement of scales in different parts of pink perch

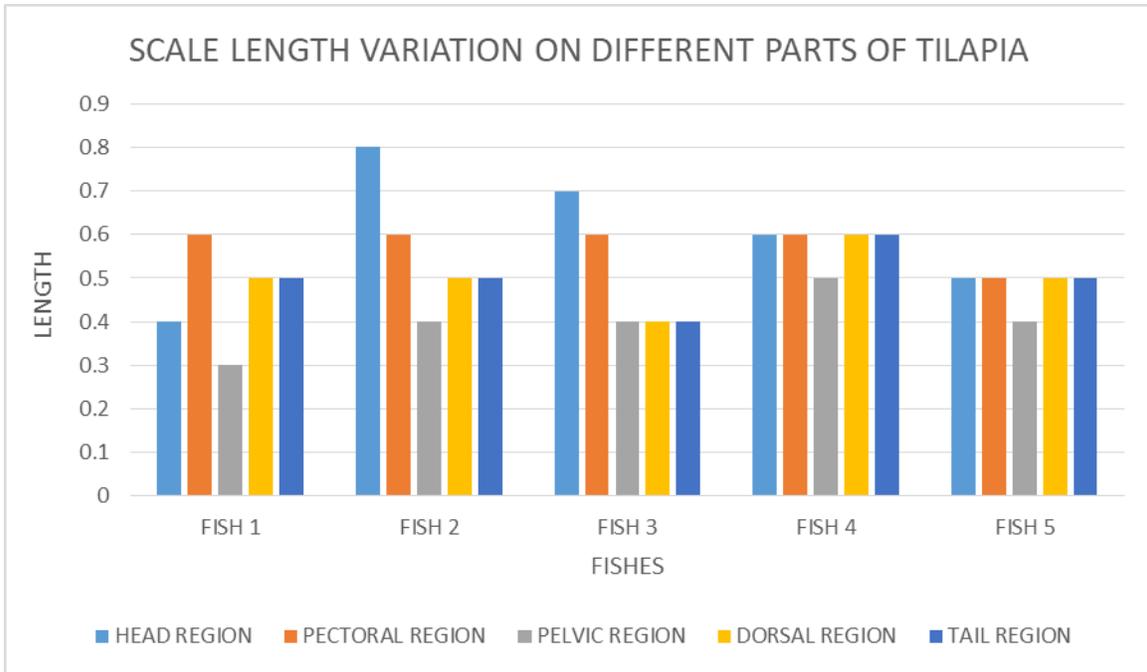
RESULT



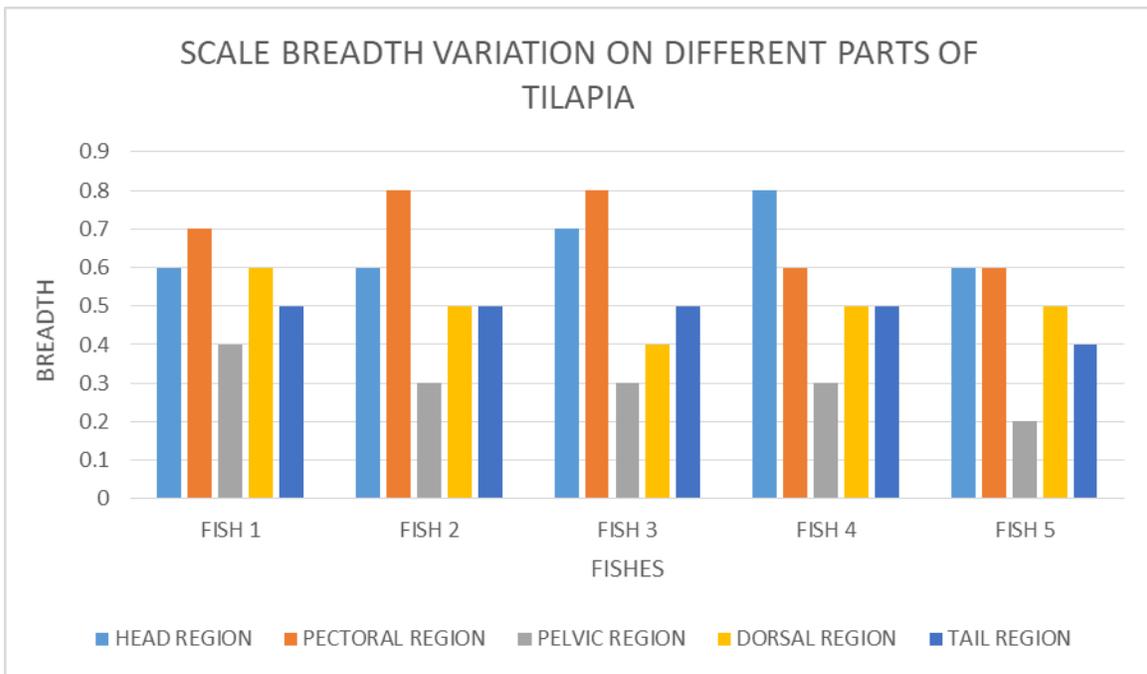
Graph 1:



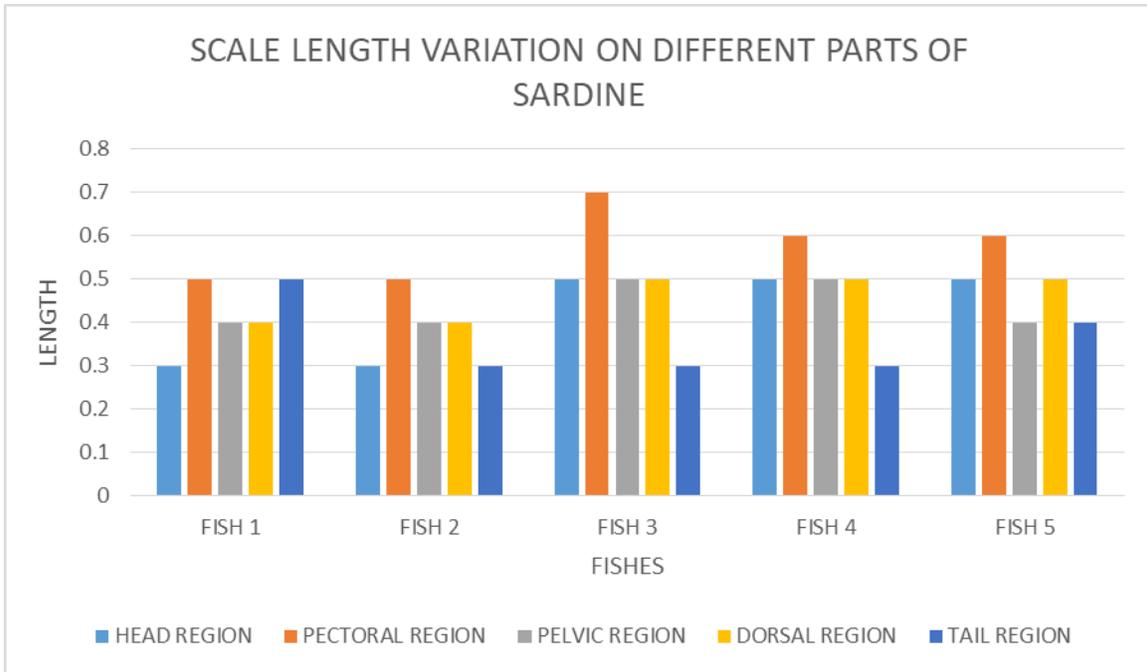
Graph 2:



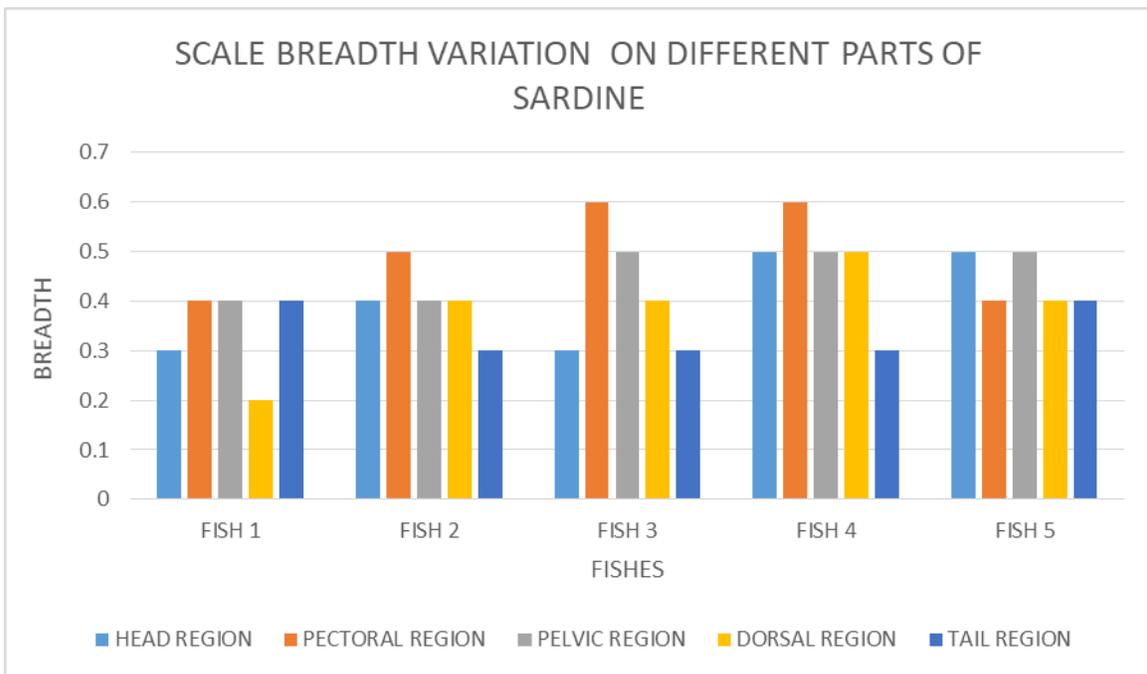
Graph 3



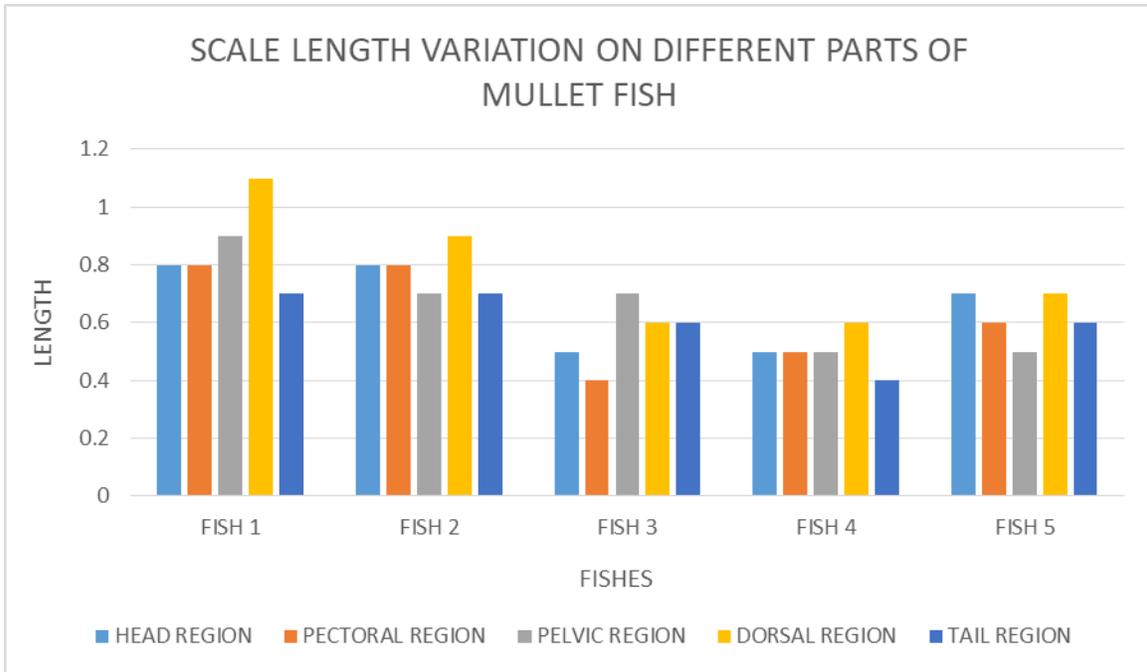
Graph 4:



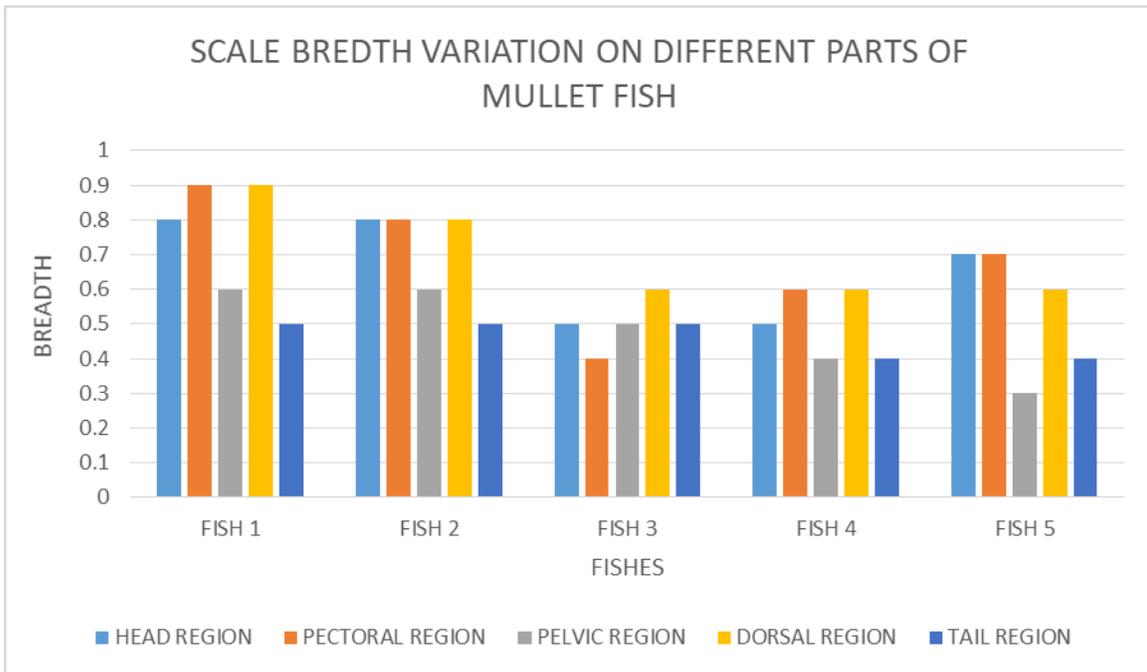
Graph 5:



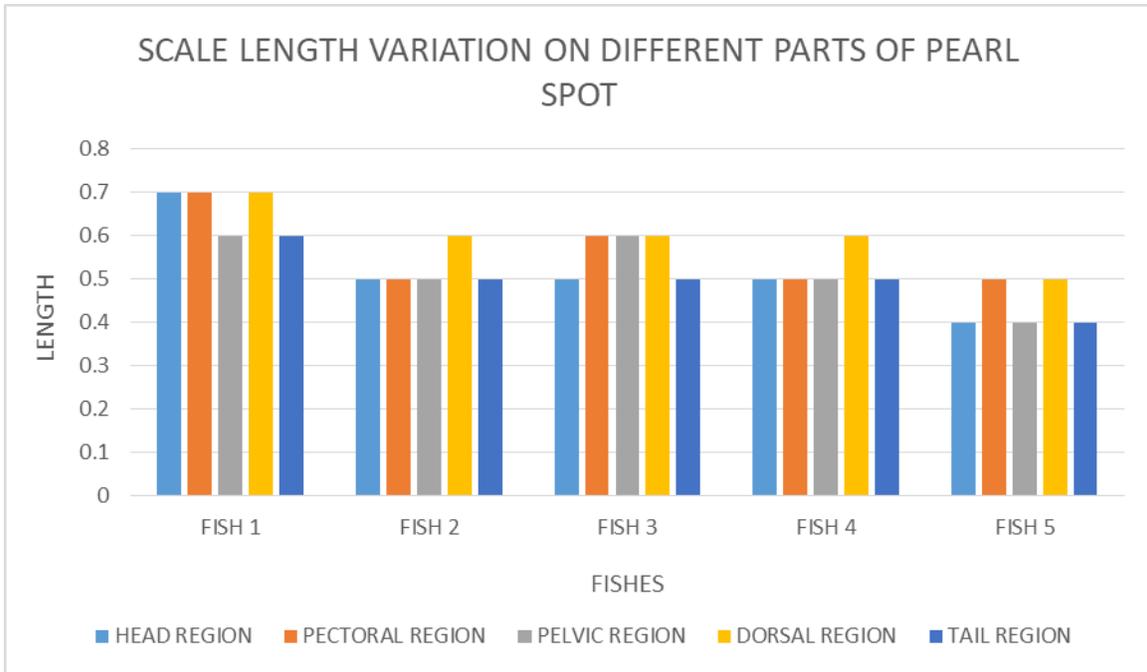
Graph 6:



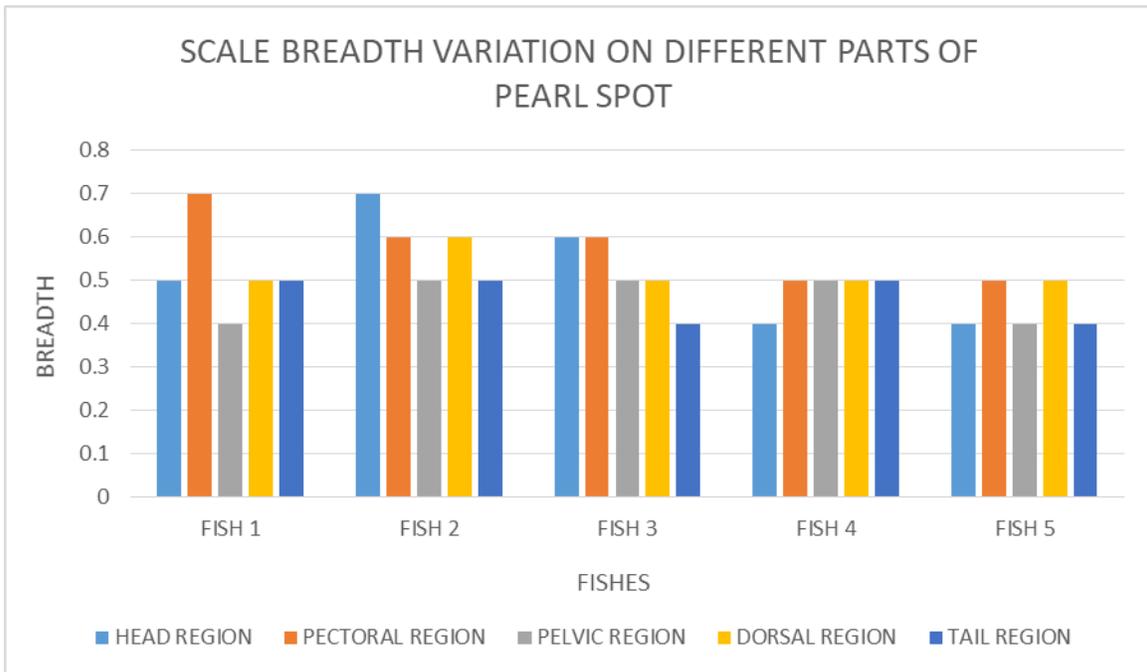
Graph 7:



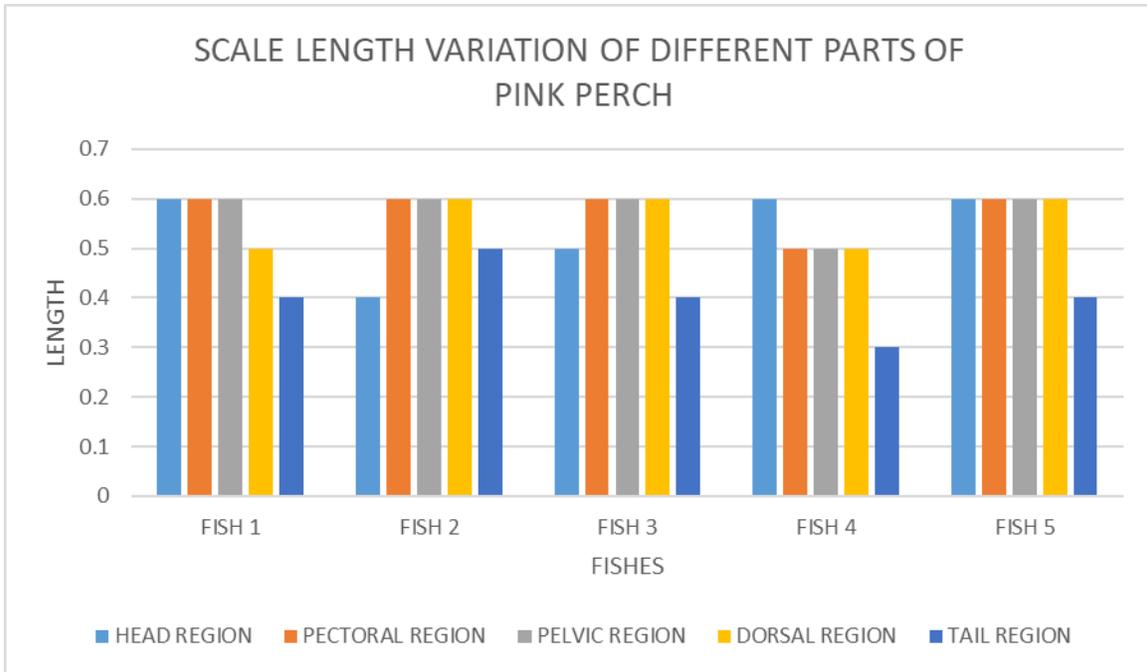
Graph 8:



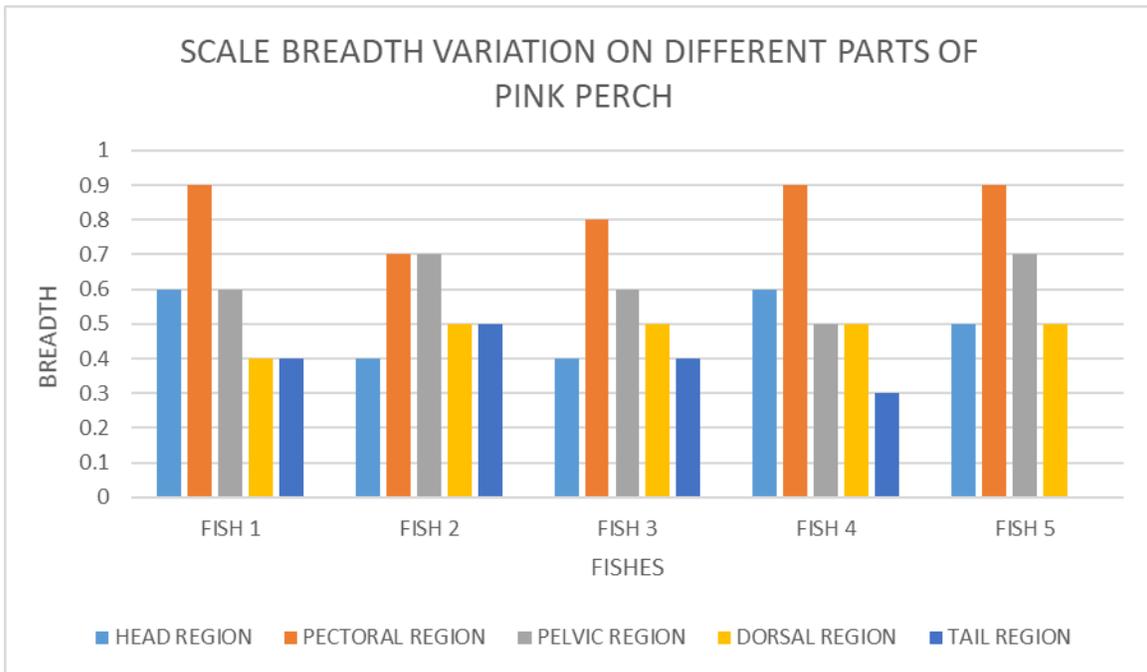
Graph 9:



Graph 10:



Graph 11:



Graph 12:

TANK GOPY		
FISHES	NO. OF ANULI	AGE OF FISH
FISH 1	1	1 YEAR
FISH 2	2	2 YEARS
FISH 3	1	1 YEAR
FISH 4	0	
FISH 5	0	
TILAPILA		
FISH 1	1	1 YEAR
FISH 2	0	
FISH 3	0	
FISH 4	<u>1</u>	1 YEAR
FISH 5	<u>1</u>	1 YEAR
SARDINE		
FISH 1	2	2 YEARS
FISH 2	3	3 YEARS
FISH 3	4	4 YEARS
FISH 4	3	3 YEARS
FISH 5	2	2 YEARS
MULLET FISH		
FISH 1	2	2 YEARS
FISH 2	2	2 YEARS
FISH 3	2	2 YEARS
FISH 4	1	1 YEAR
FISH 5	1	1 YEAR
PEARL SPOT		

FISH 1	4	4 YEARS
FISH 2	3	3 YEARS
FISH 3	2	2 YEARS
FISH 4	2	2 YEARS
FISH 5	1	1 YEAR
PINK PERCH		
FISH 1	3	3 YEARS
FISH 2	1	1 YEAR
FISH 3	4	4 YEARS
FISH 4	3	3 YEARS
FISH 5	3	3 YEARS

DISCUSSION

The body of an ideal fish is covered by thin scales. The scales develop as external growths of the epidermis or skin. The epidermis contains numerous mucus cells. These cells secrete mucus or slime, which prevents parasites, fungi, pathogens, etc. from entering the skin easily. Agnatha is an infra phylum of jawless fish in the phylum Chordata, subphylum Vertebrata, consisting of both present (cyclostomes) and extinct (conodonts and ostracoderms) species. Among recent animals, cyclostomes are sister to all vertebrates with jaws, known as gnathostomes. In modern agnathans, the body is covered in skin, with neither dermal nor epidermal scales. The skin of hagfish has copious slime glands, the slime constituting their defense mechanism. The slime can sometimes clog up enemy fishes' gills, causing them to die. In direct contrast, many extinct agnathans sported extensive exoskeletons composed of either massive, heavy dermal armour or small

mineralized scales. Most fish bear scales. The scales contain a variety of pigments that give the fish a variety of colors. The scales form a lateral line in the body of the fish along the side of the body and play an important role in detecting vibrations in the water as it acts as a sensory receptor. Cycloid and ctenoid scales are found in the majority of bony fishes (the Teleostei).

In this project two different types of scales were studied namely cycloid and ctenoid. Fishes having cycloid and ctenoid scales were collected, descaled, stained and observed. It was noticed that both the scales differed in shape and structure. Ctenoid scales have a variously developed spiny posterior margin whereas cycloid scales have a smooth posterior margin lacking ctenii. The number and distribution of scales were not same either in all the six fishes. In fishes like sardine, tank goby, pink perch it was observed that the size of the scale in the tail region was comparatively smaller than scales in other regions of the body. These fishes are active swimmers, their rapid tail movement is what allows them to swim smoothly against the friction offered by the water. The small size of scales in the tail region is an adaptation for their rapid tail movement. Scales in other regions like pectoral, dorsal and head were bigger than the ones in tail region as they are not much involved in movement. Coloration was another feature that was observed, the margin of the scale in pink perch was pink in colour.

The coloration is an adaptation for various purposes like camouflage, mating etc. Scales are of value for age determination in many of bony fishes, a broad grouping which includes most fishes of importance for food. Scales are formed when newly hatched fish complete their larval stages, and soon cover the entire body, with the exception of head and fins. In most species they lie in an overlapping pattern much like shingles on a roof and serve as a protective coat. Scale growth begins with the formation of the scale center or focus and growth is outward from this focus, though it is greatest toward the forward margin of the scale. Fine ridges called circuli are

laid down in a circular pattern around the focus as growth proceeds. Many circuli are added to the scale each year. Fish growth is reflected in scale growth. Circuli are widely spaced in warm seasons when fish growth is rapid, and closely spaced in cold seasons when it is slow. Fish growth stops in winter. The growth of a fish during one year is shown on its scales as a series of widely spaced spring and summer circuli followed by a series of closely spaced fall and winter circuli. Since fishes continue to grow throughout their lives, this pattern is repeated each year. The outer edge of a series of closely spaced circuli is generally taken to be the end of growth for that year and this point is referred to as the year mark or annulus. The age of a fish is determined by counting the number of annuli or year marks.

CONCLUSION

The scales comprise a non-growing "crown" composed of dentine, with a sometimes-ornamented enameloid upper surface and an aspidine base. Its growing base is made of cell-free bone, which sometimes developed anchorage structures to fix it in the side of the fish. They are small, thin, cornified, calcareous or bony plates which fit closely together or overlap. When the arrangement of scales on fish body is concerned, they are most often imbricated and thus, overlap like shingles on the roof, with their free margins directed towards the tail, so as to minimise the friction of water. Two different types of scales observed in the project were cycloid and

ctenoid scales. Cycloid scales are somewhat circular in appearance, in addition to being thin and translucent. The center of these scales is thicker, and can observe several concentric lines of growth. Another interesting fact about these lines of growth is that they indicate the age of the fish when counted. The greater the number of concentric rings, the older the fish. Ctenoid scales have specific comb-like projections at the back. In form, structure and arrangement, they are very similar to cycloid scales. However, ctenoid scales attach themselves more firmly to the skin. The rear parts of these scales don't overlap and they have several comb-like teeth. Some scales possess colour as in pink perch, these colouration help in mating and camouflage it also make them attractive. Scales exhibit variations in size in different regions of the body. Scales found near the tail and head regions are comparatively small than the scales in the, pectoral and dorsal regions. Size of the scales contributes much to the locomotion of the fish. Small scales in the tail region enable rapid movement of tail and move smoothly in water against the friction offered by it. More detailed studies can be conducted on fish scales, considering the structural and mechanical properties.

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EFFECT OF TEMPERATURE ON ANT MOVEMENT



Project work by
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Affiliated to Mahatma Gandhi University, Kottayam in partial
Fulfilment of requirement for the degree of Bachelor of Science
in Zoology
2021-2022

CERTIFICATE

This is to certify that the project report entitled “**EFFECT OF TEMPERATURE ON ANT MOVEMENT**” submitted by Ms. Mubashira Beegum, Reg. No. AB19Z00026 in partial fulfilment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Soja Louis and this is her original effort.

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Assistant Professor & Head of Department
Department of Zoology
St Teresa’s College (Autonomous)
Ernakulam

EXAMINERS

1)

2)

DECLARATION

I, hereby declare that this project work entitled **“EFFECT OF TEMPERATURE ON ANT MOVEMENT”** is submitted to St.Teresa’s College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science degree in Zoology. This work has been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own

Signature:

Name: Mubashira Beegum

Reg.No: AB19Z00026

ACKNOWLEDGEMENT

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Mubashira Beegum

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ABSTRACT

Ants (*Hymenoptera: formicidae*) are one of the most dominant terrestrial organisms worldwide. They are abundant, both in terms of sheer numbers and biomass, on every continent except Antarctica and are deeply embedded within a diversity of ecological networks and processes. Ants are also eusocial and colonial organisms-their lifecycle is built on the labour of sterile worker ants who support a small number of reproductive individuals. Change in the environment such as a change in temperature, rainfall, etc. may lead to the extinction of species. Melting of ice caps, forest fires, global warming all can cause extinction. It can also cause habitat loss. Due to the extinction of the species on which a species depends, coextinction may also occur. Given the climatic changes that our planet faces, the need to understand how various important taxonomic groups respond is also important, this includes the ants. The study focuses on understanding the ways in which thermal changes may affect ant colonies, populations, and communities. In general, the species living in the tropics, and in thermally variable microhabitats, such as the canopy and leaf litter environments, will be negatively impacted by rising temperatures. Species living in the temperate zones and those able to thermally buffer their nests in the soil or behaviourally avoid higher temperatures, however, are likely to be unaffected or may even benefit from a changed climate. How ants will respond to changes to other abiotic factors associated with climate change is largely unknown, as is the detail on how altered ant populations and communities will ramify through their wider ecological networks. The project discusses on how eusociality may allow ants to adapt to, or tolerate, climate change in ways that solitary organisms cannot.

INTRODUCTION

Ants are eusocial insects of the family formicidae. They are social in habit and live together in organised colonies. Ants occur worldwide but are especially common in hot climates. There are more than 10000 known species of ants living through the world. Most ants are either red or black in colour and length can be anyway from 1/3" to 1/2". They can be found in many places such as soil, leaf litter, rotting wood and dead trees and live all over the world, except for the Arctic, Antarctic, some islands and also on the coldest mountain tops. They are most abundant in the tropical rainforests and other tropical regions.

All ants are social. Infact they are the only insects in which all species are social. Large groups of ants live in colonies or communities together. In the majority of ants, colonies are families or groups of related families. These groups consist of one or more queens, who rule the colony and males whose only job is to fertilize the queen and then die soon after. The workers in the colony are only females. These workers are divided into several working classes including: enlargement and repair of the nest, taking care of the larvae, tending to the queen, defending the colony and foraging for food.

The climate change concept refers to a modification in climate conditions that is identifiable by an alteration in the mean and/or in the variability of its properties that persists for a prolonged period of time, measured in

hundreds of years (IPCC 2007). Global warming is unequivocal, and a sequence of events over the last 100 years makes this evident: increase in mean global temperature of Earth's surface (+0.74°C), retreat of glaciers and permanent snow in high mountains, rise in the sea level, and increased frequency of events such as floods and droughts on a global scale (IPCC 2007). Weather maps were made for Argentina according to the projections generated by the climate model developed by CIMA/CONICET (Nuñez *et al.* 2005). Using this model, the present climate, represented by the 1981 to 1990 decade, was compared with the 2081 to 2090 decade to show the trend in climate change in southern South America over the next 100 years (Nuñez 2009). The following greenhouse gas emission scenarios were highlighted: A2 and B2 (increasing emission of CO₂ intermediate between emission scenarios), A1F1 (more extreme), and B1 (with the least change) (IPCC 2001, 2007). The emission scenarios A2 and B2 were selected to make the model, as they are intermediate between the scenarios proposed by IPCC (2001, 2007).

In the Attini tribe, *Acromyrmex lobicornis* (Emery) has the widest geographical distribution in Argentina, being found from the north down to the 44°S parallel in the province of Chubut (Kusnezov 1978; Farji-Brener and Ruggiero 1994). Although its ample geographical distribution suggests that it harvests a wide variety of plant species, there are few studies on its diet in the different habitats where it is found (Farji Brener and Protomastro 1992; Pilati *et al.* 1997; Franzel and Farji-Brener 2000). *A. lobicornis* behaves as a generalist species: it forages both monocotyledons and dicotyledons; it uses a wide range of species for cultivating the symbiotic fungus; it is an opportunist, taking advantage of food resources when they are available

(spatially and seasonally). It also exploits habitats of low complexity and intense aridity, which has given it adaptive plasticity favouring its wide geographical distribution (Pilati *et al.* 1997; Claver 2000). Variations may be observed in the harvesting patterns of the species linked to temperature, depending on the geographic locality, although they are close to those generally cited for ants, at 10°C to 40°C (Hölldobler and Wilson 1990). Harvesting patterns in relation to soil temperature were recorded in two studies and the temperature ranges observed were 10°C to 40°C (Claver 2000) and 17°C to 28°C (Nobua Behrmann *et al.* 2010), in both cases in the desert in the centre of the Monte in the province of Mendoza, Argentina. On the other hand, in a study undertaken in the province of La Pampa, harvesting activity of *A. lobicornis* was seen over an ambient temperature range of 11.7°C to 22.6°C (Pilati and Quirán 1996). In all cases, peaks of activity were recorded during spring and at the beginning of autumn, whereas activity ceases almost completely during the winter. Moreover, in summer their foraging behavior changed from diurnal to nocturnal harvesting, always within the determined temperature limits and the same as those recorded for daytime (Pilati and Quirán 1996; Claver 2000; Nobua Behrmann *et al.* 2010).

Ant populations and their distribution are affected by many circumstances. Abiotic factors such as weather, water availability and soil characteristics may determine if a habitat is suitable for ants. Other factors such as level of disturbance, available food resources, reproductive biology and natural enemies also contribute to its distribution. Many factors such as temperature, humidity, vegetative type, level of disturbance and soil characteristics effect the microhabitat of a particular ant species. Dispersal

through mating flights, colony budding, flooding and human activity all determine the distribution and the rate of spread for many species of ants. Resource availability and ability of ants to exploit those resources faster and longer to defend their territory influences ant abundance and diversity in a habitat. Determining what factors are most likely to limit a population of ants or influence its growth and spread has been the focus of many studies. The project tries to explain the effect of temperature on the movement of ant or foraging of ant.

REVIEW OF LITERATURE

Tizón *et.al* (2014) studied the effect of increase in the temperature on the foraging of *Acromyrmex lobicornis* (Hymenoptera: Formicidae). In order to evaluate how an increase in temperature affects the activity of *A. lobicornis*, they studied the amount of foraging and the trophic preferences in two treatments under controlled humidity and temperature conditions ($\Delta 4.5^{\circ}\text{C}$). They also measured the walking speed of the workers as a function of an increase in temperature (6°C to 32°C). Their results support the hypothesis that the ants' activity changes at higher temperature, with higher rates of harvesting and a change in walking speed is observed. There is also variation in the trophic preference, selecting plant items with a higher composition of elements that are degradable by symbiotic fungi. Their results suggest that small variations in ambient temperature significantly affect certain behavior patterns in the leaf-cutting ants.

Jan Sedlacek and Josef Zeman (2013) studied the dependence of the speed movement in forest ant (*Formica rufa*) on the environmental temperature. The paper dealt with the influence of temperature on the movement activity of the ant - *Formica rufa* species. The thermo box with adjustable temperature was used for this experiment. The high-speed camera, thermo sensor and shining prisms were in this box. The ant movement was recorded at many temperatures. The observed ant was placed in an enclosed area of the visual field of the camera. The ant movement was recorded in this area at different temperatures after a five-minute acclimation. The speed of the ant was calculated by determining the number of frames between the start and the end of the selected limb movement. The software of Gimp was used

for this job. The resulting values indicate that the speed of the ant is exponentially dependent on the temperature up to 40 °C.

Wang *et.al* (2020) studied single-file movement of Ants stressed by a high temperature. The study investigated single-file movement of ants (*Camponotus japonicus*) driven by a high temperature in a narrow channel. In this work, they also investigated single-file movement and the interaction between ants in the narrow channel under high temperature conditions. Under the stimulation of high temperature, ants preferred to follow the front one closely for escaping quickly. In the channel, touching behavior played a main role on escape. It was expressed that the average speed of ants in the single-file movement experiment was faster than that in the single ant experiment.

Roeder *et.al*(2021) in his study on ant thermal tolerance discussed methods, patterns, hypotheses, and potential sources of variation in ant thermal tolerance. From understanding daily patterns in activity to testing hypotheses across geographic gradients, the field of ant thermal tolerance continues to grow.

Oldroyd (2009) studied rearing temperature affects in ant and its thermoregulatory behaviour. Differing task thresholds among workers are crucial to the efficient allocation of work in self-organized insect colonies. New evidence suggests that the rearing temperature of ant pupae causes lifelong changes in an ant's response threshold to temperature.

Chambers (2011) observed the effects of resource availability and temperature on ants. In the study, the differences in sizes of ants foraging on

different resources at different times of the day and in different temperature environments were examined. Time of day was shown to significantly affect ant foraging habits both within and between species. Specifically, different size ants forage at different times of the day. Larger individuals tend to forage at night compared to daytime foragers.

The above mentioned literatures revealed some information on the effect of temperature on ants.

MATERIALS AND METHODS

MATERIALS REQUIRED

- Ants
- Sugar
- Refrigerator
- Glass container
- Paper

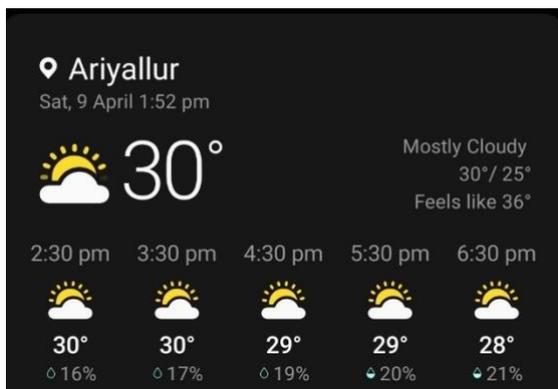
METHODOLOGY

The experiment was conducted at home itself. For the experiment some ants were collected from ground. Ants were collected carefully so as to avoid biting. They were kept in a beaker with a hole in it. The hole is to let the air come for the ants to breathe. The experiment was conducted in four different conditions.

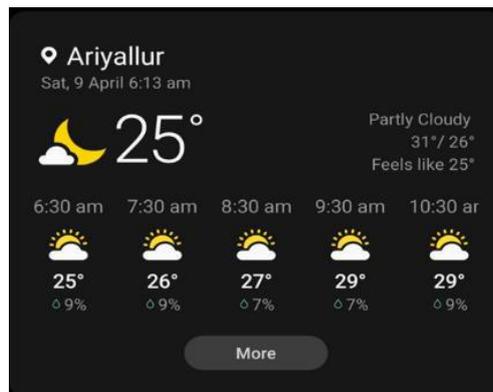
- 1) At 30°C during day time
- 2) At 25°C during night time
- 3) At medium temperature in a refrigerator. That is, approximately 37°F or 3°C
- 4) At low temperature in a refrigerator. That is approximately 40°F or -15°C

Make a circle with sugar at a radius of 5 cm. Put the ants in the centre of the circle. The ant movement towards the sugar is observed and was noted. The same procedure was adopted for 30°C and 25°C temperature ranges. At medium temperature in refrigerator, approximate 3°C, the ants were put in a transparent glass or transparent glass bowl. Cover its mouth portion using

paper to avoid ants from escaping. Make sure to put a small hole on the paper so that the cold air can enter inside the bowl. Keep the bowl in the refrigerator and note how ants move from time to time. At lower temperature in refrigerator, approximate -15°C , the procedure is same as that of medium temperature in refrigerator. After taking out the bowl from refrigerator note the ant movement.



30°C



25°C



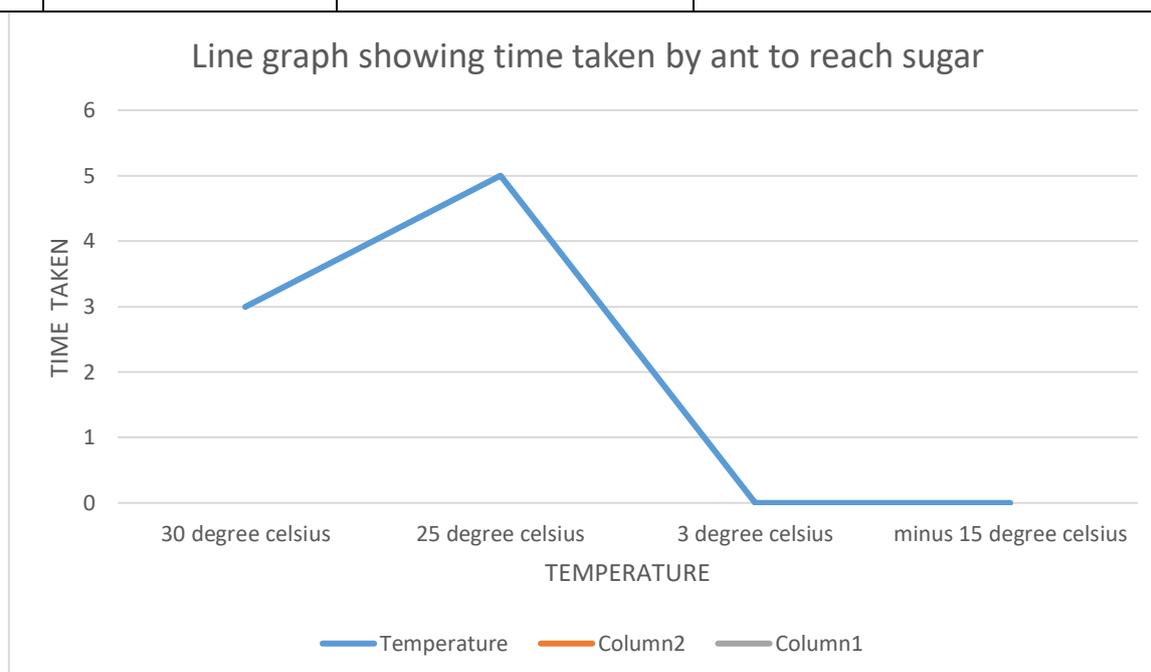
Fig.1. Experimental setup for room temperature



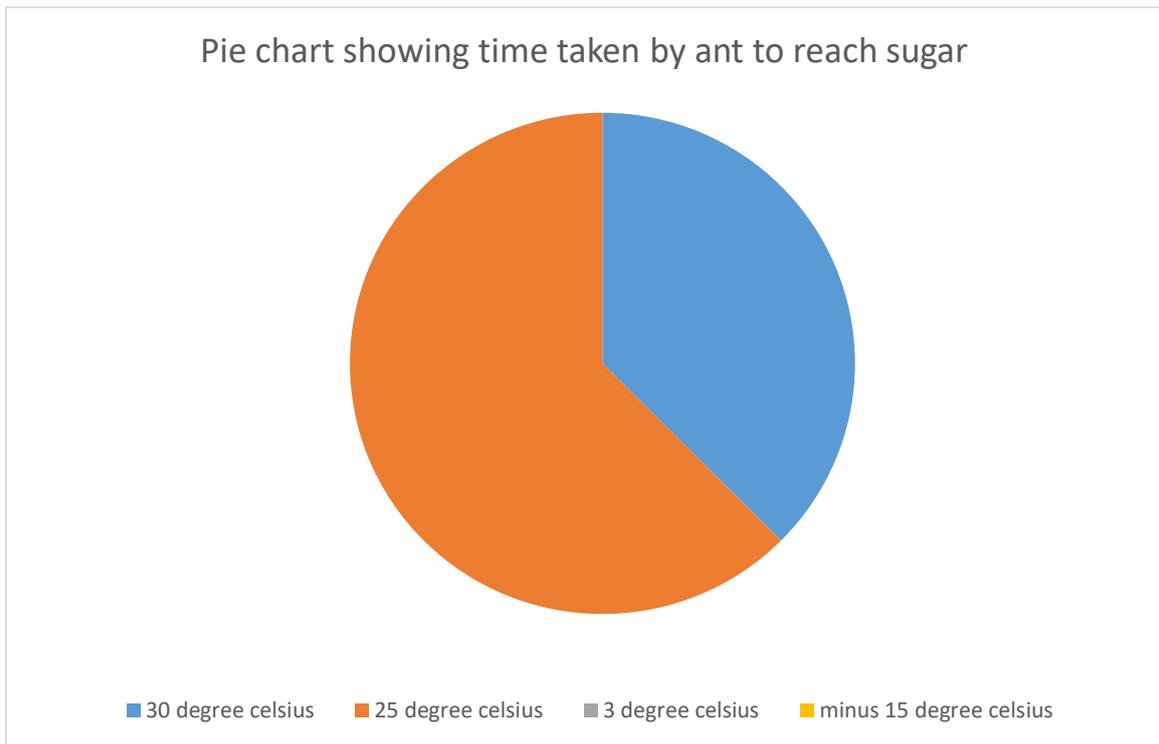
Fig.2. Experimental setup for refrigerator temperature

OBSERVATION

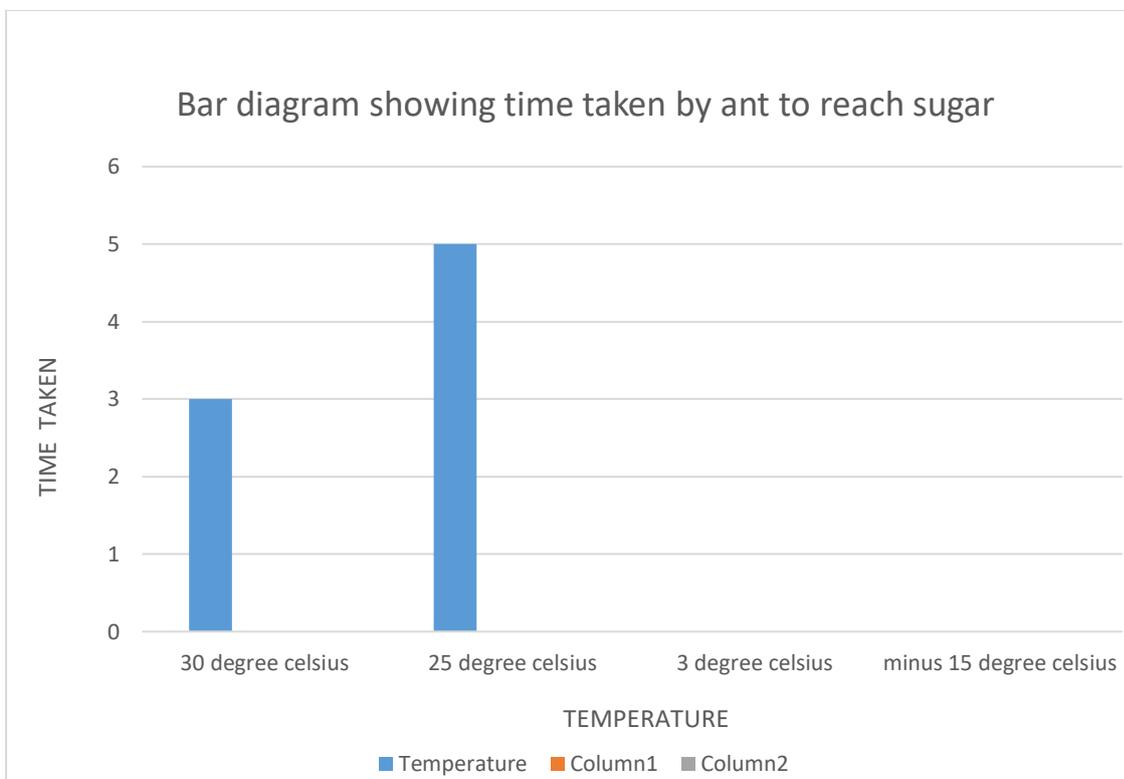
SL.NO	Temperature	Time taken by ant to reach sugar	Observation
1	30°C	3 sec	Ants move actively at this temperature
2	25°C	5 sec	Ants move a little slowly compared to 30°C
3	3°C	They don't move	Ants can't move in this temperature
4	-15°C	They don't move	Ants will be completely frozen



Graph 1:



Graph 2:



Graph 3:

RESULT

At 30°C ants move actively. They reach near sugar within 3 seconds. At 25°C ants moves a little slowly compared to that of 30°C. They take 5 seconds to reach near sugar. At 3°C ants can't move much. They start to freeze. At -15°C ants will be in their still position. They will be completely frozen.

DISCUSSIONS

Most animals including insects are ectotherms whose physiology, development, metabolism and reproduction is constrained by temperature. One of the most important physiological traits for ectotherms is their thermal tolerance.

The study animals were ants as they are an abundant, diverse, and ecologically important group of insects. There are many researches on the same topic conducted in many other countries with different environmental factors. In the experiment of Tizon *et.al*(2014), he measured the walking speed of the worker ants at the increase in temperature from 6°C to 32°C. In the experiment of Katyato Sagata and Heloise Gibb (2016), they observed high ant activity per minute at 29°C. In the experiment of Jacey Bauer (2014) they observed ant behaviour at different temperature. The lowest temperature that a change was observed was at 115°F and the highest temperature that a fire ant could withstand was 125°F. All of the above experiment gives similar results that are ants movement will slow down with the decrease in temperature. The present experiment; it was observed that the ant movement in 25°C, 30°C, -15°C and -3°C varied. The result of the present experiment is same as that of above mentioned experiments which is the movement of ant becomes slow with a decrease in temperature and it will act frozen and immovable at low temperatures. By conducting the experiment it was observed, being a cold blooded animal they go inactive in winters. Since inside temperature of refrigerator is low, ants will become inactive there. They become incapable of moving and eventually dies.

CONCLUSIONS

The results support the hypothesis that the ants' activity changes with temperature and a change in walking speed is observed. In higher temperature the ants showed greater movement and activity while in lower temperature they were disabled to move. The extreme temperatures are considered to be the most significant stress factor for ants in arid and semiarid regions. Temperature directly affects foraging activities due to its effects on oxygen consumption, water loss and transport cost.

Ants are cold-blooded just like all other insects and some other animals, like reptiles. Humans, as well as other animals, are warm-blooded. It is harder for ants to move around when they are cold. They are more active and can move much faster when they are warm. Ants are usually active during the twilight hours, so the best time to inspect a home for infestation is around sunrise and sunset.

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EFFECT OF TEMPERATURE ON ANT MOVEMENT



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Reg. No: AB19Z00027

Under the guidance of
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Department of Zoology, St.Teresa's college (Autonomous), Eranakulam
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Submitted to St. Teresa's college (Autonomous) Ernakulam
Affiliated to Mahatma Gandhi University, Kottayam in partial
Fulfilment of requirement for the degree of Bachelor of Science
in Zoology
2021-2022

CERTIFICATE

This is to certify that the project report entitled “**EFFECT OF TEMPERATURE ON ANT MOVEMENT**” submitted by Ms. Nakshathra P Dishu, Reg. No. AB19Z00027 in partial fulfilment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Soja Louis and this is her original effort.

Dr. Soja Louis

Assistant Professor & Head of Department

Department of Zoology

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(spatially and seasonally). It also exploits habitats of low complexity and intense aridity, which has given it adaptive plasticity favouring its wide geographical distribution (Pilati *et al.* 1997; Claver 2000). Variations may be observed in the harvesting patterns of the species linked to temperature, depending on the geographic locality, although they are close to those generally cited for ants, at 10°C to 40°C (Hölldobler and Wilson 1990). Harvesting patterns in relation to soil temperature were recorded in two studies and the temperature ranges observed were 10°C to 40°C (Claver 2000) and 17°C to 28°C (Nobua Behrmann *et al.* 2010), in both cases in the desert in the centre of the Monte in the province of Mendoza, Argentina. On the other hand, in a study undertaken in the province of La Pampa, harvesting activity of *A. lobicornis* was seen over an ambient temperature range of 11.7°C to 22.6°C (Pilati and Quirán 1996). In all cases, peaks of activity were recorded during spring and at the beginning of autumn, whereas activity ceases almost completely during the winter. Moreover, in summer their foraging behavior changed from diurnal to nocturnal harvesting, always within the determined temperature limits and the same as those recorded for daytime (Pilati and Quirán 1996; Claver 2000; Nobua Behrmann *et al.* 2010).

Ant populations and their distribution are affected by many circumstances. Abiotic factors such as weather, water availability and soil characteristics may determine if a habitat is suitable for ants. Other factors such as level of disturbance, available food resources, reproductive biology and natural enemies also contribute to its distribution. Many factors such as temperature, humidity, vegetative type, level of disturbance and soil characteristics effect the microhabitat of a particular ant species. Dispersal

through mating flights, colony budding, flooding and human activity all determine the distribution and the rate of spread for many species of ants. Resource availability and ability of ants to exploit those resources faster and longer to defend their territory influences ant abundance and diversity in a habitat. Determining what factors are most likely to limit a population of ants or influence its growth and spread has been the focus of many studies. The project tries to explain the effect of temperature on the movement of ant or foraging of ant.

REVIEW OF LITERATURE

Tizón *et.al* (2014) studied the effect of increase in the temperature on the foraging of *Acromyrmex lobicornis* (Hymenoptera: Formicidae). In order to evaluate how an increase in temperature affects the activity of *A. lobicornis*, they studied the amount of foraging and the trophic preferences in two treatments under controlled humidity and temperature conditions ($\Delta 4.5^{\circ}\text{C}$). They also measured the walking speed of the workers as a function of an increase in temperature (6°C to 32°C). Their results support the hypothesis that the ants' activity changes at higher temperature, with higher rates of harvesting and a change in walking speed is observed. There is also variation in the trophic preference, selecting plant items with a higher composition of elements that are degradable by symbiotic fungi. Their results suggest that small variations in ambient temperature significantly affect certain behavior patterns in the leaf-cutting ants.

Jan Sedlacek and Josef Zeman (2013) studied the dependence of the speed movement in forest ant (*Formicarufa*) on the environmental temperature. The paper dealt with the influence of temperature on the movement activity of the ant - *Formicarufa* species. The thermobox with adjustable temperature was used for this experiment. The high-speed camera, thermo sensor and shining prisms were in this box. The ant movement was recorded at many temperatures. The observed ant was placed in an enclosed area of the visual field of the camera. The ant movement was recorded in this area at different temperatures after a five-minute acclimation. The speed of the ant was calculated by determining the number of frames between the start and the end of the selected limb movement. The software of Gimp was used

for this job. The resulting values indicate that the speed of the ant is exponentially dependent on the temperature up to 40 °C.

Wang *et.al* (2020) studied single-file movement of Ants stressed by a high temperature. The study investigated single-file movement of ants (*Camponotus japonicus*) driven by a high temperature in a narrow channel. In this work, they also investigated single-file movement and the interaction between ants in the narrow channel under high temperature conditions. Under the stimulation of high temperature, ants preferred to follow the front one closely for escaping quickly. In the channel, touching behaviour played a main role on escape. It was expressed that the average speed of ants in the single-file movement experiment was faster than that in the single ant experiment.

Roeder *et.al*(2021) in his study on ant thermal tolerance discussed methods, patterns, hypotheses, and potential sources of variation in ant thermal tolerance. From understanding daily patterns in activity to testing hypotheses across geographic gradients, the field of ant thermal tolerance continues to grow.

Oldroyd (2009) studied rearing temperature affects in ant and its thermoregulatory behaviour. Differing task thresholds among workers are crucial to the efficient allocation of work in self-organized insect colonies. New evidence suggests that the rearing temperature of ant pupae causes lifelong changes in an ant's response threshold to temperature.

Chambers (2011) observed the effects of resource availability and temperature on ants. IN the study, the differences in sizes of ants foraging on

different resources at different times of the day and in different temperature environments were examined. Time of day was shown to significantly affect ant foraging habits both within and between species. Specifically, different size ants forage at different times of the day. Larger individuals to forage at night compared to daytime foragers.

The above mentioned literatures revealed some information on the effect of temperature on ants.

MATERIALS AND METHODS

MATERIALS REQUIRED

- Ants
- Sugar
- Refrigerator
- Glass container
- Paper

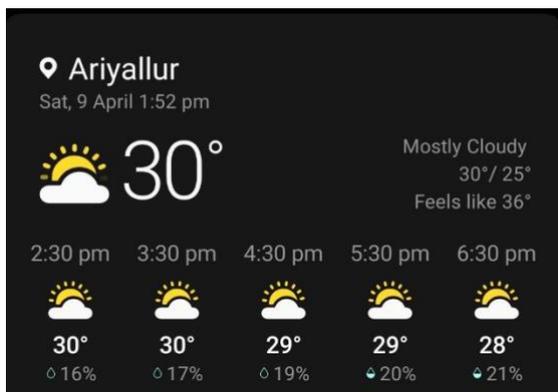
METHODOLOGY

The experiment was conducted at home itself. For the experiment some ants were collected from ground. Ants were collected carefully so as to avoid biting. They were kept in a beaker with a hole in it. The hole is to let the air come for the ants to breathe. The experiment was conducted in four different conditions.

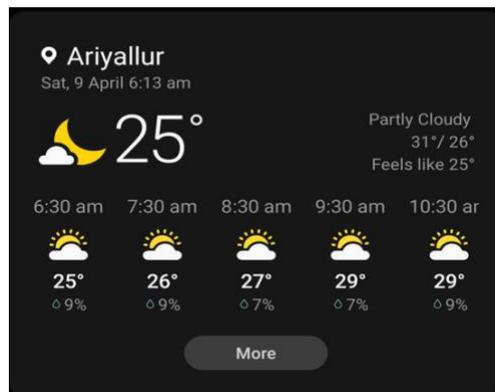
- 1) At 30°C during day time
- 2) At 25°C during night time
- 3) At medium temperature in a refrigerator. That is, approximately 37°F or 3°C
- 4) At low temperature in a refrigerator. That is approximately 40°F or -15°C

Make a circle with sugar at a radius of 5 cm. Put the ants in the centre of the circle. The ant movement towards the sugar is observed and was noted. The same procedure was adopted for 30°C and 25°C temperature ranges. At medium temperature in refrigerator, approximate 3°C, the ants were put in a transparent glass or transparent glass bowl. Cover its mouth portion using

paper to avoid ants from escaping. Make sure to put a small hole on the paper so that the cold air can enter inside the bowl. Keep the bowl in the refrigerator and note how ants move from time to time. At lower temperature in refrigerator, approximate -15°C , the procedure is same as that of medium temperature in refrigerator. After taking out the bowl from refrigerator note the ant movement.



30°C



25°C



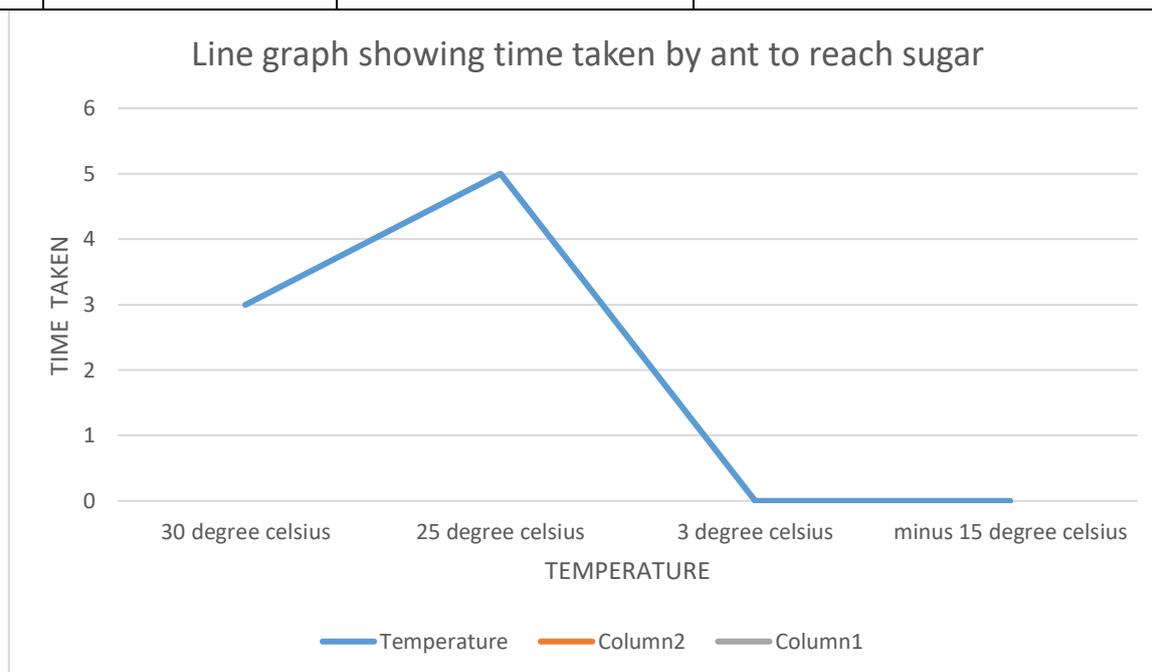
Fig.1.Experimental setup for room temperature



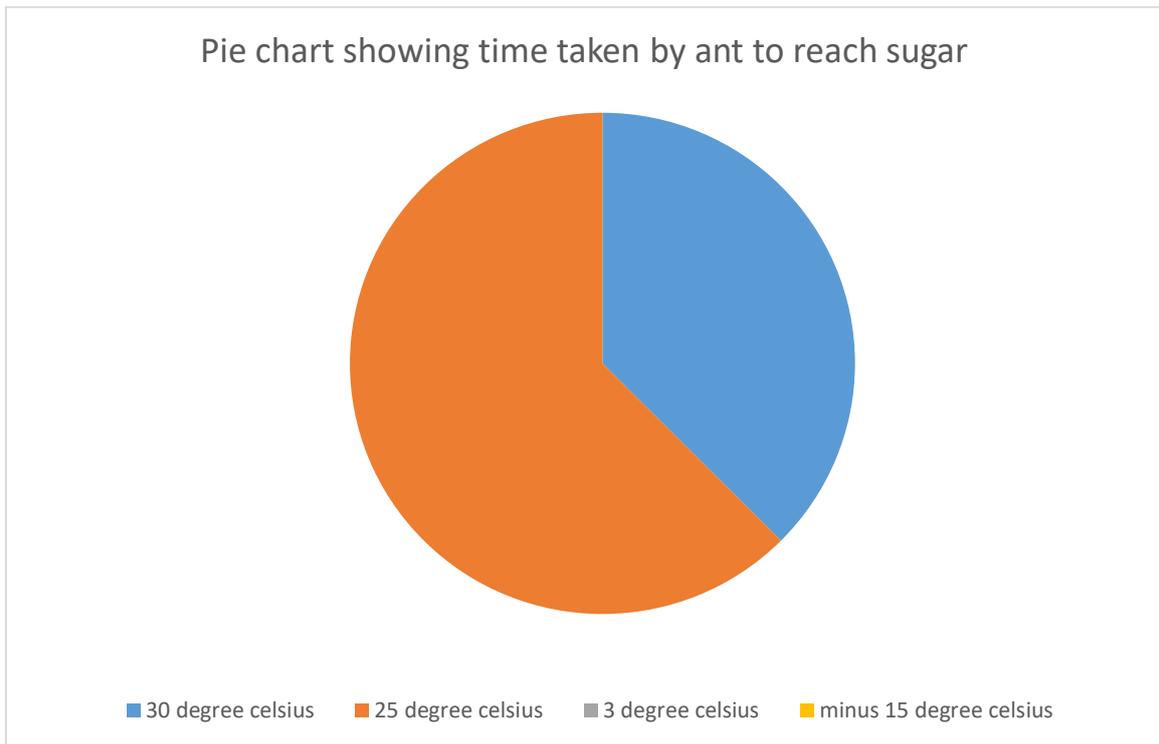
Fig.2.Experimental setup for refrigerator temperature

OBSERVATION

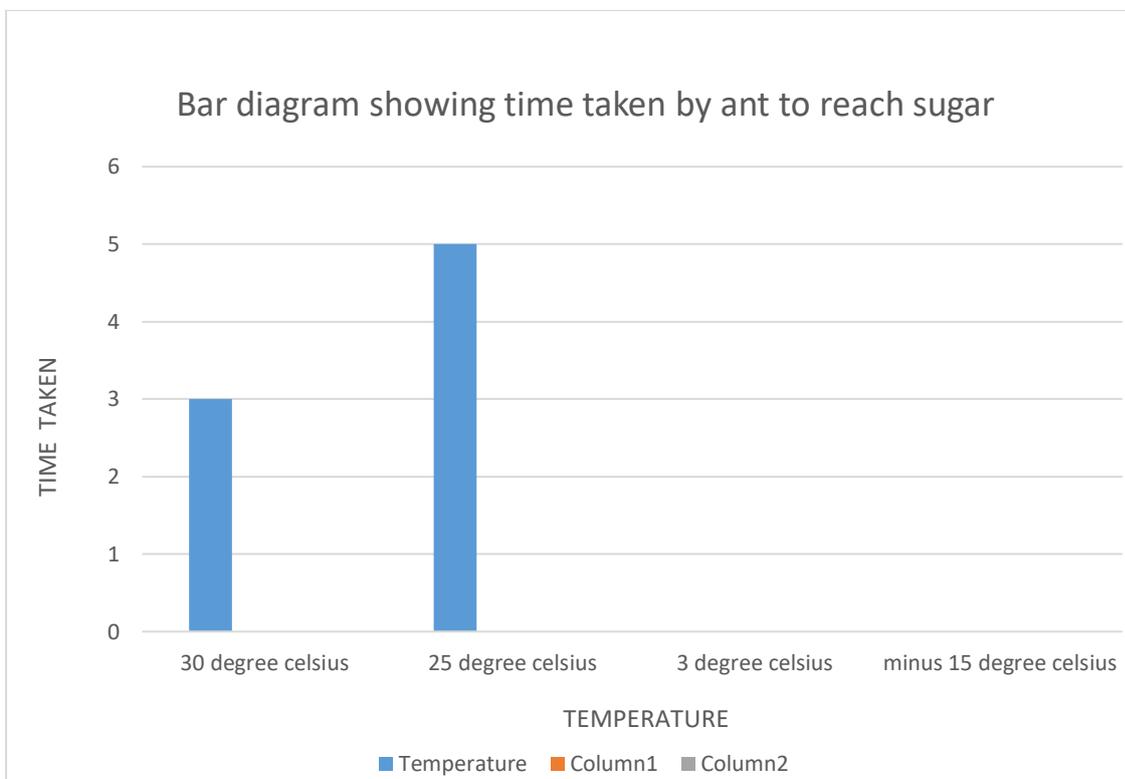
SL.NO	Temperature	Time taken by ant to reach sugar	Observation
1	30°C	3 sec	Ants move actively at this temperature
2	25°C	5 sec	Ants move a little slowly compared to 30°C
3	3°C	They don't move	Ants can't move in this temperature
4	-15°C	They don't move	Ants will be completely frozen



Graph 1:



Graph 2:



Graph 3:

RESULT

At 30°C ants move actively. They reach near sugar within 3 seconds. At 25°C ants moves a little slowly compared to that of 30°C. They take 5 seconds to reach near sugar. At 3°C ants can't move much. They start to freeze. At -15°C ants will be in their still position. They will be completely frozen.

DISCUSSIONS

Most animals including insects are ectotherms whose physiology, development, metabolism and reproduction is constrained by temperature. One of the most important physiological traits for ectotherms is their thermal tolerance.

The study animals were ants as they are an abundant, diverse, and ecologically important group of insects. There are many researches on the same topic conducted in many other countries with different environmental factors. In the experiment of Tizon *et.al*(2014), he measured the walking speed of the worker ants at the increase in temperature from 6°C to 32°C. In the experiment of Katyato Sagata and Heloise Gibb (2016), they observed high ant activity per minute at 29°C. In the experiment of Jacey Bauer (2014) they observed ant behaviour at different temperature. The lowest temperature that a change was observed was at 115°F and the highest temperature that a fire ant could withstand was 125°F. All of the above experiment gives similar results that are ants movement will slow down with the decrease in temperature. The present experiment; it was observed that the ant movement in 25°C, 30°C, -15°C and -3°C varied. The result of the present experiment is same as that of above mentioned experiments which is the movement of ant becomes slow with a decrease in temperature and it will act frozen and immovable at low temperatures. By conducting the experiment it was observed, being a cold blooded animal they go inactive in winters. Since inside temperature of refrigerator is low, ants will become inactive there. They become incapable of moving and eventually dies.

CONCLUSIONS

The results support the hypothesis that the ants' activity changes with temperature and a change in walking speed is observed. In higher temperature the ants showed greater movement and activity while in lower temperature they were disabled to move. The extreme temperatures are considered to be the most significant stress factor for ants in arid and semiarid regions. Temperature directly affects foraging activities due to its effects on oxygen consumption, water loss and transport cost.

Ants are cold-blooded just like all other insects and some other animals, like reptiles. Humans, as well as other animals, are warm-blooded. It is harder for ants to move around when they are cold. They are more active and can move much faster when they are warm. Ants are usually active during the twilight hours, so the best time to inspect a home for infestation is around sunrise and sunset.

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COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICK AND DUCK EGGS



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in partial fulfilment of requirement for the
degree of Bachelor of Science in Zoology

2021-2022

CERTIFICATE

This is to certify that the project report entitled "**COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICK AND DUCK EGGS**" submitted by Ms. Sona Thresia P.S Reg No. AB19ZOO028 in partial fulfilment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Soja Louis and this is her original effort.

Dr. Soja Louis
Assistant Professor & Head of Department
Department of Zoology
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Ernakulam

EXAMINERS

1)

2)

PLACE: ERNAKULAM

DATE: 09/05/2022

DECLARATION

I, Ms. Sona Thresia P.S, hereby declare that this project work entitled **"COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICK AND DUCK EGGS"** is submitted to St. Teresa's College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfilment of the requirements of Bachelor of Science Degree in Zoology. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own

Name: SONA THRESIA P.S

Signature

Reg.No: AB19ZOO028

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SONA THRESIA P.S

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ABSTRACT

COMPARISON OF EMBRYONIC DEVELOPMENT IN QUAIL, CHICKEN AND DUCK EGGS

This experiment was conducted to study first 5 stages of embryonic development in three different birds. Chick, Duck, and Quail were used in the study. These birds have different hatching time. Chicken is one of the most common and widespread domesticated animal. One of the greatest miracles of nature is the transformation of egg into chicken. Egg laying is stimulated by long stretches of day light that occur during the warmer months. The time between Ovulation and Egg laying is approximately 23 to 26 hours. Fertilized embryos develop quickly, and chicks hatch approximately 21 days later. Ducks are called waterfowl. A duck have water-proof feathers. Duck eggs are notable because they are almost 50% larger than a large sized hen's egg. Duck shell have tiny holes that allow it to breathe. Respiratory gases as well as water vapour travel through these pores. Duck eggs have wide range of vitamins and minerals. Quails are very small birds. They have streak and buffed feathers in either blue, black, brown, cream or white color. Quail eggs are main source of protein.

The results shows that early stages of embryonic development in these birds are somewhat similar. Embryonic development of chick in first five days shows the following features; fully formed primitive streak, appearance of optic cup, brain and spinal cord, beginning of heart beat, development of all organs and separation of three toes. In embryonic development of duck, the appearance of primitive streak which extends to center of area pellucida, neural fold development, distinct head and trunk, formation of amniotic cavity etc were observed. Enlarged blastoderm, appearance of vitelline membrane, beginning

of blood circulation, appearance of eyes and C shaped embryo were the characters observed in first five days of quail embryonic development.

INTRODUCTION

Embryonic development in birds has been widely researched. The hatching time of birds' eggs is very diverse, ranging from 11 to 100 Days, which is mainly thought to be due to differences in embryonic development. Aim of the experiment was to compare the embryonic development in three different eggs of Quail, chicken and duck, which were easily available. An egg is the organic vessel containing the zygote in which an embryo develops until it can survive on its own, at which point the animal hatches.

QUAIL

Scientific name: *Coturnix coturnix*

Kingdom: Animalia

Phylum: Chordata

Class: Aves

Order: Galliformes

Superfamily: Phasianoidea

Quail is a collective name for several genera of mid-sized birds generally placed in the order Galliforms. Quail eggs are little thicker than normal eggs because they have an extra layer of thickness where the colour sites. The egg shell was soft, with colour between white to light sand colour, and a smooth texture which allows good deposition of colour spots, with different colour levels from black to brown spots. The egg size was smaller compared to chicken and duck eggs. It was about 1 ¼ inch long and 1 inch wide and its mass is 9gm. Generally Quail takes about 18 days of incubation, but they can hatch as early as day 16 or as late as 20days.

Quail eggs are considered a delicacy in many parts of the world, including Asia, Europe, and North America.

CHICKEN

Scientific name: *Gallus gallus domesticus*

Kingdom: Animalia

Phylum: Chordata

Class: Aves

Order: Galliformes

Family: Phasianidae

Chicken is a domesticated bird. Usually white hens lay white eggs, and brown hens lay brown eggs. Eggs that are not white have pigments deposited on them as the eggs travel through the hen's oviduct. Blue and other egg colours are formed as the egg develops and the colour appears on both the inside and outside of the shell. Eggs usually become fertile about four days after the rooster has been introduced to the hens.

Chicken eggs contain within a calcium carbonate-based hard shell, which is about 11% of the weight, the egg whites (albumen) and the egg yolk, separated by membranes. Eggs are nutritionally valuable due to their content of high-value proteins, fat, lecithin, vitamins and minerals. The structural components of the egg include the shell and shell membranes the albumen or white including the thick albumen, the outer thin albumen, the inner thin albumen, and the chalazae; and the yolk. The average egg length was 34.4mm, width 24.7mm and volume 10.6cm and its mass is 50gm. Egg takes about 21 days to hatch.

DUCK

Scientific name: *Anas platyrhynchos*

Kingdom: Animalia

Phylum: Chordata

Class: Aves

Order: Anseriformes

Superfamily: Anatoidea

Family: Anatidae

Duck is the common name for numerous species of waterfowl in the family Anatidae. Ducks are generally smaller and shorter-necked than swans and geese, which are members of the same family.

Duck eggs are notable because they're almost 50% larger than a large-sized hen's egg. They have a large, golden, creamy yolk, and many people love them for their rich, extra-egg flavour. Their shells are also a treat for the eyes. The colour depends on the breed of the duck, though the shell colour sometimes varies even within the same breed. Duck egg yolks get their orange-yellow colour from natural pigments called carotenoids. Size of a duck egg is about 5-7 inches and its mass is 70gm. It takes about 28 days for hatching.

Duck eggs are an excellent source of selenium, providing almost half of the daily value in one egg. Duck eggs also provide vitamin D, the "sunshine vitamin." Low levels of vitamin D are associated with depression and seasonal affective disorder.

All these three eggs had varied features and the hatching time was also different. Quail eggs hatch early compared to hen and duck eggs. The study focuses on the different developmental stages of quail, chicken and duck eggs and comparing it.

AIM

To compare the embryonic development in Chick, Duck and Quail using incubator.

OBJECTIVE

- To compare the embryonic development in chick, duck, quail using homemade incubator.
- To check the hatching rate of eggs belong to different strain.
- To demonstrate fertility using candling method and floating test.
- To observe the variation in hatching days.

REVIEW OF LITERATURE

Anna *et al.* (2012) Article explains how to practice floating test for detecting egg viability.

Doty (2011) her study explains about the Hamburger-Hamilton stages which are sequence of images depicting 46 chronological stages in chick development. In this study the images begin with a fertilized egg and end with a fully developed chick. The stages were determined by the number of somites and each stage was at an interval of three somites. Somites or segmented blocks of mesoderm, bud off sequentially during vertebrate development and can therefore be used as a timing landmark. The embryos used for the photographs were from different varieties of chickens: white leghorns, barred Plymouth Rock, and Rhode Island reds.

Jacob (2022) explained the parts of eggs in his article and mentioned that everything that an embryo needs to develop, grow and hatch must be provided in the egg when the egg is laid. If a hen receives sub-optimal feed, there may be a lot of developmental problems with the embryos when the eggs are incubated.

Warin (2009) explained in his article about embryonic development of chick egg, from day 1 to 21 using images of embryonic stages. It also explains the distinguishing features of infertile and fertile egg.

Smith (2004) explained in his article about the following topics that improve the producer's success. They are: Selection of hatching eggs, egg care and storage, incubators, incubating conditions, sanitation trouble shooting failures.

Incubation and Hatching techniques of eggs was explained by Archer *et al.* For successful hatch one must store and incubate eggs carefully. Incubation and hatching of eggs are influenced by factors such as environmental conditions, handling, sanitation and record keeping.

Ramteke *et al.* (2013) Article helps to study the embryonic development of Japanese quail and to identify the period of incubation where the Japanese quail embryo gives the faster ontogeny than chick embryo.

Mormio explains the embryonic development using the photo presentation from the Purdue Research Institute along with the authors own photos of candling eggs throughout the 21 chicken egg incubation period.

The Article is about candling Duck eggs. Candling is an old term that means the application of bright light to an egg to see what is inside. Originally it was done by using candles in a dark room. Now a day flashlight or an actual candling light are used for candling. Article includes pictures of developing duck eggs, from Day 1 to Day 25 and also the pictures of early dead embryo.

Ghali *et al* (2010) they presented a novel way of adapting the wellknown EC culture of whole chick embryos to time lapse imaging and to functional molecular studies using blocking agents. The novelty of their method stems from the ability to apply blocking agents *ex ovo* as well as *in ovo*. They were able to study the function of a set of molecules by culturing developing embryos *ex ovo* in tissue culture media containing these molecules or by injecting them underneath the live embryo *in ovo*. The *in ovo* preparation is particularly valuable since it extends the period of time during which the developmental function of the molecule can be studied and it provides an easy,

reproducible method for screening a batch of molecules. These new techniques will prove very helpful in visualizing and understanding the role of specific molecules during embryonic morphogenesis, including blood vessel formation. They attained two objectives in this study. First, they devised a flexible ex-ovo method that combines the ability to image the live, whole embryo with the application of blocking agents. Second, they devised a simple method for studying the function of secreted factors through the application of blocking agents.

One dilemma in comparing embryo development between poultry species is the absence of a common reference of sequential stages of morphogenetic development. To counter this problem, Sellier *et al* (2006) in his paper the normal table of embryogenesis was devised to accurately assess embryo development during the oviductal and incubation phases of embryogenesis. This paper is not only important for research on the morphogenetic development of the avian embryo, but it is also useful for investigators in the poultry industry attempting to uncover the basis of fertility and hatchability problems.

Givisiez *et al* (2020) their study addresses the main changes in metabolism and intestinal development throughout incubation, and also addresses scientific advances, limitations and future perspectives associated with the use of in ovo feeding that has been regarded as an important technology by the poultry industry.

ShanshanLi *et al* (2019) their study aimed to establish a comparison of complete morphological development staging for ducks (*Anas platyrhynchos*) and geese (*Anser cygnoides*) with the embryonic staging system by Hamburger and Hamilton (HH) for the chicken (*Gallus gallus*). Their results

show that morphological development in the chicken, duck, and goose are similar in the early stages. The major differences occurred after stage 27 of embryonic development, where the beak shape in ducks and geese was wider and longer than in chickens. In addition, the second and third interdigital webs of the hind limb of the chicken were found to be degraded from stage 31, and eventually vanished at stage 35; however, they were retained in ducks and geese. They established embryonic developmental staging systems for ducks and geese, from fertilization to hatching, which can be used in comparative studies with these 3 species.

Ainsworth *et al.* The results of this study demonstrate that there are only minimal differences identified in the rate of quail embryonic development when compared with chick embryos up to 5.5 days of incubation. Therefore, up to this period, chick and quail embryos can be directly compared using either incubation times or descriptive details and the stages used are identical (stages 4–28). After 5.5 days of incubation there is a slight increase in the developmental rate of the quail embryos and hence attributing equivalent stages to both chick and quail embryos based on incubation times is no longer possible. The overall descriptions for each stage (stages 29–35) are still similar, however, so comparisons are still possible. From 8.5 days of incubation the rate of development for the quail accelerates in comparison to the rate for the chick, so there are significant anatomical differences between chick and quail embryos at these time-points. Therefore, at these later stages of development (stages 36–46) the H.H Stage series is no longer comparable to the quail series in terms of either incubation times or morphological descriptions, making the quail series completely distinct for these stages. So in this paper they developed a definitive developmental stage series for Japanese quail so that differences are fully characterized, misconceptions or

assumptions are avoided, and the results of comparative studies are not distorted.

Guryeva *et al.* Their results showed that the hatching rate of quail in the flight group made up 38 % from the laboratory and 44 % from the synchronous control. Embryonic death during the incubation period both in the control and in the flight groups was caused by their position within the egg. Flight group embryos and hatched birds did not differ from the control groups in body size either. Body length of the flight group embryos at all stages of development was slightly less than in the control groups, but within the limits of norm. In the growth dynamics of extremities, the differences between the flight and control groups exceed the diapason of individual fluctuations in earth conditions.

Saraswati *et al.* This experiment was conducted to determine the development of Japanese quail embryo (*Coturnix coturnix japonica*), through observation and measurement of embryo organ development from the age of one day until hatching. The study used 15 female quails and 5 male quails. 15 female quails were divided into 5 cages, each cage containing 3 quails females and 1 male quail. Eggs which are inserted into an egg incubator is produced when the quail began the age of 3 months. Descriptive observation has been made towards the development of organs in the embryo. Based on the results of the study, the growth and development of quail embryo organs occur in stages until hatching occurred during the 16 days.

Dupuy *et al.* In this study, an objective means to stage development of the duck embryo is lacking, Such a staging procedure, is described there .The staging scheme presented there provides objective morphological criteria describing the embryonic development of the duck.

METHODOLOGY

MATERIALS REQUIRED

Styrofoam box, bulb, holder, wire, adapter, connector, switch board, 3 inch fan, 20 chicken eggs, 20 duck eggs, 20 quail eggs, lens, water, bowl, saw dust, nylon cable ties, Thermostat.

INCUBATOR

Incubator was set in a Styrofoam box. Entire top of the box was removed and saw dust was spread on the floor of the box. A hole was cut on one side of Styrofoam box. The hole contain the light bulb and socket. Then a socket with 12 volt bulb was inserted. Socket was fixed using nylon cable wire. Then a digital thermostat was added, and it was placed on the side of the box. Then sensor of the thermostat was inserted inside the box through a hole, on the side that is opposite to the bulb. A bulb, thermostat and adaptor was connected to the circuit. Since the main function of incubator was to keep the temperature and humidity inside at an optimum level a thermostat with high rate of accuracy was selected for the experiment. Thermostat was set in such a way that the bulb turns off, when the temperature inside the box exceeds 37.6C in the case of all the 3 type of eggs. A bowl of water was placed. It was the humidity source. Light was turned on and regular inspections of incubator was done during the study to monitor the humidity and temperature.

PROCEDURE

Fertile eggs can be hatched by using an egg incubator. After setting incubator, eggs were placed inside the incubator. Here 20 chicken, Duck, and Quail eggs were used. After setting the eggs, incubation process begins. An important part of it is turning or rotating the eggs. Eggs will need to be turned a minimum of 3 times per day. Mark one side of the egg with pencil that will help us to keep track of which eggs had been turned. Proper sanitation should be followed while touching the eggs. Incubator was closed.

DATA COLLECTION

Eggs are cracked at the top and a small portion of the shell is carefully removed to observe the embryonic development under a lens. Chick, duck and quill eggs are separately observed under the lens. Clear pictures of respective eggs are taken for comparison.

Then the analysis was done using candling method from day 6. Candling of eggs was done towards the middle of the incubation period from day 7-10, eggs can be candled to determine if the embryos are growing properly. It is done by simply shining a light through an egg. White or light colored shells are easy to candle while darker shells require brighter light.

Interior is clear for infertile eggs and they are removed.

A ring of red is visible within the egg, which contains a dead embryo. It was removed.

If there is blood vessels within the egg there is a live embryo inside. If broken eggs are see, it should be removed from the incubator. Stop turning the chicken, duck and quail eggs at day 18, 25, 14 of incubation respectively.

Chicken eggs required a hatching period of 21 days, whereas duck eggs require 28 days and Quail eggs require 18 days.

OBSERVATION AND RESULT

QUAIL EGG

20 eggs were used for incubation. On first day, a small portion of egg shell was cracked and observed that the blastodisc was located on the top of the yolk and appeared irregular in shape. It was an infertile egg. Another egg was taken and when observed it was found to be a fertile egg because, the germinal disc was at the blastodermal stage. The blastoderm was enlarged and the segmentation cavity under area pellucida attained a shape of dark ring and the area pellucida and area opaca were visible. On the second day, the egg yolk had more development. The blastoderm enlarged in size; the vitelline membrane was visible and the blastoderm had a donut shaped structure. On day 3, three eggs were cracked because, the first two eggs were infertile. In the third egg embryo was lying on its left side. The beginning of blood circulation in embryo can be observed; vitelline membrane spreads over the yolk surface. Head and trunk were distinct. Cardiac structure appeared, which begins to beat.

On day 4 development of amniotic cavity, which surround the embryo, can be observed. It was filled with amniotic fluid. It protects the embryo and allows it to move. Allantoic vesicle also appeared. Eyes were visible. On fifth day of development, the embryo attained a sensible increase in size; embryo formed a C shape; the head moved closer to the tail and limbs extended. From day 8-10 the development of embryo inside the egg was observed using candling method. On day 8, when candling was done, four eggs were found to be infertile. The next egg observed was fertile. The veins were clearly visible; the whole embryo was well defined and the beak appeared. The movement of embryo can be observed.

On ninth day, the toes are completely separated from each other. The beak and feet began to keratinize and the amniotic fluid decreased in quantity. On tenth day, more veining can be seen; but it was difficult to get a clear image. On the 12th day of incubation, floating test is done for all remaining eggs to determine that the embryo was live or dead. In that test, 3 eggs were removed because they were dead and settled under water. All others float on water and were wriggling. Those eggs contained a live embryo. On 14th day, the rotation of egg was stopped. Eggs were kept for lockdown period. On 18th day three eggs were hatched and on 19th 2 eggs were hatched. Thus a total of 5 eggs were hatched at the end of experiment.

Embryonic development in first 5 days of quail embryo:



A



B



C



D



E

Plate 1: A- DAY 1; B- DAY 2; C- DAY 3; D- DAY 4; E- DAY 5

CHICK EGG

20 eggs were placed in the incubator for hatching. On 1st day, when the top portion of the shell was cracked, egg yolk was visible and it was observed that, the chick embryo was oval in shape; The primitive streak was fully formed; area opaca and area pellucida were visible; area opaca further modified into area vasculosa and area vitellina. On day two, the completion of vitelline (extra embryonic) circulatory system and formation of two pairs of aortic arches occurred. Twisting of heart and formation of chambers was also observed. Vitelline vessels were visible; formation of optic cup, brain, and spinal chord; commencement of blood circulation; presence of amnion; tail and leg buds were visible. On day 3, one egg was found to be infertile. So it was removed from the incubator. Another fertile egg was taken and in that egg, heart began to beat and appearance of blood vessels can be observed. Amniotic tail fold was developed. It extended opposite to head fold. Eyes, lungs, and liver we're clearly visible. Atrium, vitelline vein, nerve chord, cranial flexure, mesenchyme and diencephalon can be observed.

On day 4, amniotic cavity was developed, which surround the embryo and it was filled with amniotic fluid. It protects the embryo and allowed it to move. Appearance of amniotic vesicle, plays a major role in calcium reabsorption, respiration, and waste storage. By the end of fourth day of incubation, the embryo had all the organs needed to sustain life after hatching. Most of the embryo parts can be identified in this stage. On 5th day optic vesicle became distinct. In the wings demarcation of elbow and knee joint was observed and the first 3 toes were separated. From 6th day onwards, candling was done.

On day 9, candling experiment was conducted, and observed the following features; air cells can be seen, the eye of embryo can be seen as dark round

spots, blood vessels were visible, embryo appeared like a dark mass and the movement of embryo inside the shell can be seen clearly. On 10th day of incubation, the embryo resembled a solid mass. The body Differentiation can be seen; Appearance of egg-tooth; toes and beak can be identified. The embryonal movements are clearly visible. On 12th day, the air cells grow as incubation progresses. The toes were fully formed and beak was keratinized. Body was lightly covered with feathers. Now, the embryo had the aspect of a chick. On 15th day of incubation, floating experiment was done and in that experiment 4 eggs settled down. That means the eggs were decayed, infertile, or the chick formed inside was dead. Those eggs were removed. Rotation of eggs was stopped at 18th day. On 20th day 4 eggs were hatched and on 21st day 6 eggs were hatched. A total of 10 eggs were hatched at the end of experiment.

Embryonic development in the first five days of chick embryo:



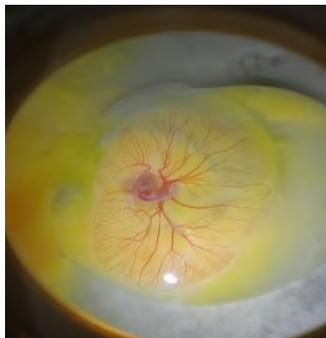
A



B



C



D



E

PLATE 2: A- DAY 1; B- DAY 2; C- DAY 3; D- DAY 4; E- DAY 5

DUCK EGG

20 eggs were used for observation. On day one, when the top portion of egg was cracked, it was observed, that the germinal disc was at the blastodermal stage. An embryonic shield was formed; primitive streak appeared and it extend to the center of pellucida. Area opaca and area pellucida can be distinguished very clearly. Area opaca further modified into area vasculosa and area vitellina. On day 2, area vasculosa and area vitellina become distinct. Primitive streak began to regress and neural fold starts to develop. On day three, first egg taken was infertile. In the next fertile egg taken, vitelline membrane spreads over the yolk surface; Embryo was lying on its left side. There was beginning of heart formation; the first indication of heart formation was defined by a paired primordia, along with primary optic vesicles. Blood circulation had started; cephalic bud, wing and leg buds were visible, neural tube developed and the head and trunk can be discerned.

On day 4, allantois appears and it became vesicular. Vitelline arteries were well developed and amniotic cavity was formed. On 5th day elbow and knee joints became distinct. On 9th, 10th, and 11th day candling experiment was done and in that it was observed that 2 eggs were infertile. So those eggs were removed. On 9th day it can be seen that the embryo became a dark mass. The eyes can be seen as a dark spot. Blood vessels spread inside the egg; and the air cell can be seen. The movement of embryo was visible, and the movement was very fast.

On 10th day, it was observed that, the embryo had development; its body was well developed. Appearance of beak and foot can be found. On day11, small feathers were formed on the body; the air cells had grown, beak and

foot growth progressed and the toes were differentiated. The movement of embryo slows down. Floating experiment was conducted on day 15 and in that experiment 3 embryos were found to be dead and they were removed. On 24th day, 3 egg had crack on shell. On 25th day floating test was again conducted and in that, 2 eggs were removed because the embryo was already dead. On 26th day of incubation, 4 eggs hatched and on 27th day 3 more eggs were hatched. A total of 7 eggs were hatched at the end of experiment.

Embryonic development in first 5 days of duck embryo:



A



B



C



D



E

PLATE 3: A- DAY 1; B- DAY 2; C- DAY 3; D- DAY 4; E- DAY 5

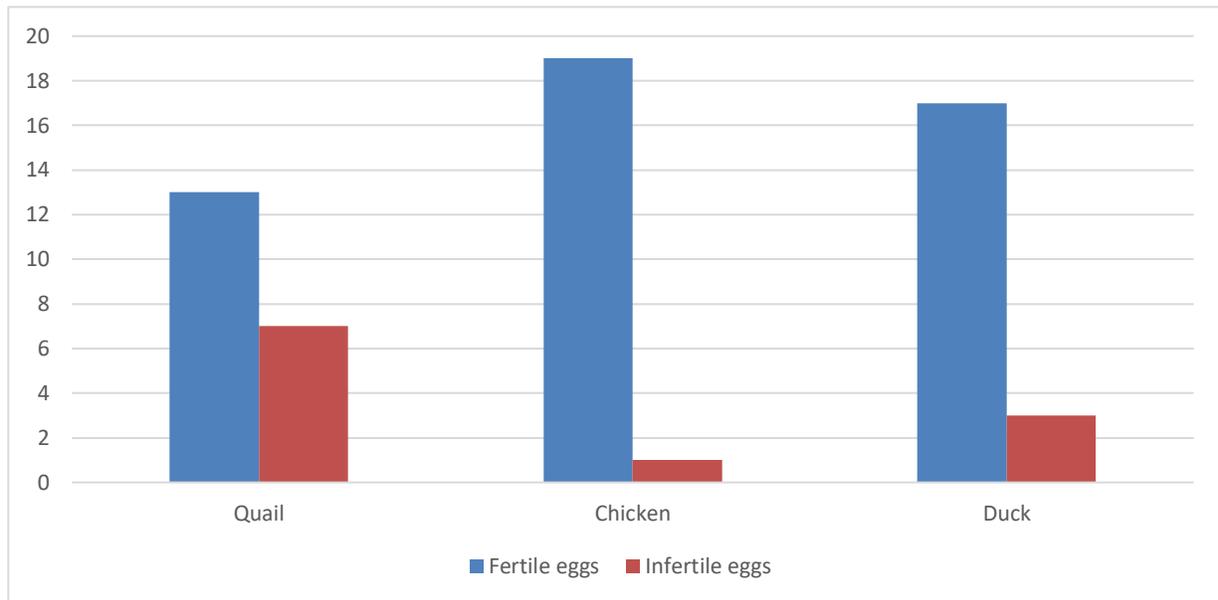
DAY	Strain		
	Quail	Chicken	Duck
Day 1	<ul style="list-style-type: none"> • Blastoderm is enlarged. • Segmentation cavity attains the shape of a dark ring. • Area opaca and area pellucida were visible. 	<ul style="list-style-type: none"> • Embryo is oval in shape • Primitive streak was fully formed • Area opaca and area pellucida were visible • Area opaca further modifies into area vasculosa and area vitellina. 	<ul style="list-style-type: none"> • Germinal disc at blastodermal stage. • Embryonic shield is formed • Primitive streak appears and extend to centre of area pellucida • Area opaca and area pellucida can be distinguished • Area opaca further modifies into area vasculosa and area vitellina
Day 2	<ul style="list-style-type: none"> • Blastoderm enlarges and attains a donut shape. • Vitelline membrane appears 	<ul style="list-style-type: none"> • Completion of vitelline circulatory system. • Formation of two pair of aortic arches. • Twisting of heart and formation of chambers • Visible Vitelline vessels • Formation of optic cup, brain, spinal chord • Commencement of blood circulation • Presence of amnion • Tail and leg bud visible 	<ul style="list-style-type: none"> • Area vasculosa and area vitellina are clearly distinguishable • Primitive streak began to regress • Neural fold starts to develop.

Day 3	<ul style="list-style-type: none"> • Embryo lying towards left • Beginning of blood circulation • Vitelline membrane spreads over yolk • Head and trunk distinguished • Cardiac structures began to beat 	<ul style="list-style-type: none"> • Starting of heart beat • Visible blood vessel • Amniotic tail fold developed • Clearly visible Eyes, lungs, and liver. • Atrium, Vitelline vein, nerve chord, cranial flexure, mesenchyme and diencephalon are visible. 	<ul style="list-style-type: none"> • Embryo lying towards left • Vitelline membrane spreads over yolk • Beginning of heart formation • Blood circulation start • Cephalic, wing and leg buds are visible. • Neural tube develops • Head and trunk are distinguishable
Day 4	<ul style="list-style-type: none"> • Amniotic cavity develops and gets filled with amniotic fluid • Allantoic vesicle appears • Eyes are visible 	<ul style="list-style-type: none"> • Amniotic cavity develops and gets filled with amniotic fluid. • Amniotic vesicle appears • Development of all organs needed to sustain life after hatching 	<ul style="list-style-type: none"> • Allantois appears and become vesicular • Amniotic cavity is formed • Vitelline arteries are well developed.
Day 5	<ul style="list-style-type: none"> • Embryo greatly increases in size • Embryo forms a c shape • Head moves closer to tail • Limbs extend 	<ul style="list-style-type: none"> • Distinct optic vesicle • Demarcation of elbow and knee joint is observable in wings • Separation of three toes 	<ul style="list-style-type: none"> • Elbow and knee joint become distinct.

Table 1: Showing embryonic development of quail, chicken, and duck during first 5 days of incubation.

SPECIES	FERTILE EGGS	INFERTILE EGGS
Quail	13	7
Chicken	19	1
Duck	17	3

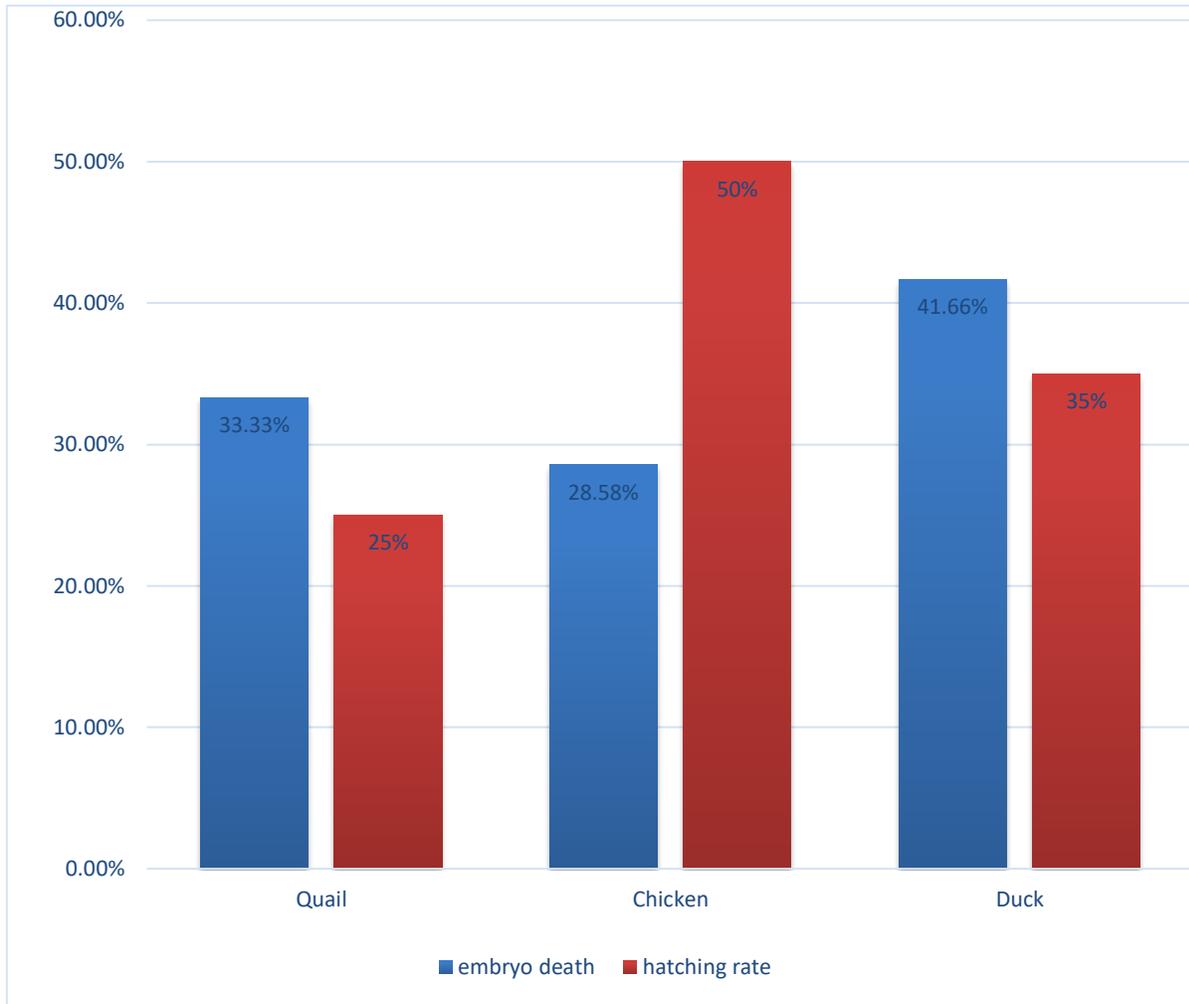
Table 2: Showing fertile and infertile eggs in each species



Graph 1: Showing the fertility and infertility of eggs

SPECIES	EMBRYO DEATH	HATCHING RATE
Quail	33.33%	25%
Chicken	28.6%	50%
Duck	41.7%	35%

Table 3: Showing embryo death and hatch rate of each species



Graph 2: Showing the embryo death and hatch rate of each species

DISCUSSION

Nearly 60 eggs were used in the development of Quail, Chicken and Duck using incubator working at same temperature. On the first day of embryonic development area opaca and area pellucida are clearly distinguishable in the three eggs. On day two vitelline membranes became visible. Commencement of blood circulation was observed on the second day in chick egg, but in quail and duck egg it was observed on day 3. Amniotic cavity develops and gets filled with amniotic fluid on day 4. On day 5 embryo forms a C shape, and head moves closer to tail in case of quail egg, whereas elbow and knee joints became distinct in chick and duck.

Similar kind of research were conducted by several scientists. The research conducted by ShanshanLi, ShibinBai, XiaQin, Junpeng Zhang, Irwin, Zhan, Zhewang (2019), aim to establish a comparison of complete morphological development staging for ducks and geese with the embryonic staging system by Hamburger and Hamilton for the chicken. The morphological development in chicken duck and goose are similar in the early stages.

According to the studies by Saraswati, Tana, faculty of science and mathematics, Diponegoro University Semarang, Indonesia, (2015)

Based on result the growth and development of quail embryo organs occur in stages until hatching occurred during the 16 days. Formation of blastoderm, differentiation of primitive streak mesoderm, circulatory system begins to develop, development of C shaped embryo were visible in the early days.

Ainsworth, Stanley and Evans, (2010) The results of this study demonstrate that there are only minimal differences Identified in the rate of quail embryonic

development When compared with chick embryos up to 5.5 days of incubation. Therefore, up to this period, chick and quail embryos can be directly compared using either incubation times or descriptive details and the stages used are identical.

Jacob explained about the parts of egg. Everything that an embryo needs to develop, grow and hatch must be provided in the egg when the egg is laid. Archer and Cartwright they explained about the important factors of incubating and hatching eggs like storage of eggs, environmental conditions, handling etc.

Warin explains about embryonic development of chick egg, from day 1 to 21 using images of embryonic stages. Also through this, we can distinguish an unfertile egg and a fertile egg.

Ramteke, charde, zade and Gabhane(2013) their article helps to study the embryonic development of Japanese Quail and to identify the period of incubation where the Japanese quail embryo gives the faster ontogeny than chick embryo.

CONCLUSION

The present work concludes by studying the specific developmental features of quail, duck and chicken embryos. Compared with the previous studies using chick, duck and quail, this study is mainly focussed on the embryonic development of these strains in first five days. The organogenetic sequence of quail, chicken and duck embryos are uniform; nevertheless, the incubation period of three species are different. Incubation period of chicken egg was 21 days whereas quail required 18 days and for duck egg it was 28 days. Fertility rate was high for chicken eggs when compared to quail and duck eggs. Out of 20 eggs 19, 17, 13 eggs were fertile for chick, duck and quail eggs respectively. Embryo death was mostly observed in late stages in duck eggs, with the reason being insufficient evaporation of water and the presence of comparatively thick shell. Hatching rate was found to be maximum in chicken eggs with 50% hatchability whereas, it is only 35% and 25% for duck and quail eggs respectively. Embryonic development in these three species is similar at early stages and difference occur only in late stages. Observation on the development of chick, duck, and, quail embryos can be used for the maintenance and management of these species.

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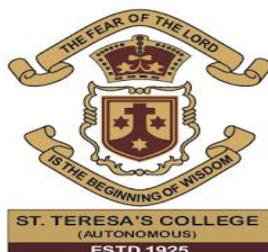
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STUDY ON ROLE OF BIOPRESERVATIVES TO INHIBIT FUNGAL GROWTH IN BREAD



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Affiliated by Mahatma Gandhi University, Kottayam

in partial fulfilment of requirement for the

Degree of Bachelor of Science in Zoology

2021-2022

CERTIFICATE

This is to certify that the dissertation titled, “*Study on role of biopreservatives to inhibit fungal growth in bread*” is an authentic record of work carried out by **TINU H.** under the supervision and guidance of **Dr. HELVIN VINCENT**, Assistant Professor, Department of Zoology, St. Teresa’s College (Autonomous), Ernakulam in partial fulfilment of the requirement for the Bachelor’s Degree of Science in Zoology.

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Ernakulam

Ernakulam

Examiners

1).....

2).....

PLACE: ERNAKULAM

DATE: 09/ 05/ 2022

DECLARATION

I, Ms. Tinu H, hereby declare that this project report entitled **STUDY ON ROLE OF BIOPRESERVATIVES TO INHIBIT FUNGAL GROWTH IN BREAD** is a bonafide record work done by me during the academic year 2021-2022 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam.

This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

TINU H.

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Place: Ernakulam

Date: 09/05/2022

TINU H.

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ABSTRACT

This study aims to identify and evaluate the role of biopreservatives to inhibit fungal growth in bread.

Nine common biopreservatives were applied to bread slices and kept in a plastic bag and left undisturbed till the appearance of mould. One slice of bread was maintained as control. The day at which mould occurred was noted. Antifungal activity of different biopreservatives was studied from the results obtained from the experiment. Among the applied nine biopreservatives lemon is more effective and clove oil is second most effective one. Garlic is also effective compared to other 6 samples used in the study. Citric acid in lemon, eugenol in clove and allicin in garlic is responsible for their antifungal activity. This study can be applied to replace artificial preservatives used in manufacturing of bread and other similar products to inhibit fungal activity and to increase shelf life.

INTRODUCTION

Food spoilage is a critical issue facing nowadays. Microbial spoilage of food happens by the means of bacteria, fungus, protozoa, etc. Fungi is one of the common group of food spoilers by microbial attack. They occur in form of mould, yeast etc.

Bread is one of the staple food used in day to day life. Bread and other bakery products are subjected to various spoilage problems, viz., physical, chemical and microbial; the latter is the most serious one particularly bacterial (*Bacillus* sp.) and mold growth. Various moulds involved in spoilage of bread include *Rhizopus*, *Mucor*, *Penicillium*, *Eurotium*, *Aspergillus* and *Monilia* (Saranraj and Geetha, 2012).

Food safety and extend shelf life of food products are essential to meet demands by consumers. An interesting alternative is the use of naturally derived antimicrobials called biopreservatives. A wide range of natural products can be used as antifungal agent.

Biopreservatives can be used to inhibit fungal growth in bread. Biopreservatives can replace artificial preservatives used in manufacturing of bread and other similar products to inhibit fungal activity and to increase shelf life. Biopreservatives or its derived forms can applicable in future to increase shelf life by inhibiting fungal growth.

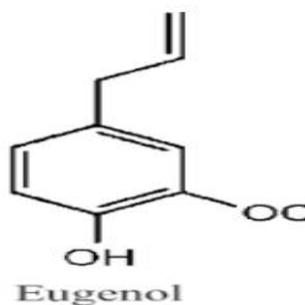
In order to increase the period of preservation of bread, it is necessary to add substances that slow down or inhibit fungal activity. The purpose of this project was to study the role of nine different commonly available biopreservatives to preserve bread and other similar products.

1.Cinnamon

The major components of cinnamon is cinnamaldehyde could be used as a natural fungistat.

2. Clove Oil

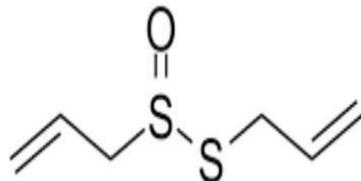
Eugenol is the main bioactive compound of clove. It is an effective antifungal compound against phytopathogenic *Aspergillus*, *Penicillium*, *Emericella*, and *Fusarium* species.



3. Garlic

Allicin is one of the main active compounds derived from garlic has antifungal activity.

Allicin



4. Gingelly oil

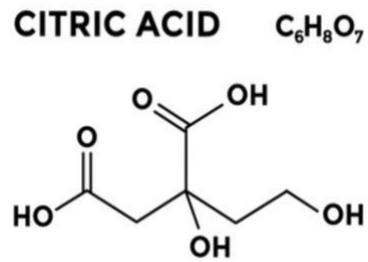
They reported that sesame oil can inhibit the growth of both yeast and mycelium of fungi.

5. Ginger

Antifungal effect of ginger is due to gingerone, dihydrogingerone and dehydroshogaol. There are also some other alkaloids, terpenes and terpenoid derivatives having antimicrobial activities on different bacterial pathogens.

6. Lemon

Citric acid were able to inhibit the mycelial growth of the pathogen



7. Raisin

It contains phenolic and poly phenolic components which help to inhibit fungal growth.

8. Salt

It is one of the commonly used agent against antimicrobial activity.

9. Turmeric

Rich in different phytochemicals. *Curcuma longa* shows the antifungal activity against *Aspergillus* sp. and *Fusarium* sp.

REVIEW OF LITERATURE

Bread is one of the staple food used in day to day life. Bread and other bakery products are subjected to various spoilage problems, viz., physical, chemical and microbial; the latter is the most serious one particularly bacterial (*Bacillus* sp.) and mould growth. Various moulds involved in spoilage of bread include *Rhizopus*, *Mucor*, *Penicillium*, *Eurotium*, *Aspergillus* and *Monilia* (Saranraj and Geetha, 2012).

From a microbiology point of view the most important factor common to different breads is a high moisture content (Legan, 1993). This characteristics make bread highly susceptible to microbial attack. The common sliced and wrapped bread is more likely to be spoiled by mould than unwrapped bread. In this type of bread, slicing provides more surfaces for mould to grow on and wrapping prevents moisture loss (Mariona Arroyo, 2002)

Evandro *et al.* (2005) in his paper discussed the inhibitory activity of spices against bacteria, fungi, etc. Antifungal activity of spices and derivatives has been studied in this paper by means of viable cells count, mycelial growth and mycotoxins synthesis. Paper concludes by the necessary awareness we should take before applying spices or their derivatives. Before including spices or their derivatives in food conservation systems, some evaluations about microbiological quality, economic feasibility, antimicrobial effect for a long time and toxicity should be carried out. Paper also evaluates the reports regarding essential oils containing carvacrol, eugenol and thymol (phenolic compounds) had highest antibacterial performances.

Suhr and Nielsen (2003) studied screening of essential oils as potential antimicrobials. Larger phenolic compounds such as thymol and eugenol (thymine, cinnamon and clove) had best effect when applied directly to the medium whereas smaller compounds such as allyl isothiocyanate and citral (mustard and lemon grass) were most efficient when added as volatile.

Naresh *et al.* (2003) studied that when an essential oil is directly incorporated into bakery products, less efficacy is observed. This may be due to the fact that specific components of the food products such as protein or fat can bind essential oil components, inactivating them (Mc Neil and Schminct, 1993).

Hitokoto *et al.* (1980) discussed inhibitory effect of 29 commercial powdered spices on inhibition of 3 species of *Aspergillus*. Eugenol from clove and thymol from thyme caused inhibition of 2 *Aspergillus* species. The inhibitory effects are interesting in connection with the prevention of mycotoxin contamination in many foods, and these compounds represent possible alternatives to the food additives in use at present.

Bhawana *et al.* (2014) published the antifungal activity of five common Indian spices namely clove, ajwain, turmeric, dalchini and black pepper against two bacteria *Aspergillus niger* and *Trichoderma* sp. The results revealed that the methanol extracts of spices have high antimicrobial activities on all test organisms. Results concluded that these spices contain high amount of secondary metabolites due to which they have high antimicrobial activity and it can be used as good bio-preservator and it can also be used for medicinal purpose.

Jonathan *et al.* (2015) reviewed the various effects of food additives and preservatives on man. Food additives plays a vital role in the food industries, but the various adverse effects associated with them remain a problem that need to be fought by us. This report also discussed about the role of Natural substances such as salt, sugar, vinegar, alcohol, and diatomaceous earth in food preservation.

Meining (2018) discussed about usage of essential oils and plant extracts as natural preservatives in food industries instead of chemicals. Essential oils are aromatic and volatile liquid extracted from plants including cinnamon, thyme, oregano, clove, rosemary, garlic, sage, basil, marjoram, savory, pepper, and cardamon, etc. Magan *et al.* (2012) and aimed at developing an essential oil-based active packaging to prolong the shelf life of bread products.

Ritika *et al.* (2016) studied the effects of spice extracts on the quality characteristics and storage life of bread. The objective of the study was to prepare antimicrobial bread using different natural antimicrobials (aqueous extracts of clove, ginger and cinnamon in different concentrations) to obtain an acceptable product. Maximum hardness was observed in bread with ginger extract (2%) whereas minimum found in cinnamon extract (4%). Bread containing 2-4% clove extract, ginger extract and cinnamon extract had a mould free shelf life for more than 6 days whereas control bread spoiled within 4 days.

Patil and Kukade (2020) studied fungal spoilage of bakery products and its control measures. Six different types of bakery products i.e. pizza base, bread, toast, cupcake, bun, cookies were used in the study. Clove, Lemongrass, Cardamom, Citrus and Edible oil are usually used in food product. These product also acts as controlling measures for these fungi. Among these Cardamom powder showed the poor effect on the growth of inhibition of fungi and Clove powder showed the excellent effect on fungi.

Naili *et al.* (2019) published bakery science of bread by discussing the effect of salt reduction in quality of bread. It also discussed about the usage of salt for preservation. It is a big challenge to the bread industry when salt is reduced in the bread products. This is because, salt is cheap, and any replacement will incur a high cost with new testing and new technology. Salt has been used to preserve foods for a long time. In bread, salt also acts as a preservative. The use of higher concentration of salt produces higher osmotic pressure which causes bread cells to lose water to the environment, and thus retarding the microbial growth. Based on the facts, derived from these study, it was concluded that salt play many important roles in improving bread quality and safety.

Melogen and Roberta (2018) published inhibitory effect of garlic against fungal growth. In this study the garlic possesses an inhibitory potential against the test organism (*Penicillium* sp). Allicin is the component of garlic. Allicin is considered as the most important biologically active compound in garlic since it decomposes to other sulfur containing molecules, thiosulfonates and disulfides.

Abouzeed *et al.* (2018) published identification of phenolic compounds, antibacterial and antioxidant activities of raisin extracts. Raisins (dried grapes; *Vitis vinifera*), are very popular plant in Mediterranean area and are widely used as a traditional and natural biomedicine in several countries where it is well adapted to the climate. The extract from grape's flesh did not have antimicrobial effect (Yigit *et al.*, 2009).

MATERIALS AND METHODS

Materials required: Slice of bread (10), plastic bags, 9 common bio- preservatives.

The 9 common biopreservatives used were:

1. Cinnamon
2. Clove oil

3. Garlic
4. Gingelly oil
5. Ginger
6. Lemon
7. Raisin
8. Salt
9. Turmeric

Methodology:

Nine different biopreservatives were prepared as follows:

- Cinnamon powder - About 21grams (1½ tablespoon) is mixed in about 3ml of water.
- Clove oil - About 3ml is taken.
- Garlic - About 75 grams taken, mashed into a paste using mortar and pestle and mixed in 3ml of water.
- Gingelly oil - About 3ml is taken.
- Ginger - About 100 grams taken and mashed into paste using mortar and pestle and mixed in 3 ml of water.
- Lemon - 3 ml of squeezed lemons is taken.
- Raisin - About 100 grams taken and extracted with 1ml of water.
- Salt - About 15 grams dissolved in 3ml of water.
- Turmeric - About 8 grams grinded and mixed with 3ml of water.

On ten slices of bread, nine different biopreservatives were added and 1 slice was kept as control (without adding any biopreservative).

The bread slices were put in separate plastic bags and sealed and kept undisturbed till mould appears on bread slices. The growth of mould was tracked by checking the bread slices everyday.

OBSERVATIONS

The growth of mould on bread slices were noticed and observation collected during December, 2021 – February, 2022 as shown in figures 1 to 10.



Figure 1 showing Control (without biopreservative)



Figure 2 showing bread slice with cinnamon

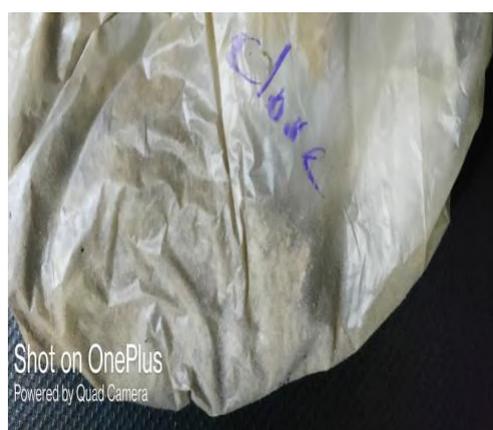


Figure 3 showing bread slice with clove oil



Figure 4 showing bread slice with Garlic



Figure 5 showing bread slice with gingelly oil



Figure 6 showing bread slice with ginger



Figure 7 showing bread slice with lemon



Figure 8 showing bread slice with raisin



Figure 9 showing bread slice with salt

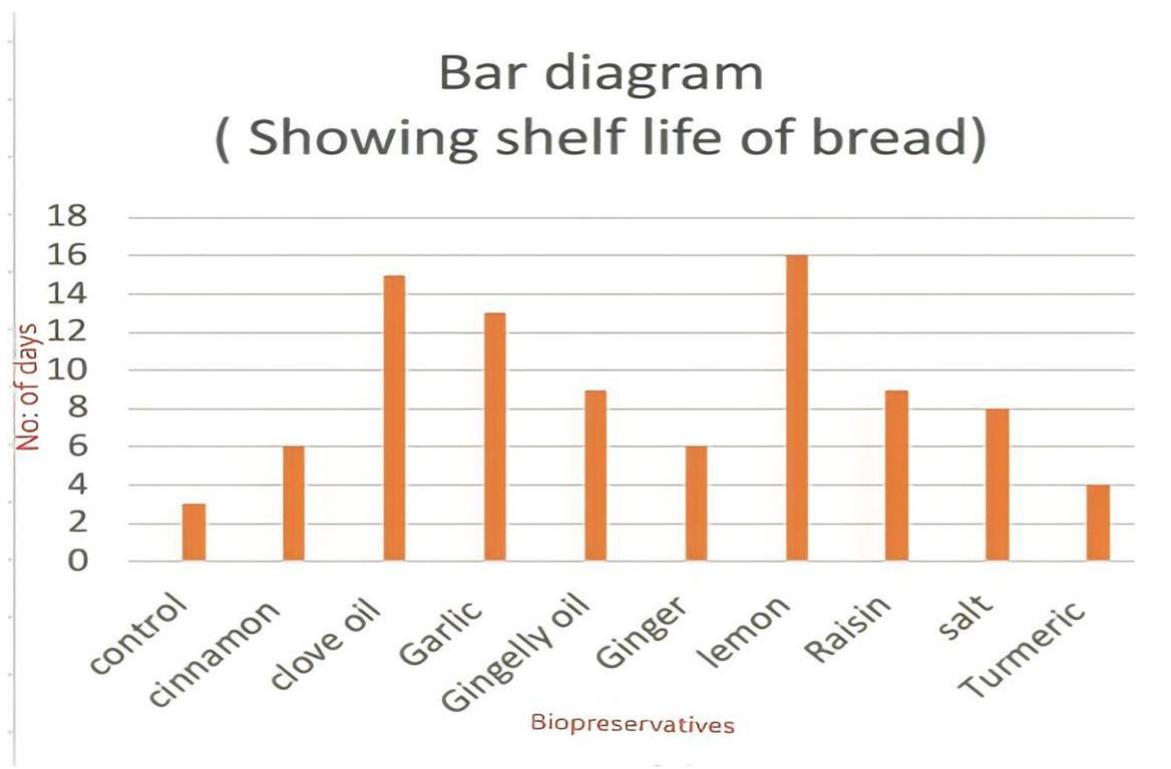


Figure 10 showing bread slice with turmeric

Table 1 showing the effect of biopreservatives.

SI. No:	Samples	Duration	No. of days / shelf life
1	Control (without bio preservative)	DEC 27 – DEC 29	Mould appeared on 3rd day
2	Cinnamon	JAN 29 – FEB 3	Mould appeared on 6th day
3	Clove oil	JAN 26-FEB 9	Mould appeared on 15th day
4	Garlic	JAN 26 - FEB 7	Mould appeared on 13th day
5	Gingelly oil	JAN 26 - FEB 3	Mould appeared on 9th day
6	Ginger	JAN 29- FEB 3	Mould appeared on 6th day
7	Lemon	Dec 26 - JAN 11	Mould appeared on 16th day
8	Raisin	JAN 24 - FEB 1	Mould appeared on 9th day
9	Salt	DEC 26 - JAN 2	Mould appeared on 8th day

10	Turmeric	JAN 29 –FEB 1	Mould appeared on 4th day



RESULT

From the observations it was noticed that Lemon is the most effective biopreservative followed by Clove oil. Lemon helps to increase shelf life of bread

about 16 days and clove oil about 15 days. Turmeric was found to be the least effective one. It has shelf life of about 4 days which is followed by ginger and cinnamon with shelf life of about 6 days.

DISCUSSION

Mould spoilage of bread is a serious issue facing nowadays. Contamination of food products like bread and its products may leads to serious food poisoning. Various substances can be added to food items to increase shelf life, texture, taste, etc.

Food additives are substances that food manufacturerers intentionally add to increase the shelf life, flavour, apperance of the food. Preservatives are substances which are capable of inhibiting the growth of microorganisms. When the food is to be stored for a prolonged period, use of additives and preservatives is essential to maintain its quality, wholesomeness, taste, appearance, flavour, freshness etc. Additives and preservatives prevent the spoilage of food due to the growth of bacteria and fungi. Synthetic food additives react with cellular components of body leading to various defects. So it should be natural one with minimal effect and those that are considered as safe and in case of those not generally recognized as safe, the acceptable daily intake should not be exceeded (Inetianbor *et al.*, 2015)

The results from our studies revealed that all the breads using extracts of ginger, clove, garlic, raisin etc. showed some activity against food associated pathogens. This results can be supported with the study by Ritika *et al.* (2016). This study concludes that bread of acceptable quality can be prepared with the use of natural antimicrobials as it increases the shelf life of bread and enhances the flavour also. Bread containing cinnamon extract, ginger extract and clove extract in concentration up to 2-4% had a mould free shelf life for 6 days. Spices are one of the most commonly used natural antimicrobial agents in foods and have been used traditionally for thousands of years by many cultures for preserving foods and as food additives to enhance aroma and flavour.

Inhibitory action of natural products on mould involves cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of intercellular and extracellular enzyme as said by Souza *et al.* (2005). In our studies second most effective antifungal sample is clove oil. Its antifungal activity is reported in earlier studies. It was supported in other studies also (Lattaoui and Tantaoui-Elaraki, 1994; Kim *et al.* (1995). They made a statement that essential oils containing carvacrol, eugenol and thymol (phenolic compounds) had highest antibacterial performances.

This clearly emphasizes the antifungal effect of clove oil and many other essential oils that are commonly available.

Spices have antimicrobial property besides contribute piquancy of food and beverages. They have been recognized for their value of preserving foods & medicinal values due to the presence of bioactive antimicrobial compounds. The antimicrobial effect of spice extract helps to prevent diseases and cater the healing effect. The result concluded that due to the high amount of secondary metabolites in the spices, they have antimicrobial activity and it can be used as a good bio-preservater (Bhawana *et al.*, 2014).

In bread, salt also acts as a preservative besides contribution to flavor. High salt level increases the time for microbial growth of some pathogens such as *Escherichia coli* and *Salmonella*. Microbial shelf stability is also affected by the existence of salts in the food products . It is a big challenge to the bread industry when salt is reduced in the bread products (Naili *et al.*, 2019). This is because, salt is cheap, and any replacement will incur a high cost with new testing and new technology. Salt also can play a role to some extent in case of antimicrobial activity.

Outcome from our study emphasizes that lemon is considered as most effective sample against fungal growth and helps mould prevention (Patil *et al.*, 2020). The citrus extract consists of many chemical compound such as geranial, Nerol, Gernyl acetate, geraniol, beta- caryophellene and Neryl acetate. Its major component is lemonene. Citrus extract has reported strong antifungal activity against many strains of fungi. It strongly inhibits spore production and germination of fungal pathogen (Patil *et al.*, 2020). Lemon is more effective biopreservative among nine samples used, because it contains citric acid as main component. Acidic pH below 4.5, inhibits fungal growth.

We can incorporate various biopreservatives to inhibit fungal growth. Before including spices and/or their derivatives in food conservation systems, some evaluations about microbiological quality, economic feasibility, antimicrobial effect for a long time and toxicity should be carried out (Souza *et al.*, 2005). The previous

studies regarding inhibition of fungal spoilage by means of spices, organic derivatives, essential oils, etc. support our results. By providing proper plannings and treatment we can eradicate fungal spoilage by means of biopreservatives and can increase shelf life to maximum.

CONCLUSION

Food spoilage with fungus and moulds is still a major problem limiting the shelf life of many high and intermediate moisture bakery products.

Normal piece of bread without applying any of the biopreservative was kept as control. Mould appeared in it within 3 days after it was kept in plastic bag.

Among the common 9 bio-preservatives studied, lemon was proved to be more effective. The fungicidal activity of lemon was due to the citric acid present in it. Slices of bread applied with lemon has more shelf life of 16 days.

Clove oil is second most effective sample of bio preservative used in experiment. Eugenol is the main bioactive compound of clove causing antifungal effect. Slice of bread with clove has shelf life of about 15 days.

Garlic was also found effective due to presence of allicin. Allicin makes it more effective against fungal activity. It increases shelf life about 13 days.

Bread slices with the samples Gingelly oil, raisin, salt, cinnamon, ginger, turmeric has shelf life of about 9, 9, 8, 6, 6 and 4 days respectively.

The study revealed the predominant efficacy of preservatives to inhibit fungal growth in bakery foods with the presence of bioactive components. Losses due to mould spoilage have been resulting in revenue loss to the baking industries. Therefore, methods to control mould growth and to extend the shelf life of bakery products is of great economic importance to the baking industry where an increased demand in global consumption exists. Other measures as good hygiene in the bakeries and if necessary complementary post packaging heat treatments or modified atmosphere packaging is the *best* alternatives.

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EFFECT OF TEMPERATURE AND pH ON THE OPERCULAR RESPIRATORY RATE (ORR) OF GOLDFISH



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Affiliated by Mahatma Gandhi University, Kottayam

in partial fulfilment of requirement for the

Degree of Bachelor of Science in Zoology

2021-2022

**EFFECT OF TEMPERATURE AND pH ON
THE OPERCULAR RESPIRATORY RATE
(ORR) OF GOLDFISH**

CERTIFICATE

This is to certify that the project entitled **EFFECT OF TEMPERATURE AND pH ON THE OPERCULAR RESPIRATORY RATE OF GOLDFISH** submitted by Ms. Anishma T M, Reg. No.AB19ZOO030 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Helvin Vincent and this is her original effort.

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Examiners

1)

2)

DECLARATION

I, Ms. Anishma T M, hereby declare that this project report entitled **EFFECT OF TEMPERATURE AND WATER ACIDTY ON THE OPERCULAR RESPIRATORY RATE OF GOLDFISH** is a bonafide record work done by me during the academic year 2021-2022 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam.

This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

ANISHMA T M

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ABSTRACT

In the past century, the world has seen an increase in the amount of carbon dioxide (CO₂) in the atmosphere. The rise in CO₂ can put stress on aquatic ecosystems due to ocean acidification and overall decrease in the pH of the waters (Adam Parker, 2015). So this project helps to determine the effect of temperature and water acidity on the opercular respiratory rate (ORR) of goldfish. For this experiment two goldfish were taken. To determine the effect of temperature five different temperatures were taken, these are as follows 25°C, 20°C, 10°C, 30°C and 35°C. At 25°C the fish 1 and fish 2 had respiratory rate slightly greater than the normal respiratory rate. At 20°C both the fishes had normal respiratory rate ie, range from 78 to 82. At 10°C the fish 1 and fish 2 had respiratory rate less than the normal respiratory rate. At 30°C the respiration rate of both was greater than the normal respiratory rate. At 35°C the respiratory rate of fish 1 and fish 2 was much greater than the normal respiratory rate. From this it was found that at higher temperatures the fishes showed high respiratory rate and at low temperature fishes showed lower respiratory rate. Thus temperature is a factor responsible in the opercular respiratory rate of aquatic fishes. The ideal temperature for the fish would be around 20°C -25°C. The second factor responsible for the ORR was acidity of water or simply pH. To determine the effect of acidity, vinegar was used to increase the acidity and four different pH were taken to find the effect of acidity and these were pH 5, pH 6, pH 7 and pH 8. At pH 5 the respiratory was very much higher than the normal respiratory rate and the fishes showed signs of stress and the breathing was rapid. At pH 6 the respiratory was around normal and also showed signs of large mouth for gasping for air. At pH 7 the respiratory rate was normal and showed no signs of defects which showed that pH 7 is the ideal pH for fish keeping. At pH 8 the respiratory rate was around to the normal respiratory rate. This concludes that the ideal pH for fishes is from pH 7- pH 8. From the experiment it was found that certain factors like the temperature and pH or water acidity has a great impact on the survival of aquatic species.

INTRODUCTION

Global warming is the process in which the earth's temperature on the atmosphere layers that are close to earth rise artificially as a result of the intense increase in some gases that occur in consequences of various human activities and that are qualified as greenhouse gases in the atmosphere. Oceans and seas are mostly affected by the process of change caused by global warming. A temperature increase of only a few degrees does not cause an increase in the temperature of large water masses such as oceans, seas, lakes and ponds but it also causes hydrological events that cause a change in the physical and chemical characteristics of water. Water temperature is the most important environmental parameter that affects the life cycle, physiology and behaviours of aquatic living beings (Tekiney, 2007). Due to global warming it can adversely affect the respiration rate of aquatic organisms, particularly the fishes. Global warming tends to increase the temperature of the water bodies and the waters of the water bodies starts to heat up. Resulting in the decrease of dissolved oxygen. Therefore fishes have difficulties in breathing in such an environment. Additionally such warming low-oxygen waters tends to increase fish's oxygen demand because their metabolism speeds up. As fish grows the demand for oxygen also increases, therefore the fish move to waters whose temperatures resembles those of their original habitats and satisfy their oxygen needs. But due to global warming the fishes find difficulty living in such waters of less oxygen and eventually dies.

Water acidity or pH is another factor responsible for the survival of aquatic organisms. By releasing carbon dioxide to the atmosphere, humans are rapidly altering the chemistry of the water bodies and affecting the aquatic life. Unpolluted deposition (or rain), in balance with atmospheric carbon dioxide has a pH of 5-6. Almost everywhere in the world the pH of rain is lower than this. The main pollutants responsible for acid deposition (or acid rain) are sulfur dioxide (SO_x) and nitrogen oxides (NO_x). Acid deposition influences mainly the pH of freshwater.

Nitrogen and sulfuric emissions come from natural and anthropogenic sources. Natural emissions include Volcano emissions, lightning and microbial processes. Power stations and industrial plants like the mining and smelting of high sulfur and nitrogen oxides and other acidic compounds. These compounds mix with the water vapour at unusual proportions to cause acid deposition with a pH of 4.2 to 4.7. That is 10 or more times the acidity of natural deposition.

The acidification of freshwater in an area is dependent on the quantity of calcium carbonate (limestone) in the soil. Limestone can buffer (neutralize) the acidification of the freshwater. The effects of acid deposition are much greater on lakes with little buffering capacity. Much of a damage to aquatic life in sensitive areas with this little buffering capacity is a results of acid shock. This is caused by the sudden runoff of large amounts of highly acidic water and aluminium ions into lakes and streams, when snow melts in the spring or after unusually heavy rains. Most freshwater lakes, streams and ponds have a natural pH in the range of 6 to 8.

Acid deposition has many harmful ecological effects when the pH of most aquatic systems falls below 5 and increase above pH 9. Below pH 5 fish population begin to disappear, the bottom is covered with un-decayed material and mosses may dominate near shore areas. It can kill many fishes by stimulating excessive mucus formation. This asphyxiates the fishes by clogging their gills. It can also cause chronic stress that may not kill individual fish, but leads to lower body weight and smaller size and makes fish less able to compete for food and habitat. The most serious chronic effect of increased acidity in surface waters appears to be interference with the fish reproductive cycle. Calcium levels in the female fish may be lowered to the point where she cannot produce eggs or the eggs fail to pass from the ovaries or if fertilized the eggs and the larvae develop abnormally.

Extreme pH can kill adult fish and invertebrate life directly and can also damage developing juvenile fish. It will strip a fish of its slime coat and high pH level chaps the skin of fish because of its alkalinity. When the pH of freshwater becomes highly alkaline, the effects on fish may include death, damage to outer surfaces like gills, eyes and skin and an inability to dispose of metabolic wastes. High pH may also increase the toxicity of other substances. For example the toxicity of ammonia is ten times more severe at a pH of 8 than it is at pH 7. It is directly toxic to aquatic life when it appears in alkaline conditions.

This topic was selected for study because of the rising issues of water bodies due to global warming and acidification.

GOLDFISH

Goldfish (*Carassius auratus*) are members of the cyprinid family (carp and their relatives). They originated in south-east Asia, although the cyprinid family covers a far wider global range. Because of accidental and deliberate introduction into the wild (Angeler *et al*, 2002). Goldfish are now found worldwide with a few exceptions such as Greenland and Antarctica. *C.auratus* are classified as least concern (IUCN2015). Goldfish are usually considered a temperate water fish; however they may survive in temperatures below 10°C and up to 30°C. Collectively their broad environmental tolerances mean that they can be found inhabiting a very wide range of habitats. Their natural diet is very varied, with wild goldfish eating anything from terrestrial insects to vegetation to detritus (Richardson *et al*, 1995; Pinto *et al*, 2005).

Goldfish show a variety of social behaviours and are often found in the company of other goldfish (Pitcher and Magurran, 1983). Breeding typically occurs in spring and males chase and court gravid females. Mate attraction and species recognition involves pheromones (Sisler and Sorensen, 2008). Females lay eggs in aquatic conditions and the eggs are adhesive and hatch in 2 to 3 days. Under optimal conditions, fry grow rapidly and can be sexually mature within a year. As shallow water fish, goldfish vision is most sensitive to red, green, blue and ultra violet wavelengths (Neumeyer, 1992).

Goldfish can differentiate certain shapes, colours and sounds (Wyzisk and Neumeyer, 2007). They can also sense vibrations and their hearing capabilities are sensitive across a broad range of frequencies (Fay and Popper, 1974).

Goldfish can also detect different odours in the water which they can use to find food, avoid predators or preferentially associate with one another. All goldfishes need adequate space for shoaling keeping adequate distances between individuals maintaining adequate water quality and allowing all goldfishes to reach their full size potential. Although goldfish may tolerate some variations in water parameters, poor water quality can be fatal and some fancy varieties are at particular risk.

AIM

This project work aim to determine the opercular respiratory rate of goldfish by the effect of temperature and water acidity.

OBJECTIVE

Global warming and acidification are the major problems faced by all living organisms on this planet. Due to this, temperature as well as acidity of water is increasing day by day which is affecting the water bodies at a higher rate. Objective of this project is to determine the effects of temperature and water acidity on the opercular respiratory rate of goldfish. This project gives the optimal temperature and water acidity where the fish can survive.

REVIEW OF LITERATURE

Global warming and acidification are the major problem of this century, which alters the natural environment conditions including quality of water, abrupt increase/decrease in temperature and increase level of pollutions. These changes in environmental conditions directly affect the natural biodiversity (Sudesh Rani, 2016).

Fishes are aquatic, craniate, gill bearing that lack limbs with digits. Most fishes are ectothermic (cold blooded) allowing their body temperatures to vary as ambient temperatures change. Factors affecting fish production in freshwater aquatic systems can be classified as physical and chemical factors. The physical properties that are important for the fish production and growth include temperature and important chemical parameters include pH, alkalinity, hardness etc.

Water temperature is one of the most important physical factors affecting the respiration of fishes, also chemical factors such as water acidity (pH) also has a role in the respiratory rate of fish. The respiration of fish is through certain organs called the gills, which has a covering called the operculum. Operculum is a bony plate and serves as a water pump. Each time the fish respire the operculum moves, operculum movement allows one to evaluate the opercular respiratory rate (ORR).

Due to global warming, increase in atmospheric temperature is the matter of great discussing issues among the scientists. Temperature as an important abiotic factor showed influence on the physiochemical parameters of all living organism on earth.

The increase and decrease in the environmental temperature and water acidity can directly and indirectly affect the living organisms. These variations become more prominent for aquatic animals. Change in the pH and temperature of water show adverse effects on the fishes and other aquatic animals (Sudesh Rani, 2016). As the adverse conditions of water increases it produce high stress on fishes (Capkin *et al*, 2006; Singh and Mishra, 2009; Sudesh Rani, 2016).

Temperature variations in water bodies depend largely on their geographical location (Latitude, Longitude and Altitude). In the tropics, marked variations in temperature and rainfall between the rainy and dry season affect the physico- chemical characteristics of the water. Cold water has more dissolved oxygen than warm water thus as temperature increases less oxygen is available to the biota. Therefore, temperature has a pronounced effect on rate of chemical and biological processes in aquatic habitats. Temperature is one of the most fundamental environmental stressors, altering almost all biological processes through its actions on basic chemical reactions supporting physiological processes (Murugain *et al*, 2008)

Most fish exchange gases using gills on either side of the pharynx. Gills are tissues which consist of threadlike structures called filaments. These filaments have many functions and are involved in ion and water transfer as well as oxygen, carbon dioxide, acid and ammonia exchange. (Randall, 1984; Adesola *et al*, 2020). Fish exchange gases by pulling oxygen-rich water through their mouths and pumping it over their gills. The gills push the oxygen-poor water out through openings in the sides of the pharynx.

Most species employ a counter-current exchange system to enhance the diffusion of substances in and out of the gill, with blood and water flowing in opposite directions to each other (Andrews *et al*, 2010). The temperature of the aquatic medium in which the fish is cultured determines the respiratory rate of the fish and consequently, its survival, productivity, distribution and normal biological activities (Anita and Pooja, 2013). Inability of fishes to adapt to temperature fluctuations is responsible for the inability of fishes to respond physiologically to the environment and hence result to death (Ayanwale *et al*, 2014). As the degree of water temperature increases it produce highly stress conditions on fishes, the degree of toxicity produced is dependent upon environmental conditions such as temperature, pH of water, oxygen content and presence of residue molecules (Tantanpale *et al*, 2009).

The rise in CO₂ can put stress on aquatic ecosystems due to ocean acidification, an overall decrease in the pH of the ocean's waters. Freshwater ecosystems, already stressed by pollution and recent increases in the number of invasive species are also showing signs of acidification due to the increase in CO₂. The effect of the rise in acidity is known to be harmful to calcifying organisms.

As more and more fossil fuel burning cars and factories are being built around the world, the amount of CO₂ being released into the atmosphere is increasing. The increasing amount of CO₂ not only causes a problem for people and animals but also aquatic ecosystems around the world (Adam Parker, 2015).

Carbon dioxide rapidly diffuses into the water causing the pH to become more acidic (Arnold *et al*, 2012). In the marine environment, the CO₂ reacts with seawater, forming carbonic acid and can ultimately result in ocean acidification. Increase in atmospheric CO₂ concentrations have led to an increased rate of CO₂ diffusion into the oceans. Ultimately, this would lead to the oceans becoming more and more acidic. If the pH of oceans rises at a rate quicker than the vegetation can adapt, submerged aquatic vegetation species could be endangered.

While marine ecosystems seem to be the main concern, it is plausible that freshwater ecosystems will also experience increasing pH as CO₂ diffuses from the atmosphere into lakes and rivers (Strumm and Morgan, 1996).

METHODOLOGY

MATERIALS

- Fahrenheit thermometer - used for measuring the temperature (range = -0°F to 400°F, least count =2.0)
- Measuring cylinder-used for measuring the solutions (5mL)
- pH test paper- used for determining the pH of the water
- Test tube - in which sampled water was taken for testing the pH
- Regular fish bowl aquarium
- 2 Gold fish
- Vinegar
- Baking soda
- Cold water
- Hot water
- Normal tap water

METHOD

This project experiment was conducted at Parakattu house near Pulleppady bridge, Ernakulam, Kerala for 7 days from 6th February 2022 to 12th February 2022. The goldfishes (*Carassius auratus*) were purchased from tropical aquarium, South Ernakulam, Kerala. The water used for the experiment was regular tap water from Cochin co-orporation.

A regular fish bowl aquarium filled with about 1 litre of water was taken. The current temperature of the water was equal to that of the room temperature (20°C (68°F)). The opercular respiratory rate (ORR) was observed for 1 minute and the result was noted. The opercular respiratory rate of the fish was calculated by counting the number of times the gills of the fish was opened. Three trials were taken and the average of 3 trials was taken as the final result. The fish was removed from the bowl carefully.

Next the second fish was introduced into the bowl naming it the fish 2. The opercular respiratory rate of the fish at (20°C (68°F)) was noted down. The fish 2 was removed carefully from the bowl.

The temperature was then reduced to 10°C (50°F) by adding required amount of ice cold water and the experiment was repeated as above using fish 1 and fish 2.

The temperature was then set to 25°C (77°F) by adding hot water (5ml/min) over 5 mins into the bowl and the experiment was repeated with both fishes and the observation noted down. The experiment was carried out at 30°C (86°F) and 35°C (95°F) also in the same manner.

The effect of water acidity on the opercular respiratory rate of the fish was studied by introducing the fish to water at different pH. The pH was tested using a pH paper and was found to be neutral (around pH 7). The ORR of both fishes were noticed. The pH of the water was set to pH 5 by adding vinegar 1ml/min over 5mins. The fish 1 was introduced and the opercular respiratory rate was calculated. The fish 1 was then removed and fish 2 was introduced and the observation was noted.

The pH of water was then changed to pH 6 by adding water 5ml/min over 5mins and the experiment was carried out. The pH of water was again increased to pH 8 by adding a little bit of baking soda. The ORR of both fishes were calculated and formulated into tables for all 7 days. The graph was plotted using the average ORR value for 7 days.

OBSERVATION

Table 1 showing the effect of temperature on ORR of fish 1 for 7 days

SL NO:	TEMPERATURE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	20°C	76.3	77	76	75.6	77	75.6	74.3	75.9
2.	10°C	61	59.6	60.3	67.6	64	57.6	58	61.1
3.	25°C	79	83	77.3	82	83	82.3	78	80.6
4.	30°C	82.3	86	84.6	88	87.3	88	83.3	85.6
5.	35°C	101.3	106	97.6	106	99.3	103.3	100.6	102

From Table 1 it was noticed that the ORR of fish 1 was 75.9 at room temperature which reduced to 61.1 when temperature was reduced to 10°C. The ORR increased when the temperature was increased to 25°C, 30°C and 35°C.

Table 2 showing the effect of temperature on ORR of fish 2 for 7 days

SL NO:	TEMPERATURE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	20°C	76.6	76.6	78.3	76.6	78.6	77	77.6	77.3
2.	10°C	58.6	61.6	64.6	65	65.3	62.3	64	63
3.	25°C	80	80	80	81.6	80.6	81	78.6	80.2
4.	30°C	85.6	82.6	86.6	87	86.6	85	86	85.6
5.	35°C	101.6	98.3	101.3	103.6	100.3	100.3	101.6	101

From Table 2 it was noticed that the ORR of fish 2 was 77.3 at room temperature which reduced to 63 when temperature was reduced to 10°C. The ORR increased when the temperature was increased to 25°C, 30°C and 35°C.

Table 3 showing the effect of pH on ORR of fish 1 for 7 days

SL NO:	pH	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	5	112.6	99.3	104.3	115	103.6	115.3	112	108.8
2.	6	109.3	104.3	99.6	111	101	108.6	110.6	106.3
3.	7	74.6	79.3	75.6	79	80	79	77.6	77.8
4.	8	79	80	77.6	81.6	78.6	78.3	80	90.5

From table 3 it was observed that the ORR of fish 1 at pH 5 was 108.8, when pH was increased to 6, ORR was found to be 106.3, when the pH was 7 ORR was noted 77.8. when the pH was increased to 8 ORR was noted as 90.5.

Table 4 showing the effect of pH on ORR of fish 2 for 7 days

SL NO:	pH	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	5	102.6	108.3	105.6	104.3	108.3	102.3	101	104.6
2.	6	103.3	104.6	97.3	100.3	101	99	99.3	100.6
3.	7	79	80.3	79	76.3	80.3	77	81.3	79
4.	8	80.3	81.6	82.6	79	80	82.6	79	80.7

From Table 4 it was observed that the ORR of fish 2 was 104.6 at pH 5, then it decreased to 100.6 at pH 6. At pH 7 the ORR was found to be 79 and when this pH was increased to 8 the ORR changed to 80.7

RESULT

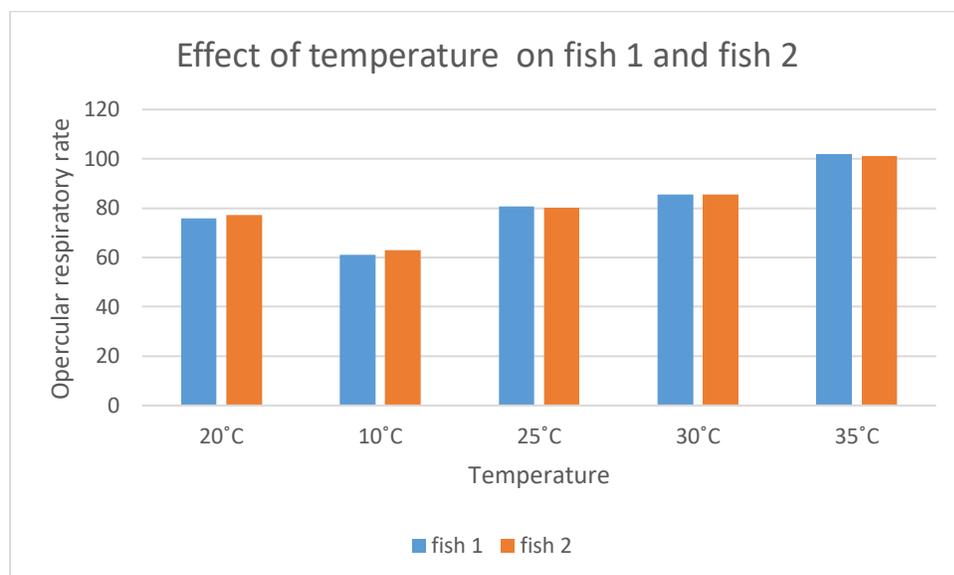


Figure 1 showing the effect of temperature on ORR of fish 1 & fish 2

Figure 1 shows when temperature is 20°C, both the fishes had a ORR between 75-78, when the temperature was reduced to 10°C the ORR was between 61-63. The temperature was increased to 25°C and th ORR was between 80.2-80.6. Again the temperature was increased to 30°C and 35°C and their ORR was found to be 85.6 and 101-102 respectively.

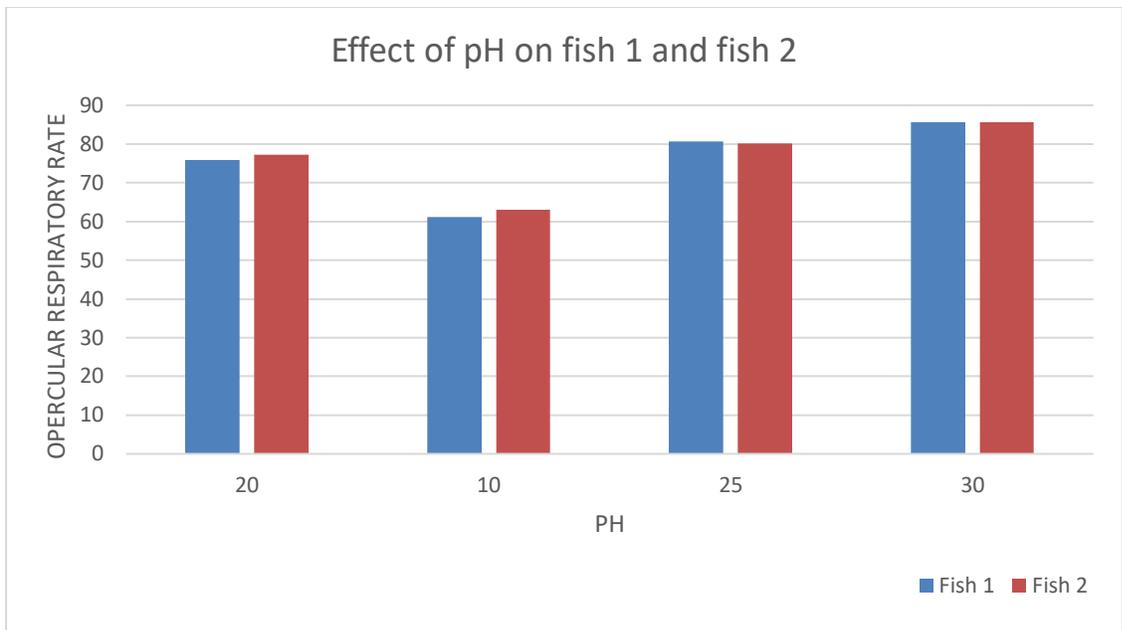


Figure 2 showing the effect of pH on ORR of fish 1 and fish 2

Figure 2 shows that at normal pH (pH 7) fish 1 and fish 2 had an opercular respiratory rate between 60-80. The respiratory rate was normal and showed no signs of defects which showed that pH 7 is the ideal pH for fish keeping.

When pH is at 5, fish 1 and fish 2 has an opercular respiratory rate between 100 - 120. At this pH the respiratory rate was very much higher than the normal respiratory rate (which was around 75-80) and the fishes showed signs of stress and the breathing was rapid.

When pH was at 6, fish 1 and fish 2 has the opercular respiratory rate between 80-120. At this pH the respiration of the fishes were not normal and also showed signs of stress like large mouth gasping for air.

When pH was at 8, fish 1 and fish 2 has opercular respiratory rate between 60-100 respectively. At pH 8 the respiratory rate was around to the normal respiratory rate. At pH 8 the water was alkaline therefore respiratory rate was also increased.

DISCUSSION

Global Warming and acidification has caused drastic effects on the water bodies across the world. Due to Global Warming, fish species has been extremely affected. As the water warms, fish need more oxygen to perform daily activities, like feeding. Change in temperatures, therefore, affects fish metabolic activities.

The surrounding water bodies absorbs most of the excess heat from greenhouse gas emissions, leading to a rise in water temperature. Increase in temperatures affect marine and freshwater species and ecosystems. Also leads to coral bleaching and the loss of breeding grounds for fishes.

Fishes don't have shells, therefore they will feel the effects of acidification. Because the surrounding water has a lower pH, a fish's cells often come into balance with the water by taking in carbonic acid. This changes the pH of the fish's blood, a condition called acidosis.

At lower pH levels, adult fishes start dying, fish eggs are unable to hatch, absence of fishes in highly acidic waters. Even if a particular species of fish is able to survive in that acidic environment, the animals feeding on these fishes won't be able to survive long, as accumulation of acid takes place in their body and leads to ultimate death.

In this present experiment, two goldfishes belonging to same species were taken and opercular respiratory rate was measured at different temperatures and pH. The experiment was done with regular tap water. About 1 liter of water was filled in a regular aquarium. The current temperature of water was 20°C (68°F). The ORR of both fishes were observed at this temperature. Similarly this procedure was repeated for 10°C, 25°C, 30°C and 35°C, and the observations and results were formulated into tables and graphs. To increase the temperature hot water was used and for decreasing the temperature ice cold water was used.

The effect of pH on ORR of fishes was the second parameter observed, both fishes were introduced to different pH. The pH were pH 5, pH 6, pH 7 and pH 8. Vinegar was used for making the water acidic and baking soda was used for making alkaline condition.

Several kinds of research were conducted by several scientists with the objective to determine how temperature as well as water acidity have an impact on the opercular respiratory rate (ORR) of fishes.

According to the research conducted by Ahmed *et al.* (2015) says that temperature is regarded as an environmental factor that can affect activity, behaviour, feeding, growth, survival, appetite and reproduction in all fishes. Temperature variation in water bodies depend largely on their geological location (latitude, longitude and altitude). In the tropics and sub tropics, marked variations in temperature and rainfall between the rainy and dry season affect the physico - chemical characteristics of the water.

Akin (2003) reported that Cold water has more dissolved oxygen than warm water thus as temperature increases less oxygen is available to the biota. Therefore it was found that at higher temperatures fish respiratory rate also increased as demanding for more oxygen.

In the research conducted by Kazumasa *et al* (2000) it says that the phenomenon called acid rain results from industrial activities where sulfuric and nitric acid are produced by the release of sulfuric oxides (SO_x) and nitrogen oxides (NO_x) into the atmosphere. Acid rain induces the acidification of inland waters which results in damage to aquatic ecosystems, including fish.

Fish have the ability to regulate their acid-base balance in order to maintain normal pH of their body fluids under acidic ambience. When fish are exposed to a low pH, chloride cells in the gill tissue take up bicarbonate (HCO_3^-) ion from the outside to neutralize the hydrogen (H^+) ion flowing in the body. At this time, the loss of sodium (Na^+) and chloride (Cl^-) ions from the body fluids occurs, and plasma osmotic pressure decreases. This process is considered to be one of the major reasons why freshwater fish die under acidic conditions.

Therefore all these research papers have similarity with this present work that increase or decrease in the water temperature and water acidity have a major effect on the opercular respiratory rate of fishes. Opercular respiratory rate of fish is directly proportional to the water temperature i.e., respiratory rate increases with the increase in the temperature and vice versa. This is because as temperature increases availability of oxygen decreases and leads the fish to demand for more oxygen. Water acidity is another factor responsible for opercular respiratory rate, at lower pH fish shows signs of difficulties and slowly leads to death. Therefore both temperature and pH are essential factors responsible for the opercular respiratory rate (ORR) of fishes.

CONCLUSION

The world has seen an increase in the amount of carbon dioxide (CO₂) in the atmosphere. The rise in CO₂ can put stress on aquatic ecosystems due to ocean acidification, an overall decrease in the pH of the ocean's waters. This is caused by global warming and acidification. Through this study we found the favourable temperature and optimal pH of the water. Temperature and water acidity are some major factor responsible in the opercular respiratory rate of aquatic fishes. From this study we can conclude the optimal temperature and pH where an aquatic animals can survive.

Temperature is an essential factor which is involved in the respiration of aquatic fishes. Nowadays temperature is getting increased day by day because of global warming. By this study it shows that at higher temperatures the fishes showed high respiratory rate and at low temperature fishes showed lower respiratory rate, this is because at lower temperature (cold water) the amount of dissolved oxygen is greater and at higher temperature the amount of dissolved oxygen is less, therefore the fish needed to take more amount of oxygen to survive. At 20°C of temperature it was found that the respiratory rate was around 75-80 range and we can conclude that this is probably the normal temperature in which the fish can survive without having any problems. At high temperature i.e., 35°C when temperature is increased respiratory rate also increased. Likewise at lower temperature i.e., 10°C and it showed lower respiratory rate. From this experiment it was found that optimal temperature where the fish can survive is 20°C.

Acidity is the next major factor that causing the variation in respiratory rate. Due to acidification the acidity of water is increased day by day. At pH 7 the respiratory rate of fish was normal and it showed no signs of stress, therefore this can be the optimal pH where the fish can survive. At pH-8 the respiratory rate ranges from 80-100 and 60-80 respectively. At a pH of 5 the fishes showed signs of difficulties and was having a wide mouth for gasping more air. Thus the optimal pH of the water that fishes can survive is pH 7 -pH 8. As CO₂ gets dissolved in the waters, the water become acidic and the oxygen content of the water decreases thus making difficulty for the survival of aquatic organisms.

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STUDY ON ROLE OF BIOPRESERVATIVES TO INHIBIT FUNGAL GROWTH IN BREAD



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in partial fulfilment of requirement for the

Degree of Bachelor of Science in Zoology

2021-2022

CERTIFICATE

This is to certify that the dissertation titled, “*Study on role of biopreservatives to inhibit fungal growth in bread* ” is an authentic record of work carried out by **ANJANA S.** under the supervision and guidance of **Dr. HELVIN VINCENT**, Assistant Professor, Department of Zoology, St. Teresa’s College (Autonomous), Ernakulam in partial fulfilment of the requirement for the Bachelor’s Degree of Science in Zoology.

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Ernakulam

Ernakulam

Examiners

1).....

2).....

PLACE: ERNAKULAM

DATE: 09/ 05/ 2022

DECLARATION

I, Ms. Anjana S, hereby declare that this project report entitled **STUDY ON ROLE OF BIOPRESERVATIVES TO INHIBIT FUNGAL GROWTH IN BREAD** is a bonafide record work done by me during the academic year 2021-2022 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam.

This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

ANJANA S.

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Place: Ernakulam

Date: 09/05/2022

ANJANA S..

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ABSTRACT

This study aims to identify and evaluate the role of biopreservatives to inhibit fungal growth in bread.

Nine common biopreservatives were applied to bread slices and kept in a plastic bag and left undisturbed till the appearance of mould. One slice of bread was maintained as control. The day at which mould occurred was noted. Antifungal activity of different biopreservatives was studied from the results obtained from the experiment. Among the applied nine biopreservatives lemon is more effective and clove oil is second most effective one. Garlic is also effective compared to other 6 samples used in the study. Citric acid in lemon, eugenol in clove and allicin in garlic is responsible for their antifungal activity. This study can be applied to replace artificial preservatives used in manufacturing of bread and other similar products to inhibit fungal activity and to increase shelf life.

INTRODUCTION

Food spoilage is a critical issue facing nowadays. Microbial spoilage of food happens by the means of bacteria, fungus, protozoa, etc. Fungi is one of the common group of food spoilers by microbial attack. They occur in form of mould, yeast etc.

Bread is one of the staple food used in day to day life. Bread and other bakery products are subjected to various spoilage problems, viz., physical, chemical and microbial; the latter is the most serious one particularly bacterial (*Bacillus* sp.) and mold growth. Various moulds involved in spoilage of bread include *Rhizopus*, *Mucor*, *Penicillium*, *Eurotium*, *Aspergillus* and *Monilia* (Saranraj and Geetha, 2012).

Food safety and extend shelf life of food products are essential to meet demands by consumers. An interesting alternative is the use of naturally derived antimicrobials called biopreservatives. A wide range of natural products can be used as antifungal agent.

Biopreservatives can be used to inhibit fungal growth in bread. Biopreservatives can replace artificial preservatives used in manufacturing of bread and other similar products to inhibit fungal activity and to increase shelf life. Biopreservatives or its derived forms can applicable in future to increase shelf life by inhibiting fungal growth.

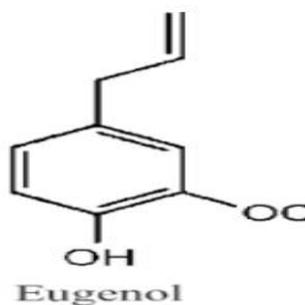
In order to increase the period of preservation of bread, it is necessary to add substances that slow down or inhibit fungal activity. The purpose of this project was to study the role of nine different commonly available biopreservatives to preserve bread and other similar products.

1.Cinnamon

The major components of cinnamon is cinnamaldehyde could be used as a natural fungistat.

2. Clove Oil

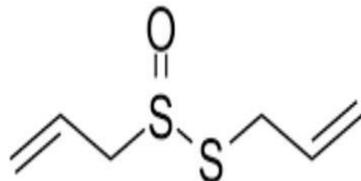
Eugenol is the main bioactive compound of clove. It is an effective antifungal compound against phytopathogenic *Aspergillus*, *Penicillium*, *Emericella*, and *Fusarium* species.



3. Garlic

Allicin is one of the main active compounds derived from garlic has antifungal activity.

Allicin



4. Gingelly oil

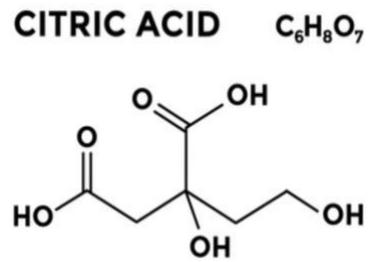
They reported that sesame oil can inhibit the growth of both yeast and mycelium of fungi.

5. Ginger

Antifungal effect of ginger is due to gingerone, dihydrogingerone and dehydroshogaol. There are also some other alkaloids, terpenes and terpenoid derivatives having antimicrobial activities on different bacterial pathogens.

6. Lemon

Citric acid were able to inhibit the mycelial growth of the pathogen



7. Raisin

It contains phenolic and poly phenolic components which help to inhibit fungal growth.

8. Salt

It is one of the commonly used agent against antimicrobial activity.

9. Turmeric

Rich in different phytochemicals. *Curcuma longa* shows the antifungal activity against *Aspergillus* sp. and *Fusarium* sp.

REVIEW OF LITERATURE

Bread is one of the staple food used in day to day life. Bread and other bakery products are subjected to various spoilage problems, viz., physical, chemical and microbial; the latter is the most serious one particularly bacterial (*Bacillus* sp.) and mould growth. Various moulds involved in spoilage of bread include *Rhizopus*, *Mucor*, *Penicillium*, *Eurotium*, *Aspergillus* and *Monilia* (Saranraj and Geetha, 2012).

From a microbiology point of view the most important factor common to different breads is a high moisture content (Legan, 1993). This characteristics make bread highly susceptible to microbial attack. The common sliced and wrapped bread is more likely to be spoiled by mould than unwrapped bread. In this type of bread, slicing provides more surfaces for mould to grow on and wrapping prevents moisture loss (Mariona Arroyo, 2002)

Evandro *et al.* (2005) in his paper discussed the inhibitory activity of spices against bacteria, fungi, etc. Antifungal activity of spices and derivatives has been studied in this paper by means of viable cells count, mycelial growth and mycotoxins synthesis. Paper concludes by the necessary awareness we should take before applying spices or their derivatives. Before including spices or their derivatives in food conservation systems, some evaluations about microbiological quality, economic feasibility, antimicrobial effect for a long time and toxicity should be carried out. Paper also evaluates the reports regarding essential oils containing carvacrol, eugenol and thymol (phenolic compounds) had highest antibacterial performances.

Suhr and Nielsen (2003) studied screening of essential oils as potential antimicrobials. Larger phenolic compounds such as thymol and eugenol (thymine, cinnamon and clove) had best effect when applied directly to the medium whereas smaller compounds such as allyl isothiocyanate and citral (mustard and lemon grass) were most efficient when added as volatile.

Naresh *et al.* (2003) studied that when an essential oil is directly incorporated into bakery products, less efficacy is observed. This may be due to the fact that specific components of the food products such as protein or fat can bind essential oil components, inactivating them (Mc Neil and Schminct, 1993).

Hitokoto *et al.* (1980) discussed inhibitory effect of 29 commercial powdered spices on inhibition of 3 species of *Aspergillus*. Eugenol from clove and thymol from thyme caused inhibition of 2 *Aspergillus* species. The inhibitory effects are interesting in connection with the prevention of mycotoxin contamination in many foods, and these compounds represent possible alternatives to the food additives in use at present.

Bhawana *et al.* (2014) published the antifungal activity of five common Indian spices namely clove, ajwain, turmeric, dalchini and black pepper against two bacteria *Aspergillus niger* and *Trichoderma* sp. The results revealed that the methanol extracts of spices have high antimicrobial activities on all test organisms. Results concluded that these spices contain high amount of secondary metabolites due to which they have high antimicrobial activity and it can be used as good bio-preservator and it can also be used for medicinal purpose.

Jonathan *et al.* (2015) reviewed the various effects of food additives and preservatives on man. Food additives plays a vital role in the food industries, but the various adverse effects associated with them remain a problem that need to be fought by us. This report also discussed about the role of Natural substances such as salt, sugar, vinegar, alcohol, and diatomaceous earth in food preservation.

Meining (2018) discussed about usage of essential oils and plant extracts as natural preservatives in food industries instead of chemicals. Essential oils are aromatic and volatile liquid extracted from plants including cinnamon, thyme, oregano, clove, rosemary, garlic, sage, basil, marjoram, savory, pepper, and cardamon, etc. Magan *et al.* (2012) and aimed at developing an essential oil-based active packaging to prolong the shelf life of bread products.

Ritika *et al.* (2016) studied the effects of spice extracts on the quality characteristics and storage life of bread. The objective of the study was to prepare antimicrobial bread using different natural antimicrobials (aqueous extracts of clove, ginger and cinnamon in different concentrations) to obtain an acceptable product. Maximum hardness was observed in bread with ginger extract (2%) whereas minimum found in cinnamon extract (4%). Bread containing 2-4% clove extract, ginger extract and cinnamon extract had a mould free shelf life for more than 6 days whereas control bread spoiled within 4 days.

Patil and Kukade (2020) studied fungal spoilage of bakery products and its control measures. Six different types of bakery products i.e. pizza base, bread, toast, cupcake, bun, cookies were used in the study. Clove, Lemongrass, Cardamom, Citrus and Edible oil are usually used in food product. These product also acts as controlling measures for these fungi. Among these Cardamom powder showed the poor effect on the growth of inhibition of fungi and Clove powder showed the excellent effect on fungi.

Naili *et al.* (2019) published bakery science of bread by discussing the effect of salt reduction in quality of bread. It also discussed about the usage of salt for preservation. It is a big challenge to the bread industry when salt is reduced in the bread products. This is because, salt is cheap, and any replacement will incur a high cost with new testing and new technology. Salt has been used to preserve foods for a long time. In bread, salt also acts as a preservative. The use of higher concentration of salt produces higher osmotic pressure which causes bread cells to lose water to the environment, and thus retarding the microbial growth. Based on the facts, derived from these study, it was concluded that salt play many important roles in improving bread quality and safety.

Melogen and Roberta (2018) published inhibitory effect of garlic against fungal growth. In this study the garlic possesses an inhibitory potential against the test organism (*Penicillium* sp). Allicin is the component of garlic. Allicin is considered as the most important biologically active compound in garlic since it decomposes to other sulfur containing molecules, thiosulfonates and disulfides.

Abouzeed *et al.* (2018) published identification of phenolic compounds, antibacterial and antioxidant activities of raisin extracts. Raisins (dried grapes; *Vitis vinifera*), are very popular plant in Mediterranean area and are widely used as a traditional and natural biomedicine in several countries where it is well adapted to the climate. The extract from grape's flesh did not have antimicrobial effect (Yigit *et al.*, 2009).

MATERIALS AND METHODS

Materials required: Slice of bread (10), plastic bags, 9 common bio- preservatives.

The 9 common biopreservatives used were:

1. Cinnamon
2. Clove oil

3. Garlic
4. Gingelly oil
5. Ginger
6. Lemon
7. Raisin
8. Salt
9. Turmeric

Methodology:

Nine different biopreservatives were prepared as follows:

- Cinnamon powder - About 21grams (1½ tablespoon) is mixed in about 3ml of water.
- Clove oil - About 3ml is taken.
- Garlic - About 75 grams taken, mashed into a paste using mortar and pestle and mixed in 3ml of water.
- Gingelly oil - About 3ml is taken.
- Ginger - About 100 grams taken and mashed into paste using mortar and pestle and mixed in 3 ml of water.
- Lemon - 3 ml of squeezed lemons is taken.
- Raisin - About 100 grams taken and extracted with 1ml of water.
- Salt - About 15 grams dissolved in 3ml of water.
- Turmeric - About 8 grams grinded and mixed with 3ml of water.

On ten slices of bread, nine different biopreservatives were added and 1 slice was kept as control (without adding any biopreservative).

The bread slices were put in separate plastic bags and sealed and kept undisturbed till mould appears on bread slices. The growth of mould was tracked by checking the bread slices everyday.

OBSERVATIONS

The growth of mould on bread slices were noticed and observation collected during December, 2021 – February, 2022 as shown in figures 1 to 10.



Figure 1 showing Control (without biopreservative)



Figure 2 showing bread slice with cinnamon

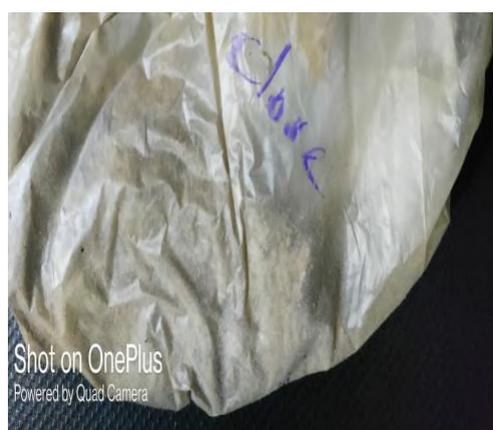


Figure 3 showing bread slice with clove oil

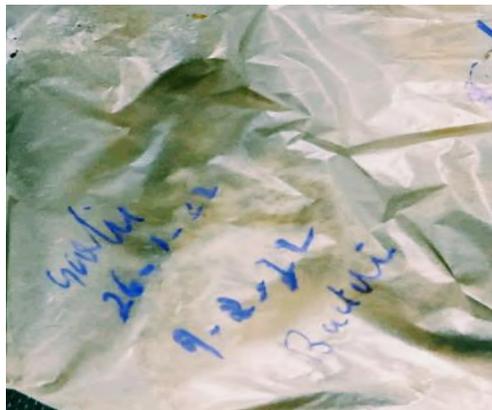


Figure 4 showing bread slice with Garlic



Figure 5 showing bread slice with gingelly oil



Figure 6 showing bread slice with ginger



Figure 7 showing bread slice with lemon



Figure 8 showing bread slice with raisin



Figure 9 showing bread slice with salt

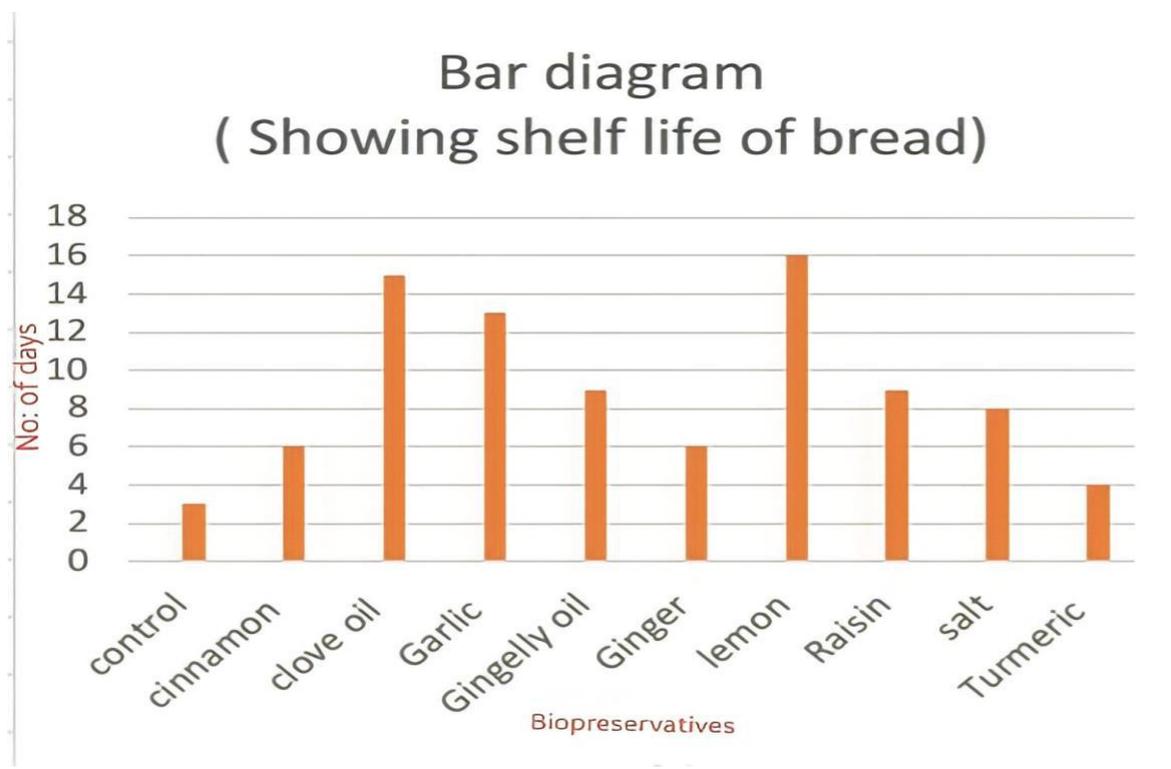


Figure 10 showing bread slice with turmeric

Table 1 showing the effect of biopreservatives.

SI. No:	Samples	Duration	No. of days / shelf life
1	Control (without bio preservative)	DEC 27 – DEC 29	Mould appeared on 3rd day
2	Cinnamon	JAN 29 – FEB 3	Mould appeared on 6th day
3	Clove oil	JAN 26-FEB 9	Mould appeared on 15th day
4	Garlic	JAN 26 - FEB 7	Mould appeared on 13th day
5	Gingelly oil	JAN 26 - FEB 3	Mould appeared on 9th day
6	Ginger	JAN 29- FEB 3	Mould appeared on 6th day
7	Lemon	Dec 26 - JAN 11	Mould appeared on 16th day
8	Raisin	JAN 24 - FEB 1	Mould appeared on 9th day
9	Salt	DEC 26 - JAN 2	Mould appeared on 8th day

10	Turmeric	JAN 29 –FEB 1	Mould appeared on 4th day



RESULT

From the observations it was noticed that Lemon is the most effective biopreservative followed by Clove oil. Lemon helps to increase shelf life of bread

about 16 days and clove oil about 15 days. Turmeric was found to be the least effective one. It has shelf life of about 4 days which is followed by ginger and cinnamon with shelf life of about 6 days.

DISCUSSION

Mould spoilage of bread is a serious issue facing nowadays. Contamination of food products like bread and its products may leads to serious food poisoning. Various substances can be added to food items to increase shelf life, texture, taste, etc.

Food additives are substances that food manufacturerers intentionally add to increase the shelf life, flavour, apperance of the food. Preservatives are substances which are capable of inhibiting the growth of microorganisms. When the food is to be stored for a prolonged period, use of additives and preservatives is essential to maintain its quality, wholesomeness, taste, appearance, flavour, freshness etc. Additives and preservatives prevent the spoilage of food due to the growth of bacteria and fungi. Synthetic food additives react with cellular components of body leading to various defects. So it should be natural one with minimal effect and those that are considered as safe and in case of those not generally recognized as safe, the acceptable daily intake should not be exceeded (Inetianbor *et al.*, 2015)

The results from our studies revealed that all the breads using extracts of ginger, clove, garlic, raisin etc. showed some activity against food associated pathogens. This results can be supported with the study by Ritika *et al.* (2016). This study concludes that bread of acceptable quality can be prepared with the use of natural antimicrobials as it increases the shelf life of bread and enhances the flavour also. Bread containing cinnamon extract, ginger extract and clove extract in concentration up to 2-4% had a mould free shelf life for 6 days. Spices are one of the most commonly used natural antimicrobial agents in foods and have been used traditionally for thousands of years by many cultures for preserving foods and as food additives to enhance aroma and flavour.

Inhibitory action of natural products on mould involves cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of intercellular and extracellular enzyme as said by Souza *et al.* (2005). In our studies second most effective antifungal sample is clove oil. Its antifungal activity is reported in earlier studies. It was supported in other studies also (Lattaoui and Tantaoui-Elaraki, 1994; Kim *et al.* (1995). They made a statement that essential oils containing carvacrol, eugenol and thymol (phenolic compounds) had highest antibacterial performances.

This clearly emphasizes the antifungal effect of clove oil and many other essential oils that are commonly available.

Spices have antimicrobial property besides contribute piquancy of food and beverages. They have been recognized for their value of preserving foods & medicinal values due to the presence of bioactive antimicrobial compounds. The antimicrobial effect of spice extract helps to prevent diseases and cater the healing effect. The result concluded that due to the high amount of secondary metabolites in the spices, they have antimicrobial activity and it can be used as a good bio-preservater (Bhawana *et al.*, 2014).

In bread, salt also acts as a preservative besides contribution to flavor. High salt level increases the time for microbial growth of some pathogens such as *Escherichia coli* and *Salmonella*. Microbial shelf stability is also affected by the existence of salts in the food products . It is a big challenge to the bread industry when salt is reduced in the bread products (Naili *et al.*, 2019). This is because, salt is cheap, and any replacement will incur a high cost with new testing and new technology. Salt also can play a role to some extent in case of antimicrobial activity.

Outcome from our study emphasizes that lemon is considered as most effective sample against fungal growth and helps mould prevention (Patil *et al.*, 2020). The citrus extract consists of many chemical compound such as geranial, Nerol, Gernyl acetate, geraniol, beta- caryophellene and Neryl acetate. Its major component is lemonene. Citrus extract has reported strong antifungal activity against many strains of fungi. It strongly inhibits spore production and germination of fungal pathogen (Patil *et al.*, 2020). Lemon is more effective biopreservative among nine samples used, because it contains citric acid as main component. Acidic pH below 4.5, inhibits fungal growth.

We can incorporate various biopreservatives to inhibit fungal growth. Before including spices and/or their derivatives in food conservation systems, some evaluations about microbiological quality, economic feasibility, antimicrobial effect for a long time and toxicity should be carried out (Souza *et al.*, 2005). The previous

studies regarding inhibition of fungal spoilage by means of spices, organic derivatives, essential oils, etc. support our results. By providing proper plannings and treatment we can eradicate fungal spoilage by means of biopreservatives and can increase shelf life to maximum.

CONCLUSION

Food spoilage with fungus and moulds is still a major problem limiting the shelf life of many high and intermediate moisture bakery products.

Normal piece of bread without applying any of the biopreservative was kept as control. Mould appeared in it within 3 days after it was kept in plastic bag.

Among the common 9 bio-preservatives studied, lemon was proved to be more effective. The fungicidal activity of lemon was due to the citric acid present in it. Slices of bread applied with lemon has more shelf life of 16 days.

Clove oil is second most effective sample of bio preservative used in experiment. Eugenol is the main bioactive compound of clove causing antifungal effect. Slice of bread with clove has shelf life of about 15 days.

Garlic was also found effective due to presence of allicin. Allicin makes it more effective against fungal activity. It increases shelf life about 13 days.

Bread slices with the samples Gingelly oil, raisin, salt, cinnamon, ginger, turmeric has shelf life of about 9, 9, 8, 6, 6 and 4 days respectively.

The study revealed the predominant efficacy of preservatives to inhibit fungal growth in bakery foods with the presence of bioactive components. Losses due to mould spoilage have been resulting in revenue loss to the baking industries. Therefore, methods to control mould growth and to extend the shelf life of bakery products is of great economic importance to the baking industry where an increased demand in global consumption exists. Other measures as good hygiene in the bakeries and if necessary complementary post packaging heat treatments or modified atmosphere packaging is the *best* alternatives.

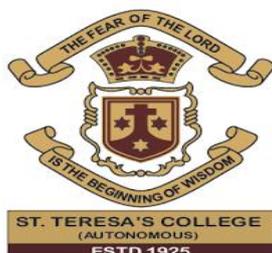
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STUDY ON ROLE OF BIOPRESERVATIVES TO INHIBIT FUNGAL GROWTH IN BREAD



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Affiliated by Mahatma Gandhi University, Kottayam

in partial fulfilment of requirement for the

Degree of Bachelor of Science in Zoology

2021-2022

CERTIFICATE

This is to certify that the dissertation titled, “*Study on role of biopreservatives to inhibit fungal growth in bread*” is an authentic record of work carried out by **ASNA K.S** under the supervision and guidance of **Dr. HELVIN VINCENT**, Assistant Professor, Department of Zoology, St. Teresa’s College (Autonomous), Ernakulam in partial fulfilment of the requirement for the Bachelor’s Degree of Science in Zoology.

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Assistant professor

Department of Zoology

Department of Zoology

St.Teresa’s College

St.Teresa’s College

Ernakulam

Ernakulam

Examiners

1).....

2).....

PLACE: ERNAKULAM

DATE: 09/ 05/ 2022

DECLARATION

I, Ms. Asna K.S , hereby declare that this project report entitled **STUDY ON ROLE OF BIOPRESERVATIVES TO INHIBIT FUNGAL GROWTH IN BREAD** is a bonafide record work done by me during the academic year 2021-2022 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam.

This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

ASNA K.S

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I place on record my sincere thanks to my parents and friends for their kindness, support and whole hearted encouragement which was a guiding in light for me throughout my project.

Place: Ernakulam

Date: 09/05/2022

ASNA K.S

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ABSTRACT

This study aims to identify and evaluate the role of biopreservatives to inhibit fungal growth in bread.

Nine common biopreservatives were applied to bread slices and kept in a plastic bag and left undisturbed till the appearance of mould. One slice of bread was maintained as control. The day at which mould occurred was noted. Antifungal activity of different biopreservatives was studied from the results obtained from the experiment. Among the applied nine biopreservatives lemon is more effective and clove oil is second most effective one. Garlic is also effective compared to other 6 samples used in the study. Citric acid in lemon, eugenol in clove and allicin in garlic is responsible for their antifungal activity. This study can be applied to replace artificial preservatives used in manufacturing of bread and other similar products to inhibit fungal activity and to increase shelf life.

INTRODUCTION

Food spoilage is a critical issue facing nowadays. Microbial spoilage of food happens by the means of bacteria, fungus, protozoa, etc. Fungi is one of the common group of food spoilers by microbial attack. They occur in form of mould, yeast etc.

Bread is one of the staple food used in day to day life. Bread and other bakery products are subjected to various spoilage problems, viz., physical, chemical and microbial; the latter is the most serious one particularly bacterial (*Bacillus* sp.) and mold growth. Various moulds involved in spoilage of bread include *Rhizopus*, *Mucor*, *Penicillium*, *Eurotium*, *Aspergillus* and *Monilia* (Saranraj and Geetha, 2012).

Food safety and extend shelf life of food products are essential to meet demands by consumers. An interesting alternative is the use of naturally derived antimicrobials called biopreservatives. A wide range of natural products can be used as antifungal agent.

Biopreservatives can be used to inhibit fungal growth in bread. Biopreservatives can replace artificial preservatives used in manufacturing of bread and other similar products to inhibit fungal activity and to increase shelf life. Biopreservatives or its derived forms can applicable in future to increase shelf life by inhibiting fungal growth.

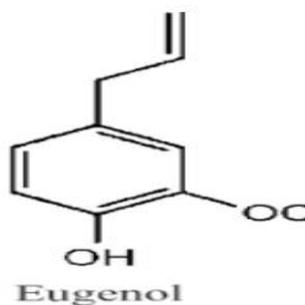
In order to increase the period of preservation of bread, it is necessary to add substances that slow down or inhibit fungal activity. The purpose of this project was to study the role of nine different commonly available biopreservatives to preserve bread and other similar products.

1.Cinnamon

The major components of cinnamon is cinnamaldehyde could be used as a natural fungistat.

2. Clove Oil

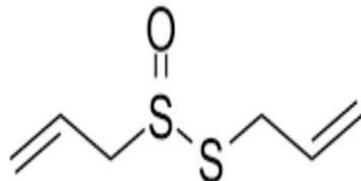
Eugenol is the main bioactive compound of clove. It is an effective antifungal compound against phytopathogenic *Aspergillus*, *Penicillium*, *Emericella*, and *Fusarium* species.



3. Garlic

Allicin is one of the main active compounds derived from garlic has antifungal activity.

Allicin



4. Gingelly oil

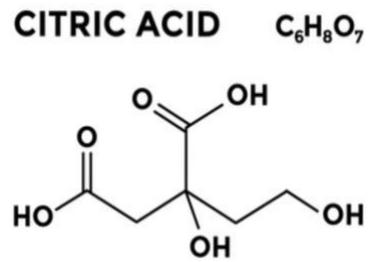
They reported that sesame oil can inhibit the growth of both yeast and mycelium of fungi.

5. Ginger

Antifungal effect of ginger is due to gingerone, dihydrogingerone and dehydroshogaol. There are also some other alkaloids, terpenes and terpenoid derivatives having antimicrobial activities on different bacterial pathogens.

6. Lemon

Citric acid were able to inhibit the mycelial growth of the pathogen



7. Raisin

It contains phenolic and poly phenolic components which help to inhibit fungal growth.

8. Salt

It is one of the commonly used agent against antimicrobial activity.

9. Turmeric

Rich in different phytochemicals. *Curcuma longa* shows the antifungal activity against *Aspergillus* sp. and *Fusarium* sp.

REVIEW OF LITERATURE

Bread is one of the staple food used in day to day life. Bread and other bakery products are subjected to various spoilage problems, viz., physical, chemical and microbial; the latter is the most serious one particularly bacterial (*Bacillus* sp.) and mould growth. Various moulds involved in spoilage of bread include *Rhizopus*, *Mucor*, *Penicillium*, *Eurotium*, *Aspergillus* and *Monilia* (Saranraj and Geetha, 2012).

From a microbiology point of view the most important factor common to different breads is a high moisture content (Legan, 1993). This characteristics make bread highly susceptible to microbial attack. The common sliced and wrapped bread is more likely to be spoiled by mould than unwrapped bread. In this type of bread, slicing provides more surfaces for mould to grow on and wrapping prevents moisture loss (Mariona Arroyo, 2002)

Evandro *et al.* (2005) in his paper discussed the inhibitory activity of spices against bacteria, fungi, etc. Antifungal activity of spices and derivatives has been studied in this paper by means of viable cells count, mycelial growth and mycotoxins synthesis. Paper concludes by the necessary awareness we should take before applying spices or their derivatives. Before including spices or their derivatives in food conservation systems, some evaluations about microbiological quality, economic feasibility, antimicrobial effect for a long time and toxicity should be carried out. Paper also evaluates the reports regarding essential oils containing carvacrol, eugenol and thymol (phenolic compounds) had highest antibacterial performances.

Suhr and Nielsen (2003) studied screening of essential oils as potential antimicrobials. Larger phenolic compounds such as thymol and eugenol (thymine, cinnamon and clove) had best effect when applied directly to the medium whereas smaller compounds such as allyl isothiocyanate and citral (mustard and lemon grass) were most efficient when added as volatile.

Naresh *et al.* (2003) studied that when an essential oil is directly incorporated into bakery products, less efficacy is observed. This may be due to the fact that specific components of the food products such as protein or fat can bind essential oil components, inactivating them (Mc Neil and Schminct, 1993).

Hitokoto *et al.* (1980) discussed inhibitory effect of 29 commercial powdered spices on inhibition of 3 species of *Aspergillus*. Eugenol from clove and thymol from thyme caused inhibition of 2 *Aspergillus* species. The inhibitory effects are interesting in connection with the prevention of mycotoxin contamination in many foods, and these compounds represent possible alternatives to the food additives in use at present.

Bhawana *et al.* (2014) published the antifungal activity of five common Indian spices namely clove, ajwain, turmeric, dalchini and black pepper against two bacteria *Aspergillus niger* and *Trichoderma* sp. The results revealed that the methanol extracts of spices have high antimicrobial activities on all test organisms. Results concluded that these spices contain high amount of secondary metabolites due to which they have high antimicrobial activity and it can be used as good bio-preservator and it can also be used for medicinal purpose.

Jonathan *et al.* (2015) reviewed the various effects of food additives and preservatives on man. Food additives plays a vital role in the food industries, but the various adverse effects associated with them remain a problem that need to be fought by us. This report also discussed about the role of Natural substances such as salt, sugar, vinegar, alcohol, and diatomaceous earth in food preservation.

Meining (2018) discussed about usage of essential oils and plant extracts as natural preservatives in food industries instead of chemicals. Essential oils are aromatic and volatile liquid extracted from plants including cinnamon, thyme, oregano, clove, rosemary, garlic, sage, basil, marjoram, savory, pepper, and cardamon, etc. Magan *et al.* (2012) and aimed at developing an essential oil-based active packaging to prolong the shelf life of bread products.

Ritika *et al.* (2016) studied the effects of spice extracts on the quality characteristics and storage life of bread. The objective of the study was to prepare antimicrobial bread using different natural antimicrobials (aqueous extracts of clove, ginger and cinnamon in different concentrations) to obtain an acceptable product. Maximum hardness was observed in bread with ginger extract (2%) whereas minimum found in cinnamon extract (4%). Bread containing 2-4% clove extract, ginger extract and cinnamon extract had a mould free shelf life for more than 6 days whereas control bread spoiled within 4 days.

Patil and Kukade (2020) studied fungal spoilage of bakery products and its control measures. Six different types of bakery products i.e. pizza base, bread, toast, cupcake, bun, cookies were used in the study. Clove, Lemongrass, Cardamom, Citrus and Edible oil are usually used in food product. These product also acts as controlling measures for these fungi. Among these Cardamom powder showed the poor effect on the growth of inhibition of fungi and Clove powder showed the excellent effect on fungi.

Naili *et al.* (2019) published bakery science of bread by discussing the effect of salt reduction in quality of bread. It also discussed about the usage of salt for preservation. It is a big challenge to the bread industry when salt is reduced in the bread products. This is because, salt is cheap, and any replacement will incur a high cost with new testing and new technology. Salt has been used to preserve foods for a long time. In bread, salt also acts as a preservative. The use of higher concentration of salt produces higher osmotic pressure which causes bread cells to lose water to the environment, and thus retarding the microbial growth. Based on the facts, derived from these study, it was concluded that salt play many important roles in improving bread quality and safety.

Melogen and Roberta (2018) published inhibitory effect of garlic against fungal growth. In this study the garlic possesses an inhibitory potential against the test organism (*Penicillium* sp). Allicin is the component of garlic. Allicin is considered as the most important biologically active compound in garlic since it decomposes to other sulfur containing molecules, thiosulfonates and disulfides.

Abouzeed *et al.* (2018) published identification of phenolic compounds, antibacterial and antioxidant activities of raisin extracts. Raisins (dried grapes; *Vitis vinifera*), are very popular plant in Mediterranean area and are widely used as a traditional and natural biomedicine in several countries where it is well adapted to the climate. The extract from grape's flesh did not have antimicrobial effect (Yigit *et al.*, 2009).

MATERIALS AND METHODS

Materials required: Slice of bread (10), plastic bags, 9 common bio- preservatives.

The 9 common biopreservatives used were:

1. Cinnamon
2. Clove oil

3. Garlic
4. Gingelly oil
5. Ginger
6. Lemon
7. Raisin
8. Salt
9. Turmeric

Methodology:

Nine different biopreservatives were prepared as follows:

- Cinnamon powder - About 21grams (1½ tablespoon) is mixed in about 3ml of water.
- Clove oil - About 3ml is taken.
- Garlic - About 75 grams taken, mashed into a paste using mortar and pestle and mixed in 3ml of water.
- Gingelly oil - About 3ml is taken.
- Ginger - About 100 grams taken and mashed into paste using mortar and pestle and mixed in 3 ml of water.
- Lemon - 3 ml of squeezed lemons is taken.
- Raisin - About 100 grams taken and extracted with 1ml of water.
- Salt - About 15 grams dissolved in 3ml of water.
- Turmeric - About 8 grams grinded and mixed with 3ml of water.

On ten slices of bread, nine different biopreservatives were added and 1 slice was kept as control (without adding any biopreservative).

The bread slices were put in separate plastic bags and sealed and kept undisturbed till mould appears on bread slices. The growth of mould was tracked by checking the bread slices everyday.

OBSERVATIONS

The growth of mould on bread slices were noticed and observation collected during December, 2021 – February, 2022 as shown in figures 1 to 10.



Figure 1 showing Control (without biopreservative)



Figure 2 showing bread slice with cinnamon

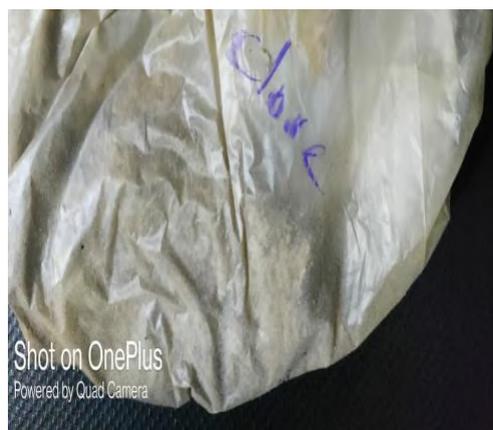


Figure 3 showing bread slice with clove oil



Figure 4 showing bread slice with Garlic



Figure 5 showing bread slice with gingelly oil



Figure 6 showing bread slice with ginger



Figure 7 showing bread slice with lemon



Figure 8 showing bread slice with raisin



Figure 9 showing bread slice with salt

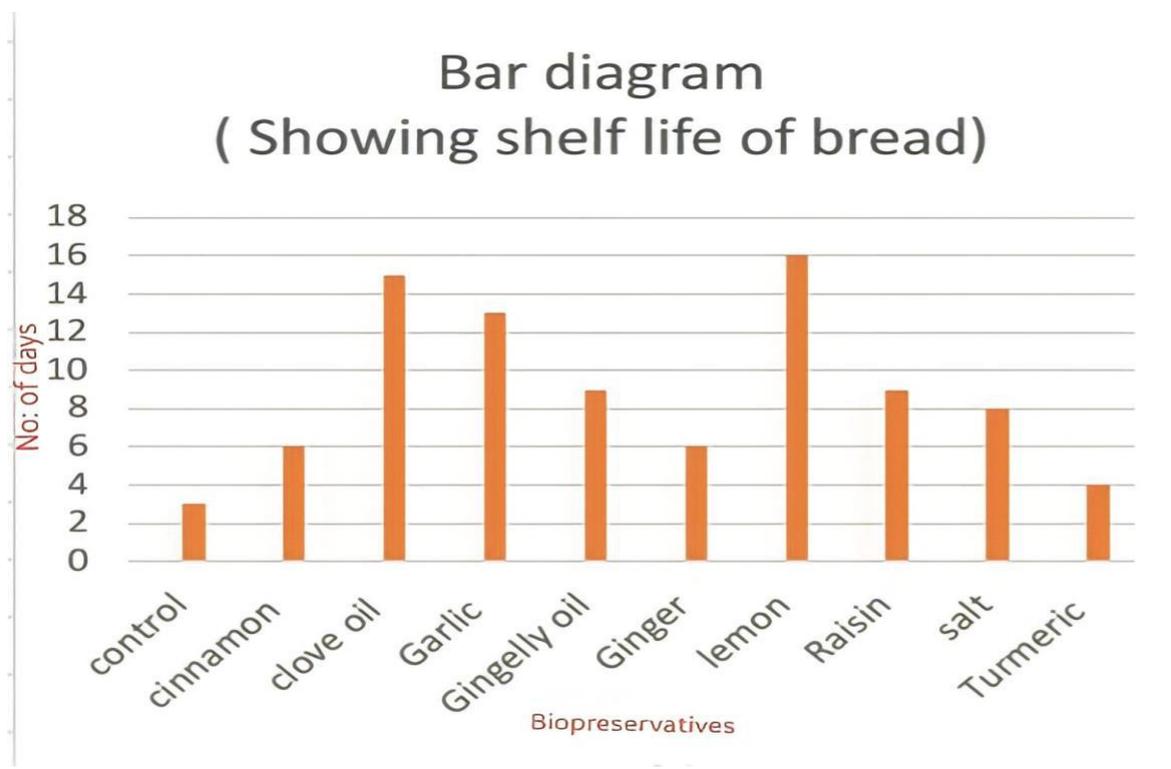


Figure 10 showing bread slice with turmeric

Table 1 showing the effect of biopreservatives.

SI. No:	Samples	Duration	No. of days / shelf life
1	Control (without bio preservative)	DEC 27 – DEC 29	Mould appeared on 3rd day
2	Cinnamon	JAN 29 – FEB 3	Mould appeared on 6th day
3	Clove oil	JAN 26-FEB 9	Mould appeared on 15th day
4	Garlic	JAN 26 - FEB 7	Mould appeared on 13th day
5	Gingelly oil	JAN 26 - FEB 3	Mould appeared on 9th day
6	Ginger	JAN 29- FEB 3	Mould appeared on 6th day
7	Lemon	Dec 26 - JAN 11	Mould appeared on 16th day
8	Raisin	JAN 24 - FEB 1	Mould appeared on 9th day
9	Salt	DEC 26 - JAN 2	Mould appeared on 8th day

10	Turmeric	JAN 29 –FEB 1	Mould appeared on 4th day



RESULT

From the observations it was noticed that Lemon is the most effective biopreservative followed by Clove oil. Lemon helps to increase shelf life of bread

about 16 days and clove oil about 15 days. Turmeric was found to be the least effective one. It has shelf life of about 4 days which is followed by ginger and cinnamon with shelf life of about 6 days.

DISCUSSION

Mould spoilage of bread is a serious issue facing nowadays. Contamination of food products like bread and its products may leads to serious food poisoning. Various substances can be added to food items to increase shelf life, texture, taste, etc.

Food additives are substances that food manufacturerers intentionally add to increase the shelf life, flavour, apperance of the food. Preservatives are substances which are capable of inhibiting the growth of microorganisms. When the food is to be stored for a prolonged period, use of additives and preservatives is essential to maintain its quality, wholesomeness, taste, appearance, flavour, freshness etc. Additives and preservatives prevent the spoilage of food due to the growth of bacteria and fungi. Synthetic food additives react with cellular components of body leading to various defects. So it should be natural one with minimal effect and those that are considered as safe and in case of those not generally recognized as safe, the acceptable daily intake should not be exceeded (Inetianbor *et al.*, 2015)

The results from our studies revealed that all the breads using extracts of ginger, clove, garlic, raisin etc. showed some activity against food associated pathogens. This results can be supported with the study by Ritika *et al.* (2016). This study concludes that bread of acceptable quality can be prepared with the use of natural antimicrobials as it increases the shelf life of bread and enhances the flavour also. Bread containing cinnamon extract, ginger extract and clove extract in concentration up to 2-4% had a mould free shelf life for 6 days. Spices are one of the most commonly used natural antimicrobial agents in foods and have been used traditionally for thousands of years by many cultures for preserving foods and as food additives to enhance aroma and flavour.

Inhibitory action of natural products on mould involves cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of intercellular and extracellular enzyme as said by Souza *et al.* (2005). In our studies second most effective antifungal sample is clove oil. Its antifungal activity is reported in earlier studies. It was supported in other studies also (Lattaoui and Tantaoui-Elaraki, 1994; Kim *et al.* (1995). They made a statement that essential oils containing carvacrol, eugenol and thymol (phenolic compounds) had highest antibacterial performances.

This clearly emphasizes the antifungal effect of clove oil and many other essential oils that are commonly available.

Spices have antimicrobial property besides contribute piquancy of food and beverages. They have been recognized for their value of preserving foods & medicinal values due to the presence of bioactive antimicrobial compounds. The antimicrobial effect of spice extract helps to prevent diseases and cater the healing effect. The result concluded that due to the high amount of secondary metabolites in the spices, they have antimicrobial activity and it can be used as a good bio-preservater (Bhawana *et al.*, 2014).

In bread, salt also acts as a preservative besides contribution to flavor. High salt level increases the time for microbial growth of some pathogens such as *Escherichia coli* and *Salmonella*. Microbial shelf stability is also affected by the existence of salts in the food products . It is a big challenge to the bread industry when salt is reduced in the bread products (Naili *et al.*, 2019). This is because, salt is cheap, and any replacement will incur a high cost with new testing and new technology. Salt also can play a role to some extent in case of antimicrobial activity.

Outcome from our study emphasizes that lemon is considered as most effective sample against fungal growth and helps mould prevention (Patil *et al.*, 2020). The citrus extract consists of many chemical compound such as geranial, Nerol, Gernyl acetate, geraniol, beta- caryophellene and Neryl acetate. Its major component is lemonene. Citrus extract has reported strong antifungal activity against many strains of fungi. It strongly inhibits spore production and germination of fungal pathogen (Patil *et al.*, 2020). Lemon is more effective biopreservative among nine samples used, because it contains citric acid as main component. Acidic pH below 4.5, inhibits fungal growth.

We can incorporate various biopreservatives to inhibit fungal growth. Before including spices and/or their derivatives in food conservation systems, some evaluations about microbiological quality, economic feasibility, antimicrobial effect for a long time and toxicity should be carried out (Souza *et al.*, 2005). The previous

studies regarding inhibition of fungal spoilage by means of spices, organic derivatives, essential oils, etc. support our results. By providing proper plannings and treatment we can eradicate fungal spoilage by means of biopreservatives and can increase shelf life to maximum.

CONCLUSION

Food spoilage with fungus and moulds is still a major problem limiting the shelf life of many high and intermediate moisture bakery products.

Normal piece of bread without applying any of the biopreservative was kept as control. Mould appeared in it within 3 days after it was kept in plastic bag.

Among the common 9 bio-preservatives studied, lemon was proved to be more effective. The fungicidal activity of lemon was due to the citric acid present in it. Slices of bread applied with lemon has more shelf life of 16 days.

Clove oil is second most effective sample of bio preservative used in experiment. Eugenol is the main bioactive compound of clove causing antifungal effect. Slice of bread with clove has shelf life of about 15 days.

Garlic was also found effective due to presence of allicin. Allicin makes it more effective against fungal activity. It increases shelf life about 13 days.

Bread slices with the samples Gingelly oil, raisin, salt, cinnamon, ginger, turmeric has shelf life of about 9, 9, 8, 6, 6 and 4 days respectively.

The study revealed the predominant efficacy of preservatives to inhibit fungal growth in bakery foods with the presence of bioactive components. Losses due to mould spoilage have been resulting in revenue loss to the baking industries. Therefore, methods to control mould growth and to extend the shelf life of bakery products is of great economic importance to the baking industry where an increased demand in global consumption exists. Other measures as good hygiene in the bakeries and if necessary complementary post packaging heat treatments or modified atmosphere packaging is the *best* alternatives.

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EFFECT OF TEMPERATURE AND pH ON THE OPERCULAR RESPIRATORY RATE (ORR) OF GOLDFISH



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Affiliated by Mahatma Gandhi University, Kottayam

in partial fulfilment of requirement for the

Degree of Bachelor of Science in Zoology

2021-2022

**EFFECT OF TEMPERATURE AND pH ON
THE OPERCULAR RESPIRATORY RATE
(ORR) OF GOLDFISH**

CERTIFICATE

This is to certify that the project entitled **EFFECT OF TEMPERATURE AND pH ON THE OPERCULAR RESPIRATORY RATE OF GOLDFISH** submitted by Ms. Avlin Rose A J, Reg. No.AB19ZOO033 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Helvin Vincent and this is her original effort.

Dr. Helvin Vincent

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Examiners

1)

2)

DECLARATION

I, Ms. Avlin Rose A J, hereby declare that this project report entitled **EFFECT OF TEMPERATURE AND WATER ACIDTY ON THE OPERCULAR RESPIRATORY RATE OF GOLDFISH** is a bonafide record work done by me during the academic year 2021-2022 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam.

This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

AVLIN ROSE A J

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ABSTRACT

In the past century, the world has seen an increase in the amount of carbon dioxide (CO₂) in the atmosphere. The rise in CO₂ can put stress on aquatic ecosystems due to ocean acidification and overall decrease in the pH of the waters (Adam Parker, 2015). So this project helps to determine the effect of temperature and water acidity on the opercular respiratory rate (ORR) of goldfish. For this experiment two goldfish were taken. To determine the effect of temperature five different temperatures were taken, these are as follows 25°C, 20°C, 10°C, 30°C and 35°C. At 25°C the fish 1 and fish 2 had respiratory rate slightly greater than the normal respiratory rate. At 20°C both the fishes had normal respiratory rate ie, range from 78 to 82. At 10°C the fish 1 and fish 2 had respiratory rate less than the normal respiratory rate. At 30°C the respiration rate of both was greater than the normal respiratory rate. At 35°C the respiratory rate of fish 1 and fish 2 was much greater than the normal respiratory rate. From this it was found that at higher temperatures the fishes showed high respiratory rate and at low temperature fishes showed lower respiratory rate. Thus temperature is a factor responsible in the opercular respiratory rate of aquatic fishes. The ideal temperature for the fish would be around 20°C -25°C. The second factor responsible for the ORR was acidity of water or simply pH. To determine the effect of acidity, vinegar was used to increase the acidity and four different pH were taken to find the effect of acidity and these were pH 5, pH 6, pH 7 and pH 8. At pH 5 the respiratory was very much higher than the normal respiratory rate and the fishes showed signs of stress and the breathing was rapid. At pH 6 the respiratory was around normal and also showed signs of large mouth for gasping for air. At pH 7 the respiratory rate was normal and showed no signs of defects which showed that pH 7 is the ideal pH for fish keeping. At pH 8 the respiratory rate was around to the normal respiratory rate. This concludes that the ideal pH for fishes is from pH 7- pH 8. From the experiment it was found that certain factors like the temperature and pH or water acidity has a great impact on the survival of aquatic species.

INTRODUCTION

Global warming is the process in which the earth's temperature on the atmosphere layers that are close to earth rise artificially as a result of the intense increase in some gases that occur in consequences of various human activities and that are qualified as greenhouse gases in the atmosphere. Oceans and seas are mostly affected by the process of change caused by global warming. A temperature increase of only a few degrees does not cause an increase in the temperature of large water masses such as oceans, seas, lakes and ponds but it also causes hydrological events that cause a change in the physical and chemical characteristics of water. Water temperature is the most important environmental parameter that affects the life cycle, physiology and behaviours of aquatic living beings (Tekiney, 2007). Due to global warming it can adversely affect the respiration rate of aquatic organisms, particularly the fishes. Global warming tends to increase the temperature of the water bodies and the waters of the water bodies starts to heat up. Resulting in the decrease of dissolved oxygen. Therefore fishes have difficulties in breathing in such an environment. Additionally such warming low-oxygen waters tends to increase fish's oxygen demand because their metabolism speeds up. As fish grows the demand for oxygen also increases, therefore the fish move to waters whose temperatures resembles those of their original habitats and satisfy their oxygen needs. But due to global warming the fishes find difficulty living in such waters of less oxygen and eventually dies.

Water acidity or pH is another factor responsible for the survival of aquatic organisms. By releasing carbon dioxide to the atmosphere, humans are rapidly altering the chemistry of the water bodies and affecting the aquatic life. Unpolluted deposition (or rain), in balance with atmospheric carbon dioxide has a pH of 5-6. Almost everywhere in the world the pH of rain is lower than this. The main pollutants responsible for acid deposition (or acid rain) are sulfur dioxide (SO_x) and nitrogen oxides (NO_x). Acid deposition influences mainly the pH of freshwater.

Nitrogen and sulfuric emissions come from natural and anthropogenic sources. Natural emissions include Volcano emissions, lightning and microbial processes. Power stations and industrial plants like the mining and smelting of high sulfur and nitrogen oxides and other acidic compounds. These compounds mix with the water vapour at unusual proportions to cause acid deposition with a pH of 4.2 to 4.7. That is 10 or more times the acidity of natural deposition.

The acidification of freshwater in an area is dependent on the quantity of calcium carbonate (limestone) in the soil. Limestone can buffer (neutralize) the acidification of the freshwater. The effects of acid deposition are much greater on lakes with little buffering capacity. Much of a damage to aquatic life in sensitive areas with this little buffering capacity is a results of acid shock. This is caused by the sudden runoff of large amounts of highly acidic water and aluminium ions into lakes and streams, when snow melts in the spring or after unusually heavy rains. Most freshwater lakes, streams and ponds have a natural pH in the range of 6 to 8.

Acid deposition has many harmful ecological effects when the pH of most aquatic systems falls below 5 and increase above pH 9. Below pH 5 fish population begin to disappear, the bottom is covered with un-decayed material and mosses may dominate near shore areas. It can kill many fishes by stimulating excessive mucus formation. This asphyxiates the fishes by clogging their gills. It can also cause chronic stress that may not kill individual fish, but leads to lower body weight and smaller size and makes fish less able to compete for food and habitat. The most serious chronic effect of increased acidity in surface waters appears to be interference with the fish reproductive cycle. Calcium levels in the female fish may be lowered to the point where she cannot produce eggs or the eggs fail to pass from the ovaries or if fertilized the eggs and the larvae develop abnormally.

Extreme pH can kill adult fish and invertebrate life directly and can also damage developing juvenile fish. It will strip a fish of its slime coat and high pH level chaps the skin of fish because of its alkalinity. When the pH of freshwater becomes highly alkaline, the effects on fish may include death, damage to outer surfaces like gills, eyes and skin and an inability to dispose of metabolic wastes. High pH may also increase the toxicity of other substances. For example the toxicity of ammonia is ten times more severe at a pH of 8 than it is at pH 7. It is directly toxic to aquatic life when it appears in alkaline conditions.

This topic was selected for study because of the rising issues of water bodies due to global warming and acidification.

GOLDFISH

Goldfish (*Carassius auratus*) are members of the cyprinid family (carp and their relatives). They originated in south-east Asia, although the cyprinid family covers a far wider global range. Because of accidental and deliberate introduction into the wild (Angeler *et al*, 2002). Goldfish are now found worldwide with a few exceptions such as Greenland and Antarctica. *C.auratus* are classified as least concern (IUCN2015). Goldfish are usually considered a temperate water fish; however they may survive in temperatures below 10°C and up to 30°C. Collectively their broad environmental tolerances mean that they can be found inhabiting a very wide range of habitats. Their natural diet is very varied, with wild goldfish eating anything from terrestrial insects to vegetation to detritus (Richardson *et al*, 1995; Pinto *et al*, 2005).

Goldfish show a variety of social behaviours and are often found in the company of other goldfish (Pitcher and Magurran, 1983). Breeding typically occurs in spring and males chase and court gravid females. Mate attraction and species recognition involves pheromones (Sisler and Sorensen, 2008). Females lay eggs in aquatic conditions and the eggs are adhesive and hatch in 2 to 3 days. Under optimal conditions, fry grow rapidly and can be sexually mature within a year. As shallow water fish, goldfish vision is most sensitive to red, green, blue and ultra violet wavelengths (Neumeyer, 1992).

Goldfish can differentiate certain shapes, colours and sounds (Wyzisk and Neumeyer, 2007). They can also sense vibrations and their hearing capabilities are sensitive across a broad range of frequencies (Fay and Popper, 1974).

Goldfish can also detect different odours in the water which they can use to find food, avoid predators or preferentially associate with one another. All goldfishes need adequate space for shoaling keeping adequate distances between individuals maintaining adequate water quality and allowing all goldfishes to reach their full size potential. Although goldfish may tolerate some variations in water parameters, poor water quality can be fatal and some fancy varieties are at particular risk.

AIM

This project work aim to determine the opercular respiratory rate of goldfish by the effect of temperature and water acidity.

OBJECTIVE

Global warming and acidification are the major problems faced by all living organisms on this planet. Due to this, temperature as well as acidity of water is increasing day by day which is affecting the water bodies at a higher rate. Objective of this project is to determine the effects of temperature and water acidity on the opercular respiratory rate of goldfish. This project gives the optimal temperature and water acidity where the fish can survive.

REVIEW OF LITERATURE

Global warming and acidification are the major problem of this century, which alters the natural environment conditions including quality of water, abrupt increase/decrease in temperature and increase level of pollutions. These changes in environmental conditions directly affect the natural biodiversity (Sudesh Rani, 2016).

Fishes are aquatic, craniate, gill bearing that lack limbs with digits. Most fishes are ectothermic (cold blooded) allowing their body temperatures to vary as ambient temperatures change. Factors affecting fish production in freshwater aquatic systems can be classified as physical and chemical factors. The physical properties that are important for the fish production and growth include temperature and important chemical parameters include pH, alkalinity, hardness etc.

Water temperature is one of the most important physical factors affecting the respiration of fishes, also chemical factors such as water acidity (pH) also has a role in the respiratory rate of fish. The respiration of fish is through certain organs called the gills, which has a covering called the operculum. Operculum is a bony plate and serves as a water pump. Each time the fish respire the operculum moves, operculum movement allows one to evaluate the opercular respiratory rate (ORR).

Due to global warming, increase in atmospheric temperature is the matter of great discussing issues among the scientists. Temperature as an important abiotic factor showed influence on the physiochemical parameters of all living organism on earth.

The increase and decrease in the environmental temperature and water acidity can directly and indirectly affect the living organisms. These variations become more prominent for aquatic animals. Change in the pH and temperature of water show adverse effects on the fishes and other aquatic animals (Sudesh Rani, 2016). As the adverse conditions of water increases it produce high stress on fishes (Capkin *et al*, 2006; Singh and Mishra, 2009; Sudesh Rani, 2016).

Temperature variations in water bodies depend largely on their geographical location (Latitude, Longitude and Altitude). In the tropics, marked variations in temperature and rainfall between the rainy and dry season affect the physico- chemical characteristics of the water. Cold water has more dissolved oxygen than warm water thus as temperature increases less oxygen is available to the biota. Therefore, temperature has a pronounced effect on rate of chemical and biological processes in aquatic habitats. Temperature is one of the most fundamental environmental stressors, altering almost all biological processes through its actions on basic chemical reactions supporting physiological processes (Murugain *et al*, 2008)

Most fish exchange gases using gills on either side of the pharynx. Gills are tissues which consist of threadlike structures called filaments. These filaments have many functions and are involved in ion and water transfer as well as oxygen, carbon dioxide, acid and ammonia exchange. (Randall, 1984; Adesola *et al*, 2020). Fish exchange gases by pulling oxygen-rich water through their mouths and pumping it over their gills. The gills push the oxygen-poor water out through openings in the sides of the pharynx.

Most species employ a counter-current exchange system to enhance the diffusion of substances in and out of the gill, with blood and water flowing in opposite directions to each other (Andrews *et al*, 2010). The temperature of the aquatic medium in which the fish is cultured determines the respiratory rate of the fish and consequently, its survival, productivity, distribution and normal biological activities (Anita and Pooja, 2013). Inability of fishes to adapt to temperature fluctuations is responsible for the inability of fishes to respond physiologically to the environment and hence result to death (Ayanwale *et al*, 2014). As the degree of water temperature increases it produce highly stress conditions on fishes, the degree of toxicity produced is dependent upon environmental conditions such as temperature, pH of water, oxygen content and presence of residue molecules (Tantanpale *et al*, 2009).

The rise in CO₂ can put stress on aquatic ecosystems due to ocean acidification, an overall decrease in the pH of the ocean's waters. Freshwater ecosystems, already stressed by pollution and recent increases in the number of invasive species are also showing signs of acidification due to the increase in CO₂. The effect of the rise in acidity is known to be harmful to calcifying organisms.

As more and more fossil fuel burning cars and factories are being built around the world, the amount of CO₂ being released into the atmosphere is increasing. The increasing amount of CO₂ not only causes a problem for people and animals but also aquatic ecosystems around the world (Adam Parker, 2015).

Carbon dioxide rapidly diffuses into the water causing the pH to become more acidic (Arnold *et al*, 2012). In the marine environment, the CO₂ reacts with seawater, forming carbonic acid and can ultimately result in ocean acidification. Increase in atmospheric CO₂ concentrations have led to an increased rate of CO₂ diffusion into the oceans. Ultimately, this would lead to the oceans becoming more and more acidic. If the pH of oceans rises at a rate quicker than the vegetation can adapt, submerged aquatic vegetation species could be endangered.

While marine ecosystems seem to be the main concern, it is plausible that freshwater ecosystems will also experience increasing pH as CO₂ diffuses from the atmosphere into lakes and rivers (Strumm and Morgan, 1996).

METHODOLOGY

MATERIALS

- Fahrenheit thermometer - used for measuring the temperature (range = -0°F to 400°F, least count =2.0)
- Measuring cylinder-used for measuring the solutions (5mL)
- pH test paper- used for determining the pH of the water
- Test tube - in which sampled water was taken for testing the pH
- Regular fish bowl aquarium
- 2 Gold fish
- Vinegar
- Baking soda
- Cold water
- Hot water
- Normal tap water

METHOD

This project experiment was conducted at Parakattu house near Pulleppady bridge, Ernakulam, Kerala for 7 days from 6th February 2022 to 12th February 2022. The goldfishes (*Carassius auratus*) were purchased from tropical aquarium, South Ernakulam, Kerala. The water used for the experiment was regular tap water from Cochin co-orporation.

A regular fish bowl aquarium filled with about 1 litre of water was taken. The current temperature of the water was equal to that of the room temperature (20°C (68°F)). The opercular respiratory rate (ORR) was observed for 1 minute and the result was noted. The opercular respiratory rate of the fish was calculated by counting the number of times the gills of the fish was opened. Three trials were taken and the average of 3 trials was taken as the final result. The fish was removed from the bowl carefully.

Next the second fish was introduced into the bowl naming it the fish 2. The opercular respiratory rate of the fish at (20°C (68°F)) was noted down. The fish 2 was removed carefully from the bowl.

The temperature was then reduced to 10°C (50°F) by adding required amount of ice cold water and the experiment was repeated as above using fish 1 and fish 2.

The temperature was then set to 25°C (77°F) by adding hot water (5ml/min) over 5 mins into the bowl and the experiment was repeated with both fishes and the observation noted down. The experiment was carried out at 30°C (86°F) and 35°C (95°F) also in the same manner.

The effect of water acidity on the opercular respiratory rate of the fish was studied by introducing the fish to water at different pH. The pH was tested using a pH paper and was found to be neutral (around pH 7). The ORR of both fishes were noticed. The pH of the water was set to pH 5 by adding vinegar 1ml/min over 5mins. The fish 1 was introduced and the opercular respiratory rate was calculated. The fish 1 was then removed and fish 2 was introduced and the observation was noted.

The pH of water was then changed to pH 6 by adding water 5ml/min over 5mins and the experiment was carried out. The pH of water was again increased to pH 8 by adding a little bit of baking soda. The ORR of both fishes were calculated and formulated into tables for all 7 days. The graph was plotted using the average ORR value for 7 days.

OBSERVATION

Table 1 showing the effect of temperature on ORR of fish 1 for 7 days

SL NO:	TEMPERATURE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	20°C	76.3	77	76	75.6	77	75.6	74.3	75.9
2.	10°C	61	59.6	60.3	67.6	64	57.6	58	61.1
3.	25°C	79	83	77.3	82	83	82.3	78	80.6
4.	30°C	82.3	86	84.6	88	87.3	88	83.3	85.6
5.	35°C	101.3	106	97.6	106	99.3	103.3	100.6	102

From Table 1 it was noticed that the ORR of fish 1 was 75.9 at room temperature which reduced to 61.1 when temperature was reduced to 10°C. The ORR increased when the temperature was increased to 25°C, 30°C and 35°C.

Table 2 showing the effect of temperature on ORR of fish 2 for 7 days

SL NO:	TEMPERATURE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	20°C	76.6	76.6	78.3	76.6	78.6	77	77.6	77.3
2.	10°C	58.6	61.6	64.6	65	65.3	62.3	64	63
3.	25°C	80	80	80	81.6	80.6	81	78.6	80.2
4.	30°C	85.6	82.6	86.6	87	86.6	85	86	85.6
5.	35°C	101.6	98.3	101.3	103.6	100.3	100.3	101.6	101

From Table 2 it was noticed that the ORR of fish 2 was 77.3 at room temperature which reduced to 63 when temperature was reduced to 10°C. The ORR increased when the temperature was increased to 25°C, 30°C and 35°C.

Table 3 showing the effect of pH on ORR of fish 1 for 7 days

SL NO:	pH	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	5	112.6	99.3	104.3	115	103.6	115.3	112	108.8
2.	6	109.3	104.3	99.6	111	101	108.6	110.6	106.3
3.	7	74.6	79.3	75.6	79	80	79	77.6	77.8
4.	8	79	80	77.6	81.6	78.6	78.3	80	90.5

From table 3 it was observed that the ORR of fish 1 at pH 5 was 108.8, when pH was increased to 6, ORR was found to be 106.3, when the pH was 7 ORR was noted 77.8. when the pH was increased to 8 ORR was noted as 90.5.

Table 4 showing the effect of pH on ORR of fish 2 for 7 days

SL NO:	pH	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	5	102.6	108.3	105.6	104.3	108.3	102.3	101	104.6
2.	6	103.3	104.6	97.3	100.3	101	99	99.3	100.6
3.	7	79	80.3	79	76.3	80.3	77	81.3	79
4.	8	80.3	81.6	82.6	79	80	82.6	79	80.7

From Table 4 it was observed that the ORR of fish 2 was 104.6 at pH 5, then it decreased to 100.6 at pH 6. At pH 7 the ORR was found to be 79 and when this pH was increased to 8 the ORR changed to 80.7

RESULT

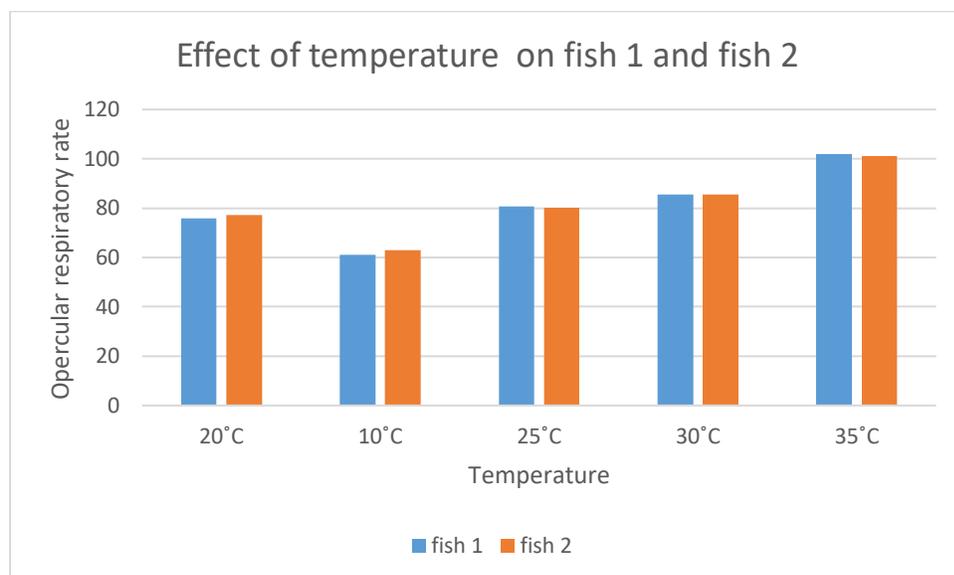


Figure 1 showing the effect of temperature on ORR of fish 1 & fish 2

Figure 1 shows when temperature is 20°C, both the fishes had a ORR between 75-78, when the temperature was reduced to 10°C the ORR was between 61-63. The temperature was increased to 25°C and th ORR was between 80.2-80.6. Again the temperature was increased to 30°C and 35°C and their ORR was found to be 85.6 and 101-102 respectively.

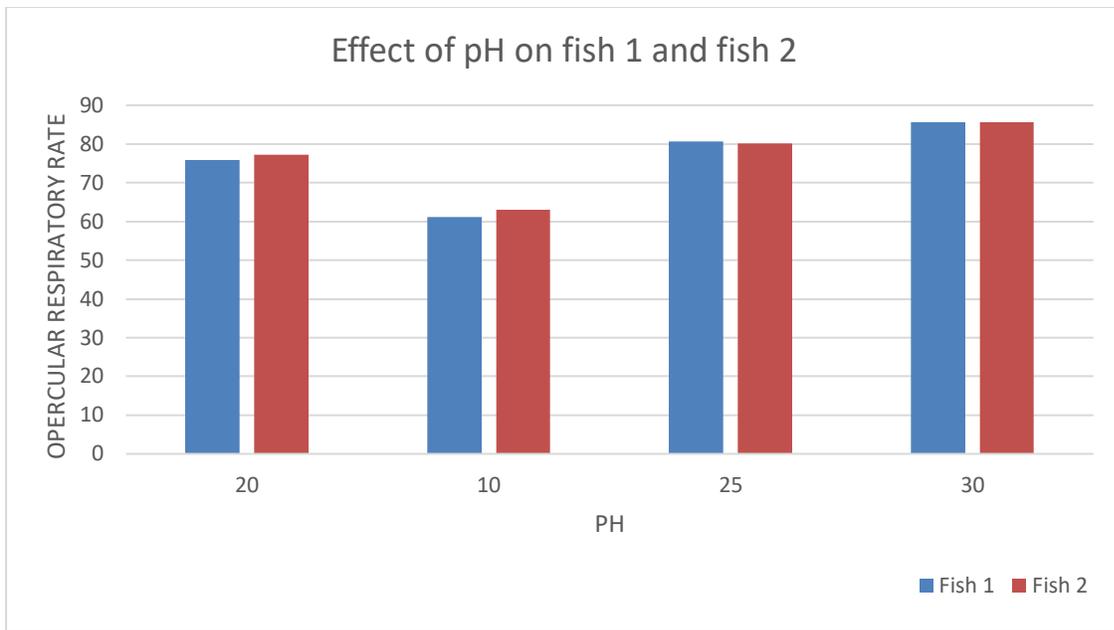


Figure 2 showing the effect of pH on ORR of fish 1 and fish 2

Figure 2 shows that at normal pH (pH 7) fish 1 and fish 2 had an opercular respiratory rate between 60-80. The respiratory rate was normal and showed no signs of defects which showed that pH 7 is the ideal pH for fish keeping.

When pH is at 5, fish 1 and fish 2 has an opercular respiratory rate between 100 - 120. At this pH the respiratory rate was very much higher than the normal respiratory rate (which was around 75-80) and the fishes showed signs of stress and the breathing was rapid.

When pH was at 6, fish 1 and fish 2 has the opercular respiratory rate between 80-120. At this pH the respiration of the fishes were not normal and also showed signs of stress like large mouth gasping for air.

When pH was at 8, fish 1 and fish 2 has opercular respiratory rate between 60-100 respectively. At pH 8 the respiratory rate was around to the normal respiratory rate. At pH 8 the water was alkaline therefore respiratory rate was also increased.

DISCUSSION

Global Warming and acidification has caused drastic effects on the water bodies across the world. Due to Global Warming, fish species has been extremely affected. As the water warms, fish need more oxygen to perform daily activities, like feeding. Change in temperatures, therefore, affects fish metabolic activities.

The surrounding water bodies absorbs most of the excess heat from greenhouse gas emissions, leading to a rise in water temperature. Increase in temperatures affect marine and freshwater species and ecosystems. Also leads to coral bleaching and the loss of breeding grounds for fishes.

Fishes don't have shells, therefore they will feel the effects of acidification. Because the surrounding water has a lower pH, a fish's cells often come into balance with the water by taking in carbonic acid. This changes the pH of the fish's blood, a condition called acidosis.

At lower pH levels, adult fishes start dying, fish eggs are unable to hatch, absence of fishes in highly acidic waters. Even if a particular species of fish is able to survive in that acidic environment, the animals feeding on these fishes won't be able to survive long, as accumulation of acid takes place in their body and leads to ultimate death.

In this present experiment, two goldfishes belonging to same species were taken and opercular respiratory rate was measured at different temperatures and pH. The experiment was done with regular tap water. About 1 liter of water was filled in a regular aquarium. The current temperature of water was 20°C (68°F). The ORR of both fishes were observed at this temperature. Similarly this procedure was repeated for 10°C, 25°C, 30°C and 35°C, and the observations and results were formulated into tables and graphs. To increase the temperature hot water was used and for decreasing the temperature ice cold water was used.

The effect of pH on ORR of fishes was the second parameter observed, both fishes were introduced to different pH. The pH were pH 5, pH 6, pH 7 and pH 8. Vinegar was used for making the water acidic and baking soda was used for making alkaline condition.

Several kinds of research were conducted by several scientists with the objective to determine how temperature as well as water acidity have an impact on the opercular respiratory rate (ORR) of fishes.

According to the research conducted by Ahmed *et al.* (2015) says that temperature is regarded as an environmental factor that can affect activity, behaviour, feeding, growth, survival, appetite and reproduction in all fishes. Temperature variation in water bodies depend largely on their geological location (latitude, longitude and altitude). In the tropics and sub tropics, marked variations in temperature and rainfall between the rainy and dry season affect the physico - chemical characteristics of the water.

Akin (2003) reported that Cold water has more dissolved oxygen than warm water thus as temperature increases less oxygen is available to the biota. Therefore it was found that at higher temperatures fish respiratory rate also increased as demanding for more oxygen.

In the research conducted by Kazumasa *et al* (2000) it says that the phenomenon called acid rain results from industrial activities where sulfuric and nitric acid are produced by the release of sulfuric oxides (SO_x) and nitrogen oxides (NO_x) into the atmosphere. Acid rain induces the acidification of inland waters which results in damage to aquatic ecosystems, including fish.

Fish have the ability to regulate their acid-base balance in order to maintain normal pH of their body fluids under acidic ambience. When fish are exposed to a low pH, chloride cells in the gill tissue take up bicarbonate (HCO_3^-) ion from the outside to neutralize the hydrogen (H^+) ion flowing in the body. At this time, the loss of sodium (Na^+) and chloride (Cl^-) ions from the body fluids occurs, and plasma osmotic pressure decreases. This process is considered to be one of the major reasons why freshwater fish die under acidic conditions.

Therefore all these research papers have similarity with this present work that increase or decrease in the water temperature and water acidity have a major effect on the opercular respiratory rate of fishes. Opercular respiratory rate of fish is directly proportional to the water temperature i.e., respiratory rate increases with the increase in the temperature and vice versa. This is because as temperature increases availability of oxygen decreases and leads the fish to demand for more oxygen. Water acidity is another factor responsible for opercular respiratory rate, at lower pH fish shows signs of difficulties and slowly leads to death. Therefore both temperature and pH are essential factors responsible for the opercular respiratory rate (ORR) of fishes.

CONCLUSION

The world has seen an increase in the amount of carbon dioxide (CO₂) in the atmosphere. The rise in CO₂ can put stress on aquatic ecosystems due to ocean acidification, an overall decrease in the pH of the ocean's waters. This is caused by global warming and acidification. Through this study we found the favourable temperature and optimal pH of the water. Temperature and water acidity are some major factor responsible in the opercular respiratory rate of aquatic fishes. From this study we can conclude the optimal temperature and pH where an aquatic animals can survive.

Temperature is an essential factor which is involved in the respiration of aquatic fishes. Nowadays temperature is getting increased day by day because of global warming. By this study it shows that at higher temperatures the fishes showed high respiratory rate and at low temperature fishes showed lower respiratory rate, this is because at lower temperature (cold water) the amount of dissolved oxygen is greater and at higher temperature the amount of dissolved oxygen is less, therefore the fish needed to take more amount of oxygen to survive. At 20°C of temperature it was found that the respiratory rate was around 75-80 range and we can conclude that this is probably the normal temperature in which the fish can survive without having any problems. At high temperature i.e., 35°C when temperature is increased respiratory rate also increased. Likewise at lower temperature i.e., 10°C and it showed lower respiratory rate. From this experiment it was found that optimal temperature where the fish can survive is 20°C.

Acidity is the next major factor that causing the variation in respiratory rate. Due to acidification the acidity of water is increased day by day. At pH 7 the respiratory rate of fish was normal and it showed no signs of stress, therefore this can be the optimal pH where the fish can survive. At pH-8 the respiratory rate ranges from 80-100 and 60-80 respectively. At a pH of 5 the fishes showed signs of difficulties and was having a wide mouth for gasping more air. Thus the optimal pH of the water that fishes can survive is pH 7 -pH 8. As CO₂ gets dissolved in the waters, the water become acidic and the oxygen content of the water decreases thus making difficulty for the survival of aquatic organisms.

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EFFECT OF TEMPERATURE AND pH ON THE OPERCULAR RESPIRATORY RATE (ORR) OF GOLDFISH



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2021-2022

**EFFECT OF TEMPERATURE AND pH ON
THE OPERCULAR RESPIRATORY RATE
(ORR) OF GOLDFISH**

CERTIFICATE

This is to certify that the project entitled **EFFECT OF TEMPERATURE AND pH ON THE OPERCULAR RESPIRATORY RATE OF GOLDFISH** submitted by Ms. Ayisha Almass P A, Reg. No.AB19ZOO034 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Dr. Helvin Vincent and this is her original effort.

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Examiners

1)

2)

DECLARATION

I, Ms. Ayisha Almass P A, hereby declare that this project report entitled **EFFECT OF TEMPERATURE AND WATER ACIDTY ON THE OPERCULAR RESPIRATORY RATE OF GOLDFISH** is a bonafide record work done by me during the academic year 2021-2022 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam.

This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

AYISHA ALMASS P A

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ABSTRACT

In the past century, the world has seen an increase in the amount of carbon dioxide (CO₂) in the atmosphere. The rise in CO₂ can put stress on aquatic ecosystems due to ocean acidification and overall decrease in the pH of the waters (Adam Parker, 2015). So this project helps to determine the effect of temperature and water acidity on the opercular respiratory rate (ORR) of goldfish. For this experiment two goldfish were taken. To determine the effect of temperature five different temperatures were taken, these are as follows 25°C, 20°C, 10°C, 30°C and 35°C. At 25°C the fish 1 and fish 2 had respiratory rate slightly greater than the normal respiratory rate. At 20°C both the fishes had normal respiratory rate ie, range from 78 to 82. At 10°C the fish 1 and fish 2 had respiratory rate less than the normal respiratory rate. At 30°C the respiration rate of both was greater than the normal respiratory rate. At 35°C the respiratory rate of fish 1 and fish 2 was much greater than the normal respiratory rate. From this it was found that at higher temperatures the fishes showed high respiratory rate and at low temperature fishes showed lower respiratory rate. Thus temperature is a factor responsible in the opercular respiratory rate of aquatic fishes. The ideal temperature for the fish would be around 20°C -25°C. The second factor responsible for the ORR was acidity of water or simply pH. To determine the effect of acidity, vinegar was used to increase the acidity and four different pH were taken to find the effect of acidity and these were pH 5, pH 6, pH 7 and pH 8. At pH 5 the respiratory was very much higher than the normal respiratory rate and the fishes showed signs of stress and the breathing was rapid. At pH 6 the respiratory was around normal and also showed signs of large mouth for gasping for air. At pH 7 the respiratory rate was normal and showed no signs of defects which showed that pH 7 is the ideal pH for fish keeping. At pH 8 the respiratory rate was around to the normal respiratory rate. This concludes that the ideal pH for fishes is from pH 7- pH 8. From the experiment it was found that certain factors like the temperature and pH or water acidity has a great impact on the survival of aquatic species.

INTRODUCTION

Global warming is the process in which the earth's temperature on the atmosphere layers that are close to earth rise artificially as a result of the intense increase in some gases that occur in consequences of various human activities and that are qualified as greenhouse gases in the atmosphere. Oceans and seas are mostly affected by the process of change caused by global warming. A temperature increase of only a few degrees does not cause an increase in the temperature of large water masses such as oceans, seas, lakes and ponds but it also causes hydrological events that cause a change in the physical and chemical characteristics of water. Water temperature is the most important environmental parameter that affects the life cycle, physiology and behaviours of aquatic living beings (Tekiney, 2007). Due to global warming it can adversely affect the respiration rate of aquatic organisms, particularly the fishes. Global warming tends to increase the temperature of the water bodies and the waters of the water bodies starts to heat up. Resulting in the decrease of dissolved oxygen. Therefore fishes have difficulties in breathing in such an environment. Additionally such warming low-oxygen waters tends to increase fish's oxygen demand because their metabolism speeds up. As fish grows the demand for oxygen also increases, therefore the fish move to waters whose temperatures resembles those of their original habitats and satisfy their oxygen needs. But due to global warming the fishes find difficulty living in such waters of less oxygen and eventually dies.

Water acidity or pH is another factor responsible for the survival of aquatic organisms. By releasing carbon dioxide to the atmosphere, humans are rapidly altering the chemistry of the water bodies and affecting the aquatic life. Unpolluted deposition (or rain), in balance with atmospheric carbon dioxide has a pH of 5-6. Almost everywhere in the world the pH of rain is lower than this. The main pollutants responsible for acid deposition (or acid rain) are sulfur dioxide (SO_x) and nitrogen oxides (NO_x). Acid deposition influences mainly the pH of freshwater.

Nitrogen and sulfuric emissions come from natural and anthropogenic sources. Natural emissions include Volcano emissions, lightning and microbial processes. Power stations and industrial plants like the mining and smelting of high sulfur and nitrogen oxides and other acidic compounds. These compounds mix with the water vapour at unusual proportions to cause acid deposition with a pH of 4.2 to 4.7. That is 10 or more times the acidity of natural deposition.

The acidification of freshwater in an area is dependent on the quantity of calcium carbonate (limestone) in the soil. Limestone can buffer (neutralize) the acidification of the freshwater. The effects of acid deposition are much greater on lakes with little buffering capacity. Much of a damage to aquatic life in sensitive areas with this little buffering capacity is a results of acid shock. This is caused by the sudden runoff of large amounts of highly acidic water and aluminium ions into lakes and streams, when snow melts in the spring or after unusually heavy rains. Most freshwater lakes, streams and ponds have a natural pH in the range of 6 to 8.

Acid deposition has many harmful ecological effects when the pH of most aquatic systems falls below 5 and increase above pH 9. Below pH 5 fish population begin to disappear, the bottom is covered with un-decayed material and mosses may dominate near shore areas. It can kill many fishes by stimulating excessive mucus formation. This asphyxiates the fishes by clogging their gills. It can also cause chronic stress that may not kill individual fish, but leads to lower body weight and smaller size and makes fish less able to compete for food and habitat. The most serious chronic effect of increased acidity in surface waters appears to be interference with the fish reproductive cycle. Calcium levels in the female fish may be lowered to the point where she cannot produce eggs or the eggs fail to pass from the ovaries or if fertilized the eggs and the larvae develop abnormally.

Extreme pH can kill adult fish and invertebrate life directly and can also damage developing juvenile fish. It will strip a fish of its slime coat and high pH level chaps the skin of fish because of its alkalinity. When the pH of freshwater becomes highly alkaline, the effects on fish may include death, damage to outer surfaces like gills, eyes and skin and an inability to dispose of metabolic wastes. High pH may also increase the toxicity of other substances. For example the toxicity of ammonia is ten times more severe at a pH of 8 than it is at pH 7. It is directly toxic to aquatic life when it appears in alkaline conditions.

This topic was selected for study because of the rising issues of water bodies due to global warming and acidification.

GOLDFISH

Goldfish (*Carassius auratus*) are members of the cyprinid family (carp and their relatives). They originated in south-east Asia, although the cyprinid family covers a far wider global range. Because of accidental and deliberate introduction into the wild (Angeler *et al*, 2002). Goldfish are now found worldwide with a few exceptions such as Greenland and Antarctica. *C.auratus* are classified as least concern (IUCN2015). Goldfish are usually considered a temperate water fish; however they may survive in temperatures below 10°C and up to 30°C. Collectively their broad environmental tolerances mean that they can be found inhabiting a very wide range of habitats. Their natural diet is very varied, with wild goldfish eating anything from terrestrial insects to vegetation to detritus (Richardson *et al*, 1995; Pinto *et al*, 2005).

Goldfish show a variety of social behaviours and are often found in the company of other goldfish (Pitcher and Magurran, 1983). Breeding typically occurs in spring and males chase and court gravid females. Mate attraction and species recognition involves pheromones (Sisler and Sorensen, 2008). Females lay eggs in aquatic conditions and the eggs are adhesive and hatch in 2 to 3 days. Under optimal conditions, fry grow rapidly and can be sexually mature within a year. As shallow water fish, goldfish vision is most sensitive to red, green, blue and ultra violet wavelengths (Neumeyer, 1992).

Goldfish can differentiate certain shapes, colours and sounds (Wyzisk and Neumeyer, 2007). They can also sense vibrations and their hearing capabilities are sensitive across a broad range of frequencies (Fay and Popper, 1974).

Goldfish can also detect different odours in the water which they can use to find food, avoid predators or preferentially associate with one another. All goldfishes need adequate space for shoaling keeping adequate distances between individuals maintaining adequate water quality and allowing all goldfishes to reach their full size potential. Although goldfish may tolerate some variations in water parameters, poor water quality can be fatal and some fancy varieties are at particular risk.

AIM

This project work aim to determine the opercular respiratory rate of goldfish by the effect of temperature and water acidity.

OBJECTIVE

Global warming and acidification are the major problems faced by all living organisms on this planet. Due to this, temperature as well as acidity of water is increasing day by day which is affecting the water bodies at a higher rate. Objective of this project is to determine the effects of temperature and water acidity on the opercular respiratory rate of goldfish. This project gives the optimal temperature and water acidity where the fish can survive.

REVIEW OF LITERATURE

Global warming and acidification are the major problem of this century, which alters the natural environment conditions including quality of water, abrupt increase/decrease in temperature and increase level of pollutions. These changes in environmental conditions directly affect the natural biodiversity (Sudesh Rani, 2016).

Fishes are aquatic, craniate, gill bearing that lack limbs with digits. Most fishes are ectothermic (cold blooded) allowing their body temperatures to vary as ambient temperatures change. Factors affecting fish production in freshwater aquatic systems can be classified as physical and chemical factors. The physical properties that are important for the fish production and growth include temperature and important chemical parameters include pH, alkalinity, hardness etc.

Water temperature is one of the most important physical factors affecting the respiration of fishes, also chemical factors such as water acidity (pH) also has a role in the respiratory rate of fish. The respiration of fish is through certain organs called the gills, which has a covering called the operculum. Operculum is a bony plate and serves as a water pump. Each time the fish respire the operculum moves, operculum movement allows one to evaluate the opercular respiratory rate (ORR).

Due to global warming, increase in atmospheric temperature is the matter of great discussing issues among the scientists. Temperature as an important abiotic factor showed influence on the physiochemical parameters of all living organism on earth.

The increase and decrease in the environmental temperature and water acidity can directly and indirectly affect the living organisms. These variations become more prominent for aquatic animals. Change in the pH and temperature of water show adverse effects on the fishes and other aquatic animals (Sudesh Rani, 2016). As the adverse conditions of water increases it produce high stress on fishes (Capkin *et al*, 2006; Singh and Mishra, 2009; Sudesh Rani, 2016).

Temperature variations in water bodies depend largely on their geographical location (Latitude, Longitude and Altitude). In the tropics, marked variations in temperature and rainfall between the rainy and dry season affect the physico- chemical characteristics of the water. Cold water has more dissolved oxygen than warm water thus as temperature increases less oxygen is available to the biota. Therefore, temperature has a pronounced effect on rate of chemical and biological processes in aquatic habitats. Temperature is one of the most fundamental environmental stressors, altering almost all biological processes through its actions on basic chemical reactions supporting physiological processes (Murugain *et al*, 2008)

Most fish exchange gases using gills on either side of the pharynx. Gills are tissues which consist of threadlike structures called filaments. These filaments have many functions and are involved in ion and water transfer as well as oxygen, carbon dioxide, acid and ammonia exchange. (Randall, 1984; Adesola *et al*, 2020). Fish exchange gases by pulling oxygen-rich water through their mouths and pumping it over their gills. The gills push the oxygen-poor water out through openings in the sides of the pharynx.

Most species employ a counter-current exchange system to enhance the diffusion of substances in and out of the gill, with blood and water flowing in opposite directions to each other (Andrews *et al*, 2010). The temperature of the aquatic medium in which the fish is cultured determines the respiratory rate of the fish and consequently, its survival, productivity, distribution and normal biological activities (Anita and Pooja, 2013). Inability of fishes to adapt to temperature fluctuations is responsible for the inability of fishes to respond physiologically to the environment and hence result to death (Ayanwale *et al*, 2014). As the degree of water temperature increases it produce highly stress conditions on fishes, the degree of toxicity produced is dependent upon environmental conditions such as temperature, pH of water, oxygen content and presence of residue molecules (Tantanpale *et al*, 2009).

The rise in CO₂ can put stress on aquatic ecosystems due to ocean acidification, an overall decrease in the pH of the ocean's waters. Freshwater ecosystems, already stressed by pollution and recent increases in the number of invasive species are also showing signs of acidification due to the increase in CO₂. The effect of the rise in acidity is known to be harmful to calcifying organisms.

As more and more fossil fuel burning cars and factories are being built around the world, the amount of CO₂ being released into the atmosphere is increasing. The increasing amount of CO₂ not only causes a problem for people and animals but also aquatic ecosystems around the world (Adam Parker, 2015).

Carbon dioxide rapidly diffuses into the water causing the pH to become more acidic (Arnold *et al*, 2012). In the marine environment, the CO₂ reacts with seawater, forming carbonic acid and can ultimately result in ocean acidification. Increase in atmospheric CO₂ concentrations have led to an increased rate of CO₂ diffusion into the oceans. Ultimately, this would lead to the oceans becoming more and more acidic. If the pH of oceans rises at a rate quicker than the vegetation can adapt, submerged aquatic vegetation species could be endangered.

While marine ecosystems seem to be the main concern, it is plausible that freshwater ecosystems will also experience increasing pH as CO₂ diffuses from the atmosphere into lakes and rivers (Strumm and Morgan, 1996).

METHODOLOGY

MATERIALS

- Fahrenheit thermometer - used for measuring the temperature (range = -0°F to 400°F, least count =2.0)
- Measuring cylinder-used for measuring the solutions (5mL)
- pH test paper- used for determining the pH of the water
- Test tube - in which sampled water was taken for testing the pH
- Regular fish bowl aquarium
- 2 Gold fish
- Vinegar
- Baking soda
- Cold water
- Hot water
- Normal tap water

METHOD

This project experiment was conducted at Parakattu house near Pulleppady bridge, Ernakulam, Kerala for 7 days from 6th February 2022 to 12th February 2022. The goldfishes (*Carassius auratus*) were purchased from tropical aquarium, South Ernakulam, Kerala. The water used for the experiment was regular tap water from Cochin co-orporation.

A regular fish bowl aquarium filled with about 1 litre of water was taken. The current temperature of the water was equal to that of the room temperature (20°C (68°F)). The opercular respiratory rate (ORR) was observed for 1 minute and the result was noted. The opercular respiratory rate of the fish was calculated by counting the number of times the gills of the fish was opened. Three trials were taken and the average of 3 trials was taken as the final result. The fish was removed from the bowl carefully.

Next the second fish was introduced into the bowl naming it the fish 2. The opercular respiratory rate of the fish at (20°C (68°F)) was noted down. The fish 2 was removed carefully from the bowl.

The temperature was then reduced to 10°C (50°F) by adding required amount of ice cold water and the experiment was repeated as above using fish 1 and fish 2.

The temperature was then set to 25°C (77°F) by adding hot water (5ml/min) over 5 mins into the bowl and the experiment was repeated with both fishes and the observation noted down. The experiment was carried out at 30°C (86°F) and 35°C (95°F) also in the same manner.

The effect of water acidity on the opercular respiratory rate of the fish was studied by introducing the fish to water at different pH. The pH was tested using a pH paper and was found to be neutral (around pH 7). The ORR of both fishes were noticed. The pH of the water was set to pH 5 by adding vinegar 1ml/min over 5mins. The fish 1 was introduced and the opercular respiratory rate was calculated. The fish 1 was then removed and fish 2 was introduced and the observation was noted.

The pH of water was then changed to pH 6 by adding water 5ml/min over 5mins and the experiment was carried out. The pH of water was again increased to pH 8 by adding a little bit of baking soda. The ORR of both fishes were calculated and formulated into tables for all 7 days. The graph was plotted using the average ORR value for 7 days.

OBSERVATION

Table 1 showing the effect of temperature on ORR of fish 1 for 7 days

SL NO:	TEMPERATURE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	20°C	76.3	77	76	75.6	77	75.6	74.3	75.9
2.	10°C	61	59.6	60.3	67.6	64	57.6	58	61.1
3.	25°C	79	83	77.3	82	83	82.3	78	80.6
4.	30°C	82.3	86	84.6	88	87.3	88	83.3	85.6
5.	35°C	101.3	106	97.6	106	99.3	103.3	100.6	102

From Table 1 it was noticed that the ORR of fish 1 was 75.9 at room temperature which reduced to 61.1 when temperature was reduced to 10°C. The ORR increased when the temperature was increased to 25°C, 30°C and 35°C.

Table 2 showing the effect of temperature on ORR of fish 2 for 7 days

SL NO:	TEMPERATURE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	20°C	76.6	76.6	78.3	76.6	78.6	77	77.6	77.3
2.	10°C	58.6	61.6	64.6	65	65.3	62.3	64	63
3.	25°C	80	80	80	81.6	80.6	81	78.6	80.2
4.	30°C	85.6	82.6	86.6	87	86.6	85	86	85.6
5.	35°C	101.6	98.3	101.3	103.6	100.3	100.3	101.6	101

From Table 2 it was noticed that the ORR of fish 2 was 77.3 at room temperature which reduced to 63 when temperature was reduced to 10°C. The ORR increased when the temperature was increased to 25°C, 30°C and 35°C.

Table 3 showing the effect of pH on ORR of fish 1 for 7 days

SL NO:	pH	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	5	112.6	99.3	104.3	115	103.6	115.3	112	108.8
2.	6	109.3	104.3	99.6	111	101	108.6	110.6	106.3
3.	7	74.6	79.3	75.6	79	80	79	77.6	77.8
4.	8	79	80	77.6	81.6	78.6	78.3	80	90.5

From table 3 it was observed that the ORR of fish 1 at pH 5 was 108.8, when pH was increased to 6, ORR was found to be 106.3, when the pH was 7 ORR was noted 77.8. when the pH was increased to 8 ORR was noted as 90.5.

Table 4 showing the effect of pH on ORR of fish 2 for 7 days

SL NO:	pH	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	AVERAGE ORR
1.	5	102.6	108.3	105.6	104.3	108.3	102.3	101	104.6
2.	6	103.3	104.6	97.3	100.3	101	99	99.3	100.6
3.	7	79	80.3	79	76.3	80.3	77	81.3	79
4.	8	80.3	81.6	82.6	79	80	82.6	79	80.7

From Table 4 it was observed that the ORR of fish 2 was 104.6 at pH 5, then it decreased to 100.6 at pH 6. At pH 7 the ORR was found to be 79 and when this pH was increased to 8 the ORR changed to 80.7

RESULT

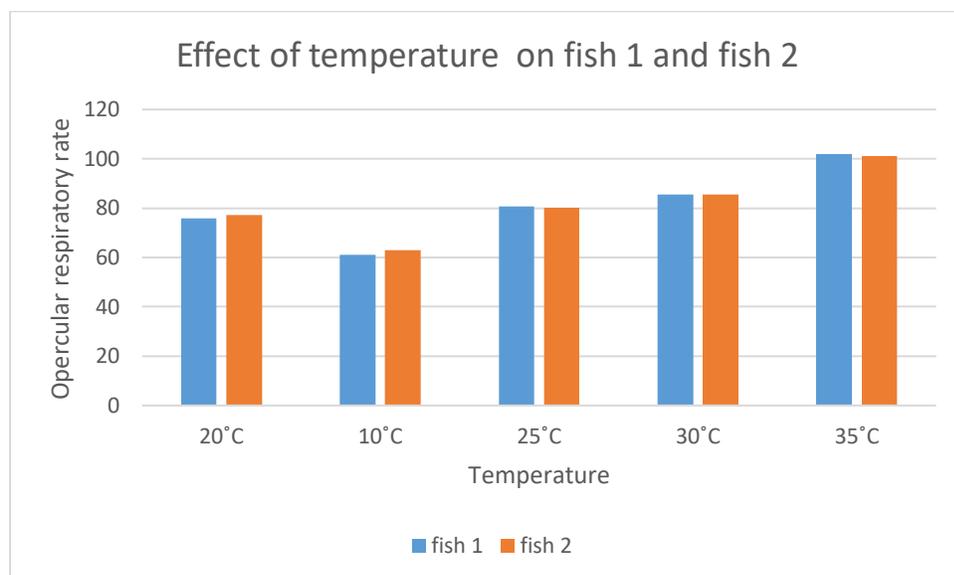


Figure 1 showing the effect of temperature on ORR of fish 1 & fish 2

Figure 1 shows when temperature is 20°C, both the fishes had a ORR between 75-78, when the temperature was reduced to 10°C the ORR was between 61-63. The temperature was increased to 25°C and th ORR was between 80.2-80.6. Again the temperature was increased to 30°C and 35°C and their ORR was found to be 85.6 and 101-102 respectively.

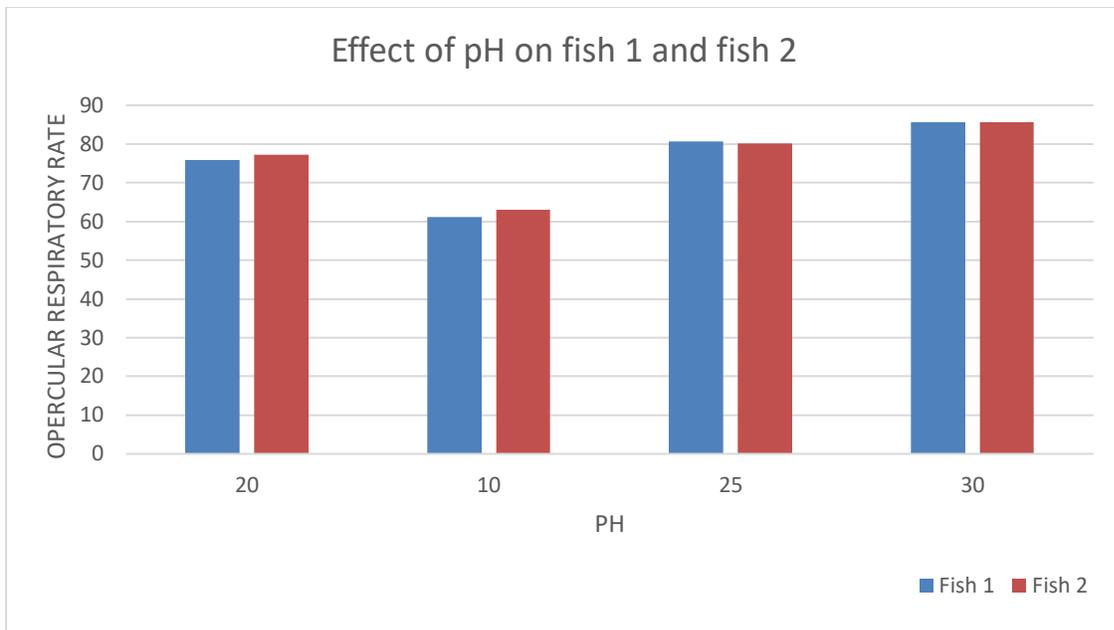


Figure 2 showing the effect of pH on ORR of fish 1 and fish 2

Figure 2 shows that at normal pH (pH 7) fish 1 and fish 2 had an opercular respiratory rate between 60-80. The respiratory rate was normal and showed no signs of defects which showed that pH 7 is the ideal pH for fish keeping.

When pH is at 5, fish 1 and fish 2 has an opercular respiratory rate between 100 - 120. At this pH the respiratory rate was very much higher than the normal respiratory rate (which was around 75-80) and the fishes showed signs of stress and the breathing was rapid.

When pH was at 6, fish 1 and fish 2 has the opercular respiratory rate between 80-120. At this pH the respiration of the fishes were not normal and also showed signs of stress like large mouth gasping for air.

When pH was at 8, fish 1 and fish 2 has opercular respiratory rate between 60-100 respectively. At pH 8 the respiratory rate was around to the normal respiratory rate. At pH 8 the water was alkaline therefore respiratory rate was also increased.

DISCUSSION

Global Warming and acidification has caused drastic effects on the water bodies across the world. Due to Global Warming, fish species has been extremely affected. As the water warms, fish need more oxygen to perform daily activities, like feeding. Change in temperatures, therefore, affects fish metabolic activities.

The surrounding water bodies absorbs most of the excess heat from greenhouse gas emissions, leading to a rise in water temperature. Increase in temperatures affect marine and freshwater species and ecosystems. Also leads to coral bleaching and the loss of breeding grounds for fishes.

Fishes don't have shells, therefore they will feel the effects of acidification. Because the surrounding water has a lower pH, a fish's cells often come into balance with the water by taking in carbonic acid. This changes the pH of the fish's blood, a condition called acidosis.

At lower pH levels, adult fishes start dying, fish eggs are unable to hatch, absence of fishes in highly acidic waters. Even if a particular species of fish is able to survive in that acidic environment, the animals feeding on these fishes won't be able to survive long, as accumulation of acid takes place in their body and leads to ultimate death.

In this present experiment, two goldfishes belonging to same species were taken and opercular respiratory rate was measured at different temperatures and pH. The experiment was done with regular tap water. About 1 liter of water was filled in a regular aquarium. The current temperature of water was 20°C (68°F). The ORR of both fishes were observed at this temperature. Similarly this procedure was repeated for 10°C, 25°C, 30°C and 35°C, and the observations and results were formulated into tables and graphs. To increase the temperature hot water was used and for decreasing the temperature ice cold water was used.

The effect of pH on ORR of fishes was the second parameter observed, both fishes were introduced to different pH. The pH were pH 5, pH 6, pH 7 and pH 8. Vinegar was used for making the water acidic and baking soda was used for making alkaline condition.

Several kinds of research were conducted by several scientists with the objective to determine how temperature as well as water acidity have an impact on the opercular respiratory rate (ORR) of fishes.

According to the research conducted by Ahmed *et al.* (2015) says that temperature is regarded as an environmental factor that can affect activity, behaviour, feeding, growth, survival, appetite and reproduction in all fishes. Temperature variation in water bodies depend largely on their geological location (latitude, longitude and altitude). In the tropics and sub tropics, marked variations in temperature and rainfall between the rainy and dry season affect the physico - chemical characteristics of the water.

Akin (2003) reported that Cold water has more dissolved oxygen than warm water thus as temperature increases less oxygen is available to the biota. Therefore it was found that at higher temperatures fish respiratory rate also increased as demanding for more oxygen.

In the research conducted by Kazumasa *et al* (2000) it says that the phenomenon called acid rain results from industrial activities where sulfuric and nitric acid are produced by the release of sulfuric oxides (SO_x) and nitrogen oxides (NO_x) into the atmosphere. Acid rain induces the acidification of inland waters which results in damage to aquatic ecosystems, including fish.

Fish have the ability to regulate their acid-base balance in order to maintain normal pH of their body fluids under acidic ambience. When fish are exposed to a low pH, chloride cells in the gill tissue take up bicarbonate (HCO_3^-) ion from the outside to neutralize the hydrogen (H^+) ion flowing in the body. At this time, the loss of sodium (Na^+) and chloride (Cl^-) ions from the body fluids occurs, and plasma osmotic pressure decreases. This process is considered to be one of the major reasons why freshwater fish die under acidic conditions.

Therefore all these research papers have similarity with this present work that increase or decrease in the water temperature and water acidity have a major effect on the opercular respiratory rate of fishes. Opercular respiratory rate of fish is directly proportional to the water temperature i.e., respiratory rate increases with the increase in the temperature and vice versa. This is because as temperature increases availability of oxygen decreases and leads the fish to demand for more oxygen. Water acidity is another factor responsible for opercular respiratory rate, at lower pH fish shows signs of difficulties and slowly leads to death. Therefore both temperature and pH are essential factors responsible for the opercular respiratory rate (ORR) of fishes.

CONCLUSION

The world has seen an increase in the amount of carbon dioxide (CO₂) in the atmosphere. The rise in CO₂ can put stress on aquatic ecosystems due to ocean acidification, an overall decrease in the pH of the ocean's waters. This is caused by global warming and acidification. Through this study we found the favourable temperature and optimal pH of the water. Temperature and water acidity are some major factor responsible in the opercular respiratory rate of aquatic fishes. From this study we can conclude the optimal temperature and pH where an aquatic animals can survive.

Temperature is an essential factor which is involved in the respiration of aquatic fishes. Nowadays temperature is getting increased day by day because of global warming. By this study it shows that at higher temperatures the fishes showed high respiratory rate and at low temperature fishes showed lower respiratory rate, this is because at lower temperature (cold water) the amount of dissolved oxygen is greater and at higher temperature the amount of dissolved oxygen is less, therefore the fish needed to take more amount of oxygen to survive. At 20°C of temperature it was found that the respiratory rate was around 75-80 range and we can conclude that this is probably the normal temperature in which the fish can survive without having any problems. At high temperature i.e., 35°C when temperature is increased respiratory rate also increased. Likewise at lower temperature i.e., 10°C and it showed lower respiratory rate. From this experiment it was found that optimal temperature where the fish can survive is 20°C.

Acidity is the next major factor that causing the variation in respiratory rate. Due to acidification the acidity of water is increased day by day. At pH 7 the respiratory rate of fish was normal and it showed no signs of stress, therefore this can be the optimal pH where the fish can survive. At pH-8 the respiratory rate ranges from 80-100 and 60-80 respectively. At a pH of 5 the fishes showed signs of difficulties and was having a wide mouth for gasping more air. Thus the optimal pH of the water that fishes can survive is pH 7 -pH 8. As CO₂ gets dissolved in the waters, the water become acidic and the oxygen content of the water decreases thus making difficulty for the survival of aquatic organisms.

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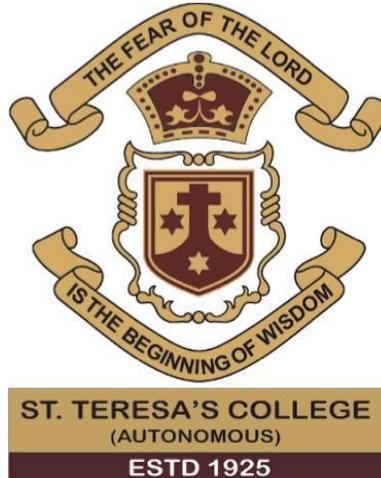
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**A STUDY ON THE EFFECT OF *CATHARANTHUS ROSEUS* EXTRACT
ON REGENERATION IN EARTHWORM**



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Submitted to St Teresa's College (Autonomous), Ernakulam**

**Affiliated to Mahatma Gandhi University, Kottayam in partial fulfillment of
requirements for the Degree of Bachelor of Science in Zoology**

2021-2022

CERTIFICATE

This is to certify that the project report entitled "**STUDY ON THE EFFECT OF *CATHARANTHUS ROSEUS* EXTRACT ON REGENERATION IN EARTHWORM**" submitted by Ms. FATHIMA FARZANA M.N, Reg. No. AB18ZOO035 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Akhila Anilkumar and this is her original effort.

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EXAMINERS

1)

2)

DECLARATION

I, Ms. FATHIMA FARZANA M.N hereby declare that this project report entitled “A STUDY ON THE EFFECT OF *CATHARANTHUS ROSEUS* EXTRACT ON REGENERATION IN EARTHWORM” is a bonafide record of work done by me during the academic year 2021-2022 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

FATHIMA FARZANA

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ABSTRACT

Earthworms are terrestrial invertebrates that belong to the phylum Annelida. They exhibit a tube-within-a-tube body plan; they are externally segmented with corresponding internal segmentation; and they usually have setae on all segments. They occur worldwide where soil, water, and temperature allow. The main aim of our study is to get a more comprehensive understanding on the effects of different concentrations of *catharanthus roseus* extract on the regeneration of earthworms, *Pheretima posthuma*. For that, 21 earthworms of the species *Pheretima posthuma* were collected. They were cut into equal halves and their growth was observed in soil containing different concentrations of the *catharanthus roseus* extract. The experimental results proved that *catharanthus roseus* in concentrations 10%, 20%, 40% and 80% promotes the regeneration of earthworms. Therefore, low concentrations of *Catharanthus roseus* extract can be used as a regeneration promoting substance for earthworms. It may be suggested that *catharanthus roseus* can be used in soils and vermicompost during vermiculture for preventing the loss of earthworms due to autotomy or other mechanical injuries and to progress their regeneration process. The promotion of better health on earthworms can be helpful in agricultural practices as they help fertilize the soil better

INTRODUCTION

An earthworm is a terrestrial invertebrate. They occur worldwide where soil, water, and temperature allow. Earthworms are commonly found in soil, eating a wide variety of organic matter. This organic matter includes plant matter, living protozoa, rotifers, nematodes, bacteria, fungi, and other microorganisms. The principal systematic features of earthworms are that they are bilaterally symmetrical, externally segmented, with a corresponding internal segmentation. They have no skeleton and a thinly pigmented cuticle, bearing setae on all segments except the first two; with an outer layer of circular muscles and an inner layer of longitudinal muscles. They are hermaphrodite and have relatively few gonads, which are situated in definite segmental positions. When mature, a swollen area of the epidermis called a clitellum, located in particular segments, forms a cocoon in which the eggs or ova are deposited, and this is then passed over the anterior segment. The eggs are usually fertilized and the young develop within the eggs without a free larval stage, the newly hatched worms resembling adults. Structurally, earthworms have large coelomic cavities containing coelomocytes, a closed vascular system with at least a dorsal and a ventral trunk and a ventral nerve cord. The alimentary canal is basically an anterior-posterior tube with excretion through the anus or specialized organs called nephridia; respiration is mainly cuticular.

Pheretima posthuma



Kingdom	Animalia
Phylum	Annelida
Class	Oligochaeta
Order	Haplotaxida
Suborder	Lumbricina
Family	Megascolecidae
Genus	Pheretima
Species	Posthuma
Nomenclature code	ICZN

Body is long, narrow and cylindrical. Length may reach upto 150 mm. Body colour is brown. Anterior end is pointed while the posterior end is blunt. Body is divided into 100-140 segments called metameres. The anteriormost segment is called Prostomium. Mouth is a crescentic aperture, present at anterior end. The segment containing mouth is called peristomium. Setae are present at all the segments except-1st and last. Each seta is embedded in a setal sac. A glandular band called clitellum is situated in 14th to 16th segments. It forms cocoon during the reproduction. Female genital pore is situated in 14th segment while male genital pore is present in 18th segment. The earthworm feeds on organic matter in the soil.

The food is sucked by the pharynx and the oesophageal glands add calcite to neutralise acidity of the soil. The food is then grinded by the horny lining of the gizzard and is absorbed in the intestine. Undigested food material passes out the anus and is deposited as worm castings. The earthworm 'breathes' by the diffusion of gases through its moist skin. The blood contains haemoglobin which transports oxygen throughout the body. Circulatory system is of closed type. Earthworms have no sense organs but they can sense light intensity by small light-sensitive cells found mainly on the upper skin surface of their body. They can also sense vibrations and chemicals by the means of tactile or chemo-receptors. The earthworms exhibit undulating

movement which takes place by alternate contraction and relaxation of circular and longitudinal muscles of each segment. Earthworms are hermaphrodites but they reproduce by cross-fertilization.

IMPORTANCE OF EARTHWORM

Of all the members of the soil food web, earthworms need the least introduction. Most people become familiar with these soft, slimy, invertebrates at a young age. An earthworm is a segmented worm, a terrestrial invertebrate belonging to phylum Annelida. Earthworms occur in most temperate soil and many tropical soils. They are major decomposers of dead and decomposing organic matter, and derive their nutrition from bacteria and fungi that grow upon these materials. They fragment organic matter and make contribution to recycling the nutrients it contains. Earthworms dramatically change soil structure, water movement, nutrient dynamics and plant growth. They are not essential to cell healthy soil systems but their presence is usually an indicator of a healthy system. Earthworms perform several beneficial functions.

Stimulate microbial activity: Earthworms derive their nutrition from microorganisms, many more microorganisms are present in their feces/casts than in organic matter that they consume. Increased microbial activity facilitates the cycling of nutrients from organic matter and their conversion into a form readily taken up by plants.

Mix and aggregate soil: As they consume organic matter and mineral particles, earth excreta wastes is the form of casts, a type of soil aggregate.

Increased infiltration: Earthworms enhance porosity as they move through the soil. Some species make permanent burrows deep into the soil. It can be a major conduit for soil drainage particularly under heavy rainfall. At the same time burrows reduce surface water erosion.

Improve water-holding capacity: Earthworms can significantly increase the water-holding capacity of soils.

Provide a channel for root growth: The channel made by deep burrowing earthworms are lined with readily available nutrients and make it easier for roots to penetrate deep into the soil.

Bury and shred plant residue: plant and crop residue are gradually buried by cast material deposited on the surface and as earthworms pull surface residue into their burrows.

Interaction of earthworm with other members of the food web: Earthworms influence soil-inhabiting invertebrates by changing the amount and distribution of organic matter and microbial population.

Economic Importance of Earthworm:

Earthworms are extremely beneficial in agriculture. They aid in the following ways. The earthworm improves the fertility of soil in different ways, they are of utmost importance in agriculture. The burrowing and soil feeding habits of earthworms make the soil porous which permit both aeration and quick absorption of water. They also reduce the alkalinity and acidity of the soil to provide better conditions for plant growth. Thus earthworms are better known as the friend of farmers. Many people earn their livelihood by catching these worms and supplying them to scientific laboratories. Ayurvedic and unani systems of therapy suggest that these worms are used in making medicines for the care of diseases like bladder stones, piles, rheumatism, jaundice etc. They are also used as baits to catch fish. It also plays a great role in Vermiculture to produce high-quality manure.

REGENERATION IN EARTHWORM

Regeneration in earthworm is an epimorphic regeneration, which is very sensitive to the environmental situations. The existence of stem cells help the worm to regenerate the lost organs. This kind of regeneration could be happened due to the low level of differentiation in these organisms to the comparison with higher level organisms in the evolutionary tree. But this ability varies between species and depends on the extent of the damage. There were significant differences in both survival rates and lengths of regeneration between immature earthworms and clitellate adult earthworms during the early stages of regeneration, but not at later stages of regeneration. The immature earthworms had a greater Regeneration potential than clitellate adults amputated at the same segment. The survival rates of earthworms were correlated significantly with the number of body segments remaining after amputation, but not with the position of the amputation. If an earthworm is split into two, it will not become two new worms. The head of the worm may survive and regenerate its tail if the animal is cut behind the clitella. But the original tail of the worm will not be able to grow a new head or the Rest of its vital organs and will instead die.

Present study focused to determine the effect of medicinal plant *catharanthus roseus* on earthworm regeneration

Common name : Cape periwinkle

Family : Apocynaceae

Scientific name : *Catharanthus roseus*

Useful part. : Leaf, root, shoot , stem

Medicinal plants have a long history of usage in traditional medicine. Ethno-botanical information on medicinal plants and their usage by indigenous cultures is useful in the conservation of traditional cultures, biodiversity, community health care and drug development. *Catharanthus roseus* L.Don, is an important medicinal plant belonging to the Apocynaceae family; this plant is a dicotyledonous angiosperm and synthesizes two terpene indole alkaloids: vinblastine and vincristine that are used to fight cancer. Peckolt, in 1910, described the use in Brazil of an infusion of the leaves to control hemorrhage and scurvy, as a mouthwash for toothache, and for the healing and cleaning of chronic wounds. In Europe related species have been used for the proprietary suppression of the flow of milk. In the British West Indies it has been used to treat diabetic ulcer and in the Philippines has been reported as being an effective oral hypoglycemic agent. More recently, Chopra et al. have reported that the total alkaloids possess a limited antibacterial activity as well as a significant and sustained hypotensive action. The hypoglycemic and antibacterial activities have not been confirmed, although one of the alkaloids isolated from this plant, ajmalicine, has been reported to possess transient depressor action on arterial blood pressure Periwinkle” or *Catharanthus roseus* (Family Apocynaceae), commonly known as “Nayantara” or “Sadabahar”, the word *Catharanthus* derives from the Greek language meaning "pure flower." While, *roseus* means red, rose or rosy.

Wound healing property : Rats treated with 100 mg /kg/day of the *Catharanthus roseus* ethanol extract had high rate of wound contraction significantly decreased epithelization period, significant increase in dry weight and hydroxyproline content of the granulation tissue when compared with the controls. Wound contraction together with increased tensile strength and hydroxyproline content support the use of *C. roseus* in the management of wound healing



Earthworms are extremely important in soil formation, maintenance and structure and turnover of dead organic matter. Because of their availability and rapid regenerative power, annelids have been used commonly to study regeneration. Among annelids, the earthworm, which is important in breaking down organic wastes, has been used commonly for research into regeneration, because it is easy to culture and handle in the laboratory. This issue of regeneration of segments by amputated earthworms also has practical application to the build up of populations, especially since it appears that amputation has greater effects on mature earthworms than immature ones. Clearly, further research is needed to clarify the mechanisms of regeneration.

OBJECTIVE

1. To investigate how much a worm can be left to regenerate a new worm or if one end is better regenerating than the other.
2. To see the effect of *Catharanthus roseus* extract on regeneration of earthworms, *Pheretima posthuma*.
3. To test whether higher concentrations of *Catharanthus roseus* would speed up the regeneration of earthworm, *Pheretima posthuma*.

REVIEW OF LITERATURE

Charles Darwin (1809–1882) published his last scientific book entitled “The formation of vegetable mold through the action of worms with observations on their habits”, the result of several decades of detailed observations and measurements on earthworms and the natural sciences. The book covers the importance of earthworm activity on a variety of topics: pedogenesis and weathering processes, soil horizon differentiation and the formation of vegetable mold (topsoil), the role of earthworm burrowing and casting (bioturbation) in soil fertility and plant growth, the burial of organic materials and soil enrichment with mineral elements, the global cycle of erosion–sedimentation with hydrologic and aerial transfers of fine particles brought up to the soil surface by earthworms and the protection of archaeological remains through their burial. Finally, Darwin also performed a series of original experiments to determine if earthworms possessed, or not, a certain “intelligence”.

Gairdner B Moment (1949) explains the variation and causation in earthworm regeneration in ‘Journal of Experimental Zoology’. The coefficient of variation for the number of segments regenerated posteriorly by earthworms rarely exceeds 10. The manner of wound healing after the transaction cannot be responsible for the variation since under the conditions of these experiments all worms healed in the open manner. Bilateral asymmetry in the number of segments regenerated indicates that the counting mechanism underlying variation can hardly be humoral because both blood and coelomic fluid are common to both sides of the body.

Clive A. Edwards *et al.*, (1996) proposed a paper on biology and ecology of earthworms. It is in the third edition of this popular text where reviews on all aspects of earthworm biology and ecology are mentioned. These include a greatly expanded treatment of earthworm community ecology, interactions between earthworms and microorganisms, and the importance of earthworms in environmental management and their use in organic waste management. The book also summarizes the toxicity to earthworms of a wide range of chemicals.

“A science fair project” by Lloyd H. Barrow (2000) is a book on small animals that live in the ground; earthworms. The book makes observations about earthworm structure and behavior,

movement, preferences, and reactions. Included in the book are easy-to-follow diagrams that offer readers exact information on performing the experiments.

Bely A and Wray G (2001) conducted a study on evolution of regeneration and fission in annelids : insights from engrailed and orthodenticle class gene expression development. They presents a detailed comparison of regulatory gene expression during regeneration and asexual reproduction (by fission) in the segmented worm *Pristina leidy* (Annelida: Oligocheta). In Situ hybridization studies on worms undergoing normal growth, regeneration and fission demonstrated that in all three processes, *Pl-en* is primarily expressed in the developing nervous system, *Pl-Otx1* and *Pl-Otx2* are expressed primarily in the anterior body wall, foregut and developing nervous system. They state that these annelids fission may have evolved by recruitment of regenerative processes. Furthermore, by comparing the existing data from leech embryos, they found evidence that embryonic processes are re-deployed during regeneration and fission.

A study was conducted by Shishin Kawamoto., *et al.*, (2005) on bipolar head regeneration induced by artificial amputation in *Enchytraeus japonensis*. As per the study, *Enchytraeus japonensis* propagates asexually by spontaneous autotomy. Normally, each of the 5-10 fragments derived from a single worm regenerates a head anteriorly and a tail posteriorly. Occasionally, however, a head is formed posteriorly in addition to the normal anterior head, resulting in a bipolar worm. Experiments to clarify how the head and the tail are determined during regeneration in this species were conducted. The results showed that (1) bipolar head regeneration occurred only after artificial amputation, and not by spontaneous autotomy, (2) anesthesia before amputation raised the frequency of bipolar head regeneration, and (3) an extraordinarily high proportion of artificially amputated head fragments regenerated posterior heads. Close microscopic observation of body segments showed that each trunk segment has one specific autotomic position, while the head segments anterior to the VIIth segment do not. Only the most posterior segment VII in the head has an autotomic position. Examination just after amputation found that the artificial cutting plane did not correspond to the normal autotomic position in most cases. As time passed, however, the proportion of worms whose cutting planes corresponded to the autotomic position increased. It was suspected that the fragments autotomized after the artificial amputation (corrective autotomy). This post-amputation autotomy

was probably inhibited by anesthesia. The rate at which amputated fragments did not autotomize corresponded roughly to the rate of bipolar regeneration. It was hypothesized then that the head regenerated posteriorly if a fragment was not amputated at the precise autotomic position from which it regenerated without succeeding in corrective autotomy.

BS Nayak *et.al.*, (2007) conducted a study on Evaluation of wound-healing potential of *Catharanthus roseus* leaf extract in rats. This study shows Significant wound-healing activity was observed in animals treated with the *C. roseus* leaf extract compared with those who received the reference standard and placebo control treatments. It shows the effects of the EtOH extract of *C. roseus* leaves, at a dose of 100 mg kg⁻¹ day⁻¹ on wound-healing activity in rats. In the excision wound model, *C. roseus*-treated animals showed a significant reduction in the wound area (Pb0.002) and period of epithelization. In the incision wound model, *C. roseus*-treated animals demonstrated high skin-breaking strength up to 435.0 ± 4.53. In the dead space wound model, the EtOH extract-treated animals showed significantly increased levels of

Sung-Jin Cho *et al.*, (2009) had explained about expression of three labial genes in earthworm head regeneration, in “The differential Expression of Three labial Genes during Earthworm Head Regeneration”. Here they report the full length cloning of three labial genes (Pex-lab01, Pex-lab02, and Pex-lab 03) in the earthworm *Perionyx excavatus*. To analyze their expression pattern during head and tail regeneration, they used the reverse transcription-polymerase chain reaction. Their results indicate that the three labial genes were expressed only in the head regenerating tissues.

A study conducted by Nengwen Xiao *et al.*, (2011) on the regeneration capacity of an earthworm, *Eisenia fetida* in relation to the site of amputation along the body. Their aim was to link the regeneration capacity of an earthworm, *Eisenia fetida* with the site of amputation, so they amputated earthworm at different body segment location along the length of the body to examine the different survival rates and regeneration length of anterior, posterior and medial sections.

Yvan Capowiez *et al.*, (2015) explains about the morphological and functional characterization of the burrow systems of six earthworm species in “morphological and functional

characterisation of the burrow systems of six earthworm species (Lumbricidae)". Earthworm burrow systems are generally described based on postulated behaviors associated with the three ecological types. In this study, they used x-ray tomography to obtain 3D information on the burrowing behavior of six very common anecic (*Aporrectodea nocturna* and *Lumbricus terrestris*) and endogeic (*Aporrectodea rosea*, *Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Aporrectodea icterica*) earthworm species, introduced into repacked soil cores for 6 weeks. A simple water infiltration test, the Beerkan method, was also used to assess some functional properties of these burrow systems. Endogeic worms make larger burrow systems, which are more highly branched, less continuous and of smaller diameter, than those of anecic worms. Regarding water infiltration, anecic burrow systems were far more efficient due to open burrows linking the top and bottom of the cores. For endogeic species, we observed a linear relationship between burrow length and the water infiltration rate ($R^2 = 0.49$, $p < 0.01$). Overall, the three main characteristics significantly influencing water infiltration were burrow length, burrow number and bioturbation volume. This last characteristic highlighted the effect of burrow refilling by casts.

A study conducted by MT Rosa *et al.*, (2017) on Aloe Extracts, Pro and Antioxidant Conditions in "Regeneration of the Planarian *Girardia tigrina*", reported that regeneration of the planarian *Girardia tigrina* was evaluated over different oxidative conditions, as pro oxidant (H₂O₂), antioxidant (vitamin C) and using aloe gel. The aloe plants have a millennial medicinal use and the succulent portion of leaf, called aloe gel, is used for wound healing. Here they analyze the action of aloe gel obtained from two species: *Aloe vera* and *A. arborescens*. The results show that ROS are important in the regeneration of *G. tigrina* and that the initial exposure to H₂O₂, soon after transection, accelerates the regeneration. However, during the regeneration process an antioxidant medium, containing vitamin C, promotes acceleration of regeneration, even if less intensely. Aloes extracts promote acceleration of regeneration in this planarian. No differences were observed among portions of cells in phases of cell cycle, with exception of worms exposed to *A.vera* extracts at 0.4% that show more cells in G2 phases, suggesting a faster cell cycling. No toxic effect was observed for the aloes extracts in planarians. Instead, an increase in the survival rate was observed in treated animals.

Jai Narayan Mishra, Navneet Kumar Verma (2017) proposed a paper on *Catharanthus roseus*. The paper gives information regarding the geographical distribution, and chemical constitution of *Catharanthus roseus*. Pharmacological and biological activity - Anticancer, Antioxidant, and wound healing property etc. of *Catharanthus roseus* are discussed.

Hanady SA Al-Shmgani *et.al.*,(2017) conducted a study on Biosynthesis of silver nanoparticles from *Catharanthus roseus* leaf extract and assessing their antioxidant, antimicrobial, and wound-healing activities. This study reveals that the *C. roseus* leaf extract-synthesized AgNPs exhibited a strong antimicrobial activity against several pathogens that were tested in present study. The tailored AgNPs prompted wound-healing potential in albino male mice using wound closure assay. The AgNPs also enhanced the wound-healing activity in mice by inhibiting the pathogenic bacterial growth in the wound area. On the basis of our previous and current findings, it is possible to conclude that the biosynthesized AgNPs could be considered as cost-effective, antioxidant, and effective therapeutic agent for controlling bacterial and fungal growth.

Yun Seon Bae *et al.*, (2020) discovered the Characterization of *Perionyx excavatus* Development and its head regeneration. The regeneration of the central nervous system is limited to specific animals including *Perionyx excavatus*. Here we set up a culture system to sustain the life cycle of *P. excavatus* and characterize the development of *P. excavatus* from embryo to juvenile, based on morphology, myogenesis and neurogenesis. Their data suggest that *P. excavatus* is a model system to study CNS regeneration.

Mini *et al.*,(2021) conducted a study on the effect of *Aloe vera* extract on regeneration in earthworm, *Lampito mauritii*. It was found that the anterior has potential to be renewed unlike the posterior which do not have any vital organs. This kind of regeneration could happen due to the low level of differentiation in these organisms. Data also revealed that *Aloe vera*, in small amounts (10-20%), has a positive effect on the regeneration of earthworm, speeding up regeneration time by about three days. More importantly, there was no significance in regeneration of earthworm in the very lower and higher concentrations of *Aloe vera*. Moreover, the higher concentrations (40-80%) of *Aloe vera* appeared to be toxic to the earthworm. Their

experimental results proved that *Aloe vera* had capability to promote regeneration of earthworms and it was suggested that *Aloe vera* can be used in vermicompost.

Neda Gholami *et al.*, (2021) had conducted a study on “In vivo assessment of APPJ discharge on the earthworm: coelomic TAC and MDA levels, cell death, and tissue regeneration”. The effective medical applications of cold atmospheric pressure plasma jet (APPJ) have been reported by many researchers including sterilization of liquid and solid surfaces, treatment of chronic wounds, cancer tumors and blood clots. Results showed APPJ induced significant effects on regeneration ability of earthworms after 20 and 30s of exposure ($p < 0.05$). Atmospheric plasma jet did not have significant effects on MDA content and TUNEL-positive cells, but this effect was significant for TAC and CAT in both species ($p < 0.05$). In conclusion the present study revealed for the first time that regeneration of missed segments in earthworms can be stimulated by plasma treatment.

METHODOLOGY

Collect the moist soil compost. Prepare 4 cups for the earthworms to live in by placing $\frac{1}{2}$ cup of moist compost in to the bottom of each cup. Choose 6 worms of good size and equal lengths, the front end of the worm will be closest to the clitellum. Using scissors cut the first 3 worms in half and place the two pieces of worm into two cups labeled, “front half” and “back half”. Using scissors cut 3 worms into a one-third piece two-third piece and place the two pieces of worm into separate cups with corresponding labels. Cover the cups with wrapping paper and secure to the tops of the cups with rubber bands. Poke several small air holes into the wrapping paper covering each cup. Keep the cups in a cool dark place for several days, make observations of worms in every 3 days and place $\frac{1}{2}$ cup of fresh moist compost into the cup. Continue to observe the worms every 3 days for 2 to 3 weeks. *Catharanthus roseus* extract is made into a paste and preserved in a refrigerator (-250C) for 3 days. Repeat the experiment by adding *Catharanthus roseus* solutions of 0%, 10%, 20%, 40% and 80% into soil in separate cups. Three worm fragments (fragments containing head region) were added to each mug containing the varying solutions of *Catharanthus roseus*. The length of each fragment was measured every 3 days.





RESULTS

Through this experiment, we observed that the regeneration of earthworm took place in the presence of *Catharanthus roseus* extract. *Catharanthus roseus* in 10% & 20% concentrations promoted regeneration in the cut area of the anterior half of equally cut earthworms. Wound healing properties of *Catharanthus roseus* may have aided in the regeneration process in the earthworms. Through this experimental study, the following results were obtained.

Table 1. Showing mean change in length of anterior regions of equal and unequal halves of three earthworms each

DAYS	Normal (equal halves)		Normal (unequal halves)	
	(Average change in length in cm)			
	Cup I	Cup II	Cup III	Cup IV
	Head region	Tail region	Head region	Tail region
1	4.6	4.6	4.6	3.6
4	4.7	4.6	4.7	3.6
7	4.8	4.6	4.9	3.6
10	5.5	4.6	5.4	3.6
13	5.4	Decayed	5.7	Decayed
16	6.3		6.2	
19	6.5		6.4	
22	6.7		6.6	
25	7.2		6.9	
28	7.4		7.1	

Fig 1. Showing the same rate of regeneration of anterior regions of both equal and unequal halves.

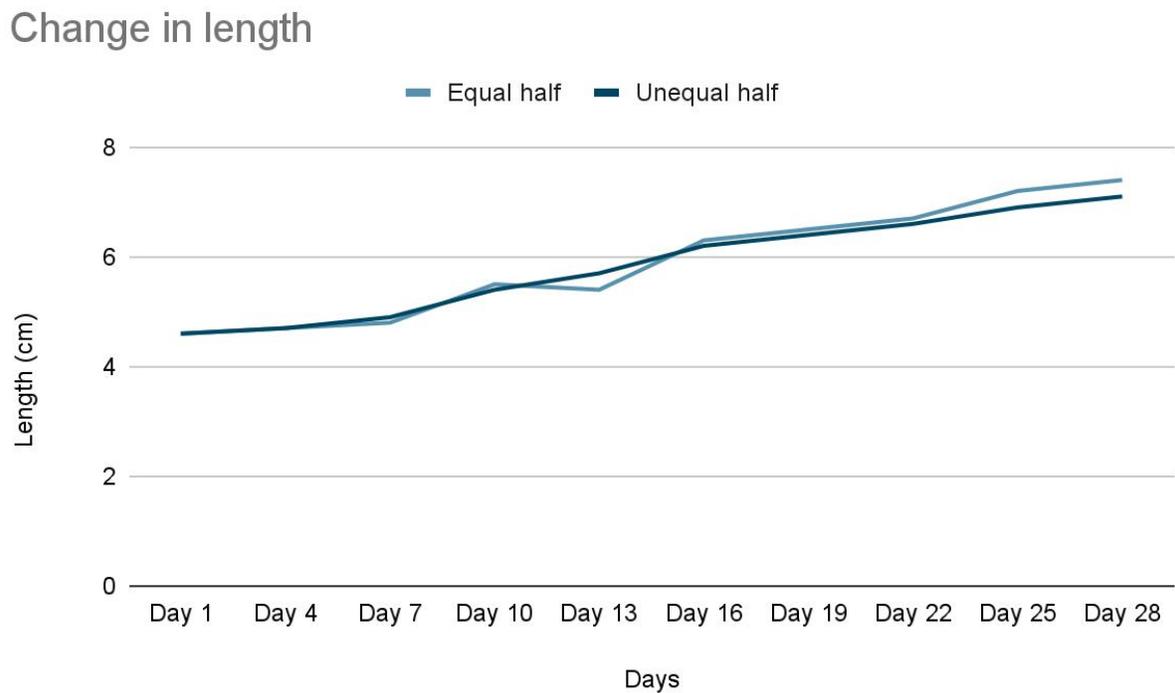
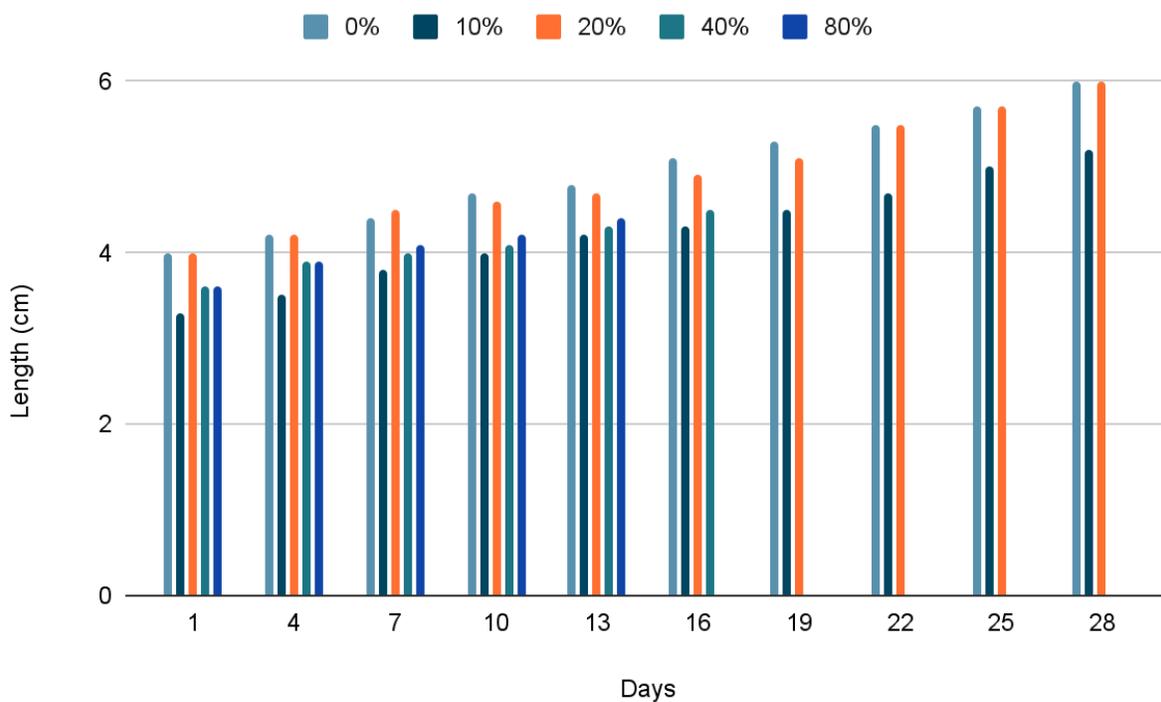


Table 2. Showings mean change in length of anterior regions of six earthworms after treatment with *Catharanthus roseus*

DAYS	% of <i>Catharanthus roseus</i> extract in moist soil compost				
	(Average change in length of front half in cm)				
	0%	10%	20%	40%	80%
1	4	3.3	4	3.6	3.6
4	4.2	3.5	4.2	3.9	3.9
7	4.4	3.8	4.5	4	4.1
10	4.7	4	4.6	4.1	4.2
13	4.8	4.2	4.7	4.3	4.4

16	5.1	4.3	4.9	4.5	Dead
19	5.3	4.5	5.1	Dead	
22	5.5	4.7	5.5		
25	5.7	5	5.7		
28	6	5.2	6		

Fig 2. Graph showing variation in growth of earthworm in different concentrations of *Catharanthus roseus*



DISCUSSION

In this experimental study, the regenerative power of earthworm in the presence of *Catharanthus roseus* extract was checked. *Catharanthus roseus* in various concentrations (10%, 20%, 40%, 80%) was added to the soil where the anterior half of equally cut earthworms were placed. It was found that worms in 10% & 20% concentrations showed regeneration. The changes in length were measured for 28 days. It can be concluded that *Catharanthus roseus* may have wound healing properties and it aids in regeneration of annelids.

In the article “The Differential Expression of Three labial Genes during Earthworm Head Regeneration” by Sung-Jin Cho *et al.*, (2009) had explained about expression of three labial genes in earthworm head regeneration. Here they report the full length cloning of three labial genes (Pex-lab01, Pex-lab02, and Pex-lab 03) in the earthworm *Perionyx excavatus*. Their results indicate that the three labial genes were expressed only in the head regenerating tissues. In my experiment also, only the head regions showed regeneration. The tail regions did not show change in length for 12 days and decayed on the 13th day.

In study conducted by Nengwen Xiao *et al.*, (2011) on the regeneration capacity of an earthworm, *Eisenia fetida* in relation to the site of amputation along the body, they amputated earthworm at different body segment location along the length of the body to examine the different survival rates and regeneration length of anterior, posterior and medial sections. My aim was to check the regenerative capacity of the worms in different concentrations of the same extract.

Jai Narayan Mishra *et.al.*, proposed a paper on *Cathranthus roseus* (2017). The paper gives information regarding the general properties, geographical distribution, and chemical constitution of *Cathranthus roseus*. Pharmacological and biological activity -Such as anti-cancer activity , anti-bacterial activity, wound healing property etc. seen in *Cathranthus roseus*. This helped in my study to know about the chemical properties of *Cathranthus roseus*. The selection of this plant was made possible because of this paper.

In the article "Evaluation of wound-healing potential of *Catharanthus roseus* leaf extract in rats" by BS Nayak *et al.*, (2017). Rats treated with 100 mg /kg/day of the *Catharanthus roseus* ethanol extract had high rate of wound contraction significantly decreased epithelization period, significant increase in dry weight and hydroxyproline content of the granulation tissue when compared with the controls. Wound contraction together with increased tensile strength and hydroxyproline content support the use of *C. roseus* in the management of wound healing. . In my experimental study, *Catharanthus roseus* increased the regenerative capacity of stem cells in earthworms. It may be due to the presence of two terpene indole alkaloids: vinblastine and vincristine it is used to fight against cancer. High amount of vinblastine and vincristine in 10 & 20% increase the regenerative capacity of the earthworm .At lower concentration, it promotes the growth which in turns at higher concentration (40 & 80%) may be toxic to the earthworm.

In the experimental study by Mini V S, *et al.*, titled "A study on the effects of *Aloe vera* extract on a regeneration in *Lampito mauritii*". They experimented to find the regenerative capacity of earthworm in *Aloe vera* extract. Their data revealed that *Aloe vera*, in small amounts (10-20%), has a positive effect on the regeneration of earthworm, speeding up regeneration time by about three days. More importantly, there was no significance in regeneration of earthworm in the very lower and higher concentrations of *Aloe vera*. Moreover, the higher concentrations (40-80%) of *Aloe vera* appeared to be toxic to the earthworm. Thus, their project revealed that higher concentrations of *Aloe vera* would become lethal to earthworms. Low or medium amount of *Aloe vera* can be used as a regeneration promoting substance for earthworms. Whereas in my experiment, the earthworms reached full potential in their regenerative capacity in 10 & 20% concentrations of *Catharanthus roseus* extract.

CONCLUSION

Through this study it can be concluded that earthworms react efficiently towards *Catharanthus roseus* extract in the property of regeneration. From high to low concentrations, it proved to be involved in the cellular processes of cell proliferation, morphogenesis and cell differentiation of the annelid body. The regenerative capacity of the annelid body combined with the chemical constituents of the *Catharanthus roseus* proved in the fast regeneration of the earthworms. Regeneration in earthworm is an epimorphic regeneration, which is very sensitive to the environmental situations, and at the optimum condition, regeneration of the anterior segments occurs but the posterior segments are destroyed. Therefore, the anterior has potential to be renewed due to the existence of stem cells, which help the worm to regenerate the lost organs, however at the tail segments of the worm the nervous, digestive and respiratory structures do not exist. This kind of regeneration could happen due to the low level of differentiation in these organisms in comparison with higher level organisms in the evolutionary tree. My data also revealed that *catharanthus roseus* extract helped significantly in the regeneration of earthworms, speeding up regeneration time. More importantly, regeneration was achieved in concentrations 10%,&20%. The experimental results proved that *catharanthus roseus* had capability to promote regeneration of earthworms and it can be suggested that *catharanthus roseus* can be used in soils and vermicompost during vermiculture for preventing the loss of earthworms due to autotomy or other mechanical injuries and to progress their regeneration process.

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MALE MATE PREFERENCE IN GUPPY (*Poecilia reticulata*):

DO MALES PREFER LARGER FEMALES.



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**Affiliated to Mahatma Gandhi University, Kottayam in partial
fulfillment of requirements for the Degree of Bachelor of Science in**

Zoology

2021-2022

CERTIFICATE

This is to certify that the project report entitled "**Male Mate Preference in Guppy:Do Males Prefer Larger Females**". Submitted by Ms. Haripriya T K, Reg No. AB19ZOO036 in partial fulfilment of the requirements of Bachelor of science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Akhila Anilkumar and this is her original effort.

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EXAMINERS

1)

2)

DECLARATION

I, Ms Haripriya T K hereby declare that this project report entitled "**Male Mate Preference in Guppy(*Poecilia reticulata*): Do Males Prefer Larger Females As Mate**". Is a bonafide record of work done by me during the academic year 2021-2022 in Partial fulfilment of the requirements of Bachelor's of Science Degree of Mahatma Gandhi University, Kottayam.

This work has not been undertaken or submitted elsewhere in connection with any academic course and the opinion furnished in this report is entirely my own.

Haripriya T K

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Haripriya T K

ABSTRACT

Guppies are Aquatic cyprinodontiformes of class Actinopterygii phylum chordata. Guppy fish were first noticed in central America and south America but now it's common in world wide as an ornamental fish. They feed on small algae and mosquito larvae etc. Distinctive feature of guppy is brightly coloured body and fins and give birth to live young ones. Guppies are also called as Rainbow fish and Million fish. More than 276 species are found. They have 2 year's of life span, and the biggest threat is water pollution and predators.

Although females are the choosier sex in most species, male mate choice is expected to occur under certain conditions. Theoretically, males should prefer larger females as mates in species where female fecundity increases with body size. However any fecundity related benefits accruing to a male that has mated with a large female may be offset by an associated fitness cost of shared paternity if large females are more likely to be multiply mated than smaller females in nature. Tested the above hypothesis and assumption using trinidad guppy, *Poecilia reticulata* by behavioral testing for male mate choice in the house with available facilities. In this study, the glass tank aquarium is divided into two compartment by glass piece itself, and it is covered by black paper. Then the male is visible to female by visual stimuli alone and full range of natural stimuli, to find out if the male prefer larger females as mates. The analysis shows that male guppies prefer relatively larger females for mating. The significance of this topic is because they produce more egg than smaller females and the female fecundity increases with body size. Reasonable thing in this study shows, mate choice was measured by giving males a choice of different-sized females, using only visual cues.

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INTRODUCTION

The social environment can shape the behaviour of animals, with important implications for their fitness, sexual selection and social Evolution . In such environment individuals may supplement their own personal information's about local ecological conditions with public informations by eavesdropping on social interactions between other animals and their behaviour. For example with public information acquired through eavesdropping, an individual may decide to copy the behaviour of other individuals or to behave independently. While copying of the behaviour of another individual can provide benefits to the copier, there can also be associated costs with copying for both the demonstrator and copier, such as increased resources competitions and risk of sperm competition. A change in the behaviour of a focal animal in response to the presence of non interacting by standers is referred to us an audience if at. Audience effect have been documented for a variety of context, including foraging, predators avoidance and mating choices.

The guppy is an important model species for the study of mate choice and sexual selection. It is an internally fertilizing, live-bearing poeciliid fish that lives in highly dynamic mixed-sex shoals, wherein both males and females exhibit mutual mate choice in a non resource-based, promiscuous mating system (Houde1997). Adult males and females are sexually dimorphic, with males being smaller and more colorful than females, which are not ornamented and uniformly pale olive. Males prefer larger (more fecund) and sexually receptive females. Females generally favor males with greater amounts of orange pigmentation in both pre copulatory mate choice and post copulatory cryptic female choice and also prefer larger males over smaller ones as mates. Most adult female guppies are multiply mated and males thus experience very high levels of sexual and sperm competition in natural populations. Consequently, male guppies are sensitive to perceived ambient changes in their risks of sexual and sperm competition including the presence of an audience of 1 or more rival males. This is as expected for species wherein females mate multiply and some degree of last-male sperm precedence occurs , such as in the guppy (Evans and Magurran 2001; Neff and Wahl 2004).

Females are generally the choosier sex, because they are relatively less abundant, invest relatively more in reproduction (Trivers 1972), or have lower potential reproductive rates than males. In polygynous specious with conventional sex roles, males should also have mating preferences and exhibit mate choice when ever female vary in quality (Parker 1983), mate

searching and mating costs vary among females, the operation sex ratio is female biased, male invest in parental care (Sargent *et.al*, 1986), and or sperm production limits male reproductive success.

Theoretically, male should mate preferentially with larger females in species where female fecundity increases with body size, because male fitness increases with the number of mating and offspring sired. However, if large females have more likely to be multiply mated than smaller females in nature, then the fecundity related benefits potentially acquiring to the male that has mated with a large females could be offset by an associated fitness cost of sperm competition and shared paternity (Wedell *et.al*, 2002). Consequently, male should access this fitness related benefits and cost when choosing to mate with females that vary in body size.

Guppies (*Poecilia reticulata*) are small small tropical fish , native to the coastal streams of north east south America. They owe their name to Robert john Lechmere guppy who introduced them into the aquarium trade . guppies have since become very popular aquarium fish ,known for colour full males and live bearing females. But guppies are also one of the premier model systems for the study of Ecology, evolution, genetics and sexual selections.

Guppies are extremely variable both Phenotypically and genetically. Sexually mature males exhibits an amazing array of differently coloured spots and strips, such that every male almost seems unique, making the guppy one of the most polymorphic vertebrates known. Although the females do not show such colouration , they vary in terms of their preferred mates both with and between population ,making the guppy a powerful system for studying sexual selection. Like other members of the family *Poecilidae*, such as swordtails and mollies female guppies are live bearing . male fertilize the egg by using a stick –like modified anal ,the so – called the gonopodium. The guppies are ovoviviparous, ie. egg develops inside the mother. However, also vivipary with placental nutrition has evolved several times within the *poecilidae*.

The Guppy (*Poecilia reticulata*),also known as **million fish** and **rainbow fish**, is one of the world's most widely distributed tropical fish and one of the most popular freshwater aquarium fish species. It is a member of the family *Poecilidae* and like almost all American members of the family, is live bearing. Guppies originate from northeast south America , but have been introduced to many environment and are now found all over the world. They are highly

adaptable and thrive in many different environment and ecological condition. Male guppies, which are smaller than females, have ornamental caudal and dorsal fins. Wild guppies are generally feed on a variety of food sources, including benthic algae and aquatic insect larvae. Guppies are used as a model organism in the field of ecology , evolution and behavioural studies.

Guppies were first described in Venezuela as *Poecilia reticulata* by Wilhelm Peters in 1859 and as *Lebistes poecilioides* in Barbados by De Filippi in 1861. It was named *Girardinus guppii* by Albert Gunther in honor of Robert John Lechmere Guppy, who sent specimens of the species from Trinidad to the Natural History Museum in London. It was reclassified as *Lebistes reticulatus* by Regan in 1913. Then in 1963, Rosen and Bailey brought it back to its original name, *Poecilia reticulata*. While the taxonomy of the species was frequently changed and resulted in many synonyms, “guppy” remains the common name even as *Girardinus guppii* is now considered as junior synonym of *Poecilia reticulata*. Guppies are native to Antigua and Barbuda, Barbados, Suriname, Guyana, Trinidad and Tobago and Venezuela. However guppies have been introduced to many countries on every continent except Antarctica . sometimes it has occurred accidentally, but most often as a means of mosquito control. The guppies are expected to eat mosquito larvae and help slow down the spread of malaria, but in many cases, these guppies have had a negative impact on native fish populations. Field study reveal that guppies have colonized almost every freshwater body accessible to them in their natural ranges, especially in the streams located near the coastal fringes of mainland South America. Although not typically found there, guppies also have tolerance of brackish water and have colonized some brackish water environment. They tend to be more abundant in smaller streams and pools than in large, deep, or fast flowing rivers. They also capable of being used to cycle salt water as well as being used to cycle saltwater aquarium as their molly cousins.

Guppies exhibit sexual dimorphism. While wild type female are grey in body colour, males have splashes, spots, or strips that can be any of a wide variety of colours. The size of the guppy vary, but males are typically 1.5-3.5cm long, while females are 3-6 cm long . A variety of guppy strains are produced through selective breeding, characterized by different colour, patterns, shapes, and size of fins, such as snakeskin and grass varieties. Many domestic strains have morphological traits that are vary distinct from the wild type antecedents. Males and females of many domestic strains usually have larger body size and are much more lavishly ornamented

than their wild type antecedents. Guppies have 23 pairs of chromosomes, including one pair of sex chromosomes, the same number as humans. The Gene responsible for male guppies ornamentations are Y chromosome linked and are heritable.

Two generations of guppies per year occur in the wild. Guppies are well developed and capable of independent existence without parental care by the time they are born. Young guppies school together and perform anti predator tactics. Brood size is extremely variable, yet some consistence differences exist among populations depending on the predation level and the other factors. Females of matching of body sizes tend to produce more numerous but smaller sized offspring in high-predation conditions. Female guppies first produce offspring at 10 – 20 weeks of age and they continue to reproduce until 20-34 months of age. Male guppies mature in seven week or less. Total life span of wild varies greatly but it is typically around two years. Variations in such life historic characteristics of guppies are observed in different populations, indicating that different evolutionary presher's exist. Guppies body sizes are positively correlated with age and their size at maturation varies highly depending on the predation risk of the occupied environments. Male and female guppies from high predation regions mature and faster and start reproducing earlier and, they devote more resources to reproduction than those from low-predation regions. Females from high predation regions reproduce more frequently and produce more offspring per litter, indicating that they are more fecund than low predation females. Female guppies reproductive success is also related to age. Older females produce offspring with reduced size and at increased interbrood intervals.

Guppies have the mating system called polyandry, where females mate with multiple males. Multiple mating is beneficial for males because male reproductive success is directly related to how many times they mate. The cost of multiple mating for males is very low because they do not provide material benefit to the females or parental care to the offspring. Conversely, multiple mating can be disadvantageous for females because it reduces foraging efficiency and increase the chance of predation and parasitic infection. However females gain some potential benefits from multiple mating. For example, females that mate multiply are found to be able to produce more offspring in shorter gestation time, and their offspring tend to have better qualities such as enhanced schooling and predator evasion abilities. Females guppies mate again more actively and delay the development of a brood when the anticipated second mate is more attractive than

the first male. Experiment show that re mating females prefer a novel male to the original male or a brother of the original male with similar phenotype. Females preference for novel males in mating can explain the excessive phenotypic polymorphism in male guppies.

When guppies encounter a potential predator, some of them approach the predator to assess the danger. The behaviour, called predator inspection, benefits the inspector since it gains information's, but puts the inspector at a risk of predation. To reduce the risk, inspectors avoid the predators mouth area called the 'attack cone'-and approach the predators from the side or back. They may also form a group for protection, the size of which is larger in high-predation populations. Although evidence indicates predators are less likely to attack the inspector than a non inspector, the inspector remains at higher risk due to the proximity predator. Risk-taking behaviour such as predator inspection can be evolutionarily stable only when a mechanism prevents selfish individuals from taking advantages from "altruistic" individuals. Guppies may adopt a conditional-approach strategy that assembles tit for tat. According to this hypothesis guppies would inspect the predator on the first move, but if their co-inspectors do not participate the predator inspection visits or do not approach the predator close enough, they can retaliate at the trailers by copying the trailers last move in the next predator inspection visit. Guppies often forage in groups because they can find food more easily. Shoaling guppies spent less time and energy on anti predatory behaviour than solitary ones and spent more times on feeding. However, such behaviour results in food that is found being shared with other members of the group. Studies also shown when an evolutionary cost exists, guppies that tent to shoal are less aggressive and less competitive with regard to scarce resources. Therefore, shoaling is preferred in high-predation regions. When guppies with a high tendencies to shoal where isolated from high predation regions and were relocated to predator-free environment, over time, they decreased their shoaling behaviour, supporting the hypothesis that the shoaling is less preferred in low-predation environment.

Guppies are highly prolific livebearers. The gestation period of guppies varies considerably, ranging from 20-60 days at 25-27 degree Celsius and depending on several environmental factors. Reproduction typically continues through the year and the females becomes ready for the consumptions again quickly after parturition. Male guppies, like other members of the family *poeciliidae*, possess a modified tubular anal fin called the gonopodium, located directly behind

the ventral fin. The gonopodium has a channel like structure through which bundles of spermatozoa, called spermatozeugmata, are transferred to females. In courted mating, where the females shows receptive behaviour following the males courtship display, the male briefly insert the gonopodium into the females genital pore for internal fertilization. Once inseminated, female guppies can store sperms in their ovaries and gonoducts, which can continue to fertilize ova upto 8 months. Because of the sperm storage mechanisms males are capable of posthumous reproduction, meaning the female mate can give birth to the male offspring long after the males death, which contribute significantly to the reproductive dynamics of the wild guppy populations.

Male mate choice had been little studied in the guppy (Houde 1997). The notable assumption in the laboratory study of Benz and Leger 1992 using domesticated guppies of unknown origin. They reported some evidence for male mate choice based on female body length. Therefore we investigated the possibility of male mate choice in the guppy, originating from wild populations in Trinidad, but ascertaining the relationship between individual body size and behaviorally testing for male mate choice. More specifically, we tested the predictions that, (a) larger females in nature should have more desire to contributing their broods than smaller females and (b) males should be selective in their choice of mates and preferentially by visual stimuli and its behaviour towards the largest females, but only when they can accurately assess whether a female has already been mated otherwise males should not be choosy.



Guppy Fish



Male and Female Guppy

REVIEW OF LITERATURE

On the basis of male mate choice in guppy Herdman *et.al*,(2015)Theoretically, males should prefer larger females as mates in species where female fecundity increases with body size. However, any fecundity-related benefits accruing to a male that has mated with a large female may be offset by an associated fitness cost of shared paternity if large females are more likely to be multiply mated than smaller females in nature. We tested the above hypothesis and assumption using the Trinidadian guppy (*Poecilia reticulata*) by behaviourally testing for male mate choice in the laboratory and by ascertaining (with the use of microsatellite DNA genotyping) patterns of male paternity in wild-caught females. Our results thus suggest that male guppies originating from the Quaré River possess mating preferences for relatively large females, but that such preferences are expressed only when males can accurately assess the mating status of encountered females that differ in body size.

Jean - Guy J Godin and aud *et.al*, (2005), Suggest that, In vertebrates, the mating preferences of individual females can be flexible and the probability of a female mating with a particular male can be significantly increased by her having previously observed another conspecific female affiliate and mate with that same male. In theory, such mate-choice-copying behaviour has potentially important consequences for both the genetic and social ('cultural') transmission of female mating preferences. For copying to result in the 'cultural inheritance' of mating preferences, individual females must not only copy the mate choice decisions of other females but they also should tend to repeat this type of behaviour (i.e. make similar mating decisions) subsequently and to generalize their socially induced preference for a particular male to other males that share his distinctive characteristics. Here, we show experimentally that individual female guppies, *Poecilia reticulata*, not only copy the observed mating preferences of other females for particular males, but that the preference now assumed via copying is subsequently repeated and generalized to other males of a similar colour phenotype. These results provide empirical evidence for social enhancement of female preference for particular phenotypic traits of chosen males rather than for the particular males possessing those traits, and thus have important implications for our understanding of the role of social learning in the evolution of female mating preferences and of male epigamic traits.

Heather L Auld, Jean - Guy J Godin (2015), In most mating systems, males and females are commonly within signalling and receiving distance of conspecifics during courtship and mating activities. Although it is well known that females who observe sexual interactions between conspecifics will use public information obtained from these interactions when making their own mating decisions, much less is known about whether males use this type of information in making mating decisions. We used the Trinidadian guppy (*Poecilia reticulata*) to test whether males use public information to (i) copy the apparent mate choice of another male and (ii) modify their mating preference for a given female in the presence of one or two sexual rivals (potential copiers). We show that males use public information to copy the mate choice of other males and that males alter their mating preferences in response to the presence of an audience of sexual rivals, but find no evidence of a stronger audience effect when the number of sexual rivals increases. Collectively, these results indicate that males pay attention to their immediate social environment in making mating decisions and suggest that they avoid having another male copy their mate choice by weakening or even reversing their initial mating preference in the presence of eavesdropping male sexual competitors. Our findings highlight the importance of social context and public information in male mate-choice decisions and have implications for the evolution of male mating preferences and of social information use in populations.

Wolf *et.al*, (1999), done a research on disassortative mating for boldness decreases reproductive success in the guppy, However, the influence of the female's personality on this mate preference and on her compatibility with a mate with particular traits has been largely neglected. Here, using the guppy *Poecilia reticulata*, we investigated the effect of female boldness on mate choice and of the combinations of this trait in the male and female of a mating pair on parturition and brood size. Our results showed that female boldness did not affect mate choice, and brood size was independent of the boldness of the male and female in a pair. However, overall, females who mated with males with a dissimilar degree of boldness to themselves had a lower parturition success than females who mated with males with a similar degree of boldness. This work suggests that the combination of boldness characteristics within a pair influences reproductive success and that individuals of similar personality are more compatible in reproduction.

Houde and Hampten *et.al*, (1987), Models of inter-sexual selection generally assume heritable variation in mating preferences among females within populations. However, little is known about the nature of such variation. The aim of this study was to characterize quantitatively the phenotypic variation in female preference for a sexually selected male trait, body colour pattern, within a population of the Trinidadian guppy, *Poecilia reticulata*. Significantly more female guppies preferred the more brightly coloured of two similar-sized males presented simultaneously as potential mates. Mating preference scores for individual females were significantly and positively correlated between two repeated trials on successive days. Females were thus individually consistent in their particular choice of mates, and the calculated repeatability of their mating preference was relatively high. These results suggest that additive genetic variation for mating preferences based on male colour pattern is maintained, and the opportunity for the further evolution of both bright male colour patterns and female preference for this trait appears to exist in the study population from the Quaré River, Trinidad.

L Aura D Dosen, Robert(2004) done a experiment on Guppies (*Poecilia reticulata*) have a promiscuous mating system in which female choice for brightly coloured males plays an important role. Consequently, much research on guppies has examined how mate choice by females has lead to the evolution of male colour patterns. Much less attention has been devoted to mate choice by males in this species. In this study, we show that male guppies are choosy when selecting a female to associate with, significantly preferring the larger female when presented with two females when presented with two females that differed by ≥ 2 mm in standard length (SL). The strength of their preference for each female increased with absolute female size. The relative sizes of the females, however, also influenced male mating preferences: males showed stronger preferences for the larger female as the difference in SL between the two females increased. Such a preference for larger females is not unexpected as fecundity generally increases with body size in female fish.

Kodrick - Brown and paul F Nicoletto,(1998), done experiment on Female choices of males, and how these choices are influenced by ecological and social factors, have been studied extensively. However, little is known about the effects of age and breeding experience on female mating decisions. We used video techniques to examine female mate choice in guppies based on the area

of carotenoid (orange) pigmentation on the body. Females were presented with paired images of males, one ornamented and the other plain. Visual preference for each male was measured. Age-related changes in the criteria of choice were examined by comparing the responses of the same mature but sexually inexperienced 6-mo-old and 12-mo-old females. Effects of breeding experience on female choice were examined by comparing mate preferences of 12-mo-old female virgins with their preferences after they had mated and produced a brood. Female preferences for ornamented males with large areas of carotenoid pigment changed with age but not with mating experience. Six-month-old virgin females preferred ornamented males, whereas 12-mo-old virgin and postpartum females did not differentiate between males based on orange coloration. The results are discussed in light of life-history theory and have important implications for studies of sexual selection as well as for the design of mate-choice studies.

Krack and Bakker (1998), Compared with female mate choice and male mate choice and the factors underlying its evolution have been little studied and are thus poorly understood. Suggest that, Male mate competition contributes to male reproductive success in the guppy, It is commonly assumed that such success depend primaly on indivitual male quality and female mate choice.

METHODOLOGY

The present study is carried out from February 2022 to March 2022, (evening and morning) at home itself. The experiment was conducted by two different aspects. To determine whether large female guppies are more likely to be preferred by male guppies. We collected females from an aquarium shop which is reared in constant physical condition. Typically and unique features were noted on the video graph taped. Different postures of females were photographed from different angles as possible. Photographs were taken in mobile camera.

The fish were large scale aquarium reared wild trinidadian guppy. They came from large, randomly outbreak, individual-sex stocked reared under constant physical conditions (28°-12 hours presence of light). The fish were fed two times per day with commercially available micro pellet. Water was exchanged once a week. The tank size was (L and B) and it separated by a glass at the centre to separate the male and female (Prior to the experiments male and female were separated by sex for one week to increase the sexual motivation). After the experiment the male and female were mixed up and growing well now.

FIRST EXPERIMENT: MATE CHOICE WITH VISUAL STIMULI ONLY

The test was conducted in a standard tank (L and B) that was filled water 3/4. Three sides of the tank were covered to avoid disturbances and it is carried out by mobile camera.

In this experiment, we tested for males mating preference by presenting individual males with two sexually mature virgin females with different body size. Females that were physically separated from the male and each other in small clear containers. Thus the test males received only the visual stimuli from the females. I was wished to ascertain whether a male mating preference was evident under the latter circumstances of limited information available.

Modeled this experiment on the mate choice experiment with visual stimuli in house itself with small experimental set up. The experimental apparatus consist of a glass aquarium (L B H) and two small glass for the separation of compartment (W L H) placed at either the sides of the aquarium. The aquarium consists of 3/4 of the water (Depth). 26°-28°C of water temperature is maintained. Observed the first through mobile camera placed at minimum distance on the sides of aquarium. Verticals line drawn on the front and back of the aquarium and demarcated 10 cm wide male 'mating zone' near each of the end compartment. The tank was covered with tan paper the front and back of the aquarium and sides and back of the compartment tl minimize the external disturbance and also to provide a uniform background for male assessment of stimulus females. The bottom of the aquarium was covered with natural gravels substratum to stimulate natural stream.

There used three size categories of stimulus females: (Small, medium and large). The size range of the female was chosen to corresponding to that found in nature. (Reznick and endler 1982). I were isolated two groups of 4-5 stimulus females in separate aquarium to provide more experimental trials.

In a typical mate choice trail, we presented an individual male with a pair of stimulus females to test for his mating preference. The female were paired as both small, one small and one medium, one small and one large, or one medium and one large. The females in a given matched pair were placed individually in each of the two end compartment, which were shielded from the view of the central portion of the aquarium. The best male was selected from the aquarium and his standard body lengths were measured. This male was placed in the central of aquarium and with

the two stimulus females, were allowed to acclimatize for one hour before testing. Then, removed the opaque screen to allow visual contact between the males and females for 10 minutes. The glass slide that separated male and female was removed and replaced the opaque screen at both ends. The male can swim freely in water. When it removed the screen and recorded from behind a blind time spent by the male in the preference end zone associated with each female for 10 minutes. Once the first preference completed, the female end compartments were switched and the procedure was repeated for 10 minutes preference test.

As above protocol, tested the mating preference of each four males separately with each of four experimental categories of stimulus females over 4 days of period.

SECOND EXPERIMENT: MATE CHOICE WITH FULL RANGE STIMULI

In this experiment, we tested for male mating preferences by presenting individual male with two sexually mature, virgin females, one smaller than the other, that were concurrently free-swimming with the male in the aquarium. Thus in contrast with the experiment one. Both the test male and female had full access to each other and to all potential natural stimuli (visual, chemical and tactile). Exchanged between them during the behavioral observation period, under this circumstance, male were assumed to be able to accurately assess the reproductive state of paired stimulus females and were thus expected to be choosy.

The test aquarium contained aged well water maintained 26-28°C, and its bottom was covered with natural aquarium gravels. Covered the back and sides of the aquarium by tan paper to minimize external disturbances and provide uniform background for male assessment of females.

At the beginning of a typical mating preference trial, we divided the aquarium into halves with clear glass partition; and placed two stimulus females into one half and the test male into the other half; the sexes could see each other through the partition. Fish were acclimatizing for one hour, during which we observed no evidence of female-female competition such as chasing. After that removed the glass slide and thereby they allowed to swim freely and interact freely.

From the behind a blind record for 10 minutes the number of approaches, nips, copulation attempt and post copulatory jerks of the male directed towards either the large or small female. After approaching a female, the male often engaged in nipping behaviour directed at her gonopores (Houde 1997 in personal observation). A sigmoid courtship display involves the male adding his body into an S shaped and quivering near a female (Houde 1997). A male copulation attempt involves the male approaching the female laterally and attempting to insert his gonopodium into her gonopore (Houde 1997). If the male's copulation attempt is successful, he typically performs post-copulatory jerks, which are short bursts of full body shakes. Repeated this experiment for two times with other stimulus females.

RESULTS

EXPERIMENT ONE: MATE CHOICE WITH VISUAL STIMULI ONLY

Although there was a tendency for male to spend more time near the larger of the paired stimulus female; the preference difference behavioral score of males did not vary significantly among the paired stimulus female.

Size of Guppy	Duration
Small	10 minutes
Medium	10 minutes
Large	10 minutes

Table.1 *Different size of guppy and the time used*

Paired stimulus	Time duration (In minutes)		
	Small	Large	Medium
Small v/s Small	<2		
Small v/s Medium	2		6
Small v/s Large	2	8	
Medium v/s Large		8	3.8

Table 2. *Paired stimulation of Female Guppy to Male*

in reference to time

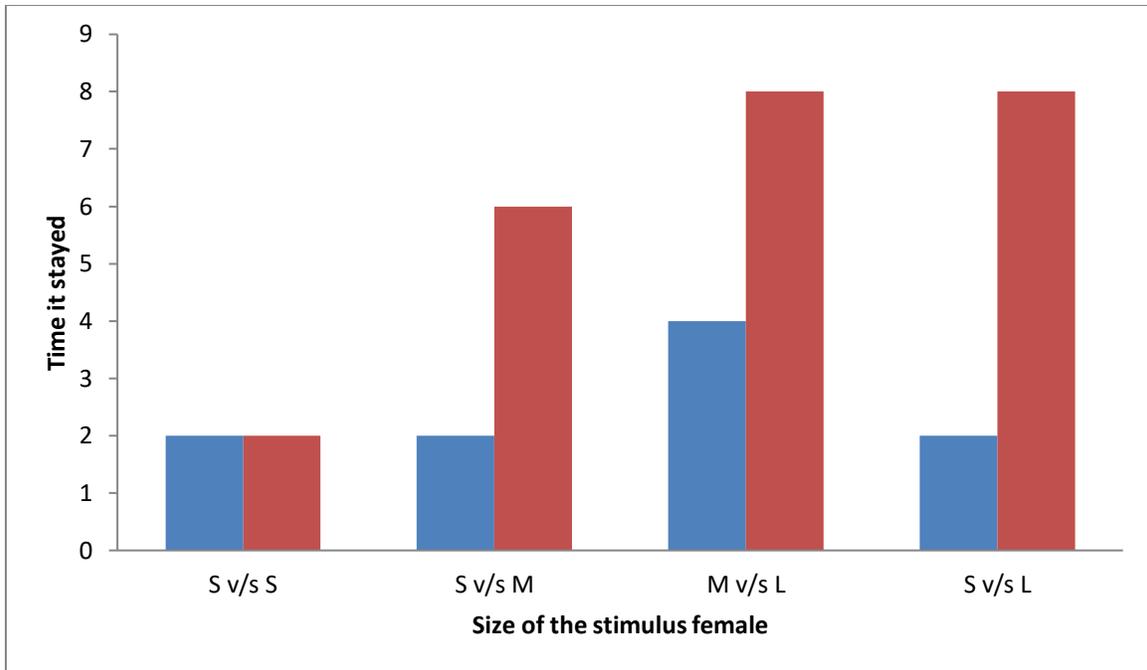


Figure 1: Mate choice with visual stimuli only

EXPERIMENT TWO: MATE CHOICE WITH FULL RANGE STIMULI

Overall male directed significantly more sexual acts including approaches, nips, copulation attempts and post copulation jerk towards larger than the smaller of the paired stimulus females. This difference can be attributed mainly to the significantly larger females (fig: 2). So, the observed male preferences for large over small female cannot be attributed simply to larger females behaving differently towards male compared with smaller females.

Mate in full range stimuli	Time they act sexually	
	Smaller	Larger
Approaches	6 times	8 times
Nipping	4-5 times	8 times
Copulation act	2 times	2-3 times
Post copulation act	0 times	1-2 times

Table 3. Male mate choice with full range of stimuli at natural environment in respect to time

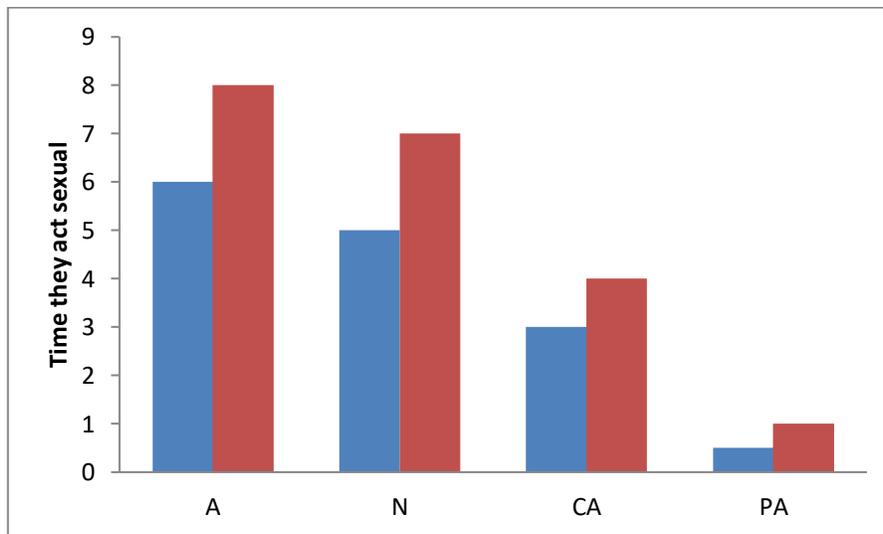


Figure 2: Mate choice with full range stimuli

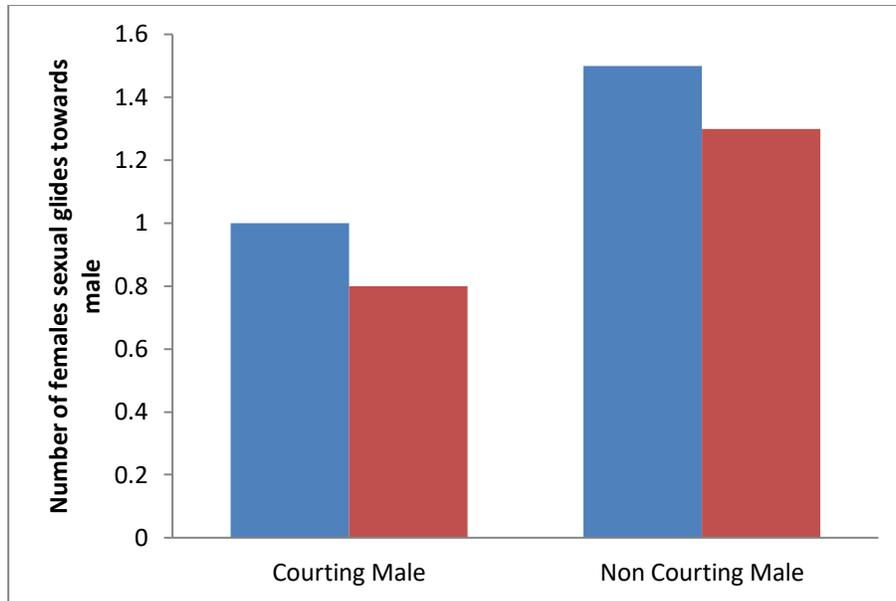


Figure 3: Number of female sexual glide towards courting and non courting male in natural environment



Fig 3. The set up of Experiments done

DISCUSSIONS

In this experiment results provide evidence for male mating preference in the Trinidadian Guppy. In each test, a preference for large of two simultaneously presented virgin females was clearly expressed in both the cases where males are exposed to full range stimuli from the females and limited stimuli alone. However, as suggested by the multiple paternity data, male choose to mate with large females may in add a large potential cost of sperm and shared paternity compared with males that mate with smaller females on average.

A similar multiple paternity – females size pattern has been previously reported for the sailfin molly, poeciliatipinna, another internally fertilizing poecilid fish species (Travis *et.al*, 1990). This data demonstrate that female guppy engage in multiple mating in nature (Kelly *et.al*, 1999) and that larger females have broods with more size on average compared with smaller females.

In my result, the behavioural mate choice experiment suggest that male guppies assess the making status of females when choosing among them as mates. When presented with paired stimulus female in binary choice trials in experiment 1(female visual stimuli available only). Male show significant behavioural preference for either one or the other females, in comparison when male were given access to free swimming females in experiment 2, they directed significantly more sexual act towards the larger of the paired females. Based on this significant trials, I conclude that the male guppies (Trinidadian guppy)shows mating preference for relatively larger females, in both the experiment. Male mating preference for the larger one of available female in behavioural assay has been previously reported for one sided live bearer, jensiamultidenata (Bisuzza *et.a,l* 2000); and japasemedalea, Oryziaslatipes (grant *et.al*,1995); In both the cases, male were given access to freely swimming females during behavioural assays.

In case of my reference project work, The laboratory test, a preference for larger of two simultaneously presented virgin female was clearly expressed only when males were exposed to the full range of stimuli from the females, but not when they are limited to visual stimuli alone, Based on these behaviour assay they concluded that male guppies originate from the quare river possess mating preference relatively larger females, but that such preference are expressed only when males concurrently encounter females of different body size and have access to the full range stimuli from them. Limitation in male production of ejaculate should favour differential

male mating behaviour towards higher quality – females (Wedell *et.al*, 2002). However, for this to occur, male must be able to assess difference in female quality. Because production of ejaculate in male guppies is rate limited (Pilastro and Bisazza 1999), they are thus expected to be discriminating in their choice of mates.

To my knowledge, the study represents the most comprehensive attempt at investigating male mate choice in Trinidadian guppy to date. In my behavioural assay and paternity result suggest that male guppies preferentially direct their mating behaviour toward and mate with the large of available females under natural conditions. Male mate choice may thus play an important yet relatively unexplored, role in the guppy mating system.

CONCLUSIONS

Guppies are a model vertebrate for studies of sexual selection and life history evolution. None the less, there have been few investigations of the factors responsible for maintaining extreme within-population genetic variation in male coloration. In this study, we tested the hypothesis that frequency-dependent mate choice contributes to the maintenance of this variation. We attempted to avoid biases inherent in earlier studies of the 'rare male effect' by familiarizing females to males bearing a particular color pattern and later presenting them with alternate male types, in equal numbers. Females were significantly more likely to mate with males having novel colour patterns than with males having a color pattern with which they were familiar. This result is consistent with the hypothesis that mate choice is frequency dependent. Other factors such as male and female size were unrelated to mate preference. Implications of the results for theories of sexual selection and the maintenance of variation are discussed.

Although females are the choosier sex in most species, male mate choice is expected to occur under certain conditions. Theoretically, males should prefer larger females as mates in species where female fecundity increases with body size. However, any fecundity-related benefits accruing to a male that has mated with a large female may be offset by an associated fitness cost of shared paternity if large females are more likely to be multiply mated than smaller females in nature. We tested the above hypothesis and assumption using the Trinidadian guppy (*Poecilia reticulata*) by behaviourally testing for male mate choice and by ascertaining patterns of male paternity in wild caught females. In this tests, a preference for the larger of two simultaneously presented virgin females was clearly expressed not only when males were exposed to the full range of natural stimuli from the females, but also when they were limited to visual stimuli alone. However, as suggested by our multiple paternity data, males that choose to mate with large females may incur a larger potential cost of sperm competition and shared paternity compared with males that mate with smaller females on average. In my results thus suggest that male guppies possess mating preferences for relatively large females, but that such preferences are expressed only when males can accurately assess the mating status of encountered females that differ in body size.

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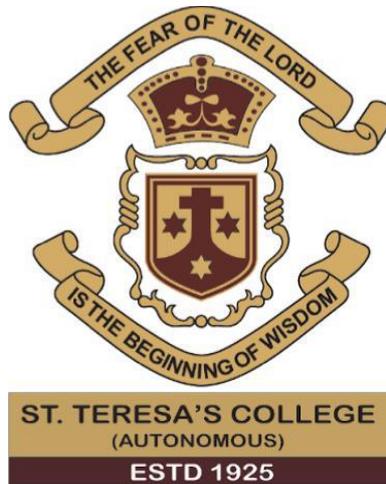
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**A STUDY ON THE EFFECT OF *PHYLLANTHUS NIRURI* EXTRACT
ON REGENERATION IN EARTHWORM**



Project Work By

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Submitted to St Teresa's College (Autonomous), Ernakulam

**Affiliated to Mahatma Gandhi University, Kottayam in partial fulfillment
of requirements for the Degree of Bachelor of Science in Zoology**

2021-2022

CERTIFICATE

This is to certify that the project report entitled "**STUDY ON THE EFFECT OF *PHYLLANTHUS NIRURI* EXTRACT ON REGENERATION IN EARTHWORM**" submitted by Ms. KRISHNAPRIYA MANOJ, Reg. No. AB18ZOO038 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Akhila Anilkumar and this is her original effort.

Akhila Anilkumar

Assistant Professor

Department of Zoology

St Teresa's College, (Autonomous) Ernakulam

EXAMINERS

1)

2)

DECLARATION

I, Ms. KRISHNAPRIYA MANOJ hereby declare that this project report entitled “A STUDY ON THE EFFECT OF *PHYLLANTHUS NIRURI* EXTRACT ON REGENERATION IN EARTHWORM” is a bonafide record of work done by me during the academic year 2021 - 2022 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam. This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

KRISHNAPRIYA MANOJ

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ABSTRACT

Earthworms are terrestrial invertebrates that belong to the phylum Annelida. They exhibit a tube-within-a-tube body plan; they are externally segmented with corresponding internal segmentation; and they usually have setae on all segments. They occur worldwide where soil, water, and temperature allow. The main aim of our study is to get a more comprehensive understanding on the effects of different concentrations of *Phyllanthus niruri* extract on the regeneration of earthworms, *Pheretima posthuma*. For that, 21 earthworms of the species *Pheretima posthuma* were collected. They were cut into equal halves and their growth was observed in soil containing different concentrations of the *Phyllanthus niruri* extract. The experimental results proved that *Phyllanthus niruri* in concentrations 10%, 20%, 40% and 80% promotes the regeneration of earthworms. Therefore, low to high concentrations of *Phyllanthus niruri* extract can be used as a regeneration promoting substance for earthworms. It may be suggested that *Phyllanthus niruri* can be used in soils and vermicompost during vermiculture for preventing the loss of earthworms due to autotomy or other mechanical injuries and to progress their regeneration process. The promotion of better health on earthworms can be helpful in agricultural practices as they help fertilize the soil better.

INTRODUCTION

An earthworm is a terrestrial invertebrate. They occur worldwide where soil, water, and temperature allow. Earthworms are commonly found in soil, eating a wide variety of organic matter. This organic matter includes plant matter, living protozoa, rotifers, nematodes, bacteria, fungi, and other microorganisms. The principal systematic features of earthworms are that they are bilaterally symmetrical, externally segmented, with a corresponding internal segmentation. They have no skeleton and a thinly pigmented cuticle, bearing setae on all segments except the first two; with an outer layer of circular muscles and an inner layer of longitudinal muscles. They are hermaphrodite and have relatively few gonads, which are situated in definite segmental positions. When mature, a swollen area of the epidermis called a clitellum, located in particular segments, forms a cocoon in which the eggs or ova are deposited, and this is then passed over the anterior segment. The eggs are usually fertilized and the young develop within the eggs without a free larval stage, the newly hatched worms resembling adults. Structurally, earthworms have large coelomic cavities containing coelomocytes, a closed vascular system with at least a dorsal and a ventral trunk and a ventral nerve cord. The alimentary canal is basically an anterior-posterior tube with excretion through the anus or specialized organs called nephridia; respiration is mainly cuticular.

Pheretima posthuma:



Kingdom	Animalia
Phylum	Annelida
Class	Oligochaeta
Order	Haplotaxida

Suborder	Lumbricina
Family	Megascolecidae
Genus	Pheretima
Species	posthuma
Nomenclatural Code	ICZN

Body is long, narrow and cylindrical. Length may reach upto 150 mm. Body colour is brown. Anterior end is pointed while the posterior end is blunt. Body is divided into 100-140 segments called metameres. The anteriormost segment is called Prostomium. Mouth is a crescentic aperture, present at anterior end. The segment containing mouth is called peristomium. Setae are present at all the segments except-1st and last. Each seta is embedded in a setal sac. A glandular band called clitellum is situated in 14th to 16th segments. It forms cocoon during the reproduction. Female genital pore is situated in 14th segment while male genital pore is present in 18th segment. The earthworm feeds on organic matter in the soil. The food is sucked by the pharynx and the oesophageal glands add calcite to neutralise acidity of the soil. The food is then grinded by the horny lining of the gizzard and is absorbed in the intestine. Undigested food material passes out the anus and is deposited as worm castings. The earthworm 'breathes' by the diffusion of gases through its moist skin. The blood contains haemoglobin which transports oxygen throughout the body. Circulatory system is of closed type. Earthworms have no sense organs but they can sense light intensity by small light-sensitive cells found mainly on the upper skin surface of their body. They can also sense vibrations and chemicals by the means of tactile or chemo-receptors. The earthworms exhibit undulating movement which takes place by alternate contraction and relaxation of circular and longitudinal muscles of each segment. Earthworms are hermaphrodites but they reproduce by cross-fertilization.

IMPORTANCE OF EARTHWORM

Of all the members of the soil food web, earthworms need the least introduction. Most people become familiar with these soft, slimy, invertebrates at a young age. An earthworm is a segmented worm, a terrestrial invertebrate belonging to phylum Annelida. Earthworms occur in most temperate soil and many tropical soils. They are major decomposers of dead and decomposing organic matter, and derive their nutrition from bacteria and fungi that grow

upon these materials. They fragment organic matter and make contribution to recycling the nutrients it contains. Earthworms dramatically change soil structure, water movement, nutrient dynamics and plant growth. They are not essential to cell healthy soil systems but their presence is usually an indicator of a healthy system. Earthworms perform several beneficial functions.

Stimulate microbial activity: Earthworms derive their nutrition from microorganisms, many more microorganisms are present in their feces/casts than in organic matter that they consume. Increased microbial activity facilitates the cycling of nutrients from organic matter and their conversion into a form readily taken up by plants.

Mix and aggregate soil: As they consume organic matter and mineral particles, earth excreta wastes is the form of casts, a type of soil aggregate.

Increased infiltration: Earthworms enhance porosity as they move through the soil. Some species make permanent burrows deep into the soil. It can be a major conduit for soil drainage particularly under heavy rainfall. At the same time burrows reduce surface water erosion.

Improve water-holding capacity: Earthworms can significantly increase the water-holding capacity of soils.

Provide a channel for root growth: The channel made by deep burrowing earthworms are lined with readily available nutrients and make it easier for roots to penetrate deep into the soil.

Bury and shred plant residue: plant and crop residue are gradually buried by cast material deposited on the surface and as earthworms pull surface residue into their burrows.

Interaction of earthworm with other members of the food web: Earthworms influence soil-inhabiting invertebrates by changing the amount and distribution of organic matter and microbial population.

Economic Importance of Earthworm:

Earthworms are extremely beneficial in agriculture. They aid in the following ways. The earthworm improves the fertility of soil in different ways, they are of utmost importance in agriculture. The burrowing and soil feeding habits of earthworms make the soil porous which permit both aeration and quick absorption of water. They also reduce the alkalinity and acidity of the soil to provide better conditions for plant growth. Thus earthworms are better known as the friend of farmers. Many people earn their livelihood by catching these worms and supplying them to scientific laboratories. Ayurvedic and unani systems of therapy suggest that these worms are used in making medicines for the care of diseases like bladder

stones, piles, rheumatism, jaundice etc. They are also used as baits to catch fish. It also plays a great role in Vermiculture to produce high -quality manure.

REGENERATION IN EARTHWORM

Regeneration in earthworm is an epimorphic regeneration, which is very sensitive to the environmental situations. The existence of stem cells help the worm to regenerate the lost organs. This kind of regeneration could be happened due to the low level of differentiation in these organisms to the comparison with higher level organisms in the evolutionary tree. But this ability varies between species and depends on the extent of the damage. There were significant differences in both survival rates and lengths of regeneration between immature earthworms and clitellate adult earthworms during the early stages of regeneration, but not at later stages of regeneration. The immature earthworms had a greater Regeneration potential than clitellate adults amputated at the same segment. The survival rates of earthworms were correlated significantly with the number of body segments remaining after amputation, but not with the position of the amputation. If an earthworm is split into two, it will not become two new worms. The head of the worm may survive and regenerate its tail if the animal is cut behind the clitella. But the original tail of the worm will not be able to grow a new head or the Rest of its vital organs and will instead die.

Present study focused to determine the effect of medicinal plant *Phyllanthus niruri* on earthworm regeneration.

Common name : Gale of the wind

Family : Phyllanthaceae

Scientific name : *Phyllanthus niruri*

Useful parts : leaves, stem, root

Phyllanthus niruri originated in India, usually occurring as a winter weed throughout the hotter parts. *Phyllanthus niruri* is a herb distributed throughout the tropical and subtropical areas that grows upto 60 cm. Phyllanthus means “leaf and flower” because the flower, as well as the fruit, seem to become one with the leaf. It is known for its liver healing properties so it is used in Chinese medicine for treatment of liver diseases. This plant is popular in folk medicine, whole plant, fresh leaves and fruits are used inthe treatment of various diseases, particularly hepatitis and other viral infection. Its wide variety of phytochemicals and their

pharmacological properties. The active phytochemicals, flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins and saponins, have been identified from various parts of *P. niruri*. Extracts of this herb have been proven to have therapeutic effects in many clinical studies. The plant is of medicinal importance for numerous ailments like dysentery, influenza, vaginitis, tumors, diabetes, diuretics, jaundice, kidney stone, dyspepsia, antihepatotoxic, anti hepatitis-B, antihyperglycemic and also as antiviral and antibacterial. In the Ayurvedic System of medicine the whole plant of *Phyllanthus niruri* can be used for medicinal purposes.

Wound healing property:

Wound healing effect of *P. niruri* was assessed using excision and dead space wound models while antiulcer activity was evaluated using indomethacin-, ethanol acid- and cold-restraint stress-induced ulcer models. Results showed that the extract (5,10%) significantly reduced the wound diameter producing 90.9 and 93.7% wound contraction respectively on day 18 post wounding. Phytochemical analysis of the extract revealed the presence of alkaloids, saponins, tannins, flavonoids, reducing sugar, carbohydrates and glycosides.

Earthworms are extremely important in soil formation, maintenance and structure and turnover of dead organic matter. Because of their availability and rapid regenerative power, annelids have been used commonly to study regeneration. Among annelids, the earthworm, which is important in breaking down organic wastes, has been used commonly for research into regeneration, because it is easy to culture and handle in the laboratory. This issue of regeneration of segments by amputated earthworms also has practical application to the build up of populations, especially since it appears that amputation has greater effects on mature earthworms than immature ones. Clearly, further research is needed to clarify the mechanisms of regeneration.

Phyllanthus niruri



OBJECTIVE

1. To investigate how much a worm can be left to regenerate a new worm or if one end is better regenerating than the other
2. To see the effect of *Phyllanthus niruri* extract on regeneration of earthworms, *Pheretima posthuma*.
3. To test whether higher concentrations of *Phyllanthus niruri* would speed up the regeneration of earthworm, *Pheretima posthuma*.

REVIEW OF LITERATURE

Charles Darwin (1809–1882) published his last scientific book entitled “The formation of vegetable mold through the action of worms with observations on their habits”, the result of several decades of detailed observations and measurements on earthworms and the natural sciences. The book covers the importance of earthworm activity on a variety of topics: pedogenesis and weathering processes, soil horizon differentiation and the formation of vegetable mold (topsoil), the role of earthworm burrowing and casting (bioturbation) in soil fertility and plant growth, the burial of organic materials and soil enrichment with mineral elements, the global cycle of erosion–sedimentation with hydrologic and aerial transfers of fine particles brought up to the soil surface by earthworms and the protection of archaeological remains through their burial. Finally, Darwin also performed a series of original experiments to determine if earthworms possessed, or not, a certain “intelligence”.

Gairdner B Moment (1949) explains the variation and causation in earthworm regeneration in ‘Journal of Experimental Zoology’. The coefficient of variation for the number of segments regenerated posteriorly by earthworms rarely exceeds 10. The manner of wound healing after the transaction cannot be responsible for the variation since under the conditions of these experiments all worms healed in the open manner. Bilateral asymmetry in the number of segments regenerated indicates that the counting mechanism underlying variation can hardly be humoral because both blood and coelomic fluid are common to both sides of the body.

Clive A. Edwards *et al.*, (1996) proposed a paper on biology and ecology of earthworms. It is in the third edition of this popular text where reviews on all aspects of earthworm biology and ecology are mentioned. These include a greatly expanded treatment of earthworm community ecology, interactions between earthworms and microorganisms, and the importance of earthworms in environmental management and their use in organic waste management. The book also summarizes the toxicity to earthworms of a wide range of chemicals.

“A science fair project” by Lloyd H. Barrow (2000) is a book on small animals that live in the ground; earthworms. The book makes observations about earthworm structure and behavior, movement, preferences, and reactions. Included in the book are easy-to-follow diagrams that offer readers exact information on performing the experiments.

Bely A and Wray G (2001) conducted a study on evolution of regeneration and fission in

annelids : insights from engrailed and orthodenticle class gene expression development. They presents a detailed comparison of regulatory gene expression during regeneration and asexual reproduction (by fission) in the segmented worm *Pristina leidyi* (Annelida: Oligocheta). In Situ hybridization studies on worms undergoing normal growth, regeneration and fission demonstrated that in all three processes, *Pl-en* is primarily expressed in the developing nervous system, *Pl-Otx1* and *Pl-Otx2* are expressed primarily in the anterior body wall, foregut and developing nervous system. They state that these annelids fission may have evolved by recruitment of regenerative processes. Furthermore, by comparing the existing data from leech embryos, they found evidence that embryonic processes are re-deployed during regeneration and fission.

A study was conducted by Shishin Kawamoto., *et al.*, (2005) on bipolar head regeneration induced by artificial amputation in *Enchytraeus japonensis*. As per the study, *t Enchytraeus japonensis* propagates asexually by spontaneous autotomy. Normally, each of the 5-10 fragments derived from a single worm regenerates a head anteriorly and a tail posteriorly. Occasionally, however, a head is formed posteriorly in addition to the normal anterior head, resulting in a bipolar worm. Experiments to clarify how the head and the tail are determined during regeneration in this species were conducted. The results showed that (1) bipolar head regeneration occurred only after artificial amputation, and not by spontaneous autotomy, (2) anesthesia before amputation raised the frequency of bipolar head regeneration, and (3) an extraordinarily high proportion of artificially amputated head fragments regenerated posterior heads. Close microscopic observation of body segments showed that each trunk segment has one specific autotomic position, while the head segments anterior to the VIIth segment do not. Only the most posterior segment VII in the head has an autotomic position. Examination just after amputation found that the artificial cutting plane did not correspond to the normal autotomic position in most cases. As time passed, however, the proportion of worms whose cutting planes corresponded to the autotomic position increased. It was suspected that the fragments autotomized after the artificial amputation (corrective autotomy). This post-amputation autotomy was probably inhibited by anesthesia. The rate at which amputated fragments did not autotomize corresponded roughly to the rate of bipolar regeneration. It was hypothesized then that the head regenerated posteriorly if a fragment was not amputated at the precise autotomic position from which it regenerated without succeeding in corrective autotomy.

Sung-Jin Cho et al., (2009) had explained about expression of three labial genes in earthworm head regeneration, in “The Differential Expression of Three labial Genes during Earthworm Head Regeneration”. Here they report the full length cloning of three labial genes (Pex-lab01, Pex-lab02, and Pex-lab 03) in the earthworm *Perionyx excavatus*. To analyze their expression pattern during head and tail regeneration, they used the reverse transcription-polymerase chain reaction. Their results indicate that the three labial genes were expressed only in the head regenerating tissues.

Tara Shanbhag *et al.*, (2010) evaluates the effect of ethanolic extract of *Phyllanthus niruri* on experimentally induced burn wound model in rats and to find whether it reverses the wound healing in steroid suppressed rats. In the burn wound model, oral and topical administration of *Phyllanthus niruri* did not show any significant effects in wound contraction and period of epithelialization when compared to control. In dexamethasone suppressed burn wound model, wound contraction rate was increased significantly by topical ($P < 0.001$) and oral ($P < 0.001$) administrations of *Phyllanthus niruri* by about 47.57% and 26.16% respectively. Topical administration has shown significant ($P < 0.05$) enhancement of wound contraction than oral dosage form. The Dexamethasone depressed epithelialization period was reversed significantly by topical ($P < 0.0001$) and oral ($P < 0.001$) administrations of *Phyllanthus niruri* by about 32.5% and 21.3% respectively. It was concluded that both topical and oral administrations of ethanolic extract of *Phyllanthus niruri* are found to reverse dexamethasone suppressed burn wound healing.

Mahmood Ameen Abdulla *et al.*,(2010) conducted a study to evaluate the gastroprotective activity of *Phyllanthus niruri* leaf extract against ethanol-induced gastric mucosal injury in rats. Six groups of Wistar rats were pre-treated, respectively, with distilled water; omeprazole 20 mg/kg; and 250, 500, 750 and 1000 mg/kg *P. niruri* leaf extract 30 min before oral administration of absolute ethanol to generate gastric mucosal injury. After one hour later, the rats were sacrificed and the ulcer areas of the gastric walls were determined. Gross evaluation has revealed that the negative control rats exhibited severe mucosal injury, whereas, pretreatment with *P. niruri* leaf extract resulted in significantly less gastric mucosal injury and flattening of the mucosal folds. Histological studies of the gastric wall revealed that negative control rats suffered very severe damage of gastric mucosa, along with edema and leucocytes infiltration of the submucosal layer compared to rats pre-treated with *P. niruri* leaf extract where there was marked gastric protection along with reduction or inhibition of

edema and leucocytes infiltration of the submucosa. The present finding suggests that *P. niruri* leaf extract promotes ulcer protection as ascertained by the comparative decreases in ulcer areas, inhibition or reduction of edema and leukocyte infiltration of the submucosa.

Paithankar V. V. *et al.*, (2011) proposed a paper on *Phyllanthus Niruri*. The paper gives information regarding the general properties, geographical distribution, and chemical constitution of *Phyllanthus niruri*. Pharmacological and biological activity - hepatoprotective effect, worldwide traditional medicinal uses, chromosome aberration inhibition, analgesic activity, antispasmodic activity etc. seen in *Phyllanthus niruri* are discussed.

A study conducted by Nengwen Xiao *et al.*, (2011) on the regeneration capacity of an earthworm, *Eisenia fetida* in relation to the site of amputation along the body. Their aim was to link the regeneration capacity of an earthworm, *Eisenia fetida* with the site of amputation, so they amputated earthworm at different body segment location along the length of the body to examine the different survival rates and regeneration length of anterior, posterior and medial sections.

Yvan Capowiez *et al.*, (2015) explains about the morphological and functional characterisation of the burrow systems of six earthworm species in “morphological and functional characterisation of the burrow systems of six earthworm species (Lumbricidae)”. Earthworm burrow systems are generally described based on postulated behaviors associated with the three ecological types. In this study, they used x-ray tomography to obtain 3D information on the burrowing behavior of six very common anecic (*Aporrectodea nocturna* and *Lumbricus terrestris*) and endogeic (*Aporrectodea rosea*, *Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Aporrectodea icterica*) earthworm species, introduced into repacked soil cores for 6 weeks. A simple water infiltration test, the Beerkan method, was also used to assess some functional properties of these burrow systems. Endogeic worms make larger burrow systems, which are more highly branched, less continuous and of smaller diameter, than those of anecic worms. Regarding water infiltration, anecic burrow systems were far more efficient due to open burrows linking the top and bottom of the cores. For endogeic species, we observed a linear relationship between burrow length and the water infiltration rate ($R^2 = 0.49$, $p < 0.01$). Overall, the three main characteristics significantly influencing water infiltration were burrow length, burrow number and bioturbation volume. This last characteristic highlighted the effect of burrow refilling by casts.

A study conducted by MT Rosa *et al.*, (2017) on Aloe Extracts, Pro and Antioxidant Conditions in “Regeneration of the Planarian *Girardia tigrina*”, reported that regeneration of the planarian *Girardia tigrina* was evaluated over different oxidative conditions, as pro oxidant (H₂O₂), antioxidant (vitamin C) and using aloe gel. The aloe plants have a millennial medicinal use and the succulent portion of leaf, called aloe gel, is used for wound healing. Here they analyze the action of aloe gel obtained from two species: *Aloe vera* and *A. arborescens*. The results show that ROS are important in the regeneration of *G. tigrina* and that the initial exposure to H₂O₂, soon after transection, accelerates the regeneration. However, during the regeneration process an antioxidant medium, containing vitamin C, promotes acceleration of regeneration, even if less intensely. Aloes extracts promote acceleration of regeneration in this planarian. No differences were observed among portions of cells in phases of cell cycle, with exception of worms exposed to *A.vera* extracts at 0.4% that show more cells in G2 phases, suggesting a faster cell cycling. No toxic effect was observed for the aloes extracts in planarians. Instead, an increase in the survival rate was observed in treated animals.

Yun Seon Bae *et al.*, (2020) discovered the Characterization of *Perionyx excavatus* Development and its head regeneration. The regeneration of the central nervous system is limited to specific animals including *Perionyx excavatus*. Here we set up a culture system to sustain the life cycle of *P. excavatus* and characterize the development of *P. excavatus* from embryo to juvenile, based on morphology, myogenesis and neurogenesis. Their data suggest that *P. excavatus* is a model system to study CNS regeneration.

Mini *et al.*,(2021) conducted a study on the effect of *Aloe vera* extract on regeneration in earthworm, *Lampito mauritii*. It was found that the anterior has potential to be renewed unlike the posterior which do not have any vital organs. This kind of regeneration could happen due to the low level of differentiation in these organisms. Data also revealed that *Aloe vera*, in small amounts (10-20%), has a positive effect on the regeneration of earthworm, speeding up regeneration time by about three days. More importantly, there was no significance in regeneration of earthworm in the very lower and higher concentrations of *Aloe vera*. Moreover, the higher concentrations (40-80%) of *Aloe vera* appeared to be toxic to the earthworm. Their experimental results proved that *Aloe vera* had capability to promote regeneration of earthworms and it was suggested that *Aloe vera* can be used in vermicompost.

Neda Gholami *et al.*, (2021) had conducted a study on “In vivo assessment of APPJ discharge on the earthworm: coelomic TAC and MDA levels, cell death, and tissue regeneration”. The effective medical applications of cold atmospheric pressure plasma jet (APPJ) have been reported by many researchers including sterilization of liquid and solid surfaces, treatment of chronic wounds, cancer tumors and blood clots. Results showed APPJ induced significant effects on regeneration ability of earthworms after 20 and 30s of exposure ($p < 0.05$). Atmospheric plasma jet did not have significant effects on MDA content and TUNEL-positive cells, but this effect was significant for TAC and CAT in both species ($p < 0.05$). In conclusion the present study revealed for the first time that regeneration of missed segments in earthworms can be stimulated by plasma treatment.

METHODOLOGY

Collect the moist soil compost. Prepare 4 cups for the earthworms to live in by placing $\frac{1}{2}$ cup of moist compost in to the bottom of each cup. Choose 6 worms of good size and equal lengths, the front end of the worm will be closest to the clitellum. Using scissors cut the first 3 worms in half and place the two pieces of worm into two cups labeled, “front half” and “back half”. Using scissors cut 3 worms into a one-third piece two-third piece and place the two pieces of worm into separate cups with corresponding labels. Cover the cups with wrapping paper and secure to the tops of the cups with rubber bands. Poke several small air holes into the wrapping paper covering each cup. Keep the cups in a cool dark place for several days, make observations of worms in every 3 days and place $\frac{1}{2}$ cup of fresh moist compost into the cup. Continue to observe the worms every 3 days for 2 to 3 weeks. From its plant, *Phyllanthus niruri* is made into a paste and preserved in a refrigerator (-250 C) for 3 days. Repeat the experiment by adding *Phyllanthus niruri* solutions of 0%, 10%, 20%, 40% and 80% into soil in separate cups. Three worm fragments (fragments containing head region) were added to each mug containing the varying solutions of *Phyllanthus niruri*. The length of each fragment was measured every 3 days.



RESULTS

Through this experiment, we observed that the regeneration of earthworm took place in the presence of *Phyllanthus niruri* extract. *Phyllanthus niruri* in various concentrations (10%, 20%, 40% and 80%) promoted regeneration in the cut area of the anterior half of equally cut earthworms. Wound healing properties of *Phyllanthus niruri* may have aided in the regeneration process in the earthworms. Through this experimental study, the following results were obtained.

Table 1. Showing mean change in length of anterior regions of equal and unequal halves of three earthworms each

DAYS	Normal (equal halves)		Normal (unequal halves)	
	(Average change in length in cm)			
	Cup I	Cup II	Cup III	Cup IV
	Head region	Tail region	Head region	Tail region
1	3.5	3.5	1.83	3.5
4	3.5	3.5	2.5	3.5
7	3.5	3.5	2.73	3.5
10	3.5	3.5	2.93	3.5
13	3.63	Dead	2.96	dead
16	3.81		3.06	
19	3.83		3.06	
22	4		3.06	
25	4.03		3.16	
28	4.13		3.16	

Fig 1. Showing the same rate of regeneration of anterior regions of both equal and unequal halves.

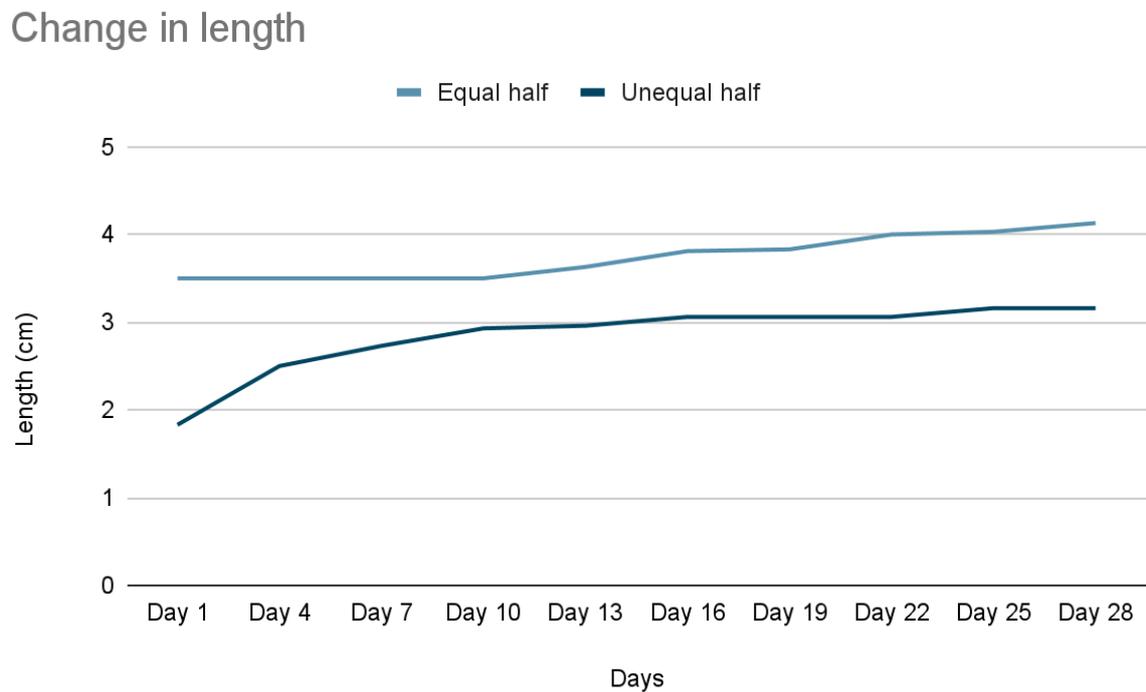
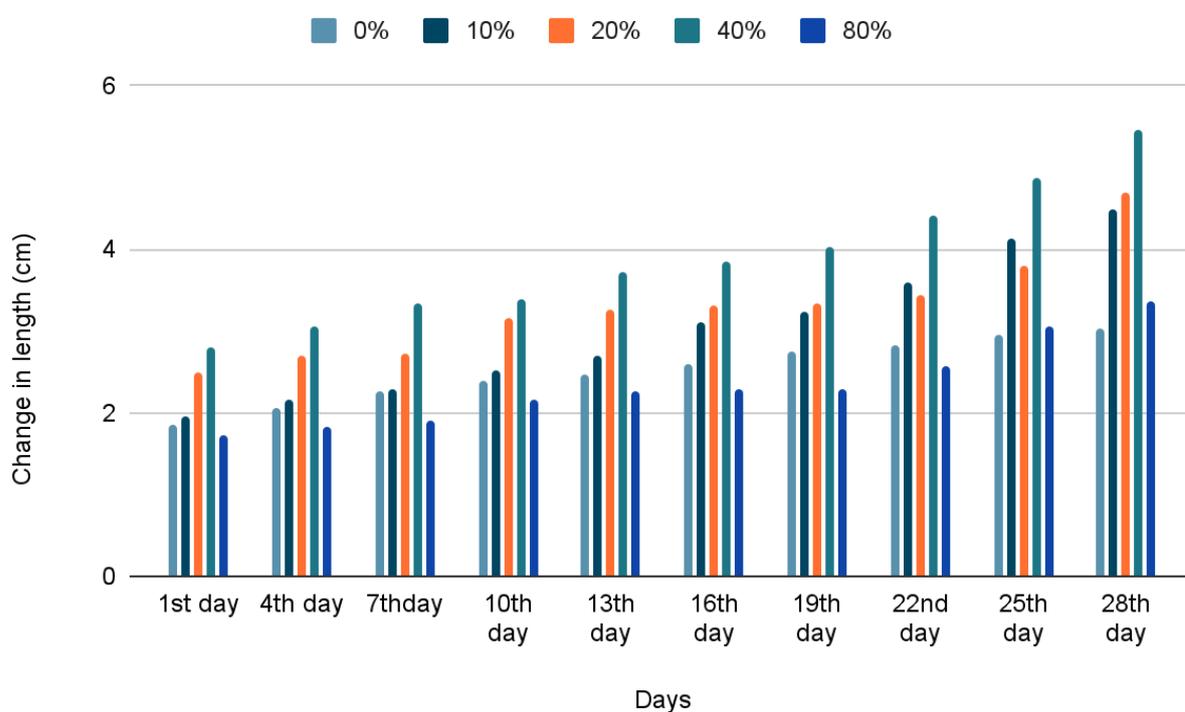


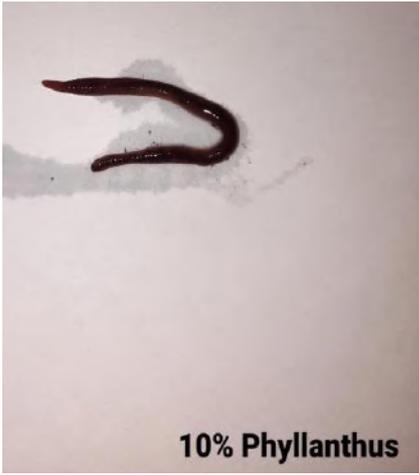
Table 2. Showings mean change in length of anterior regions of six earthworms after treatment with *Phyllanthus niruri*

DAYS	% of <i>Phyllanthus niruri</i> extract in moist soil compost				
	(Average change in length of front half in cm)				
	0%	10%	20%	40%	80%
1	1.86	1.96	2.5	2.8	1.73
4	2.06	2.16	2.7	3.06	1.83
7	2.26	2.3	2.73	3.33	1.9
10	2.4	2.53	3.16	3.4	2.16
13	2.46	2.7	3.26	3.73	2.26

16	2.6	3.1	3.3	3.86	2.28
19	2.76	3.23	3.34	4.03	2.3
22	2.82	3.6	3.43	4.4	2.56
25	2.96	4.13	3.8	4.86	3.06
28	3.02	4.5	4.7	5.46	3.36

Fig 2. Graph showing variation in growth of earthworm in different concentrations of *Phyllanthus niruri*





DISCUSSION

In this experimental study, the regenerative power of earthworm in the presence of *Phyllanthus niruri* extract was checked. *Phyllanthus niruri* in various concentrations (10%, 20%, 40% and 80%) was added to the soil where the anterior half of equally cut earthworms were placed. It was found that worms in all the above mentioned concentrations showed regeneration. The changes in length were measured for 28 days. It can be concluded that *Phyllanthus niruri* may have wound healing properties and it aids in regeneration of annelids.

In the article “Gastroprotective effect of *Phyllanthus niruri* leaf extract against ethanol-induced gastric mucosal injury in rats” by Mahmood Ameen Abdulla *et al.*, (2010), *P. niruri* leaf extract 30 min was administered into selected rats before oral administration of absolute ethanol to generate gastric mucosal injury. A few were subjected to gastric mucosal injury without administration of absolute ethanol. Gross evaluation has revealed that the negative control rats exhibited severe mucosal injury, whereas, pretreatment with *P. niruri* leaf extract resulted in significantly less gastric mucosal injury and flattening of the mucosal folds. The gastro-protective effects of *P. niruri* may be attributed to various compounds present in the plant, including acidic heteroxylan and some other polysaccharides present in the herb. In my experimental study, *Phyllanthus niruri* increased the regenerative capacity of stem cells in earthworms. It may be due to the presence of the active phytochemicals, flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins and saponins present in the various parts of the plant. It also proved to be beneficial in varying concentrations.

In the article “The Differential Expression of Three labial Genes during Earthworm Head Regeneration” by Sung-Jin Cho *et al.*, (2009) had explained about expression of three labial genes in earthworm head regeneration. Here they report the full length cloning of three labial genes (Pex-lab01, Pex-lab02, and Pex-lab 03) in the earthworm *Perionyx excavatus*. Their results indicate that the three labial genes were expressed only in the head regenerating tissues. In my experiment also, only the head regions showed regeneration. The tail regions did not show change in length for 12 days and decayed on the 13th day.

Paithankar V. V. *et al.*, proposed a paper on *Phyllanthus niruri* (2011). The paper gives information regarding the general properties, geographical distribution, and chemical constitution of *Phyllanthus niruri*. Pharmacological and biological activity - hepatoprotective

effect, worldwide traditional medicinal uses, chromosome aberration inhibition, analgesic activity, antispasmodic activity etc seen in *Phyllanthus niruri* are discussed. This helped in my study to know about the chemical properties of *Phyllanthus niruri*. The selection of this plant was made possible because of this paper.

In study conducted by Nengwen Xiao *et al.*, (2011) on the regeneration capacity of an earthworm, *Eisenia fetida* in relation to the site of amputation along the body, they amputated earthworm at different body segment location along the length of the body to examine the different survival rates and regeneration length of anterior, posterior and medial sections. My aim was to check the regenerative capacity of the worms in different concentrations of the same extract.

In the experimental study by Mini V S, *et al.*, titled “A study on the effects of *Aloe vera* extract on a regeneration in *Lampito mauritii*”. They experimented to find the regenerative capacity of earthworm in *Aloe vera* extract. Their data revealed that *Aloe vera*, in small amounts (10-20%), has a positive effect on the regeneration of earthworm, speeding up regeneration time by about three days. More importantly, there was no significance in regeneration of earthworm in the very lower and higher concentrations of *Aloe vera*. Moreover, the higher concentrations (40-80%) of *Aloe vera* appeared to be toxic to the earthworm. Thus, their project revealed that higher concentrations of *Aloe vera* would become lethal to earthworms. Low or medium amount of *Aloe vera* can be used as a regeneration promoting substance for earthworms. Whereas in my experiment, the earthworms reached full potential in their regenerative capacity in varying concentrations of *Phyllanthus niruri* extract.

CONCLUSION

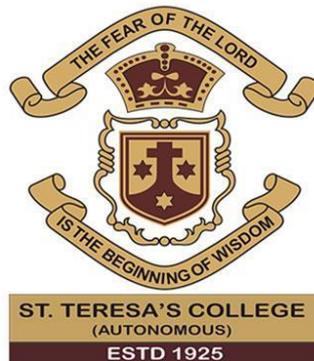
Through this study it can be concluded that earthworms react efficiently towards *Phyllanthus niruri* extract in the property of regeneration. From high to low concentrations, it proved to be involved in the cellular processes of cell proliferation, morphogenesis and cell differentiation of the annelid body. The regenerative capacity of the annelid body combined with the chemical constituents of the *Phyllanthus niruri* proved in the fast regeneration of the earthworms. Regeneration in earthworm is an epimorphic regeneration, which is very sensitive to the environmental situations, and at the optimum condition, regeneration of the anterior segments occurs but the posterior segments are destroyed. Therefore, the anterior has potential to be renewed due to the existence of stem cells, which help the worm to regenerate the lost organs, however at the tail segments of the worm the nervous, digestive and respiratory structures do not exist. This kind of regeneration could happen due to the low level of differentiation in these organisms in comparison with higher level organisms in the evolutionary tree. My data also revealed that *Phyllanthus niruri* extract helped significantly in the regeneration of earthworms, speeding up regeneration time. More importantly, regeneration was achieved in concentrations 10%, 20%, 40% as well as 80%. The experimental results proved that *Phyllanthus niruri* had capability to promote regeneration of earthworms and it can be suggested that *Phyllanthus niruri* can be used in soils and vermicompost during vermiculture for preventing the loss of earthworms due to autotomy or other mechanical injuries and to progress their regeneration process.

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**EFFECT OF *Centella asiatica* EXTRACT ON REGENERATION OF
EARTHWORM *Pheretima posthuma*.**



Project work by

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Submitted to St.Teresa's College (Autonomous) Ernakulam

Affiliated to Mahatma Gandhi University, Kottayam in partial

Fulfilment of requirement for the degree of Bachelor of science

In Zoology

2021-2022

CERTIFICATE

This is to certify that the project report entitled“**EFFECT OF *Centella asiatica* EXTRACT ON REGENERATION OF EARTHWORM *Pheretima posthuma***” submitted by Ms.MARY SANTHRA N P, Reg No. AB19ZOO039 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University,Kottayam is a bonafide work done under the guidance and supervision of **Akhila Anilkumar** and this is her original effort.

Akhila Anilkumar

Assistant Professor

Department of Zoology

St.Teresa's College (Autonomous)

Ernakulam

EXAMINERS

1)

2)

DECLARATION

I, hereby declare that this project work entitled “**EFFECT OF *Centella asiatica* EXTRACT ON REGENERATION OF EARTHWORM *Pheretima posthuma***”.is submitted to St.Teresa's College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfillment of the requirements of Bachelor of Science degree in Zoology.This work has been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own.

Name:Mary santhra N P

Signature

Reg.No: AB19ZOO039

ACKNOWLEDGEMENT

The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my project. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

I owe my deep gratitude to my project guide Ms. Akhila Anil Kumar, who took keen interest in my project work and guided me all along, till the completion of my project work by providing all necessary information for developing a good system.

I take this opportunity to express my profound gratitude to my parents and friends who helped me a lot in finishing the project within the limited time. Also, I would like to extend my sincere esteem to all staff in the laboratory for their timely support.

Last but not least I would like to thank God Almighty for the successful completion of my project.

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ABSTRACT

Earthworms are the terrestrial invertebrate belonging to phylum Annelida. Earthworms *Pheretima posthuma* species found in India as well as many regions of the world. *Pheretima posthuma* is an indigenous cheap test species, which is widely used in vermiculture, agriculture, etc. and it can be easily cultivated in laboratory conditions. So in this study we selected the species of *Pheretima posthuma* for that we cut the worms in different places and different size to find out the regeneration size or orientation of the body. The main aim of our study to get a more comprehensive understanding on the effects of different concentrations of *Centella asiatica* extract on the regeneration of the earthworm. The experimental study proves that low concentration of *Centella asiatica* extract have regeneration abilities. It may be suggested that *Centella asiatica* can be used in soils and vermicompost during vermiculture for preventing the loss of earthworms due to the autotomy or other mechanical injuries and to progress their regeneration process. The promotion of better health on earthworms can be helpful in agricultural practices as they fertilize the soil better.

Keywords: Regeneration, Earthworm, *Centella asiatica*, Extraction.

INTRODUCTION

EARTHWORM CLASSIFICATION

An earthworm is a terrestrial invertebrate. They occur worldwide where soil, water, and temperature allow. Earthworms are commonly found in soil, eating a wide variety of organic matter. This organic matter includes plant matter, living protozoa, rotifers, nematodes, bacteria, fungi, and other microorganisms. The principal systematic features of earthworms are that they are bilaterally symmetrical, externally segmented, with a corresponding internal segmentation. They have no skeleton and a thinly pigmented cuticle, bearing setae on all segments except the first two; with an outer layer of circular muscles and an inner layer of longitudinal muscles. They are hermaphrodite and have relatively few gonads, which are situated in definite segmental positions. When mature, a swollen area of the epidermis called a clitellum, located in particular segments, forms a cocoon in which the eggs or ova are deposited, and this is then passed over the anterior segment. The eggs are usually fertilized and the young develop within the eggs without a free larval stage, the newly hatched worms resembling adults. Structurally, earthworms have large coelomic cavities containing coelomocytes, a closed vascular system with at least a dorsal and a ventral trunk and a ventral nerve cord. The alimentary canal is basically an anterior-posterior tube with excretion through the anus or specialized organs called nephridia; respiration is mainly cuticular.

Pheretima posthuma:



Kingdom	Animalia
Phylum	Annelida
Class	Oligochaeta
Order	Haplotaxida
Suborder	Lumbricina
Family	Megascolecidea
Genus	Pheretima
Species	posthuma
Nomenclatural Code	ICZN

Body is long, narrow and cylindrical. Length may reach upto 150 mm. Body colour is brown. Anterior end is pointed while the posterior end is blunt. Body is divided into 100-140 segments called metameres. The anterior most segment is called Prostomium. Mouth is a crescentic aperture, present at the anterior end. The segment containing mouth is called peristomium. Setae are present at all the segments except-1st and last. Each seta is embedded in a setal sac. A glandular band called Clitellum is situated in 14th to 16th segments. It forms cocoon during the reproduction. Female genital pore is situated in 14th segment (ventral surface) while male genital pore is present in 18th segment. The earthworm feeds on organic matter in the soil.

The food is sucked by the pharynx and the oesophageal glands add calcite to neutralise acidity of the soil. The food is then grinded by the horny lining of the gizzard and is absorbed in the intestine. Undigested food material passes out the anus and is deposited as worm castings. The earthworm 'breathes' by the diffusion of gases through its moist skin. The blood contains haemoglobin which transports oxygen throughout the body. Circulatory system is of closed type. Earthworms have no sense organs but they can sense light intensity by small light-sensitive cells found mainly on the upper skin surface of their body. They can also sense vibrations and chemicals by the means of tactile or chemo-receptors. The earthworms exhibit undulating movement which takes place by alternate contraction and relaxation of circular and longitudinal muscles of each segment. Earthworms are hermaphrodites but they reproduce by cross-fertilization.

IMPORTANCE OF EARTHWORM

Of all the members of the soil food web, earthworms need the least introduction. Most people become familiar with these soft, slimy, invertebrates at a young age. An earthworm is a segmented worm, a terrestrial invertebrate belonging to phylum Annelida. Earthworms occur in most temperate soil and many tropical soils. They are major decomposers of dead and decomposing organic matter, and derive their nutrition from bacteria and fungi that grow upon these materials. They fragment organic matter and make contribution to recycling the nutrients it contains. Earthworms dramatically change soil structure, water movement, nutrient dynamics and plant growth. They are not essential to cell healthy soil systems but their presence is usually an indicator of a healthy system. Earthworms perform several beneficial functions.

Stimulate microbial activity: Earthworms derive their nutrition from microorganisms, many more microorganisms are present in their feces/casts than in organic matter that they consume. Increased microbial activity facilitates the cycling of nutrients from organic matter and their conversion into a form readily taken up by plants.

Mix and aggregate soil: As they consume organic matter and mineral particles, earth excreta wastes is the form of casts, a type of soil aggregate.

Increased infiltration: Earthworms enhance porosity as they move through the soil. Some species make permanent burrows deep into the soil. It can be a major conduit for soil drainage particularly under heavy rainfall. At the same time burrows reduce surface water erosion.

Improve water-holding capacity: Earthworms can significantly increase the water-holding capacity of soils.

Provide a channel for root growth: The channel made by deep burrowing earthworms are lined with readily available nutrients and make it easier for roots to penetrate deep into the soil. **Bury and shred plant residue:** plant and crop residue are gradually buried by cast material deposited on the surface and as earthworms pull surface residue into their burrows.

Interaction of earthworm with other members of the food web: Earthworms influence soil-inhabiting invertebrates by changing the amount and distribution of organic matter and microbial population.

Economic Importance of Earthworm

Earthworms are extremely beneficial in agriculture. They aid in the following ways. The earthworm improves the fertility of soil in different ways, they are of utmost importance in agriculture. The burrowing and soil feeding habits of earthworms make the soil porous which permit both aeration and quick absorption of water. They also reduce the alkalinity and acidity of the soil to provide better conditions for plant growth. Thus earthworms are better known as the friend of farmers. Many people earn their livelihood by catching these worms and supplying them to scientific laboratories. Ayurvedic and unani systems of therapy suggest that these worms are used in making medicines for the care of diseases like bladder stones, piles, rheumatism, jaundice etc. They are also used as baits to catch fish. It also plays a great role in Vermiculture to produce high-quality manure.

REGENERATION OF EARTHWORM

Regeneration in earthworm (*Lampito mauritii*) is an epimorphic regeneration, which is very sensitive to the environmental situations. The existence of stem cells help the worm to regenerate the lost organs. This kind of regeneration could be happened due to the low level of differentiation in these organisms to the comparison with higher level organisms in the evolutionary tree. But this ability varies between species and depends on the extent of the damage. There were significant differences in both survival rates and lengths of regeneration between immature earthworms and clitellate adult earthworms during the early stages of regeneration, but not at later stages of regeneration. The immature earthworms had a greater Regeneration potential than clitellate adults amputated at the same segment. The survival rates of earthworms were correlated significantly with the number of body segments remaining after amputation, but not with the position of the amputation. If an earthworm is split into two, it will not become two new worms. The head of the worm may survive and regenerate its tail if the animal is cut behind the clitella. But the original tail of the worm will not be able to grow a new head or the Rest of its vital organs and will instead die .

EXTRACT USED: *Centella asiatica*

Scientific name: *Centella asiatica*

Family: Apiaceae

Common name: Indian pennywort

Useful parts: The whole plant



Centella asiatica is an important medicinal plant that is belonging to the family Apiaceae commonly known as Gotu kola that has been widely used in the orient and is becoming popular in the west. Triterpenoids, saponins, the primary constituents of *Centella asiatica* are mainly believed to be responsible for its wide therapeutic actions. Apart from the wound healing properties, the herb is recommended for the treatment of various skin conditions such as leprosy, lupus, varicose ulcers, fever, diarrhea, eczema and also for the relieving anxiety and improving cognition. The medicinal plant that has been used in folk medicine for hundreds of years as well as in scientifically oriented medicine.

MORPHOLOGICAL CHARACTERISTICS

- The plant is a small trailing herb and it is the only species of *Centella* found in India.
- Stem is glabrous, pink striated and rooting at nodes.
- Leaves are fleshy, orbicular to reniform and dentate.
- Petiole is long, smooth, on the upper surface and hairy below.

FLORAL CHARACTERISTICS

- Flowers are pink and white in fascicled umbels.
- The fruits are oblong,dull brown,laterally compressed,pericarp hard,thickened and woody white.

DISTRIBUTION

- The plant occurs in marshy places throughout the country in tropical and subtropical regions.

CLIMATE AND SOIL

- Plant naturally grows over moist, fertile, loose,sandy loam and clayey soil.
- Thrives best in monsoon periods in well drained beds.

WOUND HEALING PROPERTIES

- *Centella asiatica* has antioxidant, anti inflammatory,anti cellulite and anti aging properties.
- It is effective in treatment of wounds, also in infective wounds, burns,and hypertrophic scar.
- *Asiatica* can increase the microcirculation of blood in the skin and prevent excessive accumulation of fat in cells.

OBJECTIVES

1. To see the effect of *Centella asiatica* extract on regeneration of earthworms, *Pheretima posthuma*.
2. To test whether higher concentrations of *Centella asiatica* would speed up the regeneration of earthworm, *Pheretima posthuma* .
3. To investigate how much a worm can be left to regenerate a new worm or if one end is better regenerating than the other.

REVIEW OF LITERATURE

Darwin (1809–1882) published his last scientific book entitled “The formation of vegetable mould through the action of worms with observations on their habits”, the result of several decades of detailed observations and measurements on earthworms and the natural sciences. The book covers the importance of earthworm activity on a variety of topics: pedogenesis and weathering processes, soil horizon differentiation and the formation of vegetable mould (topsoil), the role of earthworm burrowing and casting (bioturbation) in soil fertility and plant growth, the burial of organic materials and soil enrichment with mineral elements, the global cycle of erosion–sedimentation with hydrologic and aerial transfers of fine particles brought up to the soil surface by earthworms and the protection of archaeological remains through their burial. Finally, Darwin also performed a series of original experiments to determine if earthworms possessed, or not, a certain “intelligence”.

Gairdner B Moment (1949) explained the variation and its causation in earthworm regeneration. The coefficient of variation for the number of segments regenerated posteriorly by earthworms rarely exceeds 10. The manner of wound healing after the transaction cannot be responsible for the variation since under the conditions of these experiments all worms healed in the open manner. Bilateral asymmetry in the number of segments regenerated indicates that the counting mechanism underlying variation can hardly be humoral because both blood and coelomic fluid are common to both sides of the body.

Clive A. Edwards and P.J. Bohlen in (1996) proposed a paper on biology and ecology of earthworms. It is in the third edition of this popular text where reviews on all aspects of earthworm biology and ecology are mentioned. These include a greatly expanded treatment of earthworm community ecology, interactions between earthworms and microorganisms, and the importance of earthworms in environmental management and their use in organic waste management. The book also summarizes the toxicity to earthworms of a wide range of chemicals.

World Health Organization in Geneva have published a monograph on selected medicinal plant(1999) provide scientific information on the safety, efficacy, and quality control/quality assurance of widely used medicinal plants, in order to facilitate their appropriate use in Member States; provide models to assist Member States in developing their own graphs or formularies

for these or other herbal medicines; and facilitate information exchange among Member States. Each monograph contains two parts. The first part consists of pharmacopoeial summaries for quality assurance: botanical features, distribution, identity tests, purity requirements, chemical assays, and active or major chemical constituents. The second part summarizes clinical applications, pharmacology, contraindications, warnings, precautions, potential adverse reactions, and posology.

A science fair project by Lloyd H. Barrow (2000) guides the reader in exploration of one of the many small animals that live in the ground; earthworms. Barrow's detailed science experiments will help beginning biologists create winning science fair projects. Book will make observations about earthworm structure and behavior, movement, preferences, and reactions. Included in the book are easy-to-follow diagrams that offer readers exact information on performing the experiments. None of the experiments in this book in any way harm the worms.

Bely A and Wray G (2001) conducted a study on evolution of regeneration and fission in annelids : insights from engrailed and orthodenticle class gene expression development. They presents a detailed comparison of regulatory gene expression during regeneration and asexual reproduction (by fission) in the segmented worm *Pristina leidyi* (Annelida:Oligochaeta). In Situ hybridization studies on worms undergoing normal growth, regeneration and fission demonstrated that in all three processes, *Pl-en* is primarily expressed in the developing nervous system, *Pl-Otx1* and *Pl-Otx2* are expressed primarily in the anterior body wall, foregut and developing nervous system. They state that these annelids fission may have evolved by recruitment of regenerative processes. Furthermore, by comparing the existing data from leech embryos, they found evidence that embryonic processes are re-deployed during regeneration and fission.

A study was conducted by Shishin Kawamoto *et al.*, (2005) on Bipolar head regeneration induced by artificial amputation in *Enchytraeus japonensis* (Annelida, Oligochaeta). As per the study The Enchytraeida Oligochaeta *Enchytraeus japonensis* propagates asexually by spontaneous autotomy. Normally, each of the 5-10 fragments derived from a single worm regenerates a head anteriorly and a tail posteriorly. Occasionally, however, a head is formed posteriorly in addition to the normal anterior head, resulting in a bipolar worm. This phenomenon prompted us to conduct a series of experiments to clarify how the head and the tail are determined during regeneration in this species. The results showed that (1) bipolar head regeneration occurred only after artificial amputation, and not by spontaneous autotomy, (2)

anesthesia before amputation raised the frequency of bipolar head regeneration, and (3) an extraordinarily high proportion of artificially amputated head fragments regenerated posterior heads. Close microscopic observation of body segments showed that each trunk segment has one specific autonomic position, while the head segments anterior to the VIIIth segment do not. Only the most posterior segment VII in the head has an autonomic position. Examination just after amputation found that the artificial cutting plane did not correspond to the normal autotomic position in most cases. As time passed, however, the proportion of worms whose cutting planes corresponded to the autonomic position increased. It was suspected that the fragments autotomized after the artificial amputation (corrective autotomy). This post-amputation autotomy was probably inhibited by anesthesia. The rate at which amputated fragments did not autotomize corresponded roughly to the rate of bipolar regeneration. It was hypothesized then that the head regenerated posteriorly if a fragment was not amputated at the precise autonomic position from which it regenerated without succeeding in corrective autotomy.

Sung-Jin Cho *et al.* ,(2009) had explained the differential Expression of Three labial Genes during Earthworm Head Regeneration. Here they report the full length cloning of three labial genes (Pex-lab01, Pex-lab02, and Pex-lab 03) in the earthworm *Perionyx excavatus*. To analyze their expression pattern during head and tail regeneration, they used the reverse transcription-polymerase chain reaction. Their results indicate that the three labial genes were expressed only in the head regenerating tissues.

Yun Seon Bae *et al.*,(2009) discovered the Characterization of *Perionyx excavatus* Development and its head regeneration. The regeneration of the central nervous system is limited to specific animals including *Perionyx excavatus*. Here we set up a culture system to sustain the life cycle of *P. excavatus* and characterize the development of *P. excavatus* from embryo to juvenile, based on morphology, myogenesis and neurogenesis. Our data suggest that *P. excavatus* is a model system to study CNS regeneration.

A study conducted by Nengwen Xiao *et al.*,(2011) on the regeneration capacity of an earthworm, *Eisenia fetida* in relation to the site of amputation along the body. Our aim was to link the regeneration capacity of an earthworm, *Eisenia fetida* with the site of amputation, so we amputated earthworm at different body segment location along the length of the body to

examine the different survival rates and regeneration length of anterior, posterior and medial sections.

Paithankar V. V. *et al.*, (2011) proposed a paper on *Phyllanthus Niruri*. The paper gives information regarding the general properties, geographical distribution, and chemical constitution of *Phyllanthus niruri*. Pharmacological and biological activity - hepatoprotective effect, worldwide traditional medicinal uses, chromosome aberration inhibition, analgesic activity, antispasmodic activity etc seen in *Phyllanthus niruri* are discussed.

An Experimental study conducted by Juraiporn Somboonwong *et al.*, (2012) on wound healing activities of different extracts of *Centella asiatica* in incision and burn wound models on animals. Here we report the wound healing activities of sequential hexane, ethyl acetate, methanol and water extracts of *Centella asiatica* in incision and partial thickness burn wound models in rats. Male Sprague-Dawley rats weighing 250-300 g were randomly divided into incision and burn wound groups. Each group was stratified into seven subgroups: (1) untreated; (2) NSS; (3) Tween 20 (vehicle control); (4) hexane extract; (5) ethyl acetate extract; (6) methanol extract; and (7) aqueous extract -treated groups. The test substances were applied topically once daily. The tensile strength of the incision wound was measured on the seventh day after wound infliction. The degree of healing in the burn wound with four extracts were significantly higher than that of the control on Days 3, 10 and 14. Analysis by thin layer chromatography demonstrated that the phyto-constituents beta -sitosterol, asiatic acid, and asiaticoside and madecassoside were present in the hexane, ethyl acetate, and methanol extracts, respectively. All the extracts of *Centella asiatica* facilitate the wound healing process in both incision and burn wounds. Asiatic acid in the ethyl acetate extract seemed to be the most active component for healing the wound.

Wieslawa Bylka *et al.*, (2013) discovered *Centella asiatica* in cosmetology. *Centella asiatica* known as Gotu Kola is a medicinal plant that has been used in folk medicine for hundreds of years as well as in scientifically oriented medicine. The active compounds include pentacyclic triterpenes, mainly asiaticoside, madecassoside, asiatic and madecassic acids. *Centella asiatica* is effective in improving treatments of small wounds as well as burns, psoriasis and scleroderma. The mechanism of action involves promoting fibroblast proliferation and increasing the synthesis of collagen and intracellular fibronectin content and also improvement

of the tensile strength of newly formed skin as well as inhibiting the inflammatory phase of hypertrophic scars and keloids. Research results indicate that it can be used in the treatment of photoaging skin, cellulite and striae.

A pioneering study conducted by Yvan Capowiez *et al.*, (2015) explains about the Morphological and functional characterisation of the burrow systems of six earthworm species. Earthworm burrow systems are generally described based on postulated behaviors associated with the three ecological types. In this study, they used x-ray tomography to obtain 3D information on the burrowing behavior of six very common anecic (*Aporrectodea nocturna* and *Lumbricus terrestris*) and endogeic (*Aporrectodea rosea*, *Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Aporrectodea icterica*) earthworm species, introduced into repacked soil cores for 6 weeks. A simple water infiltration test, the Beerkan method, was also used to assess some functional properties of these burrow systems. Endogeic worms make larger burrow systems, which are more highly branched, less continuous and of smaller diameter, than those of anecic worms. Regarding water infiltration, anecic burrow systems were far more efficient due to open burrows linking the top and bottom of the cores. For endogeic species, we observed a linear relationship between burrow length and the water infiltration rate ($R^2 = 0.49$, $p < 0.01$). Overall, the three main characteristics significantly influencing water infiltration were burrow length, burrow number and bioturbation volume. This last characteristic highlighted the effect of burrow refilling by casts.

A study conducted by MT Rosa *et al.*, (2017) on Aloe Extracts, Pro and Antioxidant Conditions in Regeneration of the Planarian *Girardia tigrina*, reported that regeneration of the planarian *Girardia tigrina* was evaluated over different oxidative conditions, as pro oxidant (H₂O₂), antioxidant (vitamin C) and using aloe gel. The aloe plants have a millennial medicinal use and the succulent portion of leaf, called aloe gel, is used for wound healing. Here they analyze the action of aloe gel obtained from two species: *Aloe vera* and *A. arborescens*. The results show that ROS are important in the regeneration of *G. tigrina* and that the initial exposure to H₂O₂, soon after transection, accelerates the regeneration. However, during the regeneration process an antioxidant medium, containing vitamin C, promotes acceleration of regeneration, even if less intensely. Aloes extracts promote acceleration of regeneration in this planarian. No differences were observed among portions of cells in phases of cell cycle, with exception of worms exposed to *A. vera* extracts at 0.4% that show more cells in G₂ phases,

suggesting a faster cell cycling. No toxic effect was observed for the aloes extracts in planarians. Instead, an increase in the survival rate was observed in treated animals.

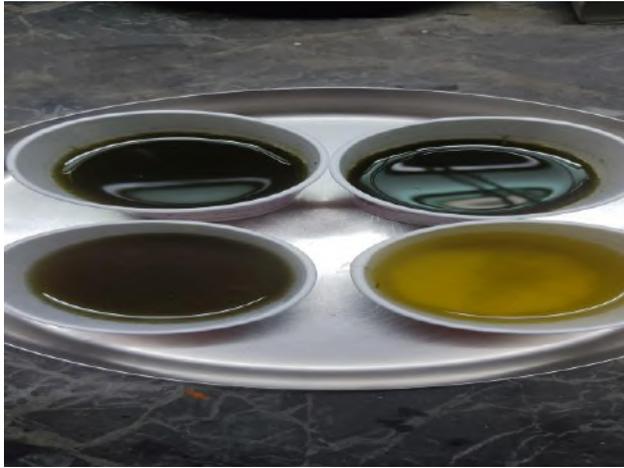
Mini V S *et al.*, (2021) conducted a study on the effect of *Aloe vera* extract on regeneration in earthworm, *Lampito mauritii*. They experimented to find the regenerative capacity of earthworm in *Aloe vera* extract. It was found that the anterior has the potential to be renewed unlike the posterior which do not have any vital organs. This kind of regeneration could happen due to the low level of differentiation in these organisms. Their data also revealed that *Aloe vera*, in small amounts (10-20%), has a positive effect on the regeneration of earthworm, speeding up regeneration time by about three days. More importantly, there was no significance in regeneration of earthworm in the lower and higher concentrations of *Aloe vera*. Moreover, the higher concentrations (40-80%) of *Aloe vera* appeared to be toxic to the earthworm. Thus, their project revealed that higher concentrations of *Aloe vera* would become lethal to earthworms. Low or medium amount of *Aloe vera* can be used as a regeneration promoting substance for earthworms. Their experimental results proved that *Aloe vera* had capability to promote regeneration of earthworms and it was suggested that *Aloe vera* can be used in vermicompost.

Neda Gholami *et al.*, (2022) had conducted a study on In vivo assessment of APPJ discharge on the earthworm: coelomic TAC and MDA levels, cell death, and tissue regeneration. The effective medical applications of cold atmospheric pressure plasma jet (APPJ) have been reported by many researchers including sterilization of liquid and solid surfaces, treatment of chronic wounds, cancer tumors and blood clots. Results showed APPJ induced significant effects on regeneration ability of earthworms after 20 and 30s of exposure ($p < 0.05$). Atmospheric plasma jet did not have significant effects on MDA content and TUNEL-positive cells, but this effect was significant for TAC and CAT in both species ($p < 0.05$). In conclusion the present study revealed for the first time that regeneration of missed segments in earthworms can be stimulated by plasma treatment.

METHODOLOGY

Collect the moist soil compost. Prepare 4 cups for the earthworms to live in by placing $\frac{1}{2}$ cup of moist compost in to the bottom of each cup. Choose 6 worms of good size and equal lengths, the front end of the worm will be closest to the clitellum. Using scissors cut the first 3 worms in half and place the two pieces of worm into two cups labeled, “front half” and “back half”. Using scissors cut 3 worms into a one-third piece two-third piece and place the two pieces of worm into separate cups with corresponding labels. Cover the cups with wrapping paper and secure to the tops of the cups with rubber bands. Poke several small air holes into the wrapping paper covering each cup. Keep the cups in a cool dark place for several days, make observations of worms in every 3 days and place $\frac{1}{2}$ cup of fresh moist compost into the cup. Continue to observe the worms every 3 days for 2 to 3 weeks. *Centella asiatica* gel is extracted from the leaves. It is made into a paste and preserved in a refrigerator (-250C) for 3 days. Repeat the experiment by adding *Aloe vera* solutions of 0%, 10%, 20%, 40% and 80% into soil in separate cups. Three worm fragments (fragments containing head region) were added to each mug containing the varying solutions of *Centella asiatica*. The length of each fragment was measured every 3 days.

IMAGES OF SAMPLES USED FOR EXTRACTION AND REGENERATION PROCESS





RESULTS

Through this experiment, we observed that the regeneration of earthworm took place in the presence of *Centella asiatica* extract. *Centella asiatica* in 20% &40% concentrations promoted regeneration in the cut area of the anterior half of equally cut earthworms. Wound healing properties of *Centella asiatica* may have aided in the regeneration process in the earthworms. Through this experimental study, the following results were obtained.

Table 1: Showing mean change in length of anterior regions of equal and unequal halves of three earthworms each.

DAYS	Normal (equal halves)		Normal (unequal halves)	
	(Average change in length in cm)			
	Cup I	Cup II	Cup III	Cup IV
	Head region	Tail region	Head region	Tail region
1	2	1.5	1.2	2
4	2.3	1.7	1.3	2.5
7	2.6	1.9	1.6	No change
10	3.1	No change	2	
13	3.5		2.2	
16	3.8		2.6	
19	4.2		2.9	
22	4.4		3.3	
25	4.7		3.6	
28	5.1		3.7	

Fig 1. Showing the same rate of regeneration of anterior regions of both equal and unequal halves.

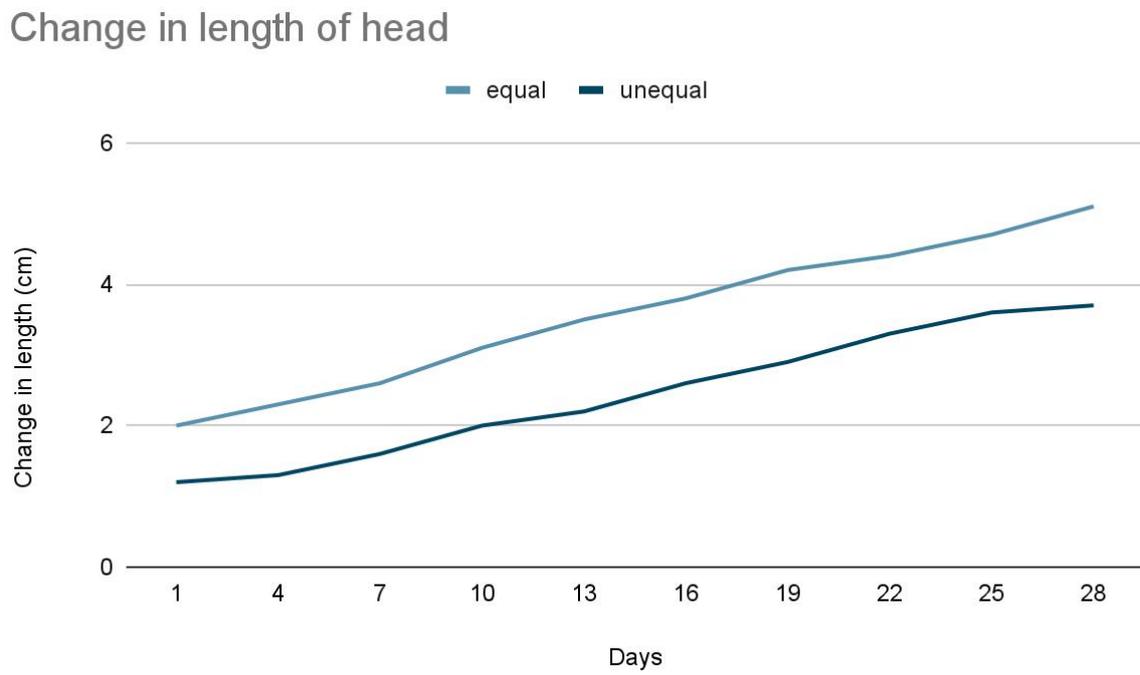
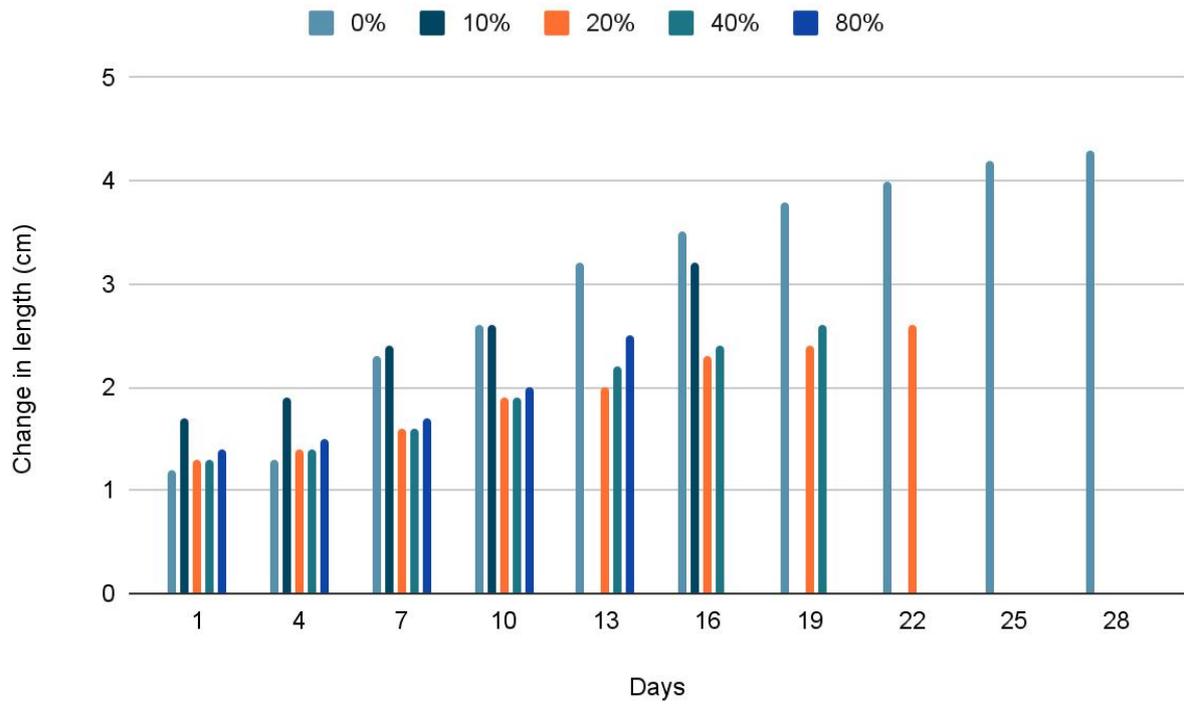


Table 2: Showing mean change in length of anterior regions of six earthworms after treatment with *Centella asiatica*.

DAYS	% of <i>Centella asiatica</i> extract in moist soil compost				
	(Average change in length of front half in cm)				
	0%	10%	20%	40%	80%
1	1.2	1.7	1.3	1.3	1.4
4	1.3	1.9	1.4	1.4	1.5
7	2.3	2.4	1.6	1.6	1.7
10	2.6	2.6	1.9	1.9	2
13	3.2	2,7	2	2.2	2.5
16	3.5	3.2	2.3	2.4	Dead
19	3.8	3,5	2.4	2.6	
22	4	Dead	2.6	Dead	
25	4.2		Dead		
28	4.3				

Fig 2: Graph showing variation in growth of earthworm in different concentrations of *Centella asiatica*.



DISCUSSION

Juraiporn Somboonwong, *et al.*, (2012) conducted an experiment on wound healing activities of different extracts of *Centella asiatica* in incision and burn wound models. Here, they report the wound healing activities of sequential hexane, ethyl acetate, methanol and water extracts of *Centella asiatica*. In this study it states that Asiatic acid in the ethyl acetate extracts seemed to be the most active component for wound healing purpose than any other extracts. Whereas in my experiment I use water extract where the regenerative capacity does not reach full potential. The regeneration ability attained small amounts in low concentrations especially at 20% and 40%.

Wieslawa Bylka, *et al.*, (2013) proposed an article on the use of *Centella asiatica* in cosmetology. *Centella asiatica* is a medical plant that has been used in folk medicine for years. This paper gives the wide information about the chemical compounds, tensile strength improvement as well as effective treatment of improving small and burns on animals. The selection of this plant was made possible because of this paper.

In the experimental study by Mini Vs, *et al.*, (2021) on the effect of *Aloe vera* Extract on regeneration in earthworms. They experimented to find the regenerative Capacity of earthworms in *Aloe vera* extract. Their data revealed that *Aloe vera*, in small amounts (10-20%), has a positive effect on the regeneration of earthworm, whereas in higher concentrations (40-80%) of *Aloe vera* appeared to be toxic to the earthworm. Thus, their project revealed that higher concentration of *Aloe vera* would become lethal to earthworms. Low or medium amount of *Aloe vera* can be used as a regeneration promoting substance for earthworms. In my experiment the earthworms do not show regenerative capacity in varying concentrations of *Centella asiatica* extract.

Nengwen Xiao, *et al.*, (2011) conducted a study on the regeneration capacity of an earthworm, *Eisenia fetida* in relation to the site of amputation along the body, they amputated earthworm at different body segment location along the length of the body to examine the different survival rates and regeneration length of anterior, posterior and medial sections. My aim was to check the regenerative capacity of the worms in different concentrations of the same extract.

In the article “The Differential Expression of Three labial Genes during Earthworm Head Regeneration “ by Sung -Jin Cho *et al.*, (2009) had explained about expression of three labial

genes in earthworm head regeneration. Here they report the full length of cloning of three labial genes (Pex-lab01, Pex-lab02, and Pex-lab 03) in the earthworm *Perionyx excavatus*. Their results indicate that three labial genes were expressed only in the head regenerating tissues. In my experiment also, only the head region showed regeneration. The tail region did not show change in length for 12 days and decayed on the 13th day.

CONCLUSION

Through this study it can be concluded that the earthworm react effectively towards *Centella asiatica* extracts for the purpose of regeneration. At low concentration, it proved to be involved in the cellular processes of cell proliferation, morphogenesis and cell differentiation of the annelid body. The regenerative capacity of the annelid body combined with chemical constituents of the *Centella asiatica* proved in the fast regeneration of the earthworm. Regeneration in earthworm is an epimorphic regeneration, which is very sensitive to the environmental situation and at the optimum condition regeneration of the anterior segments occurs but the posterior segment are destroyed. Therefore, the anterior has the potential to be renewed due to the existence of the stem cells, which help the worm to regenerate the lost organs, however at the tail sediments of the worm the nervous, digestive and respiratory structures does not exist this kind of regeneration happen due to the low level of differentiation in their organisms to the comparison with higher level organism in the evolution tree. In my data it proved that *Centella asiatica* extract lead to the regeneration of earthworm at a very fast rate. More importantly regeneration can be seen in the concentration of 10%,20%,40% but it does not reach 28 days of regeneration. The experimental study shows that *Centella asiatica* promote to the fast regeneration of earthworm at 20% and 40% of concentrations, and it suggested that aqueous extract of *Centella asiatica* does not seemed to be the most active component for the regeneration purpose. *Centella asiatica* can be used in the inhibiting the inflammatory phase of scars and can be used in soils and vermicompost, it also promotes to fertilize soil better.

SUGGESTIONS

- Improving the soil fertility.
- Better results of regeneration can be obtained when treated with ethyl acetate extract than the water extract of *Centella asiatica*.
- Improving the soil PH and temperature.
- Providing a better place and condition for the growth of earthworms.
- Sufficient space for good aeration and providing water content in the soil at frequent intervals.

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PAVLONIAN CONDITIONING IN GOLDFISH (*Carassius auratus*)



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Zoology

2021-2022

CERTIFICATE

This is to certify that the project report entitled "**PAVLONIAN CONDITONING IN GOLDFISHES**" submitted by Ms. SNEHA RENJITH , Reg. No. AB19ZOO041 in partial fulfillment of the requirements of Bachelor of Science degree, from St. Teresa's college, Ernakulam, is a bonafide work done under the guidance and supervision of Akhila Anilkumar and this is her original effort.

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EXAMINERS

1)

2)

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It is a great pleasure for me to undertake this project, I feel highly doing the project entitled – **PAVLONIAN CONDITIONING IN GOLDFISH**. I would like to express my profound gratitude towards many individuals, as without their kind support it would not be possible for me to complete this project. First and foremost, I thank God Almighty for the grace to complete the project successfully. I would like to extend my sincere thanks to my project guide **Ms. Akhila Anilkumar** for her guidance and constant supervision.

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ABSTRACT

Discovered by Russian physiologist Ivan Pavlov, classical conditioning is a type of unconscious or automatic learning. This learning process creates a conditioned response through associations between an unconditioned stimulus and a neutral stimulus. Here we have recorded our classical conditioning experiments in goldfishes. Goldfish have strong associative learning abilities, as well as social learning skills. In addition, their visual acuity allows them to distinguish between individual humans. Owners may notice that fish react favourably to them (swimming to the front bobbing of mouth, flickering of tail etc). they have strong memory and cognitive abilities equal to those of higher vertebrates Three fishes were taken in three separate bowls. The fish in the first bowl was given a neutral stimulus of colour and the one in the second bowl was given the stimulus of bell. It was provided with food immediately after the stimuli and was observed for two weeks. The response of the fishes was recorded. The fish in the third bowl was kept as control. According to our experiment, there was positive response in both the bowls. The experiment was conducted for about three weeks and after two weeks the fishes began responding to the stimuli The fishes were immediately fed after they are given the stimuli. The fish in bowl 1 responded to the colour stimulus from the 14th day of experiment. The responses in four consecutive days are recorded. As days passed the fish started responding quickly and moved to the surface mouthing for food. The goldfish in the second bowl also started showing response from the second week. It began to move faster to the surface where food was provided soon after confronting with the stimuli of bell sound. When they moved to the top of the bowl they showed movements of response like flickering the tail and bobbing of mouth. In control bowl the time remains constant. This experiment supported Pavlov's Classical Conditioning theory.

Classical conditioning has had a tremendous influence over the school of thought in psychology known as behaviorism. Behaviorism assumes that all learning occurs through interactions with the environment and that environment shapes behavior. In fish, classical conditioning has been known for decades. Studies have shown that, like mammals, fish are able not only to associate stimuli but can also associate them with with neutral stimuli. This project comprises of classical conditioning experiments in goldfishes that shows their cognitive and associative learning abilities.

INTRODUCTION

The goldfish (*Carassius auratus*) is a freshwater fish in the family Cyprinidae of order Cypriniformes. It is commonly kept as a pet in indoor aquariums and is one of the most popular aquarium fish. Native to East Asia, the goldfish is a relatively small member of the carp family (which also includes the Prussian carp and the crucian carp). It was first selectively bred for color in imperial China more than 1,000 years ago, and several distinct breeds have since been developed. Goldfish breeds vary greatly in size, body shape, fin configuration, and coloration (various combinations of white, yellow, orange, red, brown, and black are known). Goldfish can hybridize with some other *Carassius* species of carp. Koi and common carp may also interbreed with goldfish to produce sterile hybrids.



SIZE

When kept in small indoor aquariums, goldfish can grow to a size of about 1 inch (2.5 cm) to 2 inches (5.1 cm) long. Goldfish may grow larger if moved to bigger fish tanks, but they usually do not grow longer than 6 inches (15 cm).

VISION

Goldfish have one of the most studied senses of vision in fishes. Goldfish have four kinds of cone cells, which are respectively sensitive to different colors: red, green, blue and ultraviolet. The ability to distinguish between four different primary colors classifies them as tetrachromats.[1]

HEARING

Goldfish have one of the most studied senses of hearing fish. They have two otoliths, permitting the detection of sound particle motion, and Weberian ossicles connecting the swimbladder to the otoliths, facilitating the detection of sound pressure.

Goldfish can survive short periods of entirely anoxic conditions. Survival is shorter under higher temperatures, suggesting that this is a cold weather adaptation. Researchers speculate that this is specifically an adaptation to survival in frozen water bodies over winter.

Energy is obtained from liver glycogen. This process depends upon a pyruvate decarboxylase - the first known in vertebrates.

BEHAVIOUR

Goldfish are gregarious, displaying schooling behavior, as well as displaying the same types of feeding behaviors. As a fish, they can be described as “friendly” towards each other. Very rarely does a goldfish harm another goldfish, nor do the males harm the females during breeding.

COGNITIVE ABILITIES

Goldfish have strong associative learning abilities, as well as social leaning skills. In addition, their visual acuity allows them to distinguish between individual humans. Owners may notice that fish react favorably to them (swimming to the front of the glass, swimming rapidly around the tank, and going to the surface mouthing for food) while hiding when other people approach the tank. Over time, goldfish learn to associate their owners and other humans with food, often “begging” for food whenever their owners’ approach. Goldfish that have constant visual contact with humans also stop considering them to be a threat. After being kept in a tank for several weeks, sometimes months, it becomes possible to feed a goldfish by hand without it shying away.

PAVLO’S THEORY

Ivan Pavlov was a noted Russian physiologist who won the 1904 Nobel Prize for his work studying digestive processes. While studying digestion in dogs, Pavlov noted an interesting occurrence: His canine subjects would begin to salivate whenever an assistant entered the room. In his digestive research, Pavlov and his assistants would introduce a variety of edible and non-edible items and measure the saliva production that the items produced. Salivation, he noted, is a reflexive process. It occurs automatically in response to a specific stimulus and is not under conscious control. However, Pavlov noted that the dogs would often begin salivating in the absence of food and smell. He quickly realized that this salivary response was not due to an automatic, physiological process.

Based on his observations, Pavlov suggested that the salivation was a learned response. Pavlov's dog subjects were responding to the sight of the research assistants' white lab coats, which the animals had come to associate with the presentation of food. Unlike the salivary response to the presentation of food, which is an unconditioned reflex, salivating to the expectation of food is a conditioned reflex

Pavlov then focused on investigating exactly how these conditioned responses are learned or acquired. In a series of experiments, he set out to provoke a conditioned response to a previously neutral stimulus. He opted to use food as the unconditioned stimulus, or the stimulus that evokes a response naturally and automatically. The sound of a metronome was chosen to be the neutral stimulus. The dogs would first be exposed to the sound of the ticking metronome, and then the food was immediately presented. After several conditioning trials, Pavlov noted that the dogs began to salivate after hearing the metronome. A stimulus which was neutral in and of itself had been superimposed upon the action of the inborn alimentary reflex after several repetitions of the combined stimulation, the sounds of the metronome had acquired the property of stimulating salivary secretion." In other words, the previously neutral stimulus (the metronome) had become what is known as a conditioned stimulus that then provoked a conditioned response (salivation). In the present study, Goldfishes are used to study the conditioning response. While a Goldfish does not salivate, they do exhibit "excited" behavior during feeding time. This experiment will determine whether a Goldfish can be conditioned to certain stimuli such as light, color, bell and tapping. This study shows a conditional behaviour by the goldfish as in the Pavlov's Classical Conditioning experiment. Classical conditioning is used not only in therapeutic interventions, but in everyday life as well. It shows the behavioural changes and patterns in fishes, which is important in aquacultures.

OBJECTIVES

- 1.To study the classical conditioning in Goldfish
- 2.To acclimatize the fish and to note its behavioural patterns
- 3.To record the responses of the fishes to the given stimuli.

REVIEW OF LITERATURE

As we know the best known and earliest work on classical conditioning was done by Ivan Pavlov, although Edwin Twitmyer published some related findings a year earlier. During his research on physiology of digestion in dogs, Pavlov developed a procedure that enable him to study the digestive process of animals over long periods of time. Pavlov noticed that the dog began to salivate in the presence of technician for regular feed. Pavlov presented a stimulus (bell ringing sound) then gave the food. After few repetitions, the dog started to salivate in response to the stimuli. According to his theory, lot of articles were published, few articles are given below.

Experiments on classical aversive conditioning in goldfish were designed by Bitteman, M.E. *et al.*, (1967) to test hypotheses about the role of CS-US interval suggested by earlier experiments on avoidance conditioning in goldfish. Light was paired with shock, and general activity which light came to evoke was measured. The performance of independent groups trained at different CS-US intervals (0-27 sec.) was compared on interspersed testing trials at the same long interval (20 or 40 sec.) in terms both of total activity and of temporal distribution of activity on testing trials. The results help account for certain quantitative features of goldfish behavior in the shuttle box and support Pavlov's interpretation of the role of the CS-US interval in conditioning.

In their work, F. Rodriguez *et al.*, (2005) analyzed the involvement of the teleost fish cerebellum on classical conditioning of motor and emotional responses and on spatial cognition. Cerebellum lesions in goldfish impair the classical conditioning of a simple eye-retraction response analogous to the eyeblink conditioning described in mammals. Single unit extracellular electrophysiological recording and cytochrome oxidase histochemistry also reveal the involvement of the teleost fish cerebellum in classical conditioning. Autonomic emotional responses (e.g., heart rate classical conditioning) are also impaired by cerebellum lesions in goldfish. Furthermore, goldfish with cerebellum lesions present a severe impairment in spatial cognition. In contrast, cerebellum lesions do not produce any observable motor deficit as indicated by the swimming activity or obstacle avoidance and do not interfere with the occurrence of unconditioned motor or emotional responses.

These data indicate that the functional involvement of the teleost cerebellum in learning and memory is strikingly similar to mammals and suggest that the cognitive and emotional functions of the cerebellum may have evolved early in vertebrate evolution, having been conserved along the phylogenetic history of the extant vertebrate groups.

Unconditioned aggressive-display behavior elicited by the mirror image of a male Siamese Fighting Fish was brought under the control of a previously ineffective stimulus by classical conditioning. A stimulus light repeatedly paired with mirror presentation came to elicit the complex aggressive-behavior sequence. Relative rates of acquisition of four components of the display were compared. Fin erection and undulating movements were acquired most rapidly while gill-cover erection and frontal approach were acquired most slowly. A discriminative conditioning procedure revealed that the response was specifically elicited by the conditioned stimulus, and not a sensitization artifact. This experiment was performed by Travis Thompson and Thomas Sturm (1965).

V.E. Shashoua and G.E. Hesse (1989) conducted ELISA measurements showed that brain extracellular fluid (ECF) levels of endymin decreased for animals that learned to associate a paired presentation of a light stimulus (CS) with the onset of an electric shock (US), whereas no changes were obtained for control goldfish that received the same number of stimuli delivered in a random unpaired order.

Adriana Beatriz Barretto *et al.*, (2018) isolated fish in individual aquariums and introduced a water jet that caused localized water movement, followed by the introduction of a food pellet. These procedures were repeated for each fish for 20 days. After 14 days, all fish were conditioned. Moreover, in subsequent probe trials (memory retention tests) conducted within 32 days after conditioning procedures, fish responded accordingly. These findings corroborate the applicability and usefulness of the method tested herein especially under lab conditions. Therefore, we suggest that a simple water jet is a useful and reliable tool for fish conditioning in future studies. Since it is known that the length of delays and the spacing of trials are important factors with regard to deficits engendered by telencephalic ablation, these were investigated in the present work.

The present study examined the nature of the “avoidance” response in goldfish under the linear presentation procedure by Zerbolio *et al.*, (1981). With this procedure, shuttling behavior occurring during the presentation of the trial stimulus produces either CS⁻ or CS⁺, and further occurrence of shuttling within the trial interval (10 sec) changes the value of CS from negative to positive, or vice versa. If the fish remains in the compartment when the prevailing cue state is CS⁻ at the end of the interval, shock can be avoided. With this procedure fish responded to the CS⁺ more than to the CS⁻ and avoided shock. But fish in one of two control groups, in which responses had no effect in changing the cue state from CS⁺ to CS⁻, or vice versa, also showed a clear differentiation. The results were generally in line with the view that the “avoidance” response in fish is acquired through classical conditioning. The contribution of classical conditioning to the acquisition of avoidance response is discussed.

The traditional control procedures for pavlovian conditioning are examined and each is found wanting. Some procedures introduce no associative factors do not present in the experimental procedure while others transform the excitatory, experimental cs-ucs contingency into an inhibitory contingency. An alternative control procedure is suggested in which there is no contingency whatsoever between cs and ucs. This "truly random" control procedure leads to a new conception of pavlovian conditioning postulating that the contingency between cs and ucs, rather than the pairing of cs and ucs, is the important event in conditioning. The fruitfulness of this new conception of pavlovian conditioning is illustrated by 2 experimental results.

A preparation for the study of classical fear conditioning in vertebrates is described by Peter Barela (2012). Its unique features are that it is inexpensive and easy to construct and operate. The following classical conditioning phenomena are demonstrated using this preparation: excitatory conditioning, extinction, contextual conditioning, blocking, a conditioned inhibition discrimination, and latent inhibition.

The possible role of eyespot patterns in predator recognition by paradise fish was examined using a passive avoidance conditioning technique with various dummies or live goldfish. It was found that a low-intensity shock, although clearly uncomfortable, elicited exploratory behavior in the fish and that observable learning did not occur. However, if the paradise fish was shocked in the presence of a live goldfish or various fish dummies, exploration diminished and avoidance learning

was detected. This was characterized by a considerable increase in latency to enter the shocked compartment. The most effective dummies were those with two laterally arranged eye-like spots.

Scobie, S. R *et al.*, (1974) experimented on the Operant and Pavlovian control of a defensive shuttle response in goldfish. When a visual signal preceded electric shock, goldfish acquired shuttle responses both when a response avoided the shock (operant contingency) and when shock was not avoidable (Pavlovian contingency). Asymptotic levels of responding were significantly higher when shock could be avoided but were still substantial when shock was not avoidable. Response termination of the signal had little effect on performance. A control experiment showed that responding resulted from the signal-shock contingency and not from nonassociative factors. A search within the Pavlovian contingency for an UCR similar in form to the CR was inconclusive. (PsycINFO Database Record (c) 2016 APA, all rights reserved)

Drew, M. R *et al.*, (2005) conducted temporal Control of Conditioned Responding in Goldfish. The peak procedure was used to characterize response timing during acquisition and maintenance of conditioned responding in goldfish. Subjects received light-shock pairings with a 5- or 15-s interstimulus interval. On interspersed peak trials, the conditioned stimulus light was presented for 45 s and no shock was delivered. Peaks in the conditioned response, general activity, occurred at about the time of the expected unconditioned stimulus, and variability in the activity distribution was scalar. Modeling of the changes in the activity distributions over sessions revealed that the temporal features of the conditioned response changed very little during acquisition. The data suggest that times are learned early in training, and, contrary to I. P. Pavlov's (1927/1960) concept of "inhibition of delay," that timing is learning when to respond rather than learning when not to respond.

To determine whether regenerating neural pathways can support visual behavior, adult goldfish (*Carassius auratus*) were injected intraocularly with ouabain and tested for the presence of reflexive visual behaviors (dorsal light reflex and optokinetic nystagmus) and the ability to respond to visual stimuli in a classical conditioning paradigm. All visual behaviors were absent or greatly diminished until 8 to 10 weeks, when retinal layering had returned. At 10 weeks post-ouabain, reflexive behaviors to supra-threshold stimuli were near normal; however, the ability to detect supra-threshold stimuli in the conditioning paradigm did not recover until 13 weeks. Absolute dark-adapted threshold and light-adapted spectral sensitivity measured at 13 to 17 weeks were

abnormal: Dark-adapted threshold was elevated by 1.5 log units and light-adapted spectral sensitivity was markedly narrower than normal. No responses to 50% contrast sinusoidal gratings could be obtained through ouabain-treated eyes using the classical conditioning technique, even though responses through the untreated eye remained. Results demonstrate that: (a) visually mediated behaviors return in goldfish with ouabain-treated retinas; (b) the time course of recovery of reflexive responses in luminance and spatial domains parallels return of ERG function and of tectal activity; and (c) visual function that is mediated by regenerating retina appears not to be as sensitive as vision *via* normally developed retinal pathways.

The generalized activity of goldfish was measured in a classical conditioning situation using brief shock as the US and light as the CS by Gonzales *et al.*, (1999) formed by varying duration of training or the consistency of reinforcement did not differ in resistance to extinction. Groups subjected to a series of further extinctions with interpolated conditioning showed a decline in resistance on subsequent extinctions, but no differences were found between partially and consistently reinforced Ss. Results are discussed in relation to experiments on the partial-reinforcement effect in other species.

The behavioral responses of goldfish to a variety of amplitude-modulated (AM) acoustic signals were studied by Richard R. Fay using a stimulus generalization paradigm in combination with a classical conditioning method. Measurements were thus allowed of the equivalence among various complex and simple signals in controlling the conditioned respiration response. The goldfish's conditioned response to AM signals were found to be controlled by carrier frequency, modulation depth, and to a lesser extent, by modulation frequency in the 40-Hz range. On the other hand, pure-tone frequency exerted minimal behavioral control for subjects conditioned to AM signals. Subjects conditioned to a 40-Hz pure tone tended to generalize maximally to an AM signal having a 40-Hz modulation rate. The results indicate the degree to which the goldfish can use much of the information present in complex periodic acoustic signals and suggest the existence of periodicity pitch in fish.

Yoshida *et al.*, (2005) they studied on Involvement of the cerebellum in classical fear conditioning in goldfish. To investigate the emotional role of the cerebellum of fish, they conducted experiments examining effects of cerebellum manipulation on fear related classical heart rate conditioning in goldfish. They performed total ablation of the corpus carbelli to examine the effects of reversible

inactivation of the cerebellar function. Both the cardiac arousal response to the first presentation of the conditioned stimulus and the cardiac reflex to the aversive unconditioned stimulus were not impaired by the ablation or cooling of the corpus cerebelli.

Valente *et al.*, (2012) studied on Pavlov conditioning of classical and operant learning of zebra fish. They performed of developing zebra fish in both classical and operant conditioning assays wasted with a particular focus on the emergence on these behaviours during the development. Strategically positioned visual cues paired with electroshocks were used in fully automated assays to investigate both learning paradigms. These allow the evaluation of the behavioural performance of zebrafish continually throughout development from larva to adult.

Karen L Holis *et al.*,(1995) studied on Pavlov conditioning of signal- centred action patterns and autonomic behavior. This article describes the ways in which Pavlovian conditioning operates in several biological contexts. The chapter suggests that Pavlovian conditioning may transform some of the environment's capriciousness, and that the Pavlovian conditional response plays an important role in optimizing interactions with biologically important events. Through selected animal learning and animal behavior experiments the chapter highlights the ways in which an animal may incorporate a signaling operation in the various aspects of its search for food including (1) Locomotory search and approach behavior, (2) Consummatory food-procuring behavior and (3) Autonomic behavior related to food ingestion. The importance of Pavlovian conditioning in the development of food selection and in the avoidance or consumption of poisons is also discussed.

Russ E Carpenter and Cliff H Summers, they studied learning strategies during fear conditioning (Neurobiology of learning and memory 91 (4), 415-423,2009). This paper describes a model of fear learning, in which subjects have an option of behavioral responses to impending social defeat. The model generates two types of learning: social avoidance and classical conditioning, dependent upon (1) escape from or (2) social subordination to an aggressor. We hypothesized that social stress provides the impetus as well as the necessary information to stimulate dichotomous goal-oriented learning. Specialized tanks were constructed to subject rainbow trout to a conditioning paradigm where the conditioned stimulus (CS) is is cessation of tank water flow (water off) and the unconditioned stimulus (US) is social aggression from a larger conspecific. Following seven

daily CS/US pairings, approximately half of the test fish learned to consistently escape the aggression to a neutral chamber through a small escape hole available during the interaction.

Prompted by discrepant earlier findings, 3 experiments were conducted by Bitterman M.E *et al.*, (1963) on the role of the CS-US interval (0.5, 2.0, or 4.0 sec.) in the classical conditioning of fish— 1 with 54 *Mollinnesia* given massed training (1.5 min. between trials), a 2nd with 54 *Mollinnesia* given more spaced training (4 min. between trials), and a 3rd with gold-fish given massed training. Probability and magnitude of response to the CS declined from a maximum at 0.5 sec. in the 1st experiment, but were not significantly related to the CS-US interval in the other 2. Latency of response was least at the 0.5-sec. interval in each case. The results are consistent with the Pavlovian interpretation of delayed conditioning.

MATERIALS AND METHODS

MATERIALS REQUIRED

3 Goldfishes, 3 glass bowls or glasses, water (free from chlorine), fish feed, colour papers, bell, stopwatch.



METHODOLOGY

The behavioural changes were noticed for the three consecutive stages of experiments.

Stage 1

The fishes were acclimatized by keeping them for about a week in their respective bowls. During this period, they were only given food which is an unconditioned stimulus. The fishes were calmly moving in the bowl and reached the surface as soon as the feed was given.

Stage 2

In the second week the experiment of conditioning was put into force. When the neutral stimuli were given before the feeding, the fishes showed vigorous movements and swimming pattern in circular manner in the experimental bowls. When the fishes were fed after this, they reached the surface, but after a delayed time period. The experiments were continued for one week, until the fishes were somewhat conditioned to the neutral stimuli.

- 1) First fill the five bowls with water.
- 2) Then add each goldfish to the 3 bowls.
- 3) 2 bowls with goldfish kept for experiments and one bowl with goldfish as control.
- 4) Label each bowl according to the stimuli.

Bowl A - response to colour

Bowl B - response to sound

Bowl C - control bowl

Each bowl is marked according to the stimuli. The first bowl is covered by a colour paper before the time of feed. Cover it for one minute and take down the observations of goldfish. In bowl B make sound by ringing a bell and take down the observation and time. Note separately the observation of the control fish.

5) Feed each fish.

6) Make observation during feeding and note the time required for feeding. (observations like moaning of fins, swimming pattern, moving of tails etc.)

7) Repeat this for 4-6 days and twice a day each.



Response to colour stimulus



Response to sound of the bell

RESULTS

The behavioural changes were noticed for three consecutive stages of the experiments.

Stage 1

The fishes were acclimatized by keeping them for about a week in their respective bowls. During this period, they were only given food which is an unconditioned stimulus. The fishes were calmly moving in the bowl and reached the surface as soon as the feed was given.

Stage 2

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Table no. 1 Response to Colour Stimulus

Experimental day	Behaviour pattern when stimulus given	Time taken to reach the surface for feeding
Day 14	The fish was in the middle of the glass, slowly reached the top when it noticed the food	11 seconds
Day 15	The fish was in the middle of the glass, reached the top faster as it noticed the food	9 seconds
Day 16	The fish was in the middle of the glass, and quickly reached the top, bobbing the mouth and flickering its tail.	6.5 seconds
Day 17	The fish is now able to notice the type of feeding. When it sees the covered colour paper, the fish flickers its tail, bobbing the mouth and swimming in a pattern reaches the top where food is given	4 seconds

Table No. 2 Response to Sound stimulus

Experimental Day	Behaviour pattern when stimulus given	Time taken to reach the surface for feeding
Day 14	While ringing the bell the fish in the glass moves to the surface as it noticed the food	7 seconds
Day 15	While ringing the bell the fish in the glass moves to the surface faster.	5 seconds
Day 16	While ringing the bell the fish moved the surface quickly, flickering its tail and bobbing the mouth.	4 seconds
Day 17	While ringing the bell, the fish reached the surface faster, bobbing its mouth and flickering its tail.	3 seconds

Control bowl

Experimental Day	Behaviour pattern when stimulus given	Time taken to reach the surface for feeding
Day 14	The fish was in the middle of the bowl, reached the top slowly when it noticed the food	5 seconds
Day 15	The fish was in the middle of the bowl, reached the top slowly when it noticed the food	5 seconds
Day 16	The fish was in the middle of the bowl, reached the top slowly when it noticed the food	5 seconds
Day 17	The fish was in the middle of the bowl, reached the top slowly when it noticed the food	5 seconds

Fig 1. Response to Sound stimulus

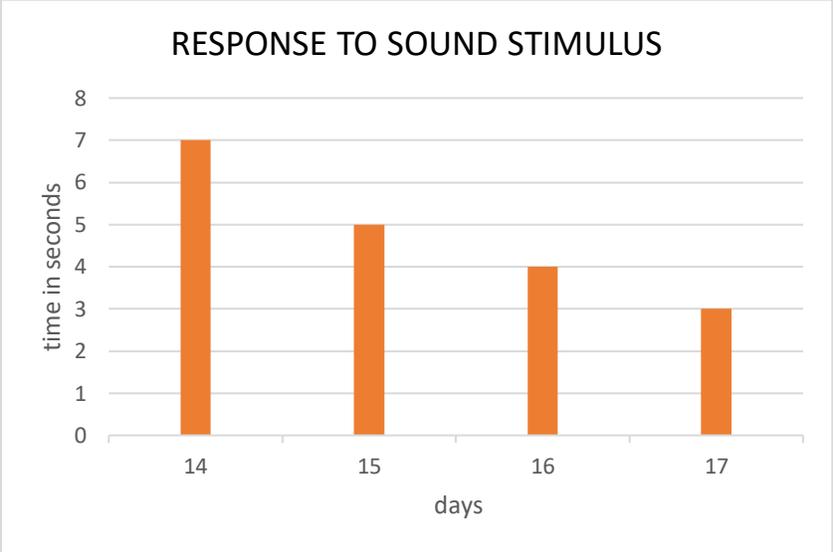
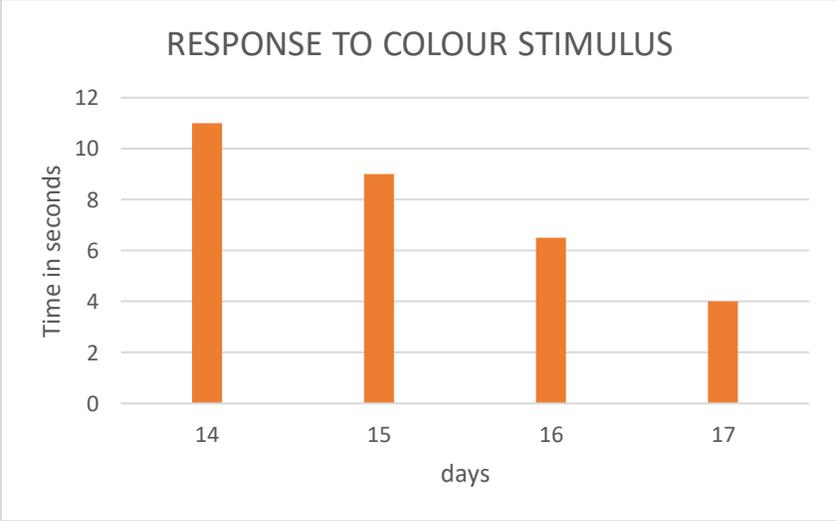
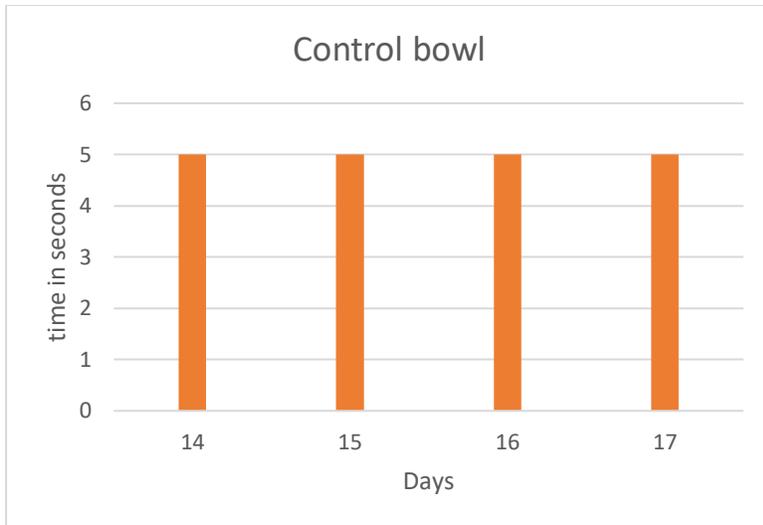


Fig 2. Response to Colour Stimulus





There was a positive result in both the bowls as the days passed on. The fish responded easily to ringing the bell and colour stimuli. The main observation noted is the positive change of the goldfish while feeding. It took lesser time to reach the surface. When they noticed the food, they started flickering their tail and bobbing the mouth. The time is also recorded while the food was given. In control bowl the time remain constant.

DISCUSSION

According to our experiment, there was positive response in both the bowls. The experiment was conducted for about three weeks and after two weeks the fishes began responding to the stimuli. The neutral stimuli used were sound of the bell and colour. The fishes were immediately fed after they are given the stimuli. The fish in bowl 1 responded to the colour stimulus from the 14th day of experiment. The responses in four consecutive days are recorded. As days passed the fish started responding quickly and moved to the surface mouthing for food. The goldfish in the second bowl also started showing response from the second week. It began to move faster to the surface where food was provided soon after confronting with the stimuli of bell sound. When they moved to the top of the bowl they showed movements of response like flickering the tail and bobbing of mouth. In control bowl the time remains constant. This experiment supported Pavlov's Classical Conditioning theory.

Bitterman's experiment with goldfish showed the effects of change in amount of reward that are predicted from reinforcement theory. This shows that the amount of reward can also bring changes in the response of the fish. Aggressive display behaviour elicited by the mirror image of fighter fish as classical conditioning by Travis Thompson et al. The movements of the fish in response where most rapid fin erection and gill- cover erection while the movements we observed were bobbing of mouth and flickering of tail. This could be because the unconditioned stimulus was the fish feed.

It is also revealed from the ELISA experiment that ependymin played a role in the learning abilities of fishes. Also there was no classical conditioning where no motor learning occurs. The response is closely associated with the motor learning of the fish, and it is made clear from our experiment too. Adriana Beatriz et al conducted experiments on goldfish by introducing a water jet that caused localized water movement, followed by the introduction of food pellet. After 14 days of experiment the fish were conditioned. In our experiment also, the fish was conditioned in the 14th or 15th day.

It is clear from the experiments that avoidance response in fish is acquired through classical conditioning. The fishes tried to avoid shocks. They also showed classical fear conditioning and

escape mechanisms. This includes changes in body movements and swimming patterns and directions. The possible role of species-specific key stimuli in avoidance learning and organizing defensive behavior was revealed by the experiments with paradise fish. It stopped its exploratory behaviour when it confronted with its predator goldfish or their dummies. Similar avoidance behaviors are seen in goldfishes also.

The cerebellum of the fish is also involved in the classical conditioning. This reminds them of the unconditioned stimuli followed by the conditioned one. In our experiment the goldfish, immediately after confronting the neutral stimuli moved to the surface of the glass, in memory of the food during the first few days. Cerebellum is also involved in the classical fear conditioning of the fish as in the experiment conducted by Valente et al. Also, social avoidance is also studied in the fishes. Social stress provides the impetus as well as necessary information to stimulate dichotomous goal-oriental learning. Goal-oriental learning and avoidance of unfamiliar situations were observed in our fishes too.

CONCLUSION

Classical conditioning is a type of unconscious or automatic learning. This learning process creates a conditioned response through associations between an unconditioned stimulus and a neutral stimulus. Colour and sound of the bell were the neutral stimuli used in this study. Soon, after the stimuli are provided, the fish were given feed as positive reinforcement. First the fishes showed a swimming pattern as if they are scared to the stimuli. After a few days the stimuli were paired with reinforcement by the fishes and they showed calm behavioral pattern, as in the control. The conditioned responses were flickering of the tail, bobbing of mouth and swimming patterns.

Goldfishes have strong associative learning abilities, as well as social learning skills. In addition, their visual acuity allows them to distinguish between individual humans. Owners may notice that fish react favorably to them (swimming to the front of the glass, swimming rapidly around the tank and going to the surface mouthing for food.) while hiding when other people approach the tank. Similarly, this study shows a conditional behaviour by the goldfish as in the Pavlov's Classical Conditioning experiment. Classical conditioning is used not only in therapeutic interventions, but in everyday life as well.

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**EFFECT OF *ALOE VERA* GEL ON REGENERATION OF
EARTHWORM *PHERETIMA POSTHUMA*.**



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Submitted to St. Teresa's college (Autonomous), Eranakulam

**Affiliated to Mahatma Gandhi University, Kottayam in partial
Fulfilment of requirement for the degree of Bachelor of Science
In Zoology**

2021 -2022

CERTIFICATE

This is to certify that the project report entitled "**EFFECT OF *ALOE VERA* GEL ON REGENERATION OF EARTHWORM *PHERETIMA POSTHUMA***" submitted by Ms.VARSHA C.J., Reg. No. AB19ZOO042 in partial fulfillment of the requirements of Bachelor of Science degree of Mahatma Gandhi University, Kottayam, is a bonafide work done under the guidance and supervision of Ms.Akhila Anilkumar and this is her original effort.

Ms. Akhila Anilkumar
Assistant Professor
Department of Zoology
St Teresa's College, (Autonomous)
Ernakulam

EXAMINERS

- 1)
- 2)

DECLARATION

I, hereby declare that this project work entitled “**EFFECT OF *ALOE VERA* GEL ON REGENERATION OF EARTHWORM *PHERETIMA POSTHUMA*.**” is submitted to St.Teresa’s College (Autonomous), Ernakulam affiliated to Mahatma Gandhi University, Kottayam in partial fulfillment of the requirements of Bachelor of Science degree in Zoology. This work has been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in the report is entirely my own .

Name:Varsha C. J.

Signature

Reg.No: AB19ZOO042

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The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my project. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

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ABSTRACT

Earthworms are the terrestrial *Oligocheates* of the class *Chaetopoda* and phylum Annelida. Earthworms *Pheretima posthuma* species found in India as well as many regions of the world. The species *Pheretima posthuma* is an experimental animal recommended by OECD in 1984. *Pheretima posthuma* is an indigenous cheap test species, which is widely used in vermiculture, agriculture, etc. and it can be easily cultivated in the laboratory conditions. The main aim of our study is to get a more comprehensive understanding on the effects of *Aloe vera* extract on the regeneration of earthworms, *Pheretima posthuma*. So, earthworms, *Pheretima posthuma* species were selected for the present study. In this study we cut worms in different places and in different sized pieces to find out if there is relationship between regeneration and size or orientation of the body. For that, 21 earthworms of the species *Pheretima posthuma* were collected. They were cut into equal halves and their growth was observed in soil containing different concentrations of the *Aloe vera* extract. The experimental results proved that *Aloe vera* in concentrations 10%, and 20% promotes the regeneration of earthworms. Therefore, low to high concentrations of *Aloe vera* extract can't be used as a regeneration promoting substance for earthworms. It may be suggested that *Aloe vera* can be used in soils and vermicompost during vermiculture for preventing the loss of earthworms due to autotomy or other mechanical injuries and to progress their regeneration process. The promotion of better health on earthworms can be helpful in agricultural practices as they help fertilize the soil better.

Keywords: Regeneration, Earth worm, *Aloe vera*, Extraction.

INTRODUCTION

An earthworm is a terrestrial invertebrate. They occur worldwide where soil, water, and temperature allow. Earthworms are commonly found in soil, eating a wide variety of organic matter. This organic matter includes plant matter, living protozoa, rotifers, nematodes, bacteria, fungi, and other microorganisms. The principal systematic features of earthworms are that they are bilaterally symmetrical, externally segmented, with a corresponding internal segmentation. They have no skeleton and a thinly pigmented cuticle, bearing setae on all segments except the first two; with an outer layer of circular muscles and an inner layer of longitudinal muscles. They are hermaphrodite and have relatively few gonads, which are situated in definite segmental positions. When mature, a swollen area of the epidermis called a clitellum, located in particular segments, forms a cocoon in which the eggs or ova are deposited, and this is then passed over the anterior segment. The eggs are usually fertilized and the young develop within the eggs without a free larval stage, the newly hatched worms resembling adults. Structurally, earthworms have large coelomic cavities containing coelomocytes, a closed vascular system with at least a dorsal and a ventral trunk and a ventral nerve cord. The alimentary canal is basically an anterior-posterior tube with excretion through the anus or specialized organs called nephridia; respiration is mainly cuticular.

Pheretima posthuma:

Kingdom	Animalia
Phylum	Annelida
Class	Oligochaeta
Order	Haplotaxida
Suborder	Lumbricina
Family	Megascolecidae
Genus	Pheretima
Species	posthuma



Pheretima posthuma

Body is long, narrow and cylindrical. Length may reach upto 150 mm. Body colour is brown. Anterior end is pointed while the posterior end is blunt. Body is divided into 100-140 segments called metameres. The anteriormost segment is called Prostomium. Mouth is a crescentic aperture, present at anterior end. The segment containing mouth is called peristomium. Setae are present at all the segments except-1st and last. Each seta is embedded in a setal sac. A glandular band called clitellum is situated in 14th to 16th segments. It forms cocoon during the reproduction. Female genital pore is situated in 14th segment while male genital pore is present in 18th segment. The earthworm feeds on organic matter in the soil.

The food is sucked by the pharynx and the oesophageal glands add calcite to neutralise acidity of the soil. The food is then grinded by the horny lining of the gizzard and is absorbed in the intestine. Undigested food material passes out the anus and is deposited as worm castings. The earthworm 'breathes' by the diffusion of gases through its moist skin. The blood contains haemoglobin which transports oxygen throughout the body. Circulatory system is of closed type. Earthworms have no sense organs but they can sense light intensity by small light-sensitive cells found mainly on the upper skin surface of their body. They can also sense vibrations and chemicals by the means of tactile or chemo-receptors. The earthworms exhibit undulating movement which takes place by alternate contraction and relaxation of circular and longitudinal muscles of each segment. Earthworms are hermaphrodites but they reproduce by cross-fertilization.

IMPORTANCE OF EARTHWORM

Of all the members of the soil food web, earthworms need the least introduction. Most people become familiar with these soft, slimy, invertebrates at a young age. An earthworm is a segmented worm, a terrestrial invertebrate belonging to phylum Annelida. Earthworms occur in most temperate soil and many tropical soils. They are major decomposers of dead and decomposing organic matter, and derive their nutrition from bacteria and fungi that grow upon these materials. They fragment organic matter and make contribution to recycling the nutrients it contains. Earthworms dramatically change soil structure, water movement, nutrient dynamics and plant growth. They are not essential to cell healthy soil systems but their presence is usually an indicator of a healthy system. Earthworms perform several beneficial functions.

Stimulate microbial activity: Earthworms derive their nutrition from microorganisms, many more microorganisms are present in their feces/casts than in organic matter that they consume. Increased microbial activity facilitates the cycling of nutrients from organic matter and their conversion into a form readily taken up by plants.

Mix and aggregate soil: As they consume organic matter and mineral particles, earth excreta wastes is the form of casts, a type of soil aggregate.

Increased infiltration: Earthworms enhance porosity as they move through the soil. Some species make permanent burrows deep into the soil. It can be a major conduit for soil drainage particularly under heavy rainfall. At the same time burrows reduce surface water erosion.

Improve water-holding capacity: Earthworms can significantly increase the water-holding capacity of soils.

Provide a channel for root growth: The channel made by deep burrowing earthworms are lined with readily available nutrients and make it easier for roots to penetrate deep into the soil.

Bury and shred plant residue: plant and crop residue are gradually buried by cast material deposited on the surface and as earthworms pull surface residue into their burrows.

Interaction of earthworm with other members of the food web: Earthworms influence soil-inhabiting invertebrates by changing the amount and distribution of organic matter and microbial population.

Economic Importance of Earthworm: Earthworms are extremely beneficial in agriculture. They aid in the following ways. The earthworm improves the fertility of soil in different ways, they are of utmost importance in agriculture. The burrowing and soil feeding habits of earthworms make the soil porous which permit both aeration and quick absorption of water. They also reduce the alkalinity and acidity of the soil to provide better conditions for plant growth. Thus earthworms are better known as the friend of farmers. Many people earn their livelihood by catching these worms and supplying them to scientific laboratories. Ayurvedic and Unani systems of therapy suggest that these worms are used in making medicines for the care of diseases like bladder stones, piles, rheumatism, jaundice etc. They are also used as baits to catch fish. It also plays a great role in vermiculture to produce high -quality manure.

REGENERATION IN EARTHWORM

Regeneration in earthworm is an epimorphic regeneration, which is very sensitive to the environmental situations. The existence of stem cells help the worm to regenerate the lost organs. This kind of regeneration could be happened due to the low level of differentiation in these organisms to the comparison with higher level organisms in the evolutionary tree. But this ability varies between species and depends on the extent of the damage. There were significant differences in both survival rates and lengths of regeneration between immature earthworms and clitellate adult earthworms during the early stages of regeneration, but not at later stages of regeneration. The immature earthworms had a greater Regeneration potential than clitellate adults amputated at the same segment. The survival rates of earthworms were correlated significantly with the number of body segments remaining after amputation, but not with the position of the amputation. If an earthworm is split into two, it will not become two new worms. The head of the worm may survive and regenerate its tail if the animal is cut behind the clitella. But the original tail of the worm will not be able to grow a new head or the Rest of its vital organs and will instead die.

Present study focused to determine the effect of medicinal plant *Aloe vera* on earthworm regeneration.



Scientific name: *Aloe vera*

Family: *Asphodelaceae*

Common name: *Aloe vera*

Useful parts: leaves, gel.

Aloe vera is one of approximately 420 species of the genus *Aloe* (Dagne *et al.*, 2000), which is variously classified as belonging to the *Asphodelaceae*, *Liliaceae*, or *Aloaceae* families. *Aloe vera* is a perennial succulent xerophyte; it has elongated and pointed leaves that are joined at the stem in a rosette pattern and that grow to about 30–50 cm in length and 10 cm in breadth at the base in the adult plant (World Health Organization, 1999). The leaf is protected by a thick, green epidermis layer (skin or rind), which surrounds the mesophyll. Immediately beneath the rind are located the vascular bundles, which are composed of three types of tubular structures: the xylem; transports water and minerals from roots to leaves, the phloem; transports starch and other synthesized products to the roots and the large pericyclic tubules; contains the yellow leaf exudates commonly referred to as “aloes,” “sap,” or “latex” (Boudreau and Beland 2006). The parenchyma is the major part of the leaf by volume, contains a clear mucilaginous gel known as *Aloe vera* gel (Femenia *et al.*, 2003). *Aloe vera* is considered to be the most biologically active of the *Aloe* species (World Health Organization 1999). More than 75 potentially active constituents have been identified in the plant including vitamins, minerals, saccharides, amino acids, anthraquinones, enzymes, lignin, saponins, and salicylic acids. The leaf exudate contains anthraquinones, particularly barbaloin, which appear to be responsible for its bitter taste and cathartic effect (Dagne *et al.*, 2000; Boudreau and Beland, 2006). Barbaloin and other products of the phenyl propanoid pathway are commonly referred to as polyphenolic compounds. These are derived from the precursor phenolic acids, and they may act as antioxidants to inhibit free

radical mediated cytotoxicity and lipid peroxidation. *Aloe vera* also contains products of the isoprenoid pathway, including carotenoids, steroids, terpenes and phytosterols (Samman, 1998). Isoprenoids can be regarded as sensory molecules because they contribute to the colour and fragrance of the products in which they exist.

Aloes extracts promotes acceleration of regeneration. No differences were observed among portions of cells in phases of cell cycle, with exception of worms exposed to *Aloe vera* extracts at 0.4% that show more cells in G2 phases, suggesting a faster cell cycling. No toxic effect was observed for the aloes extracts in planarians. Instead, was observed an increase in the survival rate in treated animals (Heber 2007). Studies also proved that *Aloe vera* extract would speed up the regeneration of Planaria but higher concentrations of *Aloe vera* would not make any difference in regeneration time (Crasnik, 2008). For tissue regeneration, angiogenesis is essentially required to provide oxygen and metabolites to the tissues. *Aloe vera* can infiltrate into the tissues and increase the transport and activities of biological factors involved in tissue regeneration such as nutrients, cells, enzymes, blood circulation, and oxygen content [58,63]. Different mechanisms are involved in the wound healing effects of *Aloe vera*, including keeping the wound moist, increase of epithelial cell migration, more rapid maturation of collagen, and reduction in inflammation [4,14]. The tissue regenerating function of *Aloe vera* is essentially a result of the synergistic mode of action of many bioactive compounds [64]. Synergy in this case is the interaction of active substances at the biochemical level, as well as the synergistic response of the body to these substances as components of *Aloe vera* consumed. Evidence shows that emodin, one of the derivatives of anthraquinones is also capable of promoting tissue regeneration.

Earthworms are extremely important in soil formation, maintenance and structure and turnover of dead organic matter. Because of their availability and rapid regenerative power, annelids have been used commonly to study regeneration. Among annelids, the earthworm, which is important in breaking down organic wastes, has been used commonly for research into regeneration, because it is easy to culture and handle in the laboratory. This issue of regeneration of segments by amputated earthworms also has practical application to the build-up of populations, especially since it appears that amputation has greater effects on mature earthworms than immature ones. Clearly, further research is needed to clarify the mechanisms of regeneration.

OBJECTIVES

1. To see the effect of *Aloe vera* extract on regeneration of earthworms, *Pheretima posthuma*.
2. To test whether higher concentrations of *Aloe vera* would speed up the regeneration of earthworm, *Pheretima posthuma*.
3. To investigate how much a worm can be left to regenerate a new worm or if one end is better regenerating than the other.

REVIEW OF LITERATURE

Mini *et al.*,(2021) conducted a study on the effect of *Aloe vera* extract on regeneration in earthworm, *Lampito mauritii*. It was found that the anterior has potential to be renewed unlike the posterior which do not have any vital organs. This kind of regeneration could happen due to the low level of differentiation in these organisms. Data also revealed that *Aloe vera*, in small amounts (10-20%), has a positive effect on the regeneration of earthworm, speeding up regeneration time by about three days. More importantly, there was no significance in regeneration of earthworm in the very lower and higher concentrations of *Aloe vera*. Moreover, the higher concentrations (40-80%) of *Aloe vera* appeared to be toxic to the earthworm. Their experimental results proved that *Aloe vera* had capability to promote regeneration of earthworms and it was suggested that *Aloe vera* can be used in vermicompost.

Neda Gholami *et al.*,(2021) had conducted a study on In vivo assessment of APPJ discharge on the earthworm: coelomic TAC and MDA levels, cell death, and tissue regeneration. The effective medical applications of cold atmospheric pressure plasma jet (APPJ) have been reported by many researchers including sterilization of liquid and solid surfaces, treatment of chronic wounds, cancer tumours and blood clots. Results showed APPJ induced significant effects on regeneration ability of earthworms after 20 and 30s of exposure ($p < 0.05$). Atmospheric plasma jet did not have significant effects on MDA content and TUNEL-positive cells, but this effect was significant for TAC and CAT in both species ($p < 0.05$). In conclusion the present study revealed for the first time that regeneration of missed segments in earthworms can be stimulated by plasma treatment.

Yun Seon Bae *et al.*, (2020) discovered the characterization of *Perionyx excavatus* development and its head regeneration. The regeneration of the central nervous system is limited to specific animals including *Perionyx excavatus*. Here they set up a culture system to sustain the life cycle of *P. excavatus* and characterize the development of *P. excavatus* from embryo to juvenile, based on morphology, myogenesis and neurogenesis. Their data suggest that *P. excavatus* is a model system to study CNS regeneration.

An interesting study conducted by Rajesh Kumar Singh *et al*; (2019) on the topic corrosion of snails in acidic environments and their protection shows the importance of *Aloe vera* in the protection of snails. The study mentions that snail's protection is essential because this species is to maintain a balanced ecology of water sources. They occur in rivers as well as ponds and balance the pH level of water. But these sources of water are contaminated by effluents, pollutants, acid rain, particulates, biological wastes etc. They can change the pH of water. Water is an absorber of carbon dioxide and it converts carbon dioxide into carbonic. Other above-mentioned wastes also increase the concentration of H⁺ ions in water. They produce a hostile environment for snails. The outer part of snails is made of CaCO₃. It produces chemical reactions in acidic medium and corrosion reaction is accelerated, and thus deterioration starts on the surface of snails. This medium makes their survival become miserable. For this work, corrosion of the snail's study in the pH values of water is 6.5 in H₂CO₃ environment. The corrosion rates of snails were calculated by gravimetric methods and potentiostat technique. *Aloe vera* was used for corrosion protection in acidic medium. The surface adsorption phenomenon was studied by Langmuir isotherm. *Aloe vera* formed thin surface film on the interface of snails which adhered with chemical bonding. It was confirmed by activation energy, heat of adsorption, free energy, enthalpy and entropy. The results of surface coverage area and inhibitor efficiency indicated that *Aloe vera* developed a strong protective barrier in the acidic medium.

A study conducted by MT Rosa *et al.*, (2017) on Aloe Extracts, Pro and Antioxidant Conditions in regeneration of the Planarian *Girardia tigrina*, reported that regeneration of the planarian *Girardia tigrina* was evaluated over different oxidative conditions, as pro oxidant (H₂O₂), antioxidant (vitamin C) and using aloe gel. The aloe plants have a millennial medicinal use and the succulent portion of leaf, called aloe gel, is used for wound healing. Here they analyses the action of aloe gel obtained from two species: *Aloe vera* and *A. arborescens*. The result shows that ROS are important in the regeneration of *G. tigrina* and that the initial exposition to H₂O₂, soon after transection, accelerate the regeneration. However, during the regeneration process an antioxidant medium, containing vitamin C, promotes acceleration of regeneration, even if less intensely. Aloe extracts promotes acceleration of regeneration in this planarian. No differences

were observed among portions of cells in phases of cell cycle, with exception of worms exposed to *A.vera* extracts at 0.4% that show more cells in G2 phases, suggesting a faster cell cycling. No toxic effect was observed for the aloes extracts in planarians. Instead, an increase in the survival rate was observed in treated animals.

A pioneering study conducted by Yvan Capowiez *et al.*,(2015) explains about the morphological and functional characterisation of the burrow systems of six earthworm species. Earthworm burrow systems are generally described based on postulated behaviors associated with the three ecological types. In this study, they used x-ray tomography to obtain 3D information on the burrowing behavior of six very common anecic (*Aporrectodea nocturna* and *Lumbricus terrestris*) and endogeic (*Aporrectodea rosea*, *Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Aporrectodea icterica*) earthworm species, introduced into repacked soil cores for 6 weeks. A simple water infiltration test, the Beerkan method, was also used to assess some functional properties of these burrow systems. Endogeic worms make larger burrow systems, which are more highly branched, less continuous and of smaller diameter, than those of anecic worms. Regarding water infiltration, anecic burrow systems were far more efficient due to open burrows linking the top and bottom of the cores. For endogeic species, we observed a linear relationship between burrow length and the water infiltration rate ($R^2 = 0.49$, $p < 0.01$). Overall, the three main characteristics significantly influencing water infiltration were burrow length, burrow number and bioturbation volume. This last characteristic highlighted the effect of burrow refilling by casts.

A study conducted by Nengwen Xiao *et al.*, (2011) on the regeneration capacity of an earthworm, *Eisenia fetida* in relation to the site of amputation along the body. Our aim was to link the regeneration capacity of an earthworm, *Eisenia fetida* with the site of amputation, so we amputated earthworm at different body segment location along the length of the body to examine the different survival rates and regeneration length of anterior, posterior and medial sections.

Paithankar V. V. *et al.*,(2011) proposed a paper on *Phyllanthus niruri*. The paper gives information regarding the general properties, geographical distribution, and chemical constitution of *Phyllanthus niruri*. Pharmacological and biological activity – hepatoprotective

effect, worldwide traditional medicinal uses, chromosome aberration inhibition, analgesic activity, antispasmodic activity etc. seen in *Phyllanthus niruri* are discussed.

Tara Shanbhag *et al.*,(2010) evaluates the effect of ethanolic extract of *Phyllanthus niruri*. Linn (Euphorbiaceae) on experimentally induced burn wound model in rats and to find whether it reverses the wound healing in steroid suppressed rats. In burn wound model, oral and topical administration of *Phyllanthus niruri* did not show any significant effects in wound contraction and period of epithelialisation when compared to control. In dexamethasone suppressed burn wound model, wound contraction rate was increased significantly by topical ($P < 0.001$) and oral ($P < 0.001$) administrations of *Phyllanthus niruri* by about 47.57% and 26.16% respectively. Topical administration has shown significant ($P < 0.05$) enhancement of wound contraction than oral dosage form. The Dexamethasone depressed epithelialization period was reversed significantly by topical ($P < 0.0001$) and oral ($P < 0.001$) administrations of *Phyllanthus niruri* by about 32.5% and 21.3% respectively. It was concluded that both topical and oral administrations of ethanolic extract of *Phyllanthus niruri* are found to reverse dexamethasone suppressed burn wound healing.

Sung-Jin Cho *et al.*, (2009) had explained the differential expression of three labial genes during Earthworm head regeneration. Here they report the full length cloning of three labial genes (Pex-lab01, Pex-lab02, and Pex-lab 03) in the earthworm *Perionyx excavatus*. To analyze their expression pattern during head and tail regeneration, they used the reverse transcription-polymerase chain reaction. Their results indicate that the three labial genes were expressed only in the head regenerating tissues.

A study was conducted by Shishin Kawamoto *et al.*,(2005) on bipolar head regeneration induced by artificial amputation in *Enchytraeus japonensis* (Annelida, Oligochaeta). As per the study the Oligochaeta *Enchytraeus japonensis* propagates asexually by spontaneous autotomy. Normally, each of the 5-10 fragments derived from a single worm regenerates a head anteriorly and a tail posteriorly. Occasionally, however, a head is formed posteriorly in addition to the normal anterior head, resulting in a bipolar worm. This phenomenon prompted us to conduct a series of experiments to clarify how the head and the tail are determined during regeneration in this

species. The results showed that (1) bipolar head regeneration occurred only after artificial amputation, and not by spontaneous autotomy, (2) anesthesia before amputation raised the frequency of bipolar head regeneration, and (3) an extraordinarily high proportion of artificially amputated head fragments regenerated posterior heads. Close microscopic observation of body segments showed that each trunk segment has one specific autotomic position, while the head segments anterior to the VIIth segment do not. Only the most posterior segment VII in the head has an autotomic position. Examination just after amputation found that the artificial cutting plane did not correspond to the normal autotomic position in most cases. As time passed, however, the proportion of worms whose cutting planes corresponded to the autotomic position increased. It was suspected that the fragments autotomized after the artificial amputation (corrective autotomy). This post-amputation autotomy was probably inhibited by anesthesia. The rate at which amputated fragments did not autotomize corresponded roughly to the rate of bipolar regeneration. It was hypothesized then that the head regenerated posteriorly if a fragment was not amputated at the precise autotomic position from which it regenerated without succeeding in corrective autotomy.

Bely A and Wray G (2001) conducted a study on evolution of regeneration and fission in Annelids: insights from engrailed and orthodenticle class gene expression development. They presents a detailed comparison of regulatory gene expression during regeneration and asexual reproduction (by fission) in the segmented worm *Pristina leidy* (Annelida:Oligocheta). In Situ hybridization studies on worms undergoing normal growth, regeneration and fission demonstrated that in all three processes, *Pl-en* is primarily expressed in the developing nervous system, *Pl-Otx1* and *Pl-Otx2* are expressed primarily in the anterior body wall, foregut and developing nervous system. They state that these annelids fission may have evolved by recruitment of regenerative processes. Furthermore, by comparing the existing data from leech embryos, they found evidence that embryonic processes are re-deployed during regeneration and fission.

A science fair project by Lloyd H. Barrow(2000) guides the reader in exploration of one of the many small animals that live in the ground; earthworms. Barrow's detailed science experiments will help beginning biologists create winning science fair projects. Book will make observations

about earthworm structure and behavior, movement, preferences, and reactions. Included in the book are easy-to-follow diagrams that offer readers exact information on performing the experiments. None of the experiments in this book in any way harm the worms.

World Health Organization in Geneva have published a monograph on selected medicinal plant (1999) provide scientific information on the safety, efficacy, and quality control/quality assurance of widely used medicinal plants, in order to facilitate their appropriate use in Member States; provide models to assist Member States in developing their own graphs or formularies for these or other herbal medicines; and facilitate information exchange among Member States. Each monograph contains two parts. The first part consists of pharmacopoeial summaries for quality assurance: botanical features, distribution, identity tests, purity requirements, chemical assays, and active or major chemical constituents.

Clive A. Edwards and P.J. Bohlen (1996) proposed a paper on biology and ecology of earthworms. It is in the third edition of this popular text where reviews on all aspects of earthworm biology and ecology are mentioned. These include a greatly expanded treatment of earthworm community ecology, interactions between earthworms and microorganisms, and the importance of earthworm in environmental management and their use in organic waste management. The book also summarizes the toxicity to earthworms of a wide range of chemicals.

Gairdner B Moment (1974) explained the variation and its causation in earthworm regeneration. The coefficient of variation for the number of segments regenerated of earthworms in environmental management and their use in organic waste management. The book also summarizes the toxicity to earthworms of a wide range of chemicals. Posteriorly by earthworms rarely exceeds 10. The manner of wound healing after the transaction cannot be responsible for the variation since under the conditions of these experiments all worms healed in the open manner. Bilateral asymmetry in the number of segments regenerated indicates that the counting mechanism underlying variation can hardly be humoral because both blood and coelomic fluid are common to both sides of the body.

Darwin (1809–1882) published his last scientific book entitled “The formation of vegetable mould through the action of worms with observations on their habits”, the result of several decades of detailed observations and measurements on earthworms and the natural sciences. .The book covers the importance of earthworm activity on a variety of topics: pedogenesis and weathering processes, soil horizon differentiation and the formation of vegetable mould (topsoil), the role of earthworm burrowing and casting (bioturbation) in soil fertility and plant growth, the burial of organic materials and soil enrichment with mineral elements, the global cycle of erosion–sedimentation with hydrologic and aerial transfers of fine particles brought up to the soil surface by earthworms and the protection of archaeological remains through their burial. Finally, Darwin also performed a series of original experiments to determine if earthworms possessed, or not, a certain “intelligence”.

METHODOLOGY

Collect the moist soil compost. Prepare 4 cups for the earthworms to live in by placing $\frac{1}{2}$ cup of moist compost in to the bottom of each cup. Choose 6 worms of good size and equal lengths, the front end of the worm will be closest to the clitellum. Using scissors cut the first 3 worms in half and place the two pieces of worm into two cups labeled, “front half” and “back half”. Using scissors cut 3 worms into a one-third piece two-third piece and place the two pieces of worm into separate cups with corresponding labels. Cover the cups with wrapping paper and secure to the tops of the cups with rubber bands. Poke several small air holes into the wrapping paper covering each cup. Keep the cups in a cool dark place for several days, make observations of worms in every 3 days and place $\frac{1}{2}$ cup of fresh moist compost into the cup. Continue to observe the worms every 3 days for 2 to 3 weeks. *Aloe vera* gel is extracted from the leaves. It is made into a paste and preserved in a refrigerator (-250C) for 3 days. Repeat the experiment by adding *Aloe vera* solutions of 0%, 10%, 20%, 40% and 80% into soil in separate cups. Three worm fragments (fragments containing head region) were added to each mug containing the varying solutions of *Aloe vera*. The length of each fragment was measured every 3 days.



RESULTS

Result of the present study shows that the regeneration of earthworm took place in the presence of *Aloe vera* and only the head regions of earthworm showed regeneration. The tail regions did not show change in length for 4-7 days . Equal posterior halves decayed on 10th day and the unequal posterior halves decayed on 7th day. Experimental data also revealed that *Aloe vera*, in small amounts (10-20%), has a positive effect on the regeneration of earthworm in the cut area of the anterior half of equally cut worms, speeding up regeneration time by about three days. More importantly, there was no significance in regeneration of Earthworm in the very lower and higher concentrations of *Aloe vera*. Moreover, the higher concentrations (40-80%) of *Aloe vera* appeared to be toxic to the earthworm. Earthworm in 40% *Aloe vera* extract died on the 13th day and those in 80% extract died on 10th day.

Table1. showing mean change in length of anterior regions of equal and unequal halves of three earthworms each.

Day	Normal(equal halves)		Normal(unequal halves)	
	(Average change in length in cm)			
	Cup I	Cup II	Cup III	Cup IV
	Head region	Tail region	Head region	Tail region
1	3.16	3.16	3.83	1.83
4	3.5	3.16	4.03	1.83
7	3.9	3.16	4.3	Decayed
10	4.3	Decayed	4.7	
13	4.6		5.03	
16	4.9		5.4	
19	5.4		5.7	
22	5.8		6	
25	6.6		6.9	
28	7		7.3	

Fig1. showing same rate of regeneration of anterior regions of both equal and unequal halves.

Regeneration of equal and unequal halves of Earthworm.

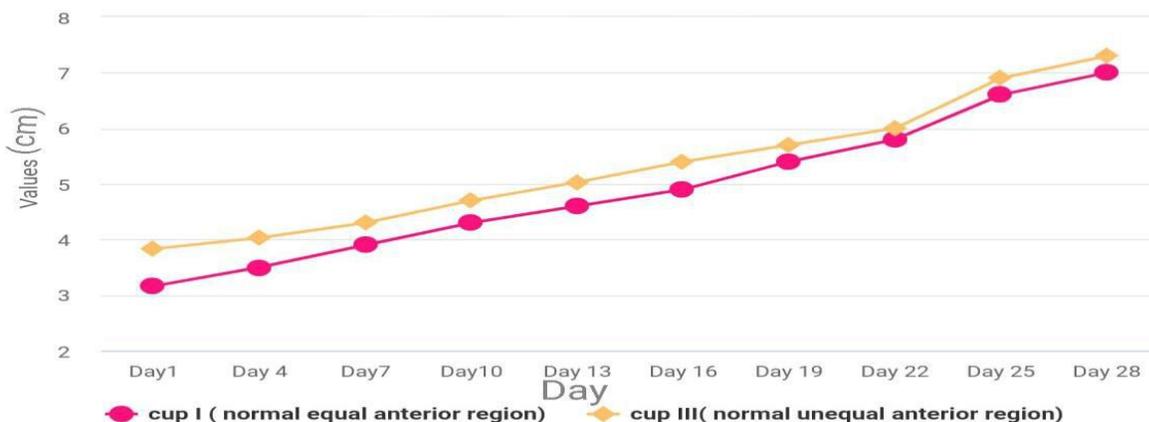
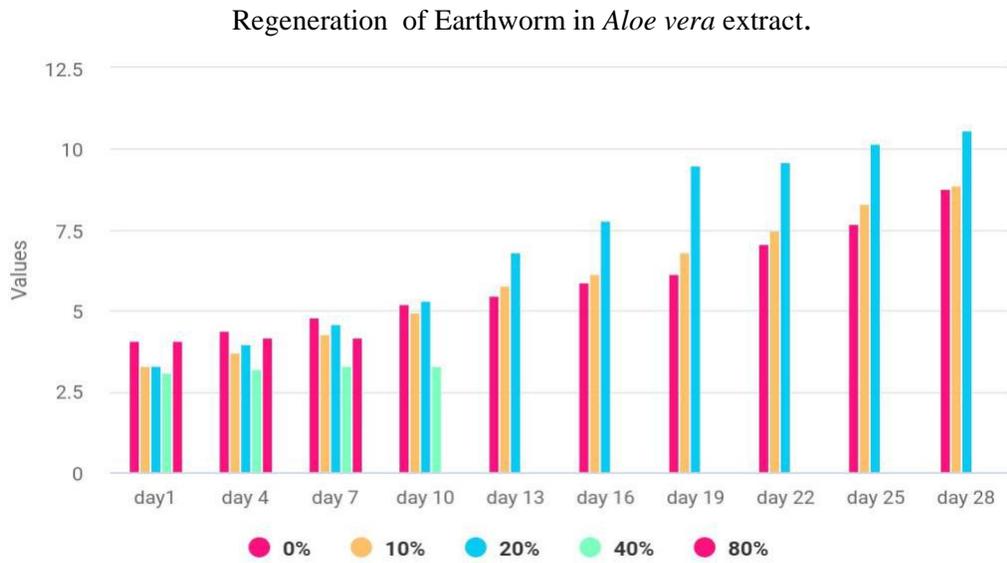


Table 2. Showing mean change in length of anterior regions of three earthworms in *Aloe vera* treatment.

% of <i>Aloe vera</i> extract in moist soil.					
DAY	(Average change in length of front half in cm)				
	0%	10%	20%	40%	80%
1	4.1	3.3	3.3	3.08	4.6
4	4.4	3.7	4	3.2	4.16
7	4.8	4.3	4.6	3.3	4.16
10	5.2	4.96	5.3	3.3	Died
13	5.5	5.8	6.8	Died	
16	5.9	6.13	7.8		
19	6.16	6.85	9.5		
22	7.1	7.5	9.6		
25	7.7	8.35	10.2		
28	8.8	8.95	10.6		

Fig2. Showing mean change in length of anterior regions of three earthworms after *Aloe vera* treatment up to 28th day.



10%



20%



normal

DISCUSSION

The results obtained in the present study explained that *Aloe vera* was a **promoter of regeneration**. Regeneration in earthworm (*Pheretima prosthuma*) is an epimorphic regeneration, which is very sensitive to the environmental situations, and at the optimum condition, regeneration of the anterior segments occurs but the posterior segments were destroyed. Therefore, the anterior has potential to be renewed due to the existence of stem cells, which help the worm to regenerate the lost organs, however at the tail segments of the worm the nervous, digestive and respiratory structures does not exist. This kind of regeneration could be due to the low level of differentiation in these organisms to the comparison with higher level organisms in the evolutionary tree. Our data also revealed that *Aloe vera*, in small amounts (10-20%), has a positive effect on the regeneration of earthworm, speeding up regeneration time by about three days. More importantly, there was no significance in regeneration of earthworm in the very lower and higher concentrations of *Aloe vera*. Moreover, the higher concentrations (40-80%) of *Aloe vera* appeared to be toxic to the earthworm.

Mini *et al.*,(2021) conducted a study on the effect of *Aloe vera* extract on regeneration in earthworm, *Lampito mauritii*. It was found that the anterior has potential to be renewed unlike the posterior which do not have any vital organs. This kind of regeneration could happen due to the low level of differentiation in these organisms. Data also revealed that *Aloe vera*, in small amounts (10-20%), has a positive effect on the regeneration of earthworm, speeding up regeneration time by about three days. More importantly, there was no significance in regeneration of earthworm in the very lower and higher concentrations of *Aloe vera*. Moreover, the higher concentrations (40-80%) of *Aloe vera* appeared to be toxic to the earthworm. Their experimental results proved that *Aloe vera* had capability to promote regeneration of earthworms and it was suggested that *Aloe vera* can be used in vermicompost. In my study also proved that *Aloe vera* in small amounts (10-20%) has a positive effect on the regeneration of earth worm *Pheretima prosthuma.*, speeding up the regeneration time by about three days and the higher concentrations of *Aloe vera* (40-80%) were lethal to earthworm.

In study conducted by Nengwen Xiao *et al.*, (2011) on the regeneration capacity of an Earthworm, *Eisenia fetida* in relation to the site of amputation along the body, they amputated Earthworm at different body segment location along the length of the body to examine the different survival rates and regeneration length of anterior, posterior and medial sections. My aim was to check the regenerative capacity of the worms in different concentrations of the same extract.

In the article “The differential expression of three labial genes during Earthworm head regeneration” by Sung-Jin Cho *et al.*, (2009) had explained about expression of three labial genes in earthworm head regeneration. Here they report the full length cloning of three labial genes (Pex-lab01, Pex-lab02, and Pex-lab 03) in the earthworm *Perionyx excavatus*. Their results indicate that the three labial genes were expressed only in the head regenerating tissues. In my experiment also, only the head regions showed regeneration. The tail regions did not show change in length for 7days and decayed on the 7th-10th day.

In the article An evaluation of the biological and toxicological properties of *Aloe barbadensis* by Boudreau, M. D. & Beland, F. A. (2006), *Aloe barbadensis* (Miller), *Aloe vera*, has a long history of use as a topical and oral therapeutic. The plant is the source of two products, gel and latex, which are obtained from its fleshy leaves. *Aloe vera* products contain multiple constituents with potential biological and toxicological activities, yet the active components elude definition. Ingestion of *Aloe vera* is associated with diarrhea, electrolyte imbalance etc. My experimental results are also supporting the toxicity of *Aloe vera* in higher concentrations.

Yoshida-Noro *et al.*, (2000) conducted an experiment on *Enchytraeus japonensis* and observed that the enchytraeid oligochaete *Enchytraeus japonensis* reproduces asexually by fragmentation under laboratory conditions. In the fragmentation process, fully-grown worms break into several fragments. Each fragment regenerates into a small but complete worm in 4 days, grows rapidly and divides again in another 10 days. We found that this fragmentation can be induced artificially by amputating the head if the worms are at least 5–6 mm in length, but not in shorter worms. The fragmentation is inducible by removing the most anterior two segments, if the worms are large enough. When a worm is cut into two, fragmentation occurs more readily in the posterior section. Moreover, even a small incision made in the ventral side of the trunk causes

fragmentation in the body posterior to the incision. Immersion of the worms in water is found to inhibit fragmentation even in decapitated worms. When a worm is placed in water immediately after decapitation, the ability to fragment is gradually lost as a new head regenerates. From these results it is postulated that the ability to fragment acquired early in the growth phase is suppressed by head-derived signal(s) until spontaneous fragmentation occurs. The signals seem to be constantly transmitted through the ventral nerve cord until the head matures and lifts its blockade of fragmentation, allowing this process to proceed. At present, however, we cannot exclude the possibility that the mature head of the worm produces fragmentation-stimulating signals. Study on *Pheretima posthuma* also revealed that when a worm is cut into two, fragmentation occur more readily in the posterior region. And also regeneration was visible in the head region.

CONCLUSION

Through this study it can be concluded that earthworms react efficiently towards *Aloe vera* extract in the property of regeneration. From high to low concentrations, it proved to be involved in the cellular processes of cell proliferation, morphogenesis and cell differentiation of the annelid body. The regenerative capacity of the annelid body combined with the chemical constituents of the *Aloe vera* proved in the fast regeneration of the earthworms. Regeneration in earthworm is an epimorphic regeneration, which is very sensitive to the environmental situations, and at the optimum condition, regeneration of the anterior segments occurs but the posterior segments are destroyed. Therefore, the anterior has potential to be renewed due to the existence of stem cells, which help the worm to regenerate the lost organs, however at the tail segments of the worm the nervous, digestive and respiratory structures do not exist. This kind of regeneration could happen due to the low level of differentiation in these organisms to the comparison with higher level organisms in the evolutionary tree. My data also revealed that *Aloe vera* extract helped significantly in the regeneration of earthworms, speeding up regeneration time. More importantly, regeneration was achieved in concentrations 10%, 20%, 40% as well as 80%. But higher concentrations (40-80%) appeared to be toxic and earthworms in those suspensions died within 10-13 day. The experimental results proved that *Aloe vera* had capability to promote regeneration of earthworms and it can be suggested that *Aloe vera* can be used in soils and vermicompost during vermiculture for preventing the loss of earthworms due to autotomy or other mechanical injuries and to progress their regeneration process.

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