AN ANALYSIS OF THE POTABILITY OF DRINKING WATER FROM SELECTED AREAS OF THOOTHUKUDI

Dissertation Submitted to

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in partial fulfilment for the award of the degree of

MASTER OF SCIENCE IN ZOOLOGY

By

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(Re-accredited with A^+ Grade by NAAC)

THOOTHUKUDI

APRIL – 2021

CERTIFICATE

This is to certify that this dissertation entitled **"AN ANALYSIS OF THE POTABILITY OF DRINKING WATER FROM SELECTED AREAS OF THOOTHUKUDI"** submitted by **A. JUDIYA**, Reg. No 19APZO01 to St. Mary's College (Autonomous) Thoothukudi affiliated to Manonmaniam Sundaranar University in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2020-2021 under my guidance and supervision. It is further certified that the dissertation or any part of this has not been submitted elsewhere for any other degree

Place: Thoothukudi

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DECLARATION

I hereby declare that the thesis entitled **"AN ANALYSIS OF THE POTABILITY OF DRINKING WATER FROM SELECTED AREAS OF THOOTHUKUDI"** Submitted by me for the degree of Master of Science in Zoology is the result of my original and independent research work carried out underthe guidance of Dr. Mrs. S. Mary Baptista Janet, M.Sc., B.Ed., M.Phil., PhD., Associate Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi, and it has not been submitted elsewhere for any other degree

Place: Thoothukudi

Date : 15.04.2021

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Signature of Candidate

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CONTENTS

INTRODUCTION

INTRODUCTION

Having access to Safe drinking water is a human right to all people, regardless of nationality, religion, colour, wealth and creed. Contaminated drinking water and poor sanitation are linked to transmission of disease such as cholera, diarrhea, dysentery and polio (WHO 2018) Poor drinking water quality significantly affecting the health of consumers. It was reported that at least 2 billion people globally used a drinking water source contaminated with faeces (WHO 2018)

People need clean water to maintain their health and dignity. Water is essential in breaking the cycle of property since it improves people's health, strength to work and ability to go to school. From 1990 to 2011, global efforts have helped 2.1 billion people gain access to improved drinking water, but not all of these new sources are necessarily safe (WHO/UNICEF, 2013). Yet 884 million people around the world live without improved drinking water and 2.5 billion people still lack access to improved sanitation, including 1.2 billion who do not have simple latrine at all (WHO/UNICEF, 2008). Families living in remote rural areas and urban slims, families displaced by war and famine, and families living in the poverty – disease trap, for whom improved sanitation and drinking water could offer a way out.

The World Health Organization (WHO) estimates that 88% of diarrheal disease is caused by unsafe, inadequate sanitation and poor hygiene.

As a result, more than 4,500 children die every day from diarrhea and other diseases. For every child that dies, countless others, including older children and adults, suffer from poor health and opportunities for work and education.

The quality of our global freshwater supplies is under increased threat of contamination. While water contains natural contaminants, it is becoming more and more polluted by human activities, such as open defecation, inadequate wastewater management, dumping of garbage, poor agricultural practices, and chemical spills at industrial sites. Faecel contamination of drinking water is a major contributor to diarrheal disease.

Water testing plays an important role in monitoring the correct operation of water supplies, verifying the safety of drinking water, investigating disease outbreaks and validating processes and preventive measures (Bain et al., 2012). The WHO/ UNICEF Joint monitoring programme for Water Supply and Sanitation (JMP) is the official United Nations Oganization responsible for monitoring progress towards the Millennium Development Goals (MDG) targets for improved drinking water and sanitation.

We find our drinking water from different places depending on where we live in the world. Three sources that are used to collect drinking water are:

- Ground water – Water that fills the spaces between rocks and soil making an aquifer. Ground water depth and quality varies

from place to place. About half of the world's drinking water comes from the ground.

- Surface water – Water that is taken directly from a stream, river, lake, pond, spring or similar source. Surface water quality is generally unsafe to drink without treatment.
- Rain water – Water that is collected and stored using a roof top, ground surface or rock catchment. The quality of rain water collect from a roof surface is usually better than a ground surface or rock catchment.

Water is in continuous movement on, above and below the surface of the earth. Water quality will vary from place to place, with the seasons and with various kinds of rock and soil which it moves through. It is important for us to judge the potability of water by taking the following three qualities into consideration:

1. Microbiological – bacteria, viruses, protozoa and worms
2. Chemical – minerals, metals and chemicals
3. Physical – temperature, colour, smell, taste and turbidity

Safe drinking water should have the following microbiological, chemical and physical qualities:

- Free of pathogens
- Low in concentrations of toxic chemicals

- Clear
- Tasteless and colourless (for aesthetic purposes)

When considering drinking water quality, in most cases microbiological contamination is the main concern since it is responsible for the majority of illnesses and deaths related to drinking unsafe water. Water intended for human consumption must be free from chemical substances and micro-organisms in amounts which would provide a hazard to health is universally accepted. Supply of drinking-water should not only be safe and free from dangers to health, but should also be aesthetically attractive as possible.

Absence of turbidity, colour and disagreeable or detectable tastes and odours is important in water supplies intended for domestic use. The location, construction, operation and supervision of water supply, its sources, reservoirs, treatment and distribution – must exclude all potential sources of pollution and contamination. Outbreaks of water borne disease could be avoided through strict control by the responsible water supply and health authorities.

Pollution of surface waters and ground waters can cause a decrease in the quality of the sources of drinking water and endanger public health. The most important issue is to protect the water use cycle in its entity. Cost arising from poor control of water quality and lack of proper maintenance are very high. It can lead to short term problems as for example, leaking water

supply systems increase not only the loss in drinking water quantity, but also the risk of water contamination. It can lead to irreversible situations as permanent pollution of ground water, harmful contamination of water resources and even lost human lives.

The demand for water has increased over the years and this has lead to water scarcity in many parts of the world. India is leading towards a fresh water crisis mainly due to improper management of water resources and environmental degradation, which has led to a lack of access of safe water millions of people (Iwuoha and Osuji, 2012).

Intensive irrigated agricultural discharge and industrial waste effluents into ground water can bring about a considerable change in the ground water quality. Once ground waste is contaminated, its quality cannot be restored by stopping the pollutants from the sources. Therefore, it becomes imperative to regularly monitor the quality of ground water and to device ways and means to protect it.

In the last few decades, there has been a tremendous increase in the demand for fresh water due to rapid growth of population and the accelerated pace of industrialization. Human health is threatened by most of the agriculture development activities like application of fertilizers and unsanitary conditions. In India several places now suffer non availability of

water for domestic and industrial use due to it's over exploitation and improper waste disposal, especially in urban areas.

Physical, chemical and Biological characters of water determine the quality of water. The water gets polluted due to increased human pollution, industrialization, use of fertilizers in agriculture and man made activity. The natural aquatic resources are causing heavy and varied pollution in aquatic environment leading to poor water quality and its depletion. Therefore it is necessary that the quality of drinking water should be checked for it's quality at regular time interval. According to WHO, about 80% of all the diseases in human beings is water borne. Despite the WHO 2008 guidelines for drinking water quality, water pollution in various sources has been increasing over resent decades in most countries (Eroula et al., 2011).

A water supply for domestic use should be free of disease causing organisms and substances which make the water unacceptable to it's users. There are several ways to find out if a water supply is safe to drink. There are five kinds of water quality analysis. All of them measure different characteristics of a water sample. the two most important types of analysis for small community water supplies are the bacteriological tests, physical and chemical tests.

Bacteriological analysis identifies organisms associated with disease. Physical and chemical analysis identify elements in a sample that make water

turbid, offensive or poisonous to users. A physical and chemical analysis include tests for turbidity, color, taste and odour, followed by tests for excess minerals, toxins and elements which may harm the system.

Drinking water supplies are prone to contamination with sewage and excreted matter which can cause outbreaks of intestinal infections such as typhoid fever. Regular bacteriological tests are crucial to ensure that drinking water supplies are microbiologically safe for human consumption. Monitoring and detecting the indicators and disease-causing microorganisms are the major parts of the sanitary microbiology. Most disease causing bacterial present in the drinking water supplies can be significantly reduced by means of chlorination method. The major concern is about the inability to consistently remove viruses and protozoa and to achieve quality standards for these microorganisms.

The presence of these bacterial in the water indicates that faecal matter has entered the water supply and the water is therefore liable to contamination with more dangerous organisms. The coliform bacilli are usually the most reliable indicators of faecal pollution. They make up approximately 10% of the intestinal microorganisms of humans. The presence of faecal coliforms, faecal *streptococci* and *clostridium perfringens*in water supplies necessitates immediate action to remove the

source of fecal pollution. These organisms are normally controlled through the disinfection of water.

Coliform bacteria are often referred to as “indicator organisms” because they indicate the potential presence of disease-causing bacteria in water. The presence of coliform bacteria in water does not guarantee that drinking the water will cause an illness. Rather, their presence indicates that a contamination pathway exists between a source of bacteria (surface water, septic, system, animal waste, etc.) and the water supply.

Disease-causing bacteria may use this pathway to enter the water supply.Most types of coliform bacteria are harmless to humans, but some can cause mild illnesses and a few can lead to serious waterborne diseases.

If disease-causing bacteria are present, the most common symptoms are gastrointestinal upset and general flu-like symptoms such as fever, abdominal cramps and diarrhea. Symptoms are most likely in children or elderly household members. In some cases, household residents acquire immunity to water borne bacteria who are common in their drinking water. In this case, visitors to the home that have not acquired immunity may become ill after drinking the water. Since the symptoms of drinking water with coliform bacteria are common to many human illness, knowing that water is the source of the problem is difficult without having the water tested.

Most bacteria in the coliform group do not cause disease, but the greater their number, greater the is likelihood that disease- causing bacteria may be present. Since coliform bacteria usually persist in water longer than most disease- causing organisms, the absence of coliform bacteria leads to the assumption that the water supply is microbiologically safe to drink. Therefore, the drinking water standard requires that no coliform bacteria be present in drinking water. Fecal coliform and *E.coli* bacteria should also be totally absent from drinking water.Lack of clean drinking water is a major problem in developing countries. Water born diseases are rampant in economically depressed rural areas because running water provided by the municipalities is simply not available. Many cities with thick population lack adequate fresh water because municipalities which should supply them are failing due to decaying infra structure and rising population.

- For instance2.2 billion people lack access to safely managed drinking water services. (WHO/UNICEF 2019)
- Over half of the global population or 4.2 billion people lack safely managed sanitation services. (WHO/UNICEF 2019)
- 297,000 children under five die every year from diarrheal diseases due to poor sanitation, poor hygiene, or unsafe drinking water. (WHO/UNICEF 2019)

- 2 billion people live in countries experiencing high water stress. (UN 2019)
- 90 per cent of natural disasters are weather-related, including floods and droughts. (UNISDR)
- 80 per cent of wastewater flows back into the ecosystem without being treated or reused. (UNESCO, 2017)
- Around two-thirds of the world's transboundary rivers do not have a cooperative management framework. (SIWI)

Childhood diarrhoea is closely associated with insufficient water supply, inadequate sanitation, water contaminated with communicable disease agents, and poor hygiene practices.

As water is a basic need for human life, access to clean safe drinking water is a basic human right. Water pollution and contamination of drinking water has been the root cause for the spread of various water borne diseases. Furthermore access to clean and safe drinking water is a dream to come true in various parts of our country.

This project aims to address the various bottleneck problems that are faced in the availability of clean and pure drinking water, and to create awareness about the same.

OBJECTIVES

OBJECTIVES

The objectives of the presents study is to perform, the following analysis to check the potability of the drinking water from four different areas of Thoothukudi

PHYSICAL ANALYSIS:

- Appearance
- Colour
- Odour
- Turbidity
- Total dissolved solids
- Electrical conductivity

CHEMICAL ANALYSIS

- P^H
- Nitrite
- Total Alkalinity
- Nitrate
- Total Hardness

- Chloride
- Calcium
- Fluoride
- Magnesium
- Sulphate
- Potassium
- Phosphate & Residual Chlorine

BACTERIOLOGICAL ANALYSIS

- ✓ Total Heterophic Bacterial Colony
- ✓ Faecal Coliformof the drinking water samples

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Analysis of six tube well water samples, which were the source of drinking water in Roorkee city of Hardwar district, was carried out along with physicochemical and biotic analysis. Presence of bacterial community in relation to biotic factors sought by Garg Dinesh *et al.,*(1991) showed an illness risk as a function of the concentration of a bacterial indicator especially faecal coliform.

SubbaRao andSubbaRao (1993) studied the chemical quality of groundwater in the industrial zones of Visakhapatnam. The report that fifteen percent of the industrial well waters recorded more than 3000 uS/cm level of conductivity and the major ions crossed the safe limits at many places.

Somasundaram *et al.,* (1993) investigated ground water quality of Madras urban aquifer to determine variations in major ions and nitrate concentration. In addition, a detailed local survey of pollution in ground water sources adjacent to sewage-polluted water course was carried out for heavy metals and bacterial populations.

Ozha *et al.,*(1993) observed that major nitrate contributing sources for the ground water in Barmer and Churu of Rajasthan appear to be of geologic origin, especially rock fossils and nitrate deposits. Further, nitrate concentration increased with total hardness, calcium and magnesium and decreased as the depth of the water table increased.

Drinking water samples were analyzed in Gandhi gram, Tamil Nadu by Mary *et al.,* (1997) to estimate the concentration of fluoride iron, hardness and bacterial population. In most of the cases the concentration of iron, total hardness and bacterial count were found to be beyond tolerance limits.

German fresh water epidemiological study by Wiedenman *et al.,* (2002) linked *E.coli* to swimmers illness risk. Naaz Abaas *et al.,* (2007) determined the bacteriological analysis of hand pump water in Pakistan for faecal contamination. He found 67% of the samples were positive for faecal streptococci.

Karthikeyen *et al.,* (2007) analyzed the physico-chemical parameters of 60 drinking water samples from Erode district, Tamil Nadu. They observed the levels of pH, electrical conductivity, TDS, alkalinity, hardness and bicarbonates, Ca, Mg, Nitrate, S, P, Na and K. The concentration of nitrate, hardness, Ca &Mg in some samples seemed to be more than the permissible limits.

Parihar *et al.,* (2007) analyzed samples from the rivers Ganga, Yamuna and their confluence (sangam) during winter for the extracellular catalase and peroxidase activity for assessment of river water quality. Maximum activity was observed at sangam with high bacterial count.

Abdul Hussain Shar *et al.,* (2008) analyzed the drinking water of Khairpur city. All the water samples were contaminated (100%) with total

coliform and faecal coliforms. The counts were higher than the maximum microbial contaminant level established by WHO.

Pathak and Gopal (2008) enumerated pollution indicator bacteria such as coliform, faecal coliform and faecal *streptococci* using a multiple-tube fermentation method in 100 treated drinking water samples from 20 locations in residential, commercial and industrial areas of a tropical city during summer.

Manjare *et al.,* (2010) investigated the physico-chemical paramenters of Tamadalge Water Tank in Kolhapur District, Maharashtra. They observed the levels of pH, electrical conductivity, TDS, alkalinity, hardness and bicrarbonates, Ca, Mg, Nitrate, S, P, Na and K. All parameters were within the permissible limits.

Abdul Hannan *et al.,* (2010) evaluated the microbial analysis of 100 samples of drinking water from Lahore by Membrane Filter Technique. It was found that the *E.coli*was grown from 42% samples and coliform organisms were grown from 54% specimens. It was alarming that 59% of drinking water was unsatisfactory for human consumption.

Anil Patel *et al.,* (2011) analyzed the physico-chemical parameters of Hosahalli Water Tank in Shimoga District, Karnataka. Monthly changes in physical and chemical parameters such as water temperature, turbidity, TDS, pH, dissolved oxygen, total hardness alkalininity, phosphate and nitrates were

analyzed for the periods of one year from 1st jan 2007 to 31st Dec 2007. All parameters were within the permissible limits.

Parihar *et al.,* (2012) evaluated the physico-chemical and microbiological characteristics of 16 drinking water samples in Gwalior, M.P India. These results showed that maximum samples were not suitable for drinking purpose.

Rajiv *et al.,* (2012) analyzed the comparative physico-chemical and microbial parameters of pond water samples collected from various sites in and around Coimbatore city, Tamil Nadu, India were analyzed to assess the quality of water for determining its suitability for drinking purpose. The results suggested that Pretreatment is needed for these waters for drinking purpose and human consumption.

Panneerselvam *et al.,* (2013) studied the physico-chemical parameters in drinking water from Vellore district, Tamil Nadu.

Geetha *et al.,* (2014) did the Physico-chemical and microbial analysis of water samples in Anakapalli municipal corporation and foundout that water in Anakapalli municipality was contaminated with various pathogenic bacteria and unfit for drinking.

Hemangi Kotibhaskar, *et al.,* (2015) conducted potability testing and microbiological analysis of drinking water at central railway stations of Mumbai suburbs. They found that the coliform count was highest in the

water sample at Thane then Dadar and was lowest at C.S.T. station. *Escherichia coli* were found in all the water samples.

Samuel Kojo Abanyie *et al.,* (2016) investigated the potability of water from dug wells of the Bolgatanga Township, Ghana.The study revealed that all 15 hand-dug wells water samples in the vicinities contained faecal and total coliforms above the WHO stipulated limits for potable water.

Arun Karnwal *et al.,* (2017) conducted the microbial analysis of potable water and its management through useful plant extracts. They found that the maximum bacterial count was in Baddi drinking water source.

Abok Elisha Onyango *et al.,* (2018) studied the microbiological Quality and Contamination Level of Water Sources in Isiolo County in Kenya. They revealed that surface, ground, and chlorinated urban water sources in Isiolo were contaminated with bacteria and cysts the to levels which are regarded as unsafe as per the standards for potable water.

Ashish Kumar Singh *et al.,* (2018) analysed the Physicochemical and microbiological profile of the potable water of Eastern Himalayan State Sikkim and reported Severe Fecal Contamination and Immediate Health Risk.

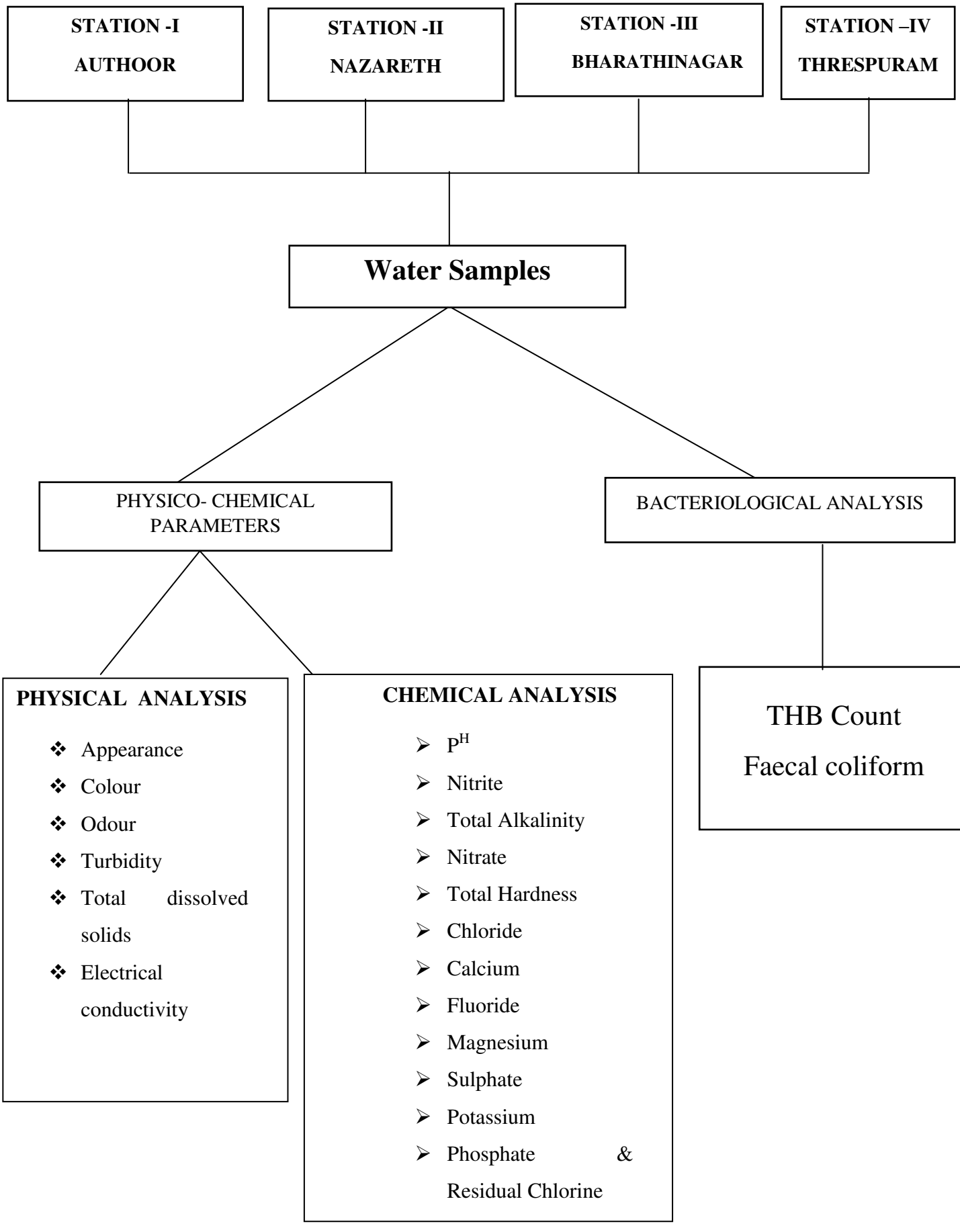
Onesmus Nzung'a Sila (2019) studied the Physico-chemical and bacteriological quality of water sources in rural settings, of Kenya, Africa. They concluded that some of the physicochemical properties of the water

sources investigated do not meet the adopted WHO and national guideline values for potable water qualities.

Roohi Rawat and Siddiqui (2019) assessed the physiochemical characteristics of drinking water in Allahabad metropolitan city, India. They reported that the drinking water in Allahabad is safe for drinking

Aaron Bivins *et al.,* (2020) who monitored the waterborne pathogens in Jaipur, India reveals potential microbial risks of urban groundwater supply. They found that the contaminated groundwater as a potential source of waterborne disease in Jaipur.

EXPERIMENTAL DESIGN



MATERIALS

AND METHODS

MATERIALS AND METHODS

STUDY AREA

For the present work, water samples were collected from four areas viz. Authoor, Nazareth, Bharathinagar and Threspuram situated in Thoothukudi District. The town depends on Thamirabarani River for water supply. It obtains water from Vallanadu (near Tirunelveli) situated at about 40km from Thoothukudi town.

STATION: 1 (AUTHOOR)

Authoor is a panchayath town in Thoothukudi district. The town is also referred as Aalanthoor. Authoor has a population of 14,470. Many of the people of this area are farmers of betal leaf, banana and rice. It is also famous for the Thamirabarani River surrounding the town. The river provides a majority of water used for irrigation in and around the area. The river flows though the area farming a big lake on one end. The people around this village, drink the river water since the are provided with panchayath water.

STATION : 2 (NAZARETH)

Nazareth was named by Christian missionaries. There is a 200+ year old church. The climate of Nazareth is usually dry. A short rainy season exists but weather is hot and humid throughout the year. It has a population

of 16,943. The main source of water in this area is the Thamirabarani River. It is polluted by people around the river.

STATION : 3 (BHARATHINAGAR)

Bharathinagar is a small village in Thoothukudi District. It comes under korampallam panchayath. It is located 8km towards Thoothukudi. In this area bore well water is generally used for drinking and other domestic purposes. The uses of fertilizers and pesticides, manure,lime, septic tank, refuse dump,etc.,are the main causes of water pollution. People residing in this area forced to use bore wells water for their domestic and drinking purposes

STATION IV (THRESPURAM)

Threspuram is a small place in Thoothukudi. The total area of the place is 4745 km2.1,750,176 people live in this area. The main occupation of people here is fishing. The source of drinking water is corporation water.

COLLECTION OF SAMPLES

Water samples were collected in sterilized bottle for bacterial analysis and physic- chemical analysis. Immediately after collection they were stored in ice box and brought to laboratory for testing. Samples taken for water testing were fresh and less than 24hours old.

ANALYSIS OF WATER SAMPLE

The collected samples were subjected to physic-chemical and bacteriological analysis.

COLOUR

Colour in drinking water may be due to the presence of coloured organic industrial wastes. Drinking water should be colourless. For the purpose of surveillance of community water supplies it is useful simply to note the presence or absence of observable colour at the time of sampling.

ODOUR

Odour in water is caused mainly by the presence of organic substances. Odour is an indicative of increased biological activity and may result from industrial pollution. Sanitary inspections should always be made to correct an odour problem.

DETERMINATION OF TURBIDITY

Turbidity was detected by using Nephleo-turbidity meter and the reading was digitally read out.

DETERMINATION OF TOTAL DISSOLVED SOLIDS

Clean the evaporating dish with distilled water and dry for an hour at 104°C. Then cool it. Weigh the evaporating dish. Add 100 ml of the filtered sample and evaporate without boiling in a hot air oven at 104°C for 16 hours. Then cool it. Weigh the dish after the water is evaporated to dryness. The difference between the two weights of the evaporating dish gives the amount of dissolved solids.

$$TDS = w_2 - w_1$$

Where

W_2 = Weight of china dish after evaporating the total volume to dryness.

W_1 = Weight of empty china dish

V = Volume of sample evaporated to dryness

ESTIMATION OF ELECTRICAL CONDUCTIVITY

Electrical conductivity is the measure of water's capacity to conduct electric current. As most of the salts in the water are present in the ionic form, are responsible to conduct electric current. Electrical conductivity is a decisive parameter in determining suitability of water for particular purpose. Electrical conductivity was measured using systronics conductivity meter and expressed in μ mohs/cm.

DETERMINATION OF pH

The pH meter was set to pH 7. The electrode was washed with distilled water and connected with pH meter. The electrode was dipped in buffer solution of pH 9.2. The selector switch was turned to pH range adjusted to set buffer knob to pH 9.2.Now the selector switch was put to zero and washed the electrode with distilled water and dipped in the water samples. Adjusted temperature compensation knob to the temperature of the sample and the pH of the samples were read between 7-14. Then turned the selector switch to zero and switched off the instrument.

DETERMINATION OF TOTAL ALKALINITY

Total alkalinity is determined by titration with a standard solution of strong mineral acid to successive bicarbonate and carbonic acid.

TOTAL HARDNESS

The sample (50 ml) was taken in a conical flask. 0.5 ml of the buffer solution was added and mixed well. 1 to 6 drops of indicator solution was introduced and mixed. Titrate immediately with EDIA solution, mixing continuously. The end point was indicated by a change from red to blue. The end point was gradual and not sudden and sharp. The blue color developed before the end point but the reddish tinge could still be seen. The end point is determined by the complete disappearance of this red tinge. The total hardness of the sample was calculated and expressed in mg/1.

CALCIUM AND MAGNESIUM

EDTA titrimetric method was adopted. 5 ml of water sample was taken in a conical flask and 5 ml of ammonium buffer was added. It was diluted with 100 ml distilled water and a pinch of erichrome black – T was added. This solution was warmed to 60^{o}C and was titrated against the EDTA in the burette. The end point was from the salmon red to blue. This titre value was taken as A and is the measure of total amount of calcium and magnesium.

Another 5 ml of the sample was taken in a conical flask and 5 ml of sodium hydroxide was added followed by a pinch of murexide as an indicator. This solution was diluted with 100 ml distilled water and it was titrated against EDTA until the colour changes from pink to deep violet. This value was taken as B and is a measure of amount of calcium.

By subtracting the titre value for calcium from the titre value of the total amount of Ca and Mg gives the value for magnesium alone and it is calculated and expressed in mg/1.

ESTIMATION OF SODIUM

Sodium is measured with the help of flame photometer. The instrument is standardized with the known concentration of sodium ion (1 to 100 mg/litre). The samples having higher concentration are suitably diluted with distilled water and the dilution factor is applied to the observed values.

ESTIMATION OF POTASSIUM

The concentration of the element in the unknown sample was calculated by reading the sample concentration from the calibration curve and multiplying it by the dilution factor.

DETERMINATION OF NITRITE

In a conical flask 25 ml of sample was taken. To this sample 0.5 ml of sulphanilamide solution was added and the solution was stirred well. After not less than 3 minutes and not longer than 8 minutes 0.5 ml of diamine solution was added and the content of the conical flask was thoroughly mixed. After 10 minutes, the OD of the red azodye developed was measured in a spectrophotometer at 543 nm. A standard curve was plotted between absorbances and concentrations of standard nitrite solution. The nitrite of the sample was calculated by comparing the absorbance with standard curve and expressed in mg/1.

DETERMINATION OF NITRATE

2 ml of NH_4CL solution was added to 100 ml of sample. Thus mixture was allowed to pass through the cadmium column. Every drop coming out of the column should not take more than 1 second. When 40 ml of the solution has been drained from the column, the remaining reduced solution was collected in a measuring cylinder. 25 ml of the reduced solution was collected in a conical flask and 0.5 ml of sulphanilamide was first added. After 3

minutes, 0.5 ml of diamine solution was added to develop azodye. The OD of the solution was then measured at 543 nm. By comparing the absorbance of the sample with that of the valves in the standard curve, the amount of NO_3 was calculated and presented in terms of mg/ litre.

ESTIMATION OF CHLORIDE

100 ml of water samples was taken in a conical flask. The sample was filtered if it contains a lot of suspended matter 1 ml potassium chromate indicator solution was added and run in silver nitrate solution taken in a burette. Swirled the flask during the addition of silver nitrate. The titration was continued until the precipitate was found and the solution turned a pink orange colour. The chloride ion in water sample was calculated expressed in mg/1.

$$\text{Chloride ion} = (A-0.2)\times N\times 354.5$$

Where,

A = Standard silver nitrate used

0.2 = Blank value attributed to potassium chromide

N = Normality of silver nitrate determined by standardization against sodium chloride which is a constant

ESTIMATION OF FLUORIDE

With the help of the standard fluoride solution (from sodium fluoride) the ion analyzer instrument is calibrated. Now known quantity of water samples are added with 5 ml of TISAB buffer in s polythene container and then the concentrations of fluoride in the samples are estimated by ion analyzer.

ESTIMATION OF SULPHATE

It is measured by nephelometric method in which the concentration of turbidity is measured against the known concentration of synthetically prepared sulphate solution. Barium chloride is used for producing turbidity due to barium sulphate and mixture od organic substance (Glycerol or Gum acetia) and sodium chloride is used to prevent the settling of turbidity.

ESTIMATION OF PHOSPHATE

These are also measured spectroscopically. Yellow colour is developed from the action of phosphates on molybdate ion under strong acidic conditions. The intensity of colour is directly proportional to the concentration of phosphate in the sample. Phosphate complexes are reduced by weak reducing agents such as ascorbic acid or tartaric acid (potassium antimonyltartarate). The colour of reduced complex is sky blue.

DETERMINATION OF RESIDUAL CHLORINE

Add 1 ml of Ki solution to a 200 ml sample and immediately add 1ml of pH 4.0 buffer solution. Immerse the electrodes in the sample and start the stirrer. Adjust the micro-ammeter pointer of the potentiometer to the high current side of the scale so the pointer can deflect counter clockwise during the analysis. Titrate using standard phenylarsine oxide solution, adding the titrant in small increamnets, and nothing the deflection of the micro ammeter pointer. Plot the progress of the titration on linear graph paper with current on the vertical axis and titrant volume on the horizontal axis.

Residual Chlorine mg/1 = 200 A/V

Where,

A = Phenylarsine oxide solution (0.00564)

V = sample used in ml

BACTERIOLGICAL ANALYSIS

TOTAL HETEROTROPHIC BACTERIAL COUNT (THB)

Total bacterial population in the water sample was estimated by the serial dilution technique. The medium was prepared and sterilized in an autoclave. Serial dilutions of water samples (10-9 ×10-5) were prepared. 1 ml of the sample of the required dilution was transferred into a petridish. The petridishes were labeled using a glass marker indicating the type of the sample, dilution and medium. About 15- 20 ml of the nutrient agar medium was poured in to the petridishes (at an ear bearing temperature- aseptically). The plates were rotated in clockwise and anti clockwise direction for thorough mixing and left undisturbed, for the agar to solidify and then incubated in an inverted position. The number of the bacterial colony per ml of the drinking water was calculated.

The total number of bacterial population was calculated by multiplying the number of colonies by the dilution factor.

FAECAL COLIFORM (MPN)

Faecal coliform was calculated by most probable number technique in water. Samples were serially diluted to the point at which there were few or no viable microorganisms. 1 ml of the water sample was added to 99 ml of water dilution bottle and shaken vigorously (1×10^{-2}). 1 ml of this dilution was transferred aseptically to a second 99 ml dilution bottle and shaken

vigorously (1×10^{-4}). From each of the three bottle (original sample, dilution I and II) the tubes were inoculated as follows.10 ml from each bottle was added to three tubes of double strength lactose broth with Durham tubes.

- 1 ml from each bottle was added to each of the three tubes of single lactose broth with Durham tubes.
- 1 ml was added from each bottle to each three tubes of single lactose broth in the Durham tubes.

The tubes were incubated at 37^{o} for 24 hours. After incubation the tubes were examined for acid and gas production. The results were recorded and the dilution in which the tubes were neither all positive nor all negatives was selected. The series with mixed results were compared with MPN table and the most probable number of coliforms were recorded and multiplied with the dilution factor.

STATISTICAL ANALYSIS
ANOVA

The results were statistically analyzed using ANOVA. ANOVA was interpreted using the following formula

$$F = \frac{\text{Variance between samples}}{\text{Variance within samples}}$$

VARIANCE WITHIN SAMPLE

Mean $= \overline{X}_1, \ \overline{X}_2, \ \overline{X}_3, \ \ldots\ldots\ldots\ldots \overline{X}_n$

Deviation from the mean $= X_1 - \overline{X}_1, \ X_2 - \overline{X}_2 \ldots\ldots\ldots$

Sum of Squares T $= (X_1 - \overline{X}_2\ )^2 \ \Sigma(X_2 - \overline{X}_2\ )^2$

Degrees of freedom $= n - 1$

VARIANCE BETWEEN THE SAMPLE

Mean

$= \overline{X}_1, \ \overline{X}_2, \ \overline{X}_3, \ \ldots\ldots\ldots\ldots \overline{X}_n$ Grand Mean $\overline{X}$

$$= \frac{\overline{X}_2, \ \overline{X}_3, \ \ldots\ldots\ldots\ldots \overline{X}_n}{N_1 + N_2 + N_3 + \ \ldots\ldots\ldots\ldots + N_n}$$

Deviation from the mean $= \overline{X}_1, \ - \overline{X}, \ \overline{X}_2 \ - \overline{X}, \overline{X}_3 \ - \overline{X} \ldots\ldots\ldots$

Sum of Squares T $= (\overline{X}_1 \ - \overline{X}\ )^2 + (\overline{X}_2 \ - \overline{X}\ )^2 \ldots\ldots (\overline{X}_n \ - \overline{X}\ )^2$

Divided the S by

Degree of freedom i.e $= \frac{s}{n-1}$

RESULTS

RESULTS

Water intended for human consumption must be free from chemical substances and microorganisms in amounts which would provide hazard to health is universally accepted

PHYSICAL PARAMETERS

The results of the analysis of the physical parameters of the water samples were presented in table -1

APPEARANCE

The results of our present study showed that among the water samples analyzed for physical parameters, station III (Bharathinagar) and station IV (Threspuram) were clear. But station I (Authoor) and station II (Nazareth) were not clear in their appearance

COLOUR

The colour of the station I (Authoor) is light brown and station II (Nazareth) is dirty white. The other two stations III & IV (Bharathinagar & Threspuram) were colourless

ODOUR

When analysed for physical parameters all the drinking water samples were found to be odourless

TURBIDITY

The turbidity of water samples ranges from 1-2 NT U. The samples I & II (Authoor&Nazareth) were found to be slightly turbid with the value of 1.5 NT U and 2 NT U respectively. But the turbidity value of samples III & IV (Bharathinagar & Threspuram) were observed to be 1 NT U (Figure 1)

TOTAL DISSOLVED SOLIDS

The total dissolved solids in the water samples under study fluctuated from 400 mg/l to 195 mg/l. The maximum amounts of dissolved solids were recorded from stationII Nazareth (400 mg/l). It was 350 mg/l in station I (Authoor) and it was found to be 195 mg/l in sample III & IV (Bharathinagar & Threspuram), (Figure -2)

ELECTRICAL CONDUCTIVITY

The results of analysis of electrical conductivity of the water samples taken for investigation showed that in water samples- III & IV (Bharathinagar & Threspuram) the electrical conductivity was 295 µ mho/ cm . The electrical conductivity of stationI (Authoor) was 320 µ mho/ cm and the maximum electrical conductivity was recorded in station II (Nazareth - 445 µ mho/ cm (Figure – 3)

TABLE : 1

PHYSICAL PARAMETERS OF WATER SAMPLES TAKEN FROM DIFFERENT STATIONS

S.No	PHYSICAL PARAMETERS	STATION -I AUTHOOR	STATION -II NAZARETH	STATION III BHARATHINAGER	STATION IV THRESPURAM
1	**Appearance**	Not Clear	Not Clear	Clear	Clear
2	**Colour**	Light brown	Dirty white	Colourless	Colourless
3	**Odour**	None	None	None	None
4	**Turbidity NT Units**	1.5	2	1.0	1.0
5.	**Total dissolved solids mg/L**	350	400	195	195
6.	**Electrical Conductivity Micro mho/cm**	320	445	295	295

CHEMICAL PARAMETERS

Chemical contamination of drinking water both naturally occurring and from contamination is a very serious problem. The results of the analysis of chemical parameters of the water samples are presented in table – II

pH

From the present results it was found out that pH of sample from station I (Authoor) was maximum (8.3) and the pH of the sample from stations II, III & IV (Nazareth, Bharathinagar and threspuram) were 7.8, 7.4 and 7.4 respectively (Figure – 4)

TOTAL ALKALINITY AS $CaCo_3$

The Total Alkalinity as $CaCo_3$ was recorded as 137 mg/l in from Nazareth (station II) and it was 105 mg/l, in station I (Authoor) 97 mg/l of total alkalinity was recorded from stations III & IV (Bharathinagar and Nazareth) (Figure- 5)

TOTAL HARDNESS

The total Hardness as CaCo3 was recorded as 115 mg/l in station II(Nazareth) and it was 91 mg/ l in station I (Authoor). 89 mg/l and 87 mg/l was recorded in stationsIII & IV (Bharathinagar and Threspuram) respectively (Figure – 5)

CALCIUM

The amount of calcium estimated was 24 mg/l from stations I & III Authoor and Bharathinagar. The amount of calcium recorded was 31 mg/lin station II (Nazareth) and 23 mg/l in stationIV (Threspuram) (Figure- 6)

MAGNESIUM

The maximum amount of magnesium was calculated from stationII (Nazareth - 9 mg/l). 7 mg/l of magnesium was estimated in all other samples analysed (Figure- 6)

SODIUM

Drinking water sample from Nazareth was found to contain 42 mg/l. The water sample from Authoor, Bharathinagar and Threspuram were found to contain 26 mg/l of sodium. (Figure-6)

POTASSIUM

As depicted in figure – 6 the amount of potassium in water from stationsIII & IV (Bharathinagar and Threspuram) was found to contain 2 mg/l and it increased to 3 mg/l in water from station I (Authoor) and the maximum amount of potassium (4 mg/l) was recorded in in water from stations III (Nazareth)

NITRITE

Our result indicates the presence of negligible amounts of nitrite in the samples analysed. The amount of nitrite present in sample – II (Nazareth) was 0.22mg/l. It was noted to be 0.20 mg/l in all the other samples (Figure -7)

NITRATE

Maximum amount of nitrate (5 mg/ l) was estimated in drinking water from Nazareth and 3 mg/l of nitrate was estimated from sample- I, II & IV (Authoor, Bharathinagar and Threspuram) (Figure- 7)

CHLORIDE

As figure- 7 shows the amount of chloride recorded in samplefrom stations I & IV (Authoor and Threspuram) was 26 mg/l and it increased to 27 mg/l in samplefrom stationIII (Bharathinagar) and the maximum amount of chloride (50 mg/l) was estimated in sample from stationII (Nazareth)

FLUORIDE

Presence of fluoride was more (0.4 mg/l) in the water sample from Bharathinagar (stationIII). The water samples from Authoor had 0.1 mg/l of fluoride and 0.2 mg/l was present in stationsII & IV (Nazareth and Threspuram) (Figure -8)

SULPHATE

The maximum amount of the sulphate was found to be present in sample II (Nazareth) 10 mg/l and 8 mg/l of sulphate were observed in all the other samples I,II & IV (Authoor, Bharathinagar and Threspuram) (Figure- 8)

PHOSPHATE

The present result indicates that there was no phosphate in sample- I (Authoor). The maximum amount of phosphate was estimated in 0.5 mg/l sample III (Bharathinagar). The quantity of phosphate in sample- II & IV (Nazareth and Threspuram) was 0.2 mg/l (Figure- 8)

RESIDUAL CHLORINE

No residual chlorine was found to be present in the water samples under study except stations III (Bharathinagar) which contained 0.2 mg/1 (Figure- 8)

TABLE :II

CHEMICAL PARAMETERS OF WATER SAMPLES TAKEN FROM DIFFERENT STATIONS

S.No	CHEMICAL PARAMETERS	STATION -I AUTHOOR	STATION -II NAZARETH	STATION III BHARATHINAGER	STATION IV THRESPURAM
1	**pH**	8.3	7.8	7.4	7.4
2	**Total Alkalinity as CaCo3**	105	137	97	97
3	**Total Hardness as CaCo3**	91	115	89	87
4	**Calcium as Ca**	24	31	24	23
5.	**Magnesium as Mg**	7	9	7	7
6.	**Sodium as Na**	26	42	26	26
7	**Potassium as K**	3	4	2	2

S.No	CHEMICAL PARAMETERS	STATION -I AUTHOOR	STATION -II NAZARETH	STATION III BHARATHINAGER	STATION IV THRESPURAM
8	**Nitrite as No_2**	0.20	0.22	0.20	0.20
9	**Nitrate as No_3**	3	5	3	3
10	**Chloride as cl**	26	50	27	26
11	**Fluoride as F**	0.1	0.2	0.4	0.2
12	**Sulphide as So_4**	8	10	8	8
13	**Phosphate as Po_4**	NIL	0.2	0.5	0.2
14	**Residual chlorine**	NIL	NIL	0.2	NIL

FIGURE – 1: TURBIDITY OF THE WATER SAMPLES FROM SELECTED AREAS

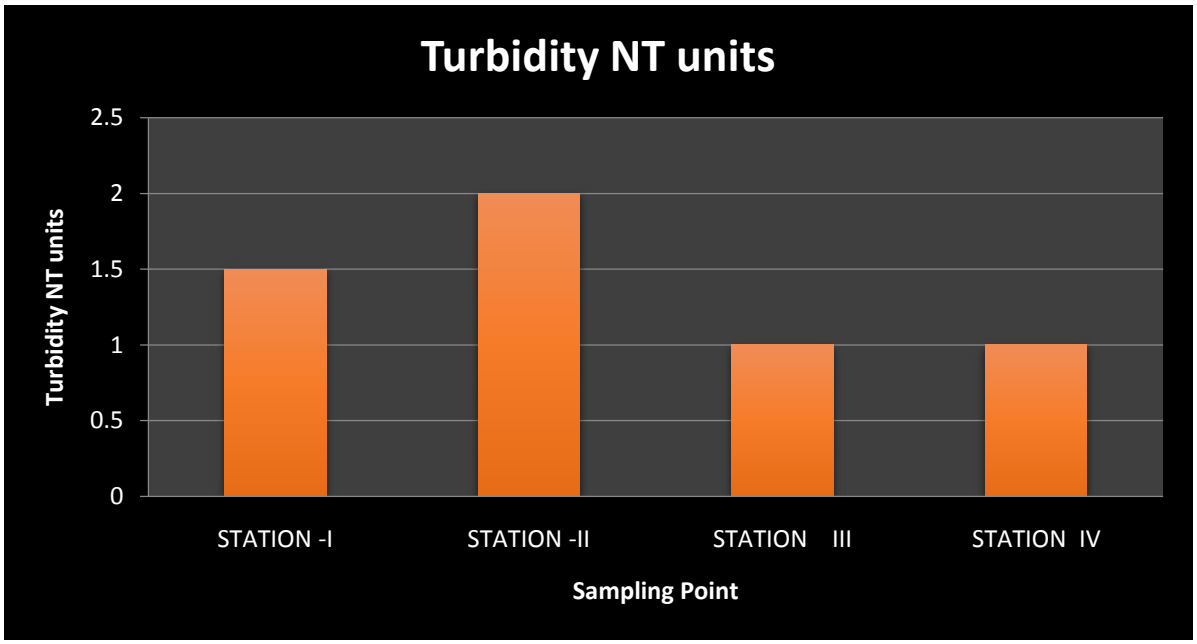


FIGURE -2 : TDS OF WATER SAMPLES COLLECTED FROM THE AREAS

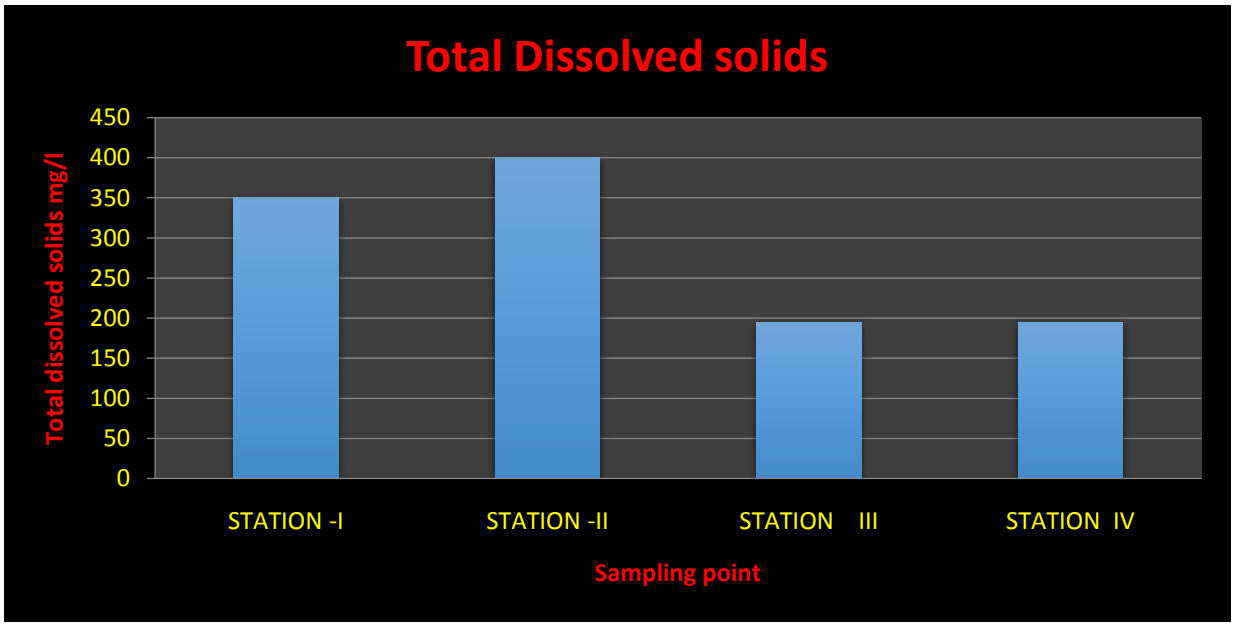


FIGURE -3 ELECTRICAL CONDUCTIVITY OF THE SAMPLES UNDER STUDY

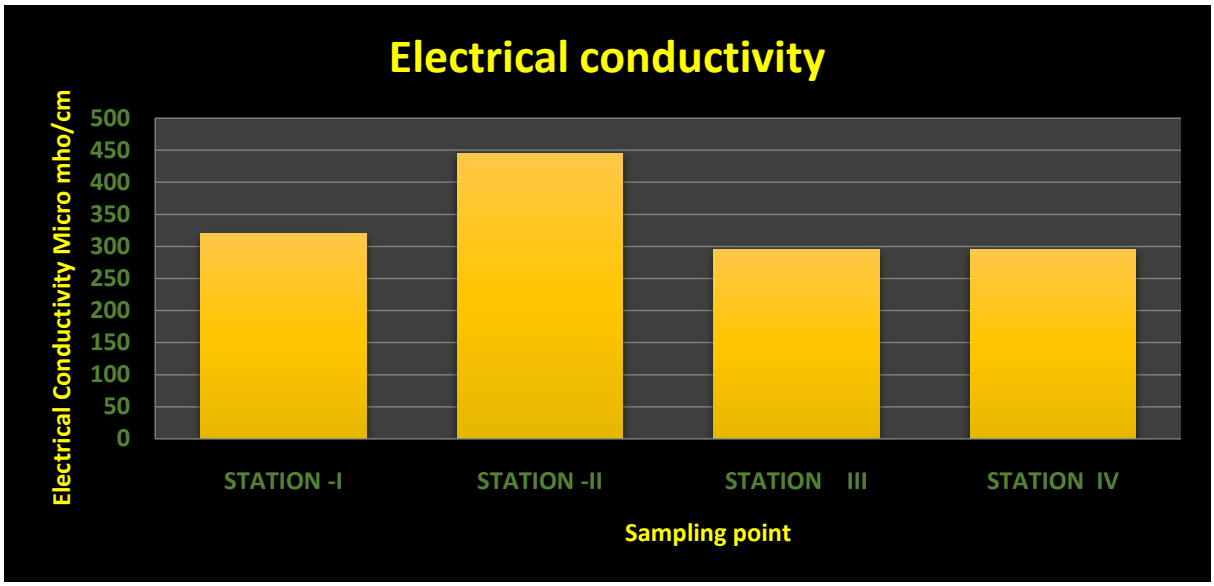


FIGURE – 4 : pH OF THE WATER SAMPLES UNDER STUDY

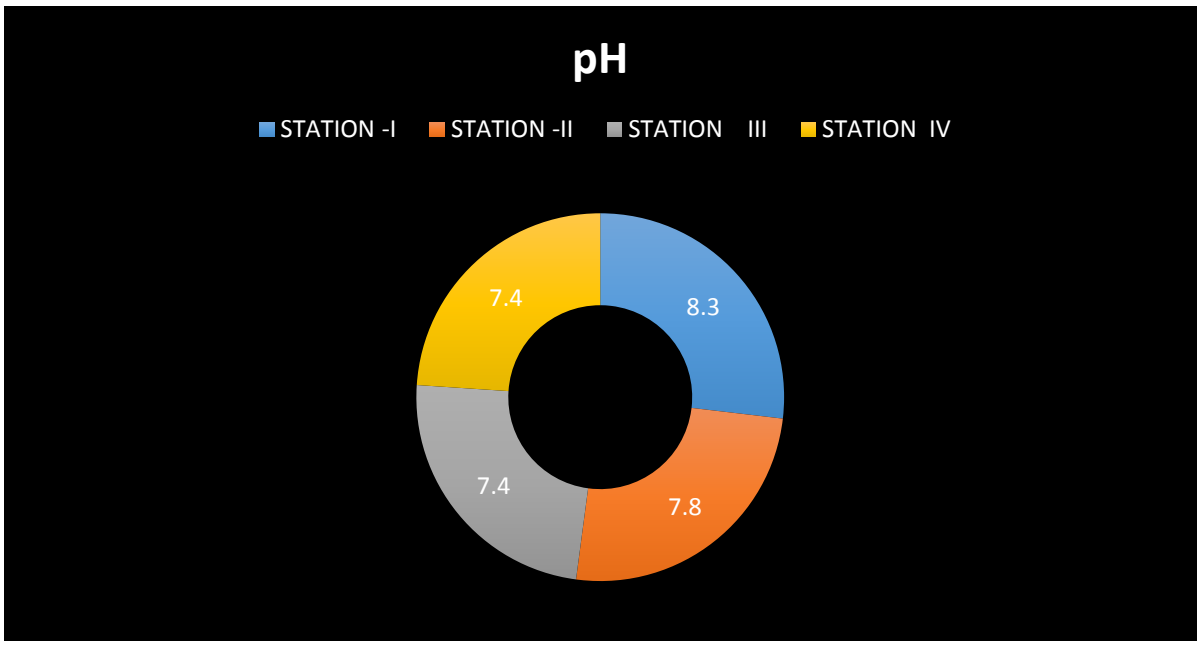


FIGURE-5 : TOTAL ALKALINITY AND HARDNESS OF THE SAMPLES TESTED

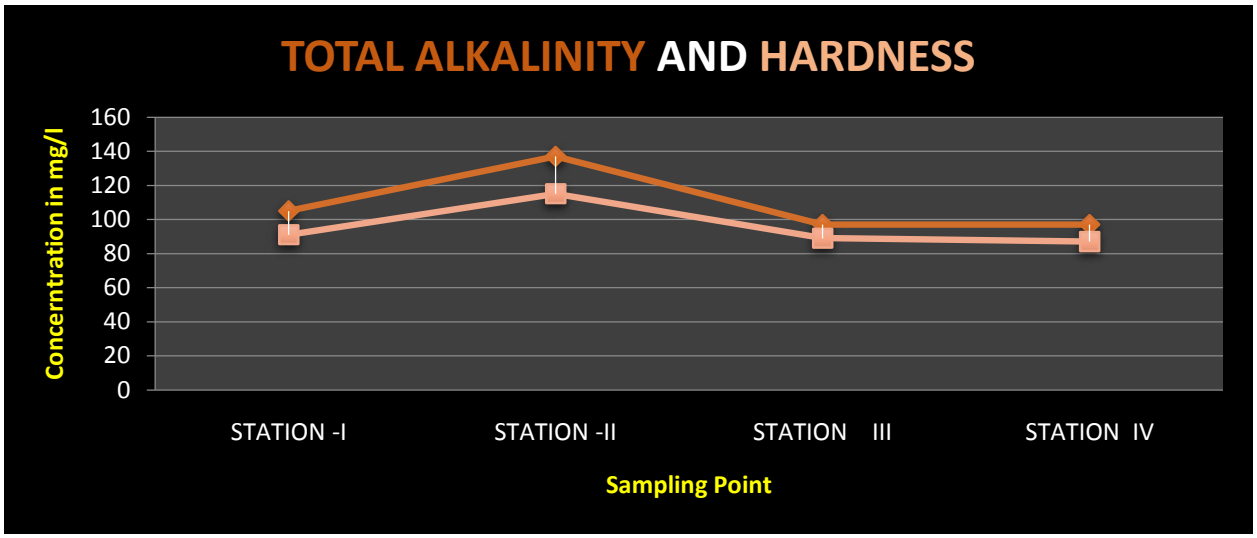


FIGURE-6: AMOUNT OF K, Na, Mg AND Ca ESTIMATED FROM THE SAMPLES

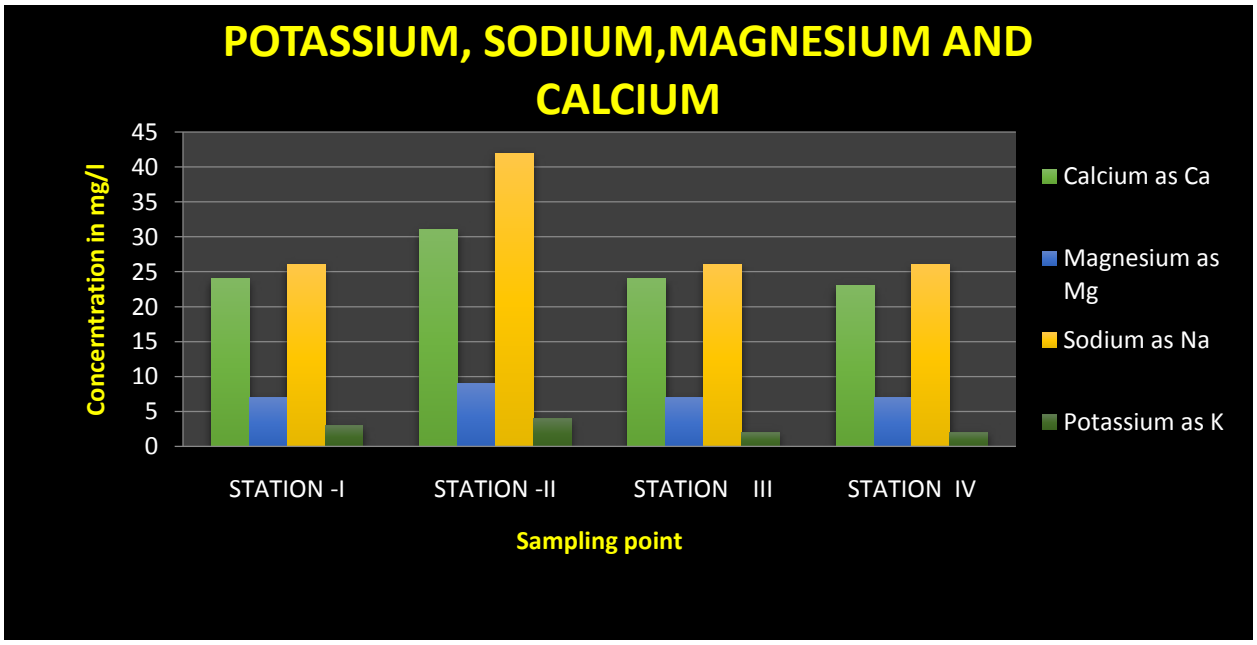


FIGURE- 7 THE AMOUNT OF NITRITE, NITRATE AND CHLORIDE ESTIMATED FROM THE SAMPLES

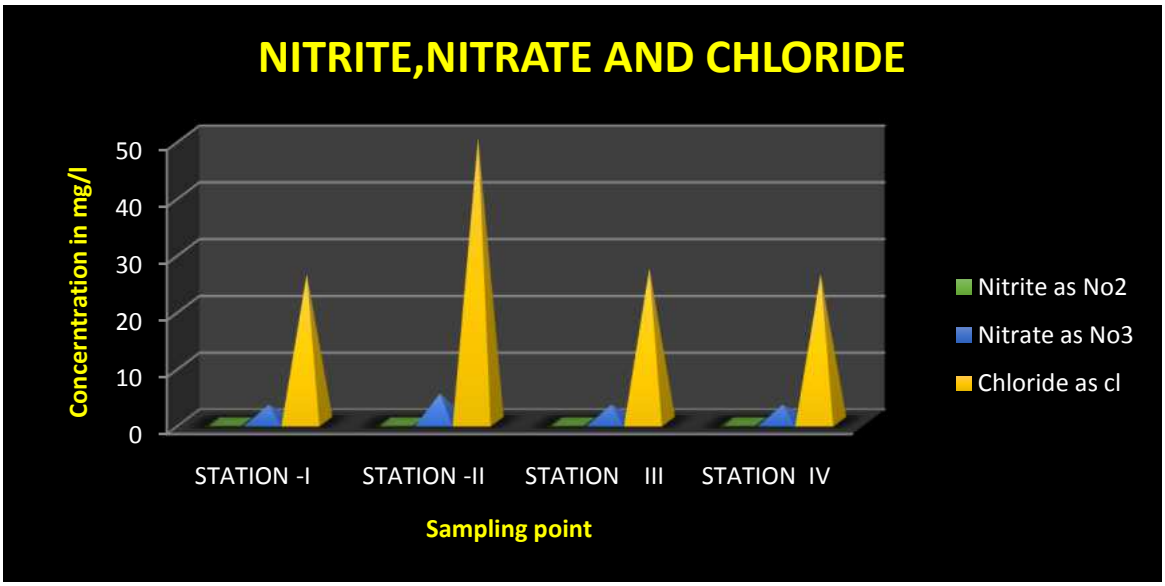
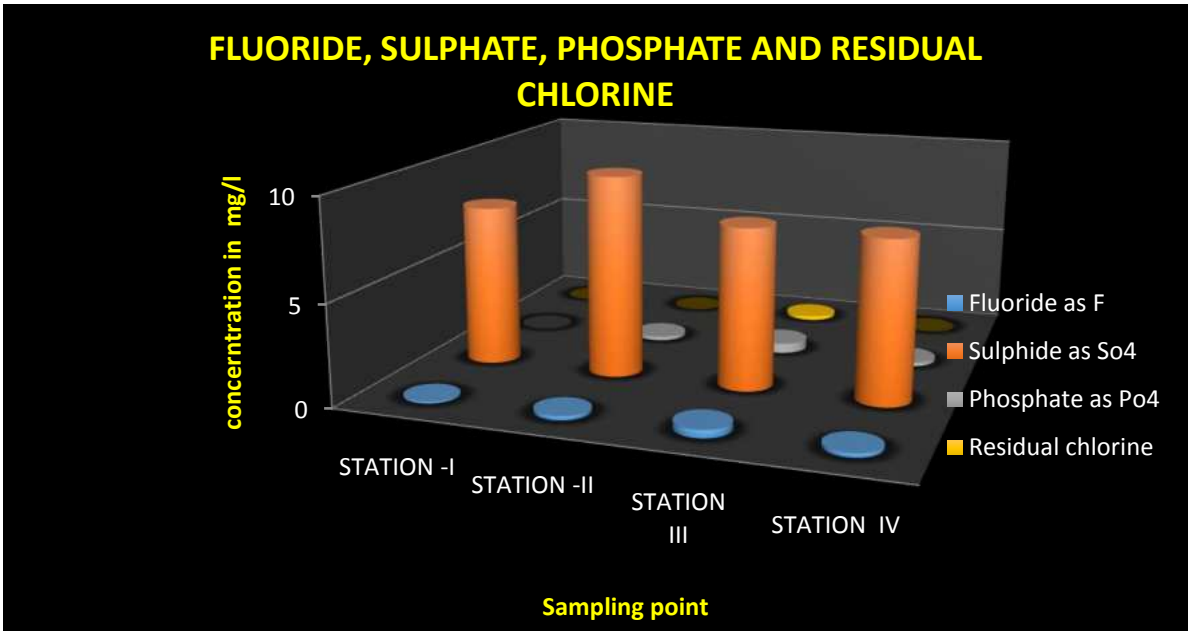


FIGURE -8 AMOUNT OF FLUORIDE, SULPHATE, PHOSPHATE AND RESIDUAL CHLORINE FROM THE WATER SAMPLES



BACTERIOLOGICAL ANALYSIS

The results of the bacteriological analysis of the water samples are presented in Table III

TOTAL HETEROTROPHIC BACTERIAL COUNT (THB)

Results of microbiological water quality analysis indicates the presence of bacterial population in the water samples under study. Total heterotrophic bacterial count was found to be more in station I from Authoor (490 x 10^{-5} CFU/ml). Total bacterial count was 370 x 10^{-5} CFU/ml in stationII (Nazareth) and 340 x 10^{-5} CFU/ml colonies in sample IV (Threspuram). The minimum number of bacterial colony (274 x 10^{-5} CFU/ml) were observed in stationIII (Bharathinagar) (Figure- 9)

FAECAL COLIFORM

In terms of total coliform the results indicates that the samples from Authoor and Nazareth (stations I&II)were slightly polluted with faecal matter. Drinking water samples from Bharathinagar and Threspuram did not show the presence of faecal coliform bacteria. The most potable number of faecal coliform in stationI (Authoor) was 2 MPN and that of station II (Nazareth) was 1 MPN (Figure- 10)

TABLE – III : BACTERIOLOGICAL ANALYSIS OF WATER SAMPLES TAKEN FROM DIFFERENT STATIONS

S.No	Bacteriological Analysis	Station I	Station II	Station III	Station IV
1	Total Heterophytic bacterial colony	490	370	274	340
2	Faecal coliform	2	1	Nil	Nil

FIGURE – 9 THE NUMBER OF THE THB COLONY IN WATER SAMPLES

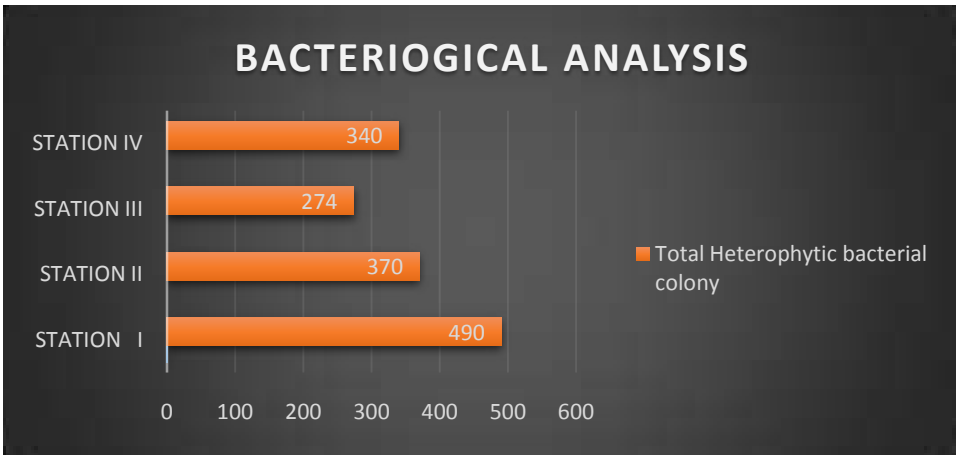


FIGURE – 10FAECAL COLIFORM BACTERIA FROM DIFFERENT WATER SAMPLES

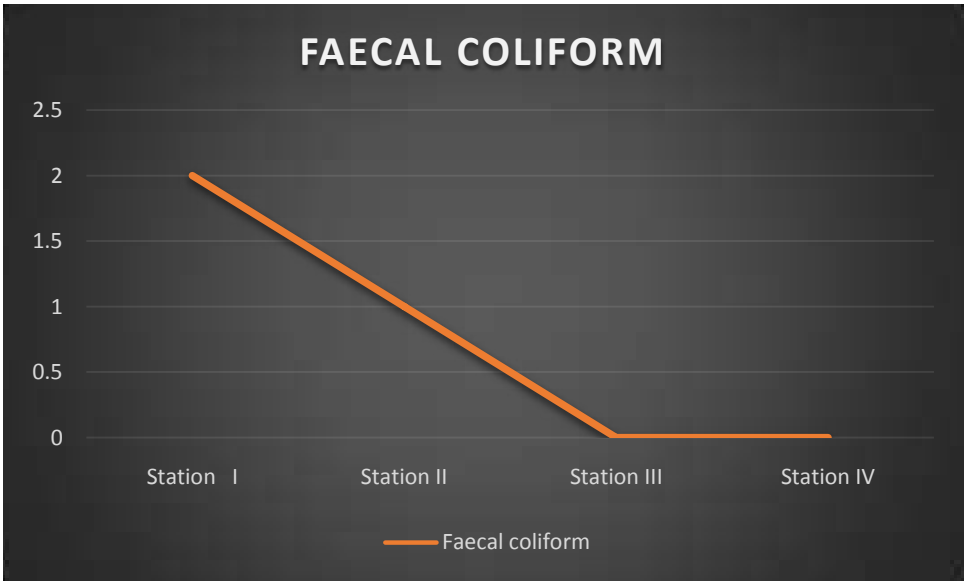


PLATE – 1

Bacterial colony in drinking water sample from Authoor

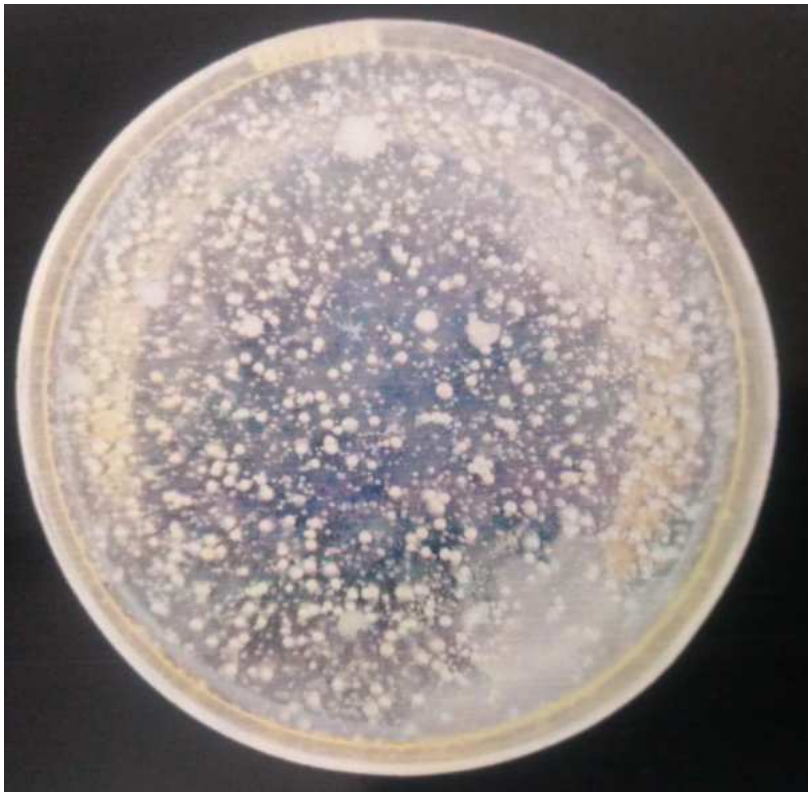


PLATE – 2

Nutrient Agar plate showing the Bacterial colony in drinking water sample from Bharathinager

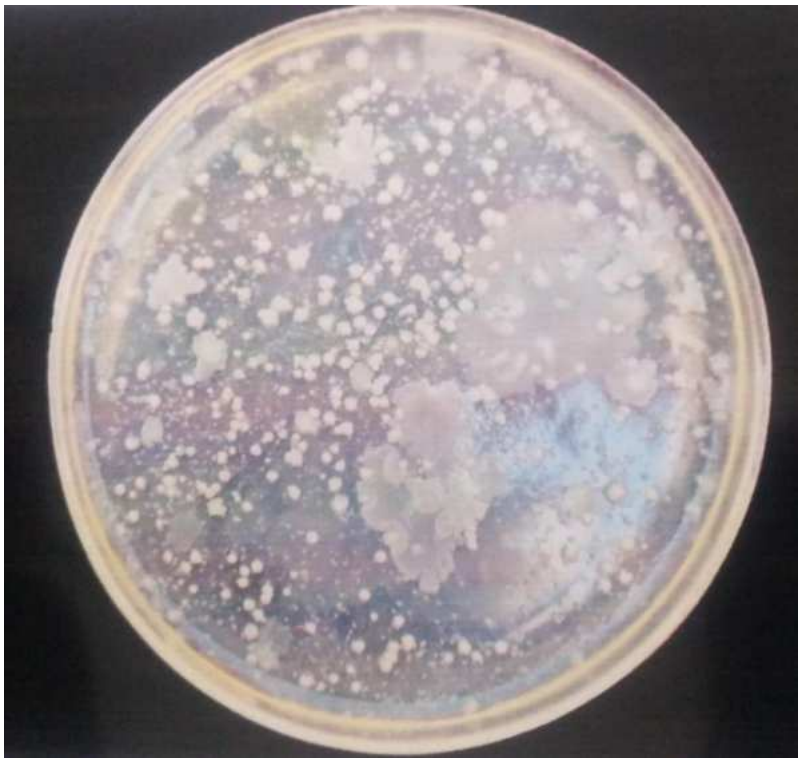


PLATE – 3

Nutrient Agar plate showing the Bacterial colony in drinking water sample from Nazareth

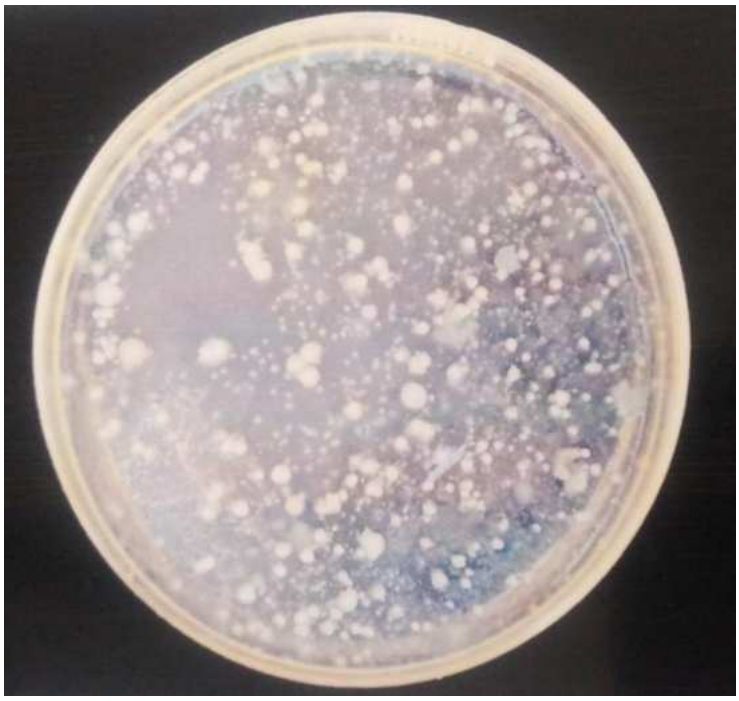
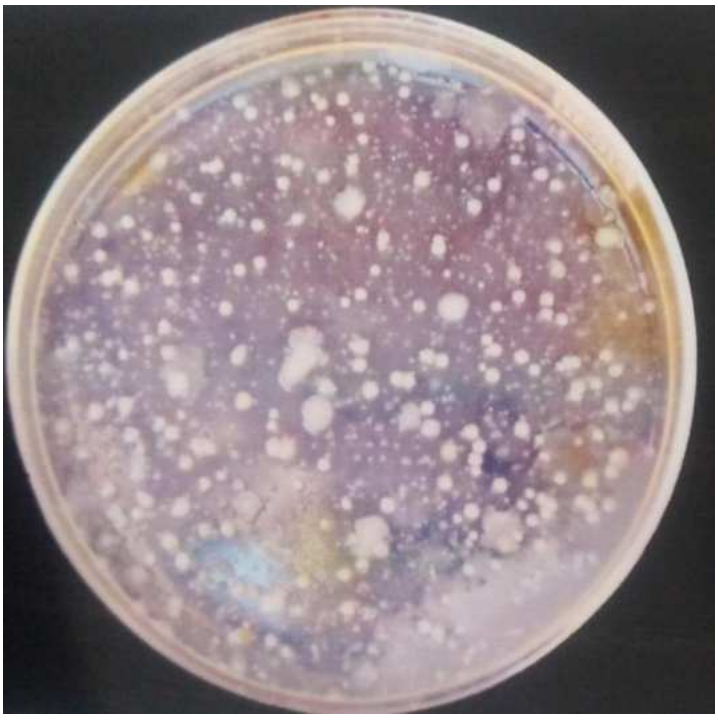


PLATE – 4

Nutrient Agar plate showing the Bacterial colony in drinking water sample from Threspuram



ANOVA

TABLE – IV : PHYSICAL ANALYSIS

Source of Variation	SS	dF	Ms	Fs	F crit
Between Groups	29119.4	3	9706.465	6.03	4.1
Within Groups	282872.2	8	35359.02		
Total	311991.61	11			

*P<0.05 Statistically Significant

TABLE – V : CHEMICAL ANALYSIS

Source of Variation	SS	dF	Ms	Fs	F crit
Between Groups	753.695	3	251.232	3.5	2.8
Within Groups	66918.977	52	1286.903		
Total	67672.671	55			

*P<0.05 Statistically Significant

TABLE – IV : PHYSICAL ANALYSIS

Source of Variation	SS	dF	Ms	Fs	F crit
Between Groups	12499.375	3	4166.458	7.3	6.6
Within Groups	282490.500	4	70622.625		
Total	294989.875	7			

*P<0.05 Statistically Significant

DISCUSSION

DISCUSSION

APPEARANCE

Observations on clarity of drinking water samples show that samples from station III & IV (Bharathinagar and Threspuram) were clear which indicates that the water from those stations were devoid of suspended and colloidal particles. So they are suitable for drinking. But samples from station I & II (Authoor and Nazareth) were not clear which may be related to their high turbidity and colloidal nature as we have studied. Since those are riverine water they appear to be not clear. So the water should be filtered before drinking.

COLOUR

Generally the colour of water does not possess a health threat. Colour may occur in drinking water due to the presence of natural organic matter, metals such as irons manganese and copper or due to the industrial wastes. The result of analysis of drinking water samples for physical parameters indicates that the samples from station I & II (Authoor and Nazareth) were slightly coloured. This may be due to the presence of high amount of dissolved salts as we have estimated in these samples. The other two water samples III & IV (Bharathinagar and Nazareth) were colourless and indicate the potability. The slight brown colour of sample I (Authoor) may be due to the presence of iron in water.

ODOUR

It was observed that all the four drinking water samples analysed were odourless. Normally the water smells only when there is biological degradation which will result in bad ad unpleasant odour. So it is learned that the samples were devoid of organic waste materials.

TURBIDITY

Turbidity makes the water aesthetically unacceptable. The turbidity values of sample I & II (Authoor and Nazareth) were beyond the acceptable limit for drinking water. This result is comparable with the other published data (Bhagavathi Perumal *et al.,*2016), analysed from kanyakumari in which turbidity was always higher in the drinking water. The impact of turbidity is that the colloidal particles which cause turbidity can harbour pathogenic micro- organisms there by making disinfection ineffective. Higher turbidity levels are often associated with higher levels of disease causing organisms such as virus, parasite and some bacteria (microbes). These organisms can cause symptoms such as nausea, cramps, diarrhea and associated headaches. So the water samples I & II (Bharathinagar and Nazareth) need treatment before drinking.

TOTAL DISSOLVEDSOLIDS (TDS) AND ELECTRICAL CONDUCTIVITY (EC)

Both the total dissolved solids and electrical conductivity results were below the guideline values. However TDS values were slightly higher in Bharathinagar and Nazareth. The similar results were obtained by Patel, *et al.,* (2012). This may be attributed to the fact that people from station I & II (Bharathinagar and Nazareth)are not provided with corporation water for drinking, so they happen to drink water from the river. TDS is the measure of all the chemical constituent dissolved in water. It is mostly influenced by the concentration of major ions like calcium, bicarbonate, magnesium, sulphate and chloride and it is also closely linked to the EC. Total dissolved solids also is related to the corrosive nature of water. So the TDS of drinking water from these two stations need treatment before consumption.

P^H

pH is an important parameter in assessing the water quality. P^H is an index of acidity, alkalinity and the acidic interaction of the number of its minerals and organic components. The P^H limit for drinking water is 6.5 – 8.5. Even though the present study shows that all the drinking water samples analyzed were having the pH within the permissible limit, sample – I (Authoor) is having the pH of 8.3 which seems to denote the alkaline nature of the sample. So water sample from station I (Authoor) indicates the

possibility of presence of metal contaminants in the drinking water. The increased pH of the sample may also be related to the increased photosynthetic assimilation and dissolved inorganic carbon by planktons. The same results was obtained by Anil Patel, *et al.,* (2011) in Hosahalli Water Tank, Shimoga district. The pH of the other water samples from Nazareth, Bharathinagar and Threspuram(stations II, III & IV) ranged from 7.8 – 7.4 which lies in the acceptable range. P^H value above 7.5 may indicate the hardness of the water. The pH below 6.5 leads to corrosion in vessels and pipes.

TOTAL ALKALINITY

Total alkalinity is due to the presence of dissolved gases like carbon dioxide. In the present study the total alkalinity ranged was from 187 mg / l to 137mg / l. The alkalinity for the standard drinking water is 200mg /l. Our samples remain in the acceptable range. Alkalinity indicates the presence of bicarbonates, carbonates and hydroxides. Above the normal value the water taste becomes unpleasant, boiled rice turns yellow and it is health concerned. Even though samples from stations I & II (Authoor and Nazareth) are river waters the alkalinity values did not coincide with the result of Panneerselvam, *et al.,* (2013) who has also done the water quality in the same type of sample. So it is understood that regarding total alkalinity, the four samples under study denotes potability.

TOTAL HARDNESS

Total hardness of water is due to the presence of calcium and magnesium ions in water. Calcium is needed for the body in small quantities and water we drink provides a small part of the total requirement. Geological survey of India uses the following classification: 1 – 60 mg /l is considered moderately hard, 110 – 180 mg/ l is considered hard and above 180 mg/ l is considered very hard. Accordingly the sample II (Nazareth). When the hardness of drinking water increases, scaling occurs in cooking vessels. The water samples analyzed ranged from 87 mg/ l to 115 mg / l in hardness. All the water samples are having the hardness within the permissible limit.

CALCIUM AND MAGNESIUM

Calcium is an important content of natural water which determines the rigidity of water. It is an essential nutrient element of human beings. Magnesium is a constituent of bones and essential for normal metabolism of calcium ion. It's deficiency leads to protein energy malnutrition. Calcium and magnesium are the most abundant elements in ground water. Calcium and magnesium dissolve readily from carbonate rocks and lime stones or leached from soils. However, dissolved magnesium ion concentration is lower the calcium ion in the ground water. Other source include primarily industrial and municipal discharges. The Indian standard for drinking water specification stated the tolerance limit of calcium as 75 mg/l. The drinking

water samples under study had the calcium amount within the acceptable limit which is same as that of the results of Roohi Rawat and A.R Siddiqui (2019) in drinking water quality of Allahabad Metropolitan city, India.The high level of calcium in water result in adverse effects on domestic use. Insufficiency in calcium causes severe rickets in human beings and excess of calcium causes concretions in the body such as kidney or bladder stones and irritation in urinary passages. It is also essential for proper functioning of nervous, muscular system and cardiac function. It also plays an important role in coagulation of blood. The samples we studied have got the calcium levels below the acceptable limit which may lead to prevalence of rickets among people using this water.

Magnesium is one of the main constituent in natural water and it is an important contributor for hardness of water. It is also co-factor for various enzymatic transformations within the cells WHO, (1984). Magnesium salts are diuretics and in high concentrations they may cause laxative effect especially in new users. The deficiency of magnesium is associated with structural and functional changes. Magnesium level in the water samples analyzed were comparatively lower than the acceptable limit which denotes less contamination of these water.

SODIUM AND POTASSIUM

Sodium and potassium are the essential nutritional elements for human life and proved to be detrimental to human beings. But in excess, potassium acts as a laxative. To people suffering from cardiac, renal and circulatory diseases, sodium turns to be harmful. From our study results, it is indicated that drinking water sample from Nazareth contains comparatively high amount of sodium (42 mg/l) than the other samples analyzed even though it's below the permissible limit (59 mg/l). The range appears to be nearing the permissible limit and high concentration of sodium in drinking water normally affects its portability and it may leads to heart problems. The level of potassium in the drinking water samples were within the permissible limit. But sample II (Nazareth) needs treatment before consumption.

NITRITE AND NITRATE

Nitrate is one of the major constituents of organisms along with carbon and hydrogen as amino acids, protein and organic compounds. The desirable amount of nitrate in drinking water is about 45 mg/1. In the present study nitrate levels in the samples were lower than the prescribed levels. Nitrates in the water may be added due to runoff from fertilizer used, leaking from septic tanks and sewage and erosion of natural deposits. Excess of nitrite and nitrate leads to shortness of breath and blue- baby syndrome.

CHLORIDE

Chlorides are the most stable component in water and their concentration is largely unaffected by most natural physical- chemical and biochemical processes. Chloride in natural water results from the leaching of chloride containing rocks and soils with which the water comes in contact. In the present study in all the water samples analyzed the chloride level was estimated to be within the permissible limit but comparatively station II (Nazareth) was found to have higher levels of chloride than the other samples. Excess of chloride level causes eye and nose irritation stomach discomfort in human beings. It also adds up corrosive nature of water WHO, (2006).

FLUORIDE

Fluoride can naturally occur in ground water and in some surface water. Drinking water is normally the major source of fluoride. High levels of fluoride can be found naturally in Indian waters, possess risk to those drinking the water. Fluoride in drinking water may be due to the erosion of natural deposits, discharge from fertilizer and aluminum factories. Fluoride although known to prevalent early stage of tooth decay, high level of its concentration in drinking water, has been found to have serious health effects in human like mottled teeth that occur in children (McDongh, *et al.,* 2004).

The samples under study were found to have the fluoride level far below permissible limit (1.5 mg/l)WHO, (2006).

SULPHATE

The source of sulphate is an abundant ion in the earth crust. According to Who, (2004) guidelines for drinking water quality, sulphate should be lower than 500mg/I but in the drinking water samples analyzed, the level falls within the acceptable limits. But when the level goes above taste of drinking water is affected. It also cause gastrointestinal irritations in human beings. It may also result in calcium sulphate scale.

PHOSPHATE

Phosphate is a natural mineral that is mined and used in fertilizers and so natural water contains more than 0.1 mg/l unless the water passes through the soil containing phosphate in the studied water samples were beyond 0.1 mg/1. High amount of phosphate in water usually indicates that there is some contamination from mining, domestic waste water or excessive fertilizers being applied in farmland. Comparatively drinking water from Bharathinagar was found to contain higher level of phosphate than the other samples. This is an indicator that protecting the ground water sources in Bharathinagar is needed to maintain a healthy drinking water system. If phosphate is consumed in excess phosphine gas is produced in the gastrointestinal tract on

reaction with gastric juice, which may lead to the deleterious effect. So water from Bharathingar needs treatment.

RESIDUAL CHLORINE

Chlorine is a chemical that we add to drinking water to kill most pathogens that can make us sick. Chlorine is not usually found naturally in the environment in large amounts that can hurt us. High amounts of chlorine can irritate the skin and eyes, if when it comes in contact. The strong smell of chlorine can also hurt our throat and lungs if we breath it in. WHO suggest that drinking water should have the residual chlorine between 0.2mg/I to 0.5mg/l to give long time protection. Among the water samples analyzed only the water from Bharathinagar was found to contain 0.2 mg/l. It is good to have chlorine in drinking water which makes it safe.

BACTERIOLOGICAL ANALYSIS

TOTAL HETEROTROPHIC BACTERIAL COLONY

Water plays a significant role in supporting human life, but if contaminated it has a great potential of transmitting a wide variety of diseases and illness. Testing of microbial quality of drinking water is an important step in public health monitoring. When considering drinking water quality in most cases microbial contamination is the main concern since it is responsible for the majority of illness and deaths related to drinking unsafe water. results of microbial analysis of drinking water samples showed that bacterial colonies were found in higher number in sample from Authoor than the other samples (plate – I). In general high levels of free co_2 may affect the bacterial counts in the sample. The presumed reason for contamination of water accounts for the microbial load from human and animal wastes. Since the source of these water samples is river, the possibility of microbial contamination is more. Sample II (Nazareth) also showed a higher number of bacterial colony (plate -3), which may also be due to the contamination with human and animal waste in riverine source. Even though in other water samples the microbial load was comparatively less there were more bacterial colony (plate –2 & 4). This might be correlated with the work of Bitton, (1994) where the ground water was found to be contaminated due to animal wastes, proximity to toilet facilities, sewage, refuse dump site and various

human activities around the well. The above possibilities of contaminations were present in sampleIII. So the drinking water samples from Authoor and Nazareth need to be treated before consumption. The sample from Threspuram was observed to contain the third maximum number of bacterial colony even though it was supplied by corporation. This needs concern to prevent the water borne diseases.

FAECAL COLIFORM

Analysis of water samples for faecal coliform contamination indicates the presence of few number of faecal coliform in sample from stations I & II (Authoor and Nazareth). These pathogens may pose a special health risk for infants, young children and people with severely compromised immune system WHO, (2008). Disease causing microbes (pathogens) in these water can cause diarrhea, cramps, nausea, headaches or other symptoms (Hiren Dandia and Harsha Chandrashekar palav2018). In some cases, house hold residents acquire immunity to water borne bacteria that are common in their drinking water. In this case, visitors to the home who have not acquired immunity may become ill after drinking water.

SUMMARY

SUMMARY

The present study on “An analysis of the portability of drinking water from selected areas of Thoothukudi” revealed that the drinking water samples from Authoor (sample I) and Nazareth (sample II) were slightly coloured. When analyzed for odour all the four samples were odourless. The drinking water samples from Authoor and Nazareth were slightly turbid than the other two samples.

The total dissolved solids of the water samples analyzed were within the permissible limit and maximum was recorded from sampleII (Nazareth).

The electrical conductivity was maximum in the sample from Authoor even though all the four samples had the electrical conductivity within the permissible limit.

The pH of the water samples analyzed were within permissible limit, but sample from Authoor had the maximum pH.

The total alkalinities recorded in the samples were within the acceptable limit and it was maximum in sample from Nazareth.

Calcium and magnesium levels recorded were within the acceptable limit and both the parameters were maximum within the drinking water from Nazareth.

The sample from Nazareth had the maximum level of sodium and potassium. Negligible amount of nitrite and nitrate were estimated from all the water samples.

Chloride was maximum in the drinking water from Nazareth and fluoride was more in the sample from Bharathinagar.

The level of sulphate was very less in all the samples analyzed compared to that of permissible limit. Comparatively sample II (Nazareth) had the maximum amount.

No residual chlorine was found to be present in the samples under study but for the samples from Bharathinagar which contained a trace amount of residual chlorine.

The total heterotrophic bacterial load was maximum in the sample from Authoor. The sample from Nazareth and Therespuram also had more bacterial count and minimum number of bacterial colony was recorded in the sample from Bharathinagar.

The drinking water samples from Authoor and Nazareth had very few faecal coliform bacteria.

CONCLUTION

AND

SUGGESTIONS

CONCLUTION AND SUGGESTIONS

The present investigation has given an insight on the quality of drinking water used by people in an around Thoothukudi. It is understood that even though all the water samples analyzed were having the physical, chemical and bacterial analysis within permissible limit the drinking water sample from Authoor and Nazareth need treatment before drinking.

Regarding bacteriological contamination water samples from Authoor, Nazareth and Thresepuram should be purified for consumption to avoid water borne diseases. So it is recommended that the people of Authoor and Nazareth should be provided with corporation drinking water to avoid health problems. If not the reverine water should be disinfected or treated before consumption.

Water sample from Threspuram needs disinfection before consumption even though they are provided with corporation water. The people residing there should be educated to be hygienic to avoid water borne disease.

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A SURVEY AND PROXIMATE COMPOSITION OF TRASH FISHES FROM THREE LANDING CENTRES IN THOOTHUKUDI COAST

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By

R. KALAISELVI

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DEPARTMENT OF ZOOLOGY

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THOOTHUKUDI – 628001

APRIL - 2021

CERTIFICATE

This is to certify that this dissertation entitled, **"A SURVEY AND PROXIMATE COMPOSITION OF TRASH FISHES FROM THREE LANDING CENTRES IN THOOTHUKUDI COAST"** submitted by **R. KALAISELVI, Reg. No. 19APZO02** to St.Mary's College (Autonomous), Thoothukudi affiliated to Manonmaniam Sundaranar University, Tirunelveli in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2020 - 2021 under my guidance and supervision. It is further certified that this dissertation or any part of this has not been submitted elsewhere for any other degree.

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EXAMINER

DECLARATION

I hereby declare that this dissertation entitled, **"A SURVEY AND PROXIMATE COMPOSITION OF TRASH FISHES FROM THREE LANDING CENTRES IN THOOTHUKUDI COAST"** submitted by me for the award of the degree of Master of Science in Zoology is the result of my original independent research work under the guidance of **Dr. S. SELVI, M.Sc., B.Ed., M.Phil., Ph.D.,** Assistant Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date : 15 04 2021

R Malarselvi

Signature of the candidate

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CONTENTS

1. INTRODUCTION

Ocean covers almost 71% of the earth's surface and it is a complex ecosystem has a vast array of ocean organisms include plants, mammals, fishes crustaceans, animals, and microorganism. Over 20,000 known species of vertebrates are fishes. 90% of these belong to the class bony fishes and some are exclusively marine.

The sea is immense almost unexploited source of new potentially useful biologically active substance (Faulkner, 1999). Recently marine microorganism has been found to produce a variety of chemically interesting and biologically significant secondary metabolites some of them being expected to serve a lead compound for drug development or pharmacological tools for basic studies in life science (Kobayashi 1989 and Jansen 1994)

In India, trawling is one of the most important commercial fishing methods followed in mechanized sector. A major portion of the world's fish supply for human consumption is provided by trawl (Sains burry, 1996) in terms of investment and yield.

Fisheries play an important role in Indian economy and it is one of the most important activities along the coastal area (FAO yearbook, 2002 and Jayaraman, 2004). According to FAO (FAO fisheries and aquaculture organization of US, 2010) total capture fisheries production of India was estimated about 93 million tons and the contribution from marine capture fisheries was about 90%. Annual

marine fishery potential of India EEZ is estimated as 3.93 million tons (Sudarsan, 1990) and the sector - wise contribution of marine fish landings are 71 % in mechanized sector , 24% in motorized sector and 5% in artisanal sector (CMFRI , annual reports 2006 2007, Cochin).

The commercial fisher man often method and processed large quantities of fish in their boats for scale heavily loaded boats often had to be lightened quickly by removal of internal organ of fish. Thus originally a 'rough fish' was a fish of any species that had been only partly processed and which could not have sold for full price. Trash fish include damage sardine, anchovies, leather jackets etc., these fishes are produced and used as fertilizer and feed stock in poultry and aquaculture. Low value trash fish is a loosely used term that describes fish species with various characteristics, but they are generally small in size, have low consumer preference and have little or no direct commercial value. They are either used for human consumption or used to food livestock/fish either directly or through reduction of fish meal/ oil

In general, the biochemical composition of the whole body indicates the fish quality. Therefore, proximate biochemical composition of a species helps to assess its nutritional and edible value in terms of energy units compared to other species. Variation of biochemical composition of fish fleshy may occur within the same species depending upon the fishing ground, fishing season, age, sex of the individual and reproductive status. The spawning cycle and food supply are

the main factors responsible for this variation. Commercial fishing not only affects the targeted species but also several other species that are caught incidentally. Apart from the targeted species fishing during trawl fishing, non-targeted species commonly referred as bycatches were also caught. Bycatches includes species of little commercial importance, which are dumped into the sea or on the shore after reaching the fish landing sites. The estimate of 2006 suggests that 7.3 million tons of the global catch is discarded annually as bycatch (Kelleher, 2005). The unutilized sub-set form in the bycatches is known as 'discards'. The bycatch and discards were collectively referred as trash fishes (Clucas, 1997). The term of the trash has been used to denote fish usually non targeted that are caught as by catch and normally has no price in the market (Nunoo, *et al*., 2009).

Conventional trawlers are poor selective fishing gears and so retain large quantities of the non-target species (Saila, 1983). The commercial marine fish catch from these trawlers generally consist of edible fishes and inedible species. The collection of inedible low value fishes and juveniles in of commercially important fishes are referred to trash fishes and locally known as 'kalasal'. These trash fishes where caught as 50% of total catch generally lacking economic value but rich in nutritional value are often not utilized properly discarded as waste. (Chandrspal, 2005; Cluscas, 1997; Immaculate *et al*., 2013).

FAO estimates suggest that there has been reduction in the amount of discards over time (Chattopathay *et al*., 2004). One factor contributing to this trend is an increase in the landing of previously discarded trash fishes (Adewoye and Omotosho, 1996) that is processed as feed for aquaculture and livestock, used as farm manure, and in some cases, sold for human consumption (Rakhsana, *et al*., 2005.), but there is no much knowledge on species composition of trash fishes landed in the currently selected study area. There is a need to study in detail of the species landing statistics and size composition of trash fish including juveniles of commercially important species appearing in the catch.

According to FAO the global trend has been towards a proper and better utilization of noncommercial fishes. Trash fishes are widely used in coastal areas either directly for human consumption and unhygenically dried and used as poultry feed, trash fishes are freshly prepared and carefully. Managed can be a very good and inexpensive, source of food for culturing aquatic animals. Sadly, this is not in practical due to its unknown nutritional components. The nutritional values of the discarded fishes are very important to initiate proper use of these trash fishes in a desirable way.

The problem of "bycatch" is recognized as one of the most pressing conservation challenges associated with the world's fisheries today and one for which there are still no clear solutions. Bycatch commonly refers to the total catch of non – target animals that takes place in fisheries which set out to capture a

particular species (e.g., shrimp) or a group of species. However, there has been considerable confusion pertaining to its definition. These definitional difficulties reflect the changes in our understanding of the composition of fish catch, the changing values in catch evolving fisheries economies, and concept such as 'trash fish', and high- and- low value species. These definitions are also influenced by an ecological understanding of marine species, and their order and interaction at various scales and tropic levels.

For example, the term "discard" (the portion of the catch that is discarded at sea) is commonly regarded as being synonymous with bycatch in some countries and is conceptually considered to be a subset of term bycatch (the total non-targeted catch landed on boat vessel). The term 'trash fish' is often also used synonymously with discards or bycatch but generally refers to very low-value fish (usually caught in trawl fisheries). All these definitions are highly dependent on ecological, economic, legal, and social influences.

Bycatch related mortality is also among the main causes od declines of threatened marine mega fauna including sea turtles, crustaceans and seabirds (Lewison *et al*., 2004). Even seemingly resilient species can be threatened as by catch, e.g., juvenile fish constitute a major proportion of the catch in tropical shrimp trawlers (Foster and Vincent, 2010). This continuous removal of large quantities of juveniles as by catch can impede the recovery of both target and bycatch stocks. Checklist on trash fishes' constituents is helpful in improving

knowledge regarding the biodiversity of the region and is an important tool for fisheries management. It also contributes to knowledge of distribution of non-conventional species, which are poorly represented in the catch statistics based on commercial landings. (Qasim, 1972).

Bycatch is a major threat to marine biodiversity. An estimate (derived primarily from fisheries) suggests that bycatch constitutes approximately 40.4% of the estimated annual global marine catch of 95 million tons (Davies *et al*., 2009). However, bycatch biomass estimates by themselves underplay the real \

tude of this problem. A diversity of species encountered as bycatch is differentially affected by the fisheries that capture them.

By catch also has a strong moral dimension to it since it has long between associated with discarding large quantities of useful fish protein (Kelleher, 2005). Interestingly what has considered bycatch by the industrial fisheries sector was the commercially important targeted catch of the small-scale fisheries sector. In tropical countries like India with a diversity of fisheries and social group, the problem of bycatch is certainly a normative one, raising questions about its implications for equity and justice in fisheries.

Appropriate feed composition is important for growth, disease resistance, and immune activity in aquaculture fish. A nutritionally adequate diet satisfies the need: fuel (chemical energy), the organic raw materials for biosynthesis and a supply of essential nutrients. The level of dietary lipids beyond about 9% - 10%

did not improve fish growth rate, but instead reduced the fish appetite in humpback grouper (Cromileptes altivelis) (Williams *et al*., 2004). Worldwide there is enormous interest in performance diet, both to improve the efficiency of growth and to reduce N- excretion to the environment (Eng *et al*., 1989 and Naylor *et al*., 2000).

The digestion and metabolism of feed ingredients id dependent on fish species and on the source inclusion level and treatment of the ingredient (Krogdahl *et al*., 2005). Knowledge of the capacity to utilize carbohydrate in the diet is an essential pre requisite for appropriate formulation of fish feed (Wilson, 1994).

Osmoregulation and locomotion to playing a possible immunological role (Flocher, 1978). Controls the intra specific chemical communication (saglie and Balance, 1989). Over the years it has also been shown that mucus plays a vital role in the prevention of colonization by parasites, bacteria and fungi (Pragatheeswaran, 2011). Man researches have screened the antibacterial effect of mucus against the marine microbial satins. It has been reported that epithelial tissues produce antimicrobial molecules which have serve as the first line of a host defense against microbial invasion in a variety of vertebrates including humans. (Ganz, 1999).

Fish consumption is associated to health benefits because if rich content in proteins of high nutritional value, mineral, vitamins and distinctive lipids, the

major fishing countries, indicated that proved more than 2.6 billion people with at least 20% of their average animal protein intake. However, fish stocks are decreasing and the annual world fish catch is stabilized as the yearly averaged discards and estimated to 7.3 million tonnes. Fishes are most important source of animal protein and usually consuming at several place of world due to its having high contents of protein, amino acid and saturated fatty acid. It is more essential for human diet to raise the utilization of marine fish and its products. (Burr 1989 and Sargent, 1997).

Lipid and carbohydrates are the critical components of the diet for supplying the carbon skeleton and energy. Fish may convert protein into an energy source if non protein energy source (carbohydrates and fats) are not present in sufficient quantities in the diet. Therefore, a certain amount of lipids is usually included in diets as a source and also to increase the protein efficiency (Cho and Kaushik 1990, Williams *et al*., 2003.

Fish use carbohydrates and lipid as energy in order to maintain daily activities and convert protein energy source (Carbohydrates or Fat) are not sufficient. It is known that, certain amount of lipid and carbohydrates are usually included in diet as energy (Cho and Kaushik 1990, Williams *et al*., 2003). Carbohydrates are often chief dietary energy source then protein and lipids. However fish species show different ability to digest and metabolize alternative

dietary components in particular the carbohydrates fraction (Dabrowski and Guderley 2002).

Fish protein Hydrolyses (FPH) obtained by controlled enzymatic hydrolysis are among the best protein hydrolysis in term of nutritional properties balanced amino acid composition high digestibility, but mainly used for animal nutrition because of their bitter flavor and fishy odor (Kristinson and Rasco, 2000).

Variation of biochemical composition of fish flesh may occur within the same species depending upon the fishing ground, fishing season, sex, age of the individual and reproductive status. The spawning cycle and food supply are the main factors responsible for this variation (Love, 1980).

Considering that the small-scale fisheries sector contributes food to local domestic markets, the problem of bycatch is seen as the depletion of this food source for local consumption and therefore a threat to livelihoods and food security in general. In this project, an attempt has been made to study the trash fishes landing details of three major landing areas of Thoothukudi.

OBJECTIVES

The objectives of the present study are

- To know the quantum of trash fishes available during the study period from December 2020 to March 2021.
- To estimate the amount of carbohydrate, protein and lipid, content of trash fish from random sample.
- To create awareness among the people about the use of trash Fishes.

2. REVIEW OF LITERATURE

Fish contributes significantly to the overall production of animal protein. A survey of literature revealed that a reasonable amount of work has been done to determine the nutritional requirement, prepare a commercial fish feed exact dosage and feed formula to get maximum production of different fish species other than major carps around the world.

Globally there is evidence, the majority of the stock will be over fished and increasingly impacted by degraded aquatic environmental mainly due to anthropogenic activities, climate and environment factors, newer technologies have been put in place to improve the state of fish production of capacity and to manage natural stocks in better ways, thus increasing and sustaining the supply of fish for consumption and socio – economics benefits while still ensuring conservation of genetic biodiversity (Laber, 2004).

The indispensability of the fishery resource in contributing to food and nutrition security and other socio – economic development has led to its increase in production and trade (Dutta, 2000; Philips, 2009) production from aquaculture augmented the experienced decreased supply from the wild (Hardy, 1999; Laing, 2009; phillips 2009).

The major fish feed were trash fishes and artificial feed that were gives in the form of dry or moist pellets very high survival rates were recorded through the use of indoor hatchery system (Lin *et al*., 1979; Tang *et al*., 1979 – Liu and

Hu, 1980). Faster and cleaner means of transport has increased trade in live fish globally value addition in the fishery resources such as drying, smoking, filtering and packaging in various ways have all opened upgrade in the fishery sub – sector.

Nevertheless, the nutritive value of fish meal is affected by the high variability in the protein content, which varies from 57 to 77% as a consequence of the different fish species used in the variability are very important in the feed compound composition and quality is to be produced from inherently variable raw materials and products (Fouz, *et al*., 1990). Hence, analytical control to assess the protein content in fish meal.

Marine based ingredients, especially fish meal are highly bought after as the protein sources of choice for many formulated diets. That is because fish meals provide feeds with high in carbohydrate, thus being usually well digested and mainly use by feeds industry as a rich source of protein (Ebran *et al*., 2000). Hence fish meal industry is widely spread across the world and almost one third of the total global fish and shellfish catch is used by this industry (Fletcher, 1978). Approximately 65% of the average annual global production of fish meal is used by the aqua feed industry (Austin and Melntosh, 1988). It is also used in poultry swine, ruminant, companion animal and even in human foods as a protein feedstuff. Thereby, the percentage of fish meal in poultry and mammalian feeds is small but the total quality of such feed is very large.

On the other hand, other vital parameter in fishmeal is the moisture content since it plays a major role in the shelf life and storage time of fishmeal. Indeed, it has been shown that the decrease in the moisture content induces an increase in the shelf – life and decreases in the spoilage phenomenon (Ingram, 1980). The number of trawlers operating in Indian waters has been recently estimated as 29,241 with a maximum in Gujarat (27.4%) followed by Tamil Nadu (18.1%), Maharashtra (14.4%), Kerala (13.6%), Karnataka (8.6%), Andhra Pradesh (6.2%), Orissa (4.6%), Goa (2.8%), west Bengal (2.1%) Pondicherry (1.1%) and Daman and Diu (1.1%) (Fisheries college and research Institute, Thoothukudi, 2012). However, the trawlers are an efficient gear led to a fairly rapid fishing of commercially targeted stocks (Bavinck, 2001 and Bhathal, 2000).

Trawl fishing begin in 1956 at export markets through funding provided by foreign aid organizations. (Deveraj Vivekananda, 1999). Introduction of trawl fishing led to an increase in commercial landings in the initial years. In India, 67.9 and 32.1% of trawlers are operated in west and east coast respectively.

Vivekananda *et al*., (2009) studied on the trophic level of fishes occurring along the Indian coast. Bijukumar and deepthi (2009) studied the mean trophic index of fish fauna of south west coast of India. There is pressure of indiscriminate catch of all varieties of fish throughout the year resulting in decline in fish biodiversity and annual yield of fish from the floodplain lakes of Eastern India (Mondal and Kaviraj, 2009).

Rukhsana Talat *et al*., (2005) reported the commercial and industrial catch of marine fishes generally consists of edible and inedible species. Among inedible species the bulk catch of small size fishes were also included and these small fishes were commonly referred to as trash fish. Twenty-three species of small fishes from fish trash were identified and studied for their mineral compositions which were collected bimonthly from fish harbour Karachi Minerals like phosphorus, calcium, sodium, potassium, iron and magnesium were found in the trash which were analysed by spectrophotometry, flame photometry and atomic absorption techniques. The results have been explained in relation of importance of minerals found in edible fish trash and their utilization as poultry feed and other useful by product.

Immaculate *et al*., (2013) observed the analysis the utilization of trash fishes as edible fish powder and its quality characteristics and consumer acceptance. Jayakody, (2016) reported that assessment on the present status of coastal fisheries at Gurunagar. Duglas Sathees *et al*., (2020) observed the poximate composition analysis of trash fish from the selected landing sites of Jaffna district, Sri Lanka.

3. MATERIALS AND METHODS

For the experimental purpose the data about the trash fish were collected from the following study area

I. Vembar fish landing centre

II Thoothukudi fishing harbor

III Keezhavaippar fish landing centre

The informations about the trash fishes were collected from Mr. Rojar (Jackson boat accountant, Vembar), Mr. Xavier (Mariyea vazgha boat accountant, Thoothukudi) and Mr. Panimayam (God's grace boat accountant, Keezhavaippar) during the study period (Decembar 2020 to March 2021).

STUDY AREA – 1 (Vembar fish landing centre)

Vembar is a village panchayat in Thoothukudi district in the Indian state of Tamil Nadu. Vembar is a village situated along the East coast road at the north end of Thoothukudi district. Vembar is located near the district's border with Ramanathapuram District, about 13 km south of Sayalkudi and around 7 km from Melmandai. The vembar coastal area has 265 steel mechanized boats and 200 country boats. The fisherman collected large amount of trash fishes along with edible fishes.

STUDY AREY-II (Thoothukudi fishing Harbour)

Thoothukudi Fishing harbour is one of the most important fishing area in Thoothukudi. It lies in the coromandal coast of Bay of Bengal. It is located about 590 kilometres southwest of Chennai, 190 kilometres northeast of Thiruvananthapuram and 580 kilometres southeast of Bengaluru. Thoothukudi is also known by the name 'Pearl city'. It is also called as "Sea Gateway of Tamil Nadu". Thoothukudi is part of the Pearl Fishery Coast, and is known for its pearl fishing and ship building industries. Thoothukudi is a port town situated in the Gulf of Mannar about 125 km. The trash fishes are brought by mechanized boats.

STUDY AREA – III (Keezhavaippar landing Centre)

Keezhavaipar is a village in Vilathikulam Block in Thoothukudi district of Tamil Nadu state, India. It is located 31km towards from North from District headquarters Thoothukudi. It is near to bay of Bengal. The trash fishes are brought both mechanized and non- mechanized boats, but mostly by wooden boats.

Proximate composition analysis:

Estimation of Carbohydrate:

Carbohydrate was estimated by anthrone method.

10 mg of trash fish muscle was taken and homogenized separately with 2 ml of 10% trichloroacetic acid and 8 ml of distilled water. The homogenate was centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and measured and it was used for analysis of carbohydrate. 1 ml of the supernatant was taken in a test tube and to this 4ml of anthrone reagent was added and mixed well. The test tube containing the mixture was kept in boiling water bath for 10 minutes. The test tubes were then cooled at room temperature and optical density was measured in a spectrophotometer at 620 nm.

$$\% \text{ of Carbohydrate} = \frac{\text{standard OD value x OD value of the sample X 1000}}{\text{Weight of the sample}}$$

Estimation of Protein:

The protein was estimated by lowery *et al*., (1951) method.

100 mg of trash fish muscle was taken and homogenized separately with 5 ml of 10 % trichloroacetic acid in a motor and pestle. The homogenate was centrifuged for 15 minutes at 3000 rpm. The supernatant was discarded. The precipitate was dissolved thoroughly in 5 ml of o.1N sodium hydroxide solution

and kept in a water bath at 60-70 c for 10 minutes. From this 0.5 ml of solution was pipette out and poured into a clean dry test tube.

To this 4 ml of carbonate copper solution was added. It was mixed well by through lateral shaking and kept in room temperature for 15 minutes. To this 4 ml of folin phenol reagent was added. The test tube were shaken well for uniform mixing and kept in room temperature for another 30 minutes. The blue color appeared, the O.D was evaluated against the blank at 640nm.

$$\% \text{ of Protein} = \frac{\text{standard OD value x OD value of the sample X 1000}}{\text{Weight of the sample}}$$

Estimation of Lipid:

Lipid was estimated by the method of Bragdon (1951).

10 mg of trash fish muscle was taken and homogenized separately with 5 ml of chloroform and solution was centrifuged at 3000 rpm for 15 minutes. The supernatant was evaporated to dryness. The 3 ml of 2% potassium dichromate in concerted sulphuric acid was added which was followed by 3 ml of distilled water. The developed colour was read in spectrophotometre at 640nm.

$$\% \text{ of Lipid} = \frac{\text{standard OD value x OD value of the sample X 1000}}{\text{Weight of the sample}}$$

4. RESULTS

The survey was conducted from December 2020 to March 2021 to know the availability of trash fishes. It was found nearly 199 kg trash fishes day/ boat were reaching the study area - I. 99 kg of trash fishes day/ boat were reaching the study area - II and 71 kg of trash fishes day / boat reaching study area-III

The weight of the fish was obtained after the marketable edible fishes were separated. It was also noticed that the quantity varied from season to season and also dependent on the availability of species. In study area I per day input of trash fishes from a single boat found to be 42 - 199 kg (Table - I, figure -1) in the study area - II the weight ranged from 32 - 99 kg (Table - 2, figure - 2), In the study area - III the weight ranged from 16 - 71 kg (Table - 3, figure - 3)

From the comparison of data, it was found that the amount of trash fishes collected was higher in Vembar landing centre than Thoothukudi fishing harbour and Keezhavaippar landing centre.

Based on random sample the carbohydrate content of the trash fish collected from the study area - I was found to be 5.6 mg/g, the area - II was found to be 8.26 mg/g and the area - III was found to be 9.52 mg/g. This result revealed the presence of quantity of protein in trash fishes (Table – 4, Figure - 4).

The protein content of the trash fish collected from the study area - I was found to be 19.372 mg/g, the area - II was found to be 35.07 mg/g, and the area - III was found to be 32.398 mg/g. This result revealed the presence of quantity of protein in trash fishes (Table – 4, Figure - 4).

The lipid content of the trash fish collected from the study area - I was found to be 7.875 mg/g, the area - II was found to be 5.875 mg/g, and the area - III was found to be 7.125 mg/g. This result revealed the presence of quantity of lipid in trash fishes (Table – 4, Figure - 4).

TABLE-1

QUANTITATIVE DATA ON TRASH FISHES FROM

THE STUDY AREA - I

(VEMBAR LANDING CENTRE)

Boats	1 day (kg)	2 day (kg)	3 day (kg)	4 day (kg)	5 day (kg)	6 day (kg)	Average
A	70	100	120	70	49	120	88.1
B	60	95	78	101	199	121	109
C	89	121	50	60	99	100	86.5
D	81	191	98	121	173	199	143.8
E	58	150	101	42	57	81	81.5

Figure - 1

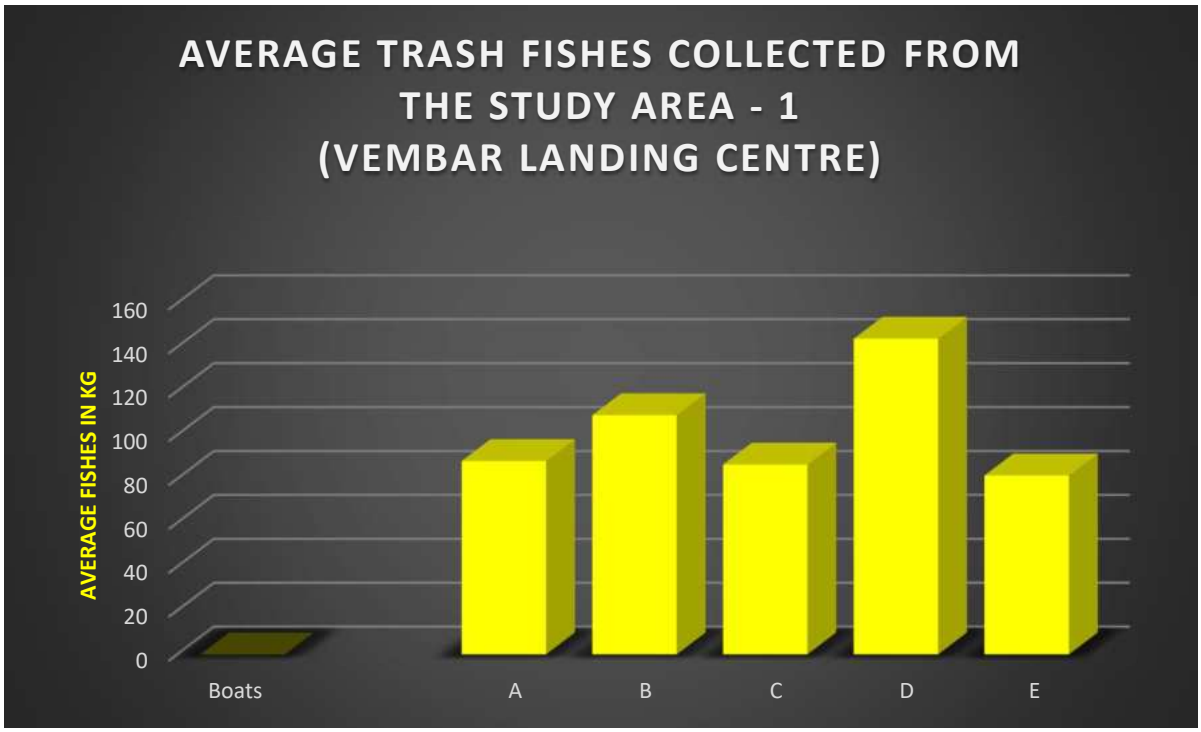

AVERAGE TRASH FISHES COLLECTED FROM THE STUDY AREA - 1 (VEMBAR LANDING CENTRE)
AVERAGE FISHES IN KG
160
140
120
100
80
60
40
20
0
Boats
A
B
C
D
E

TABLE-2

QUANTITIVE DATA ON TRASH FISHES FROM THE STUDY AREA - II (THOOTHUKUDI FISHING HARBOUR)

Boats	1 day (kg)	2 day (kg)	3 day (kg)	4 day (kg)	5 day (kg)	6 day (kg)	Average
A	88	89	33	57	50	57	62.3
B	70	60	63	53	54	77	62.8
C	96	99	72	36	41	42	64.3
D	61	54	39	61	80	32	54.5
E	55	75	58	69	41	33	55.1

Figure – 2

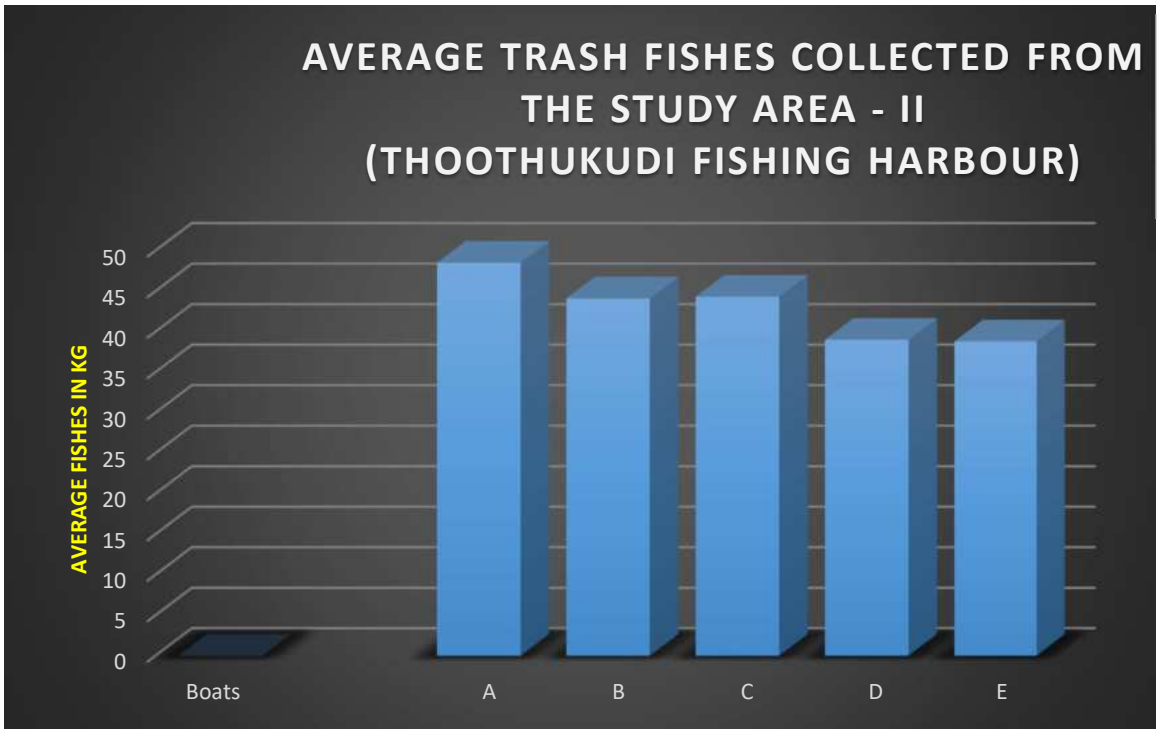

AVERAGE TRASH FISHES COLLECTED FROM THE STUDY AREA - II (THOOTHUKUDI FISHING HARBOUR)
AVERAGE FISHES IN KG
50
45
40
35
30
25
20
15
10
5
0
Boats
A
B
C
D
E

TABLE – 3

QUANTITATIVE DATA ON TRASH FISHES FROM

THE STUDY AREA - III

(KEEZHAVAIPPAR LANDING CENTRE)

Boats	1 Day (kg)	2 Day (kg)	3 Day (kg)	4 Day (kg)	5 day (kg)	6 day (kg)	Average
A	48	31	70	31	43	68	48.5
B	32	51	46	39	71	26	44.1
C	26	51	70	26	37	56	44.3
D	67	43	28	26	34	36	39
E	40	41	56	51	29	16	38.8

Figure - 3

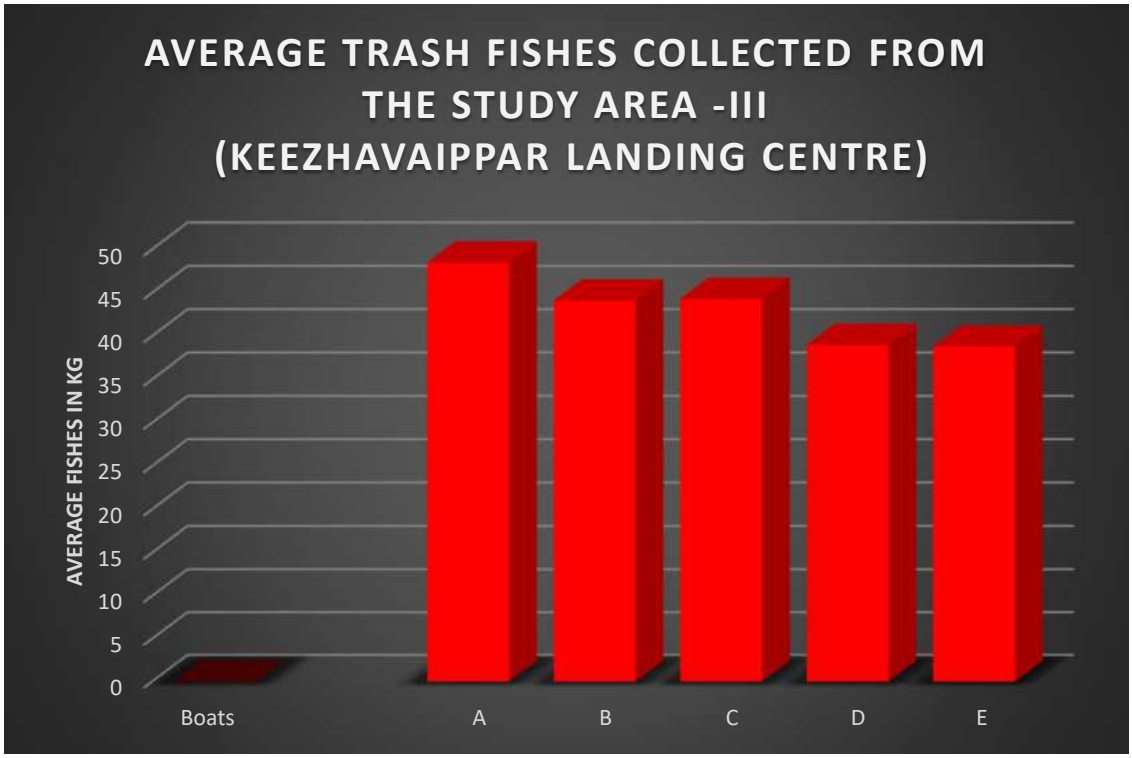

AVERAGE TRASH FISHES COLLECTED FROM THE STUDY AREA -III (KEEZHAVAIPPAR LANDING CENTRE)
AVERAGE FISHES IN KG
50
45
40
35
30
25
20
15
10
5
0
Boats
A
B
C
D
E

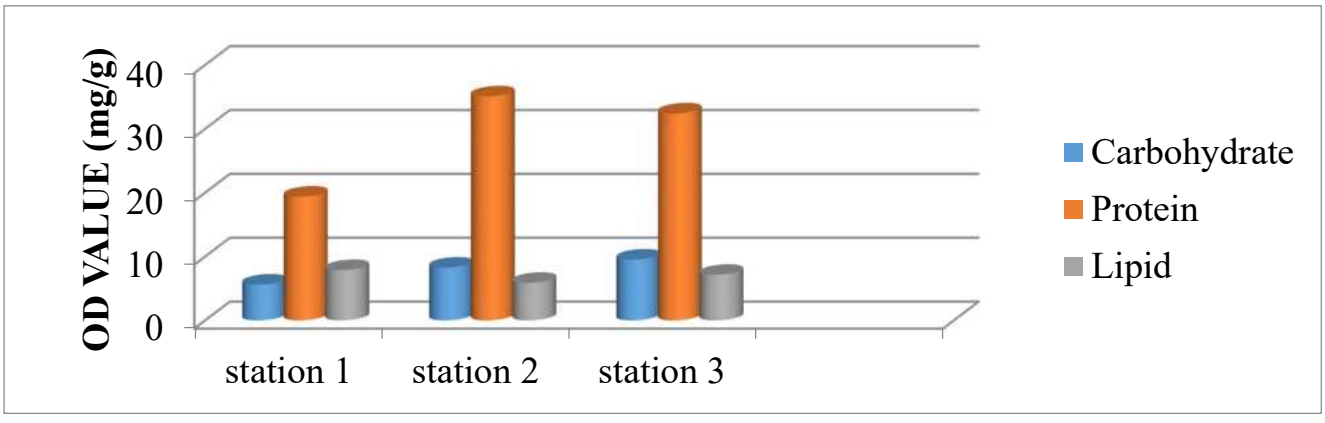
TABLE - 4

Proximate composition of trash fishes from station I, II & III

Parameter	Station -1	Station - II	Station - III
Carbohydrate	5.6 mg/ g	8.26 mg/g	9.52 mg/g
Protein	19. 372 mg/g	35.07 mg/g	32. 398 mg/g
Lipid	7.875 mg/g	5.875 mg/g	7.125 mg/g

FIGURE-4

Carbohydrate, protein and lipid contents of muscle of trash fish from the study area I, II & III



5. DISCUSSION

The trash was caught by fisherman using trawl nets. The discarded fish has little small size, poor quality or out of a variety of fin and shell fish. Limited appeal to consumers and are called ‘trash fish’. The low cost fishes are separated from other fishes.

Trash fishes are locally called as ‘kalasal’. Marine fish species like 2.0 to 10.0 cm and the average total length was 8.5 cm. Fish powder is mainly used as food in poultry and other industry. The solar drying is used to remove the water content from the trashes. Then they were powdered to use as a feed. About 40% of trash used to surimi production is ‘waste’ while about 20% of the used for drying is ‘waste’. Waste or by products are used for fish meal production. There were about 15 sites for traditional fish processing in large site. In addition, there were seven modern fish processing factories for squid. Some fish by products were being fed to pigs locally but most was destined for fish meal factories.

India produces 6.57 MMT (Million Metric tons) of fish annually, out of which 55% is from fresh water (FAO, 2008). Fish processing yields a wide variety of fish by products such as scales, head, skin, fat, viscera and roes in large quantities. Most of these by- products are discarded as waste, without processing them into value added products either for industrial application or for human consumption. In addition, indiscriminate disposal of fish by products is a serious cause of environmental pollution. Among fish by products fish eggs are highly

perishable in nature, nutritious material rich in essential minerals, amino acid and fatty acids. By – products utilization will improve the economic aspects of processing industry and further their nutritional beneficiations through valuable essential mineral, amino acids and fatty acids components. Particularly by-products with high protein content, suitable functional characteristics and specially, antioxidant activity has immense importance in the food processing industry. Marine by- products were reported to be good sources of nutraceuticals and functional food ingredients (Barrow and shahidi, 2007) . In our present study the amount of trash fish collected was higher in Vembar landing centre than Thoothukudi fishing harbour and Keezhavaippar landing centre.

Proximate content is an important indicator that determines the quantity of the raw material freshness. This method is used to determine the protein, carbohydrate and lipid of trash fish.

Vaikundamoorthy *et al*., (2014) reported the potential use of trash fish manures in agricultural fields. They analysed nutrient and minerals in trash fish samples. High amount of nitrogen (6%), phosphorous (5%) and potassium (4%) were present in trash fish and used for plant growth study. They selected three commercial plants viz. *Lycoperscon esculantum, Hibiscus esculenta* and *Solanum melongena* for analysis. The shoot length, root length, total length, number of leaves, leaf length, biomass of the plant and roots division were measured in every

15 days interval upto 45 days. After 45 days, the percentage of root length growth of *L. esculantum, H. esculenta* and *S. melongena* in experimental plants showed 84, 99 & 82% and the shoot length growth were 50, 45 & 66% higher than the control plants. The outcome of the result in the experimental plants showed fast growth than the control plants.

Soundarapandian (2016), analysed the nutritional value of Trash fish,in Parangipettai. In this study he analysed the proximate composition of three different trash fish such as *Stolephorus commersoni*, *Thryssa mystax* and *Leiognathus dussumiri* . Biochemical composition of moisture, carbohydrates and protein levels were highly in *Stolephorus commersoni*. Fatty acid profiles such saturated, mono saturated and poly saturated fatty as analysis through gas chromatography. In this result all the three fatty acid profiles were highly presented in *Leiognathus dussumiri.*

In the present study the carbohydrate content is 5.6 mg/g, 8.26 mg/g and 9.52 mg/g in study area I, II and III respectively. Higher plasma glucose level (up to a 1.7fold increase) were detected in grouper fed the high fat diets as compared to the moderate fat groups. The higher dietary starch resulted in higher plasma glucose levels is smaller in (Salmo salcerl) (Hemre *et al*., 1996). In the present investigation the protein content is 19.372 mg/g, 35.07 mg/g and 32.398 mg/g in study area I, II and III respectively. The significant interactive effect

between protein and fat on plasma triglycerides might also have contributed to the obviously high plasma triglycerides in fish fed the LP-HP diet.

In our present study the lipid content is 7.875 mg/g, 5.875 mg/g and 7.125 mg/g in study area I, II and III respectively. Fat (50% fish oil and 50% corn oil) supplementation up to 1b% in a 50% protein diet did not inhabit superoxide anion production in grouper. (Lin and shiau 2003): decreased respiratory bursts were found in sea bass (Dicentnarchus labrax L) fed a high fat diet (17%) (Sitja – Bobadilla and Perez – Sanche, 1999) and an increase in highly unsaturated fatty acid in diet resulted in increased phagocytosis and respiratory bursts (superoxide anion production) in grouper (Wu *et al.,* 2002).

6. SUMMARY

A study on trash fish will throw more light on fate of trash fishes as well as scope for future self- employment opportunity. As the prospects for increased production of quality fish meals do not look promising the future development of strongly influenced by the available and price of fish meal on the international market.

The trash fishes from a single boat found to be 42 - 199 kg, 32 – 99 kg and 16 – 71kg in study area I, II and III respectively.

From the comparison of data, it was found that the amount of trash fishes collected was higher in Vembar landing centre than Thoothukudi fishing harbour and Keezhavaippar landing centre.

Based on random sample the carbohydrate content of the trash fish was found to be 5.6 mg/g, 8.26 mg/g and 9.52 mg/g in study area I, II and III respectively. The protein content was found to be 19.372 mg/g, 35.07 mg/g, and 32.398 mg/g in study area I, II and III respectively. Lipid content was found to be 7.875 mg/g, 5.875 mg/g and 7.125 mg/g in study area I, II and III respectively.

7. CONCLUSION AND SUGGESTIONS

Biodiversity, the life sustaining systems of the biosphere has intrinsic value and its components have ecological, social, scientific, educational, cultural and aesthetic values. India being the mega diversity country has a vast coastal line 8500kms encompassed with estuaries. Backwaters sandy beaches near shore environments coral reefs, sea grass meadows, algal communities, mangrove forests and many small island has the vast potential of biodiversity.

Fish meal constitutes a cheap source of protein and also an important source of crucial micro nutrients, not easily available in other protein alternatives such as soya bean. Although fish meal makes up only a small proportion of the chicken's diet, the poultry has seen a major growth in country and can potentially result in over fishing of near shore marine resources.

Fish meal depends on its protein contents. The low value fishes are being processed accordingly before adding the key ingredient with poultry feed. Fish meal centres are functioning on a small scale basis in Thoothukudi. There are over 100 species of "marine trash fish'' that are used as an aquaculture feed or aquaculture feed ingredients. The product as a result of aquaculture farm stated by several companies has resulted in demands for fish meal.

If certain companies divide to no longer fish in unsustainable ways catching huge amount of trash fishes by catch as well as their commercial take, they will properly have to spend more money to fish in better more sustainable

ways. Trash fishes are sales using online. We can get the complete detailed information of huge number of listed dried fish meal whole sales and supplies and their products with proper description. We understanding ever increase demand of these categorized products. So that we have enlisted the group.

The trash fishes are used in various industries as fish oil, fish powder and fish meal. In our study area (Vembar landing centre, Thoothukudi fishing harbour and Keezhavaipar landing centre) the trash fishes were caught and stored out. The tried trash fishes from Thoothukudi areas were found to be exported to other areas such as Madurai, Kovai etc., for making fish meal powder was believed to be having very good scope for the preparation of poultry and other fields because of the presence of rice protein. Recently the fish meal is also found to be having a place in aquaculture industry as well as for human consumption traditional small scale pig rearing uses trash fish.

In conclusion the finding of the present study will contribute towards reducing the marine fish farmer's dependency on trash fish as the main input and further contributes towards the sustainability of the thriving grouper poultry, aquaculture and other industry.

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SCREENING AND CHARACTERIZATION OF KERATINOLYTIC BACTERIA ISOLATED FROM THE SKIN OF PUFFER FISH *AROTHRON HISPIDUS*

Dissertation submitted to

ST. MARY'S COLLEGE (Autonomous), THOOTHUKUDI

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in partial fulfilment for the award of the degree of

MASTER OF SCIENCE IN ZOOLOGY

By

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THOOTHUKUDI

APRIL – 2021

CERTIFICATE

This is to certify that this dissertation entitled **"SCREENING AND CHARACTERIZATION OF KERATINOLYTIC BACTERIA ISOLATED FROM THE SKIN OF PUFFER FISH *AROTHRON HISPIDUS*"** submitted by **A. METHONISA,** Reg. No. 19APZO04 to St. Mary's College (Autonomous), Thoothukudi, affiliated to Manonmaniam Sundaranar University in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2020-2021 under my guidance and supervision. It is further certified that the dissertation or any part of this has not been submitted elsewhere for any other degree.

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Examiner

DECLARATION

I do hereby declare that this thesis entitled **"SCREENING AND CHARACTERIZATION OF KERATINOLYTIC BACTERIA ISOLATED FROM THE SKIN OF PUFFER FISH *AROTHRON HISPIDUS*"** submitted by me for the award of the degree of Master of Science in Zoology is the result of my original and independent research work carried out under the guidance of **Dr. Mrs. P.J. Joslin, M.Sc., M.Phil., PhD.,** Associate Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi, and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date : 15.04.2021

A. Methonisa

Signature of Candidate

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CONTENTS

INTRODUCTION

1. INTRODUCTION

The leather and leather products industry is one of India's oldest manufacturing industries that catered to the international market right from the middle of the nineteenth century, the demand for its products being both domestic as well as international right from the beginning (Sumangala Damodaran and Pallavi Mansingh, 2008). The Indian leather industry has undergone a drastic change, from being an exporter of mere raw materials in the early 60's and 70's to an exporter of finished, value added leather products today. The Leather Industry holds a very prominent place in the Indian economy. The leather industry is an employment intensive sector, providing jobs to about 2.5 million people in the country and has an annual turnover of approximately USD 5,000,000 (Ashish Kumar *et al*., 2015).

Although the leather industry is considered as an important field, there are a number of issues, which have to improve by the government, business people and other stakeholders, including higher education institutions (Raden Lukas Martindro Satrio Ari Wibowo *et al*., 2017). The basic raw materials needed for the leather industry are hides and skins. The industry is currently predicting a nationwide shortage of hides and skins. In the future, this will lead to scarcity of basic raw material i.e. leather for the product sector of the industry. The supply of hides and skins being dependent on the livestock population, its birthrate, demand for red meat,

mortality rate and recovery from carcasses, is highly inelastic in nature. One of the major reasons is the banning of slaughter houses in the country. Due to the religious significance of some animals, the leather industry is still underdeveloped. It also due to the natural calamities and marginal growth/decline of livestock in some states.

Large quantities of raw hides and skins around 35% to 40% are smuggled out to neighboring countries and making the problem more acute. So, inelastic supply of raw materials to manufacturers and exporters makes it difficult for them to perform and compete in the international market and hampers their productivity and thus it is a serious concern for the Indian leather industry and thus the bargaining power of suppliers is weak because of inelastic supply of raw materials (Shailja Singh, 2018).

Oceans are the world's single largest ecosystem, covering nearly three-fourths of the earth's surface (Joris Larik *et al*., 2017). Among the world's oceanic divisions, the Indian Ocean is the third largest, covering an area of more than 70 million sq km that includes extensive Exclusive Economic Zones (EEZ) of different countries and large "high seas" (Lyndon E. Llewellyn *et al*., 2016). The deep ocean supports a surprisingly high diversity of species (Grassle, 1989).

The Gulf of Mannar can be found in the Indo-Pacific region. The Gulf of Mannar Biosphere Reserve is located on the south eastern tip of

India and is near Sri Lanka. The Gulf of Mannar was established as a biosphere reserve in 1989 by the Indian Government and the State of Tamil Nadu (Marine Conservation Society, 2009). The bio resources present in the marine ecosystem have potent biomolecules which includes many natural organic compounds (Rajamanikandan *et al*., 2011).

Fishes are a vital component of marine habitats. Fisheries play an important role in Indian economy and it is one of the most important activities along the coastal areas (FAO, 2002). Puffer fish is an underutilized fish in any fishing. It has many generic names such as blow fish, balloon fish, toad fish and globe fish. There are 120 species of puffer fish belonging to tetraodontidae family exist and most of them live in tropical seas (Aydin, 2011). It has a widespread distribution throughout the tropical Indian and Pacific Oceans; from Japan, Australia and Hong Kong in the east to Mozambique and southern African shores, as well as the Red Sea (Smith and Heemestra, 1986; Watson *et al*., 2003). Puffer fish is commonly known as a fatal fish due to the presence of a neurotoxin called tetrodotoxin (TTX) in its body organelles like liver, gonad, skin, muscle and testis. On consumption of this fish without proper processing may be highly fatal and lead to death (Kan *et al*., 1987; Sabrah *et al*., 2006; Krumme *et al*., 2007; Arakawa *et al*., 2010). The family name, literally meaning four teeth in Greek, refers to their fused jaw teeth which are very

sharp. Some puffers carry the strongest paralytic toxin known today i.e. tetrodotoxin (Sabrah *et al*., 2006).

One of the utilization of the puffer fish skin is for tannery business. The tanning of puffer fish leather can be alternative to tanning industry is currently limited to the production of cattle leather. Fish tanning business doesn't only give value added to leather waste but also become an alternative in fulfilling leather materials in the leather industry in India. Additionally, puffer fish also has a uniqueness that lies in its rounded body shape (Mohamed *et al*., 2013).

The quality of leather from puffer fish can be improved by removing its spines. One of the ways to destroy the spines is to degrade the protein keratin. Keratins are insoluble in water and exhibit high resistance to physical and chemical treatments, as well as typical proteolytic enzymes. The degradation of these proteins is possible with the participation of specific microbial proteolytic enzymes-keratinases, frequently supported by chemical or enzymatic reducing agents (Lange *et al*., 2016). Keratinase is an important for the pre-tanning process in leather industry so the skin tanning can be an eco-friendly process by reducing the use of sodium sulfate (Thanikaivelan *et al*., 2005).

Keratinolytic microorganisms and their enzymes may be used to enhance the digestibility of keratin. Keratinase that is produced by

microbes is an enzyme capable of degrading the structural protein that is generally found in feathers, hair and wool. They may have important applications in processing keratin-containing wastes from poultry and leather industries through the development of non-polluting method (Onifade *et al*., 1998).Generally, an increase in keratinolytic activity is associated with thermophilic organisms, which require high energy inputs to achieve maximum growth and the decomposition of keratin wastes (Nam *et al*., 2002). As biotechnological methods are considered as cost effective and environment-friendly, an interesting alternative to these techniques is microbial degradation, due to the lower cost, mild process conditions, lack of the ecological hazard and the output of potentially relevant products. Microorganisms break down keratin to peptides and amino acids that accumulate in culture medium, and are partially metabolized as basic building elements like carbon and nitrogen (Vasileva-Tonkova *et al*., 2009).

Keratinases and the follow-on keratin hydrolysates may also be applied in obtaining cheap, useful products, such as nitrogen-rich fertilizers, compostable films, biodegradable materials and reinforced fabrics (Singh and Kushwaha, 2015). Keratinases could be effective as components of detergents, in manufacturing of personal care products and modification of fibers, such as wool or silk. Their prospective applications

are also their use in medicine for the treatment of psoriasis and acne, as an adjunct in the nails diseases treatment, as well as in prion proteins degradation (Gupta and Ramnani, 2006; Selvam and Vishnupriya, 2012). Moreover, keratin hydrolysis products may be considered as a potential source of bioactive peptides (Choinska *et al*., 2011). Nevertheless, other applications of keratinases should be denoted as exceptionally promising in industrial circumstances. One of the target areas is leather industry, where keratinases support or carry out the dehairing process, allowing to at least partially replacing lime-sulfide treatment. Also, application of keratin hydrolysates allowed for the reduction of chromium effluents from the process of tanning (Balaji *et al*., 2008). Another vital area is the introduction of keratinolytic microorganisms the initial biodegradation stage, preceding the bioconversion keratin hydrolysates into biogas (Patinvoh *et al*., 2016).

Therefore, this study was performed to isolate and to characterize the keratinolytic bacteria from the skin of puffer fish (*Arothron hispidus*) collected from Thoothukudi coast.

OBJECTIVES

2. OBJECTIVES

The present study has been undertaken keeping the following objectives.

1. To isolate bacteria from the skin of puffer fish (*Arothron hispidus*).
2. To screen the bacteria that shows proteolytic activity.
3. To identify the isolated bacterial strains based on their cultural, morphological and biochemical characteristics.
4. To screen the bacteria that shows keratinolytic activity.
5. GC-MS analysis of culture fluid of keratinolytic bacteria.

REVIEW OF LITERATURE

3. REVIEW OF LITERATURE

Keratinolytic enzymes derived from bacteria have attained far more attention from researchers. Worldwide production of keratinous waste can be treated or recycled to minimize pollution (Mukhopadyay *et al*., 1990). Mohamed *et al*., (2013) reported that using keratinase or keratinolytic microorganisms is the alternative for recycling of keratinous waste as well as to produce cheap and supplementary protein feed stuff. Keratinase is an extracellular enzyme used for biodegradation of keratin.

Keratinolytic activity has been reported by various persons in species of *Bacillus* (Williams *et al*., 1990); *Vibrio* (Sangeeta Lal *et al*., 1999); *Actinomycetes: Streptomycetes* (Noval *et al*., 1959); *Pilimelia* and Fungi. Various microorganisms like *Bacillus lichenformis* (Sarita Agarhari and Neeraj Wadhwa, 2010); *Chryseobacterium* sp. (Takami *et al*., 1992); Actinomycetes (Dalev *et al*., 1997) have been studied which produces keratinase.

Some species of deramatophytes including *trichophyton rubum*, *Microsoproum gypseum* have been reported to produce enzyme keratinase by Thanikaivelan *et al*., (2004). Keratinase producing microorganisms have ability to degrade chicken feather, nails, wool etc. was described by Gradisar H *et al*., (2005). This enzyme has been produced by bacteria *Bacillus licheniformis, Burkholderia* sp., *Chryseobacterium,*

Pseudomonos, Microbacterium sp. Fungi including the species of *Aspergillus, Onygea, Absidia,* and *Rhizomucor* are also keratinolytic producing keratinase.

Actinomycetes (Bockle et al., 1995) and *Streptomyces* (Noval J.J.and Nickerson, 1959) are reported to produce keratinase enzyme. Selvam and Vishnipriya (2012) reported that feather degrading bacteria are morphologically and physiologically diverse and require different methods of cultivation. It has been reported that mesophilic bacteria can efficiently degrade feathers. Microorganisms which produces keratinolytic enzyme may have important application in poultry and leather industry. Use of microbial keratinase for hydrolyzing keratin is one of the efficient alternatives for bioconversion of poultry waste into feed stuff of nutritive value.

Revathi *et al*., (2013); Savita Joshi *et al*., (2007) described that keratin hydrosylate have potential use as organic fertilizers and production of rare amino acids. William *et al*., (1990) described that feather hydrolysates produced by bacterial keratinase have been used as animal feed. Scott *et al*., (2004) also described that feather hydrolysate produced by bacterial keratinase has been used as additives for animal feed as well as organic fertilizers and keratinase have another application in dehairing processes in leather industry.

Scott *et al*., (2004) reported that keratinase is a potential enzyme for removing hairs and feathers in the poultry industry as well as in clearing obstructions in sewage system during waste water treatment. Keratinolytic enzymes have been studied by Lin *et al*., (1995) for de-hairing processes in the leather industry and hydrolysis of feather keratin which is a byproduct generated in huge amount by the poultry industry.

Tamilmani *et al*., (2008) described that potential keratin degraders will have biotechnological use in industrial processes involving keratin hydrolysis. The aim of this study was to obtain keratinolytic protease with high ability to degrade the keratinous waste like native feathers. Characterization of enzyme keratinase and its application in waste resources is also studied. Keratinase enzyme is a proteolytic enzyme capable of hydrolyzing keratin was explained by Riffel *et al*., (2003).This enzyme selectively degrade beta keratin found in feathers. Sarita Agrahari and Neeraj Wadhwa (2010) reported that β-keratinase is an enzyme capable of degrading β-keratin.

Pillai *et al*., (2011) reported on the statistical optimization of production and tannery applications of a keratinolytic serine protease from *Bacillus subtilis* P13. Bioutilization of chicken feather waste by newly isolated keratinolytic bacteria and conversion of protein hydrolysates with improved functionalities was studied by Prajapati *et al*., (2021). A potent

keratinolytic bacterial strain *Bacillus* NDS-10 was isolated by Akram *et al*., (2021), which is an efficient green biocatalyst for poultry waste management, detergent formulations and hide-depilation application.

Nonso *et al*., (2020) isolated proteolytic bacteria from agro-waste industries produced keratinolytic enzymes. Nnolim *et al*., (2020) also isolated *Bacillus* sp. CSK2 from agro-wastes produced thermostable alkaline keratinase. Bhari *et al*., (2019) reported that thermostable and halotolerant keratinase produced from *Bacillus aerius* NSMK2 have remarkable dehairing and laundry applications. Tamreihao *et al*., (2019) reported that feather degradation by keratinolytic bacteria having antagonistic and plant growth promoting activities and it is an alternative tool to promote and improve organic farming, agro-ecosystem, environment, human health and soil biological activities.

Jagadeesan *et al*., (2020) studied the sustainable production, biochemical and molecular characterization of thermo-and-solvent stable alkaline serine keratinase from novel *Bacillus pumilis* AP 57 for promising poultry solid waste management. Wu *et al*., (2017) discovered novel heat stable keratinases from *Meiothermus taiwanensis* WR-220 and other extremophiles.

Mamangkey Jendri *et al*., (2020) studied the antibacterial and antioxidant activity of new keratinolytic bacteria, *Azotobacter*

chroococcum B4. Pintubala kshetri *et al*., (2019) isolated a new multifaceted keratinolytic bacterium *Chryseobacterium sediminis* RCM-SSR-7 that involved in transforming chicken feather waste into feather protein hydrolysate. Park and Son (2009) studied the keratinolytic activity of *Bacillus megaterium* F7-1, a feather- degrading mesophilic bacterium. Hong *et al*., (2015) isolated, identified and characterized the keratin-degrading bacterium *Chryseobacterium* sp.

Agrahari and Wadhwa (2010) reported the degradation of chicken feather which is a poultry waste product by keratinolytic bacteria isolated from dumping site at Ghazipur poultry processing plant. Jeong *et al*., (2010) discovered the production of keratinolytic enzyme by a newly isolated feather- degrading *Stenophomonas maltophilia* that produces plant growth- promoting activity. Paul *et al*., (2013) described the exploitation of chicken feather waste as a plant growth promoting agent using keratinase producing novel *Paenibacillus woosongensis*.

Optimization of the biotechnological process for Hide unhairing in substitution of toxic sulfides by Dettmer *et al*., (2012). Eco-benign enzymatic dehairing of goat skins utilizing a protease from a *Pseudomonas fluorescens* species isolated from fish visceral waste was reported by Kandasamy *et al*., (2011).

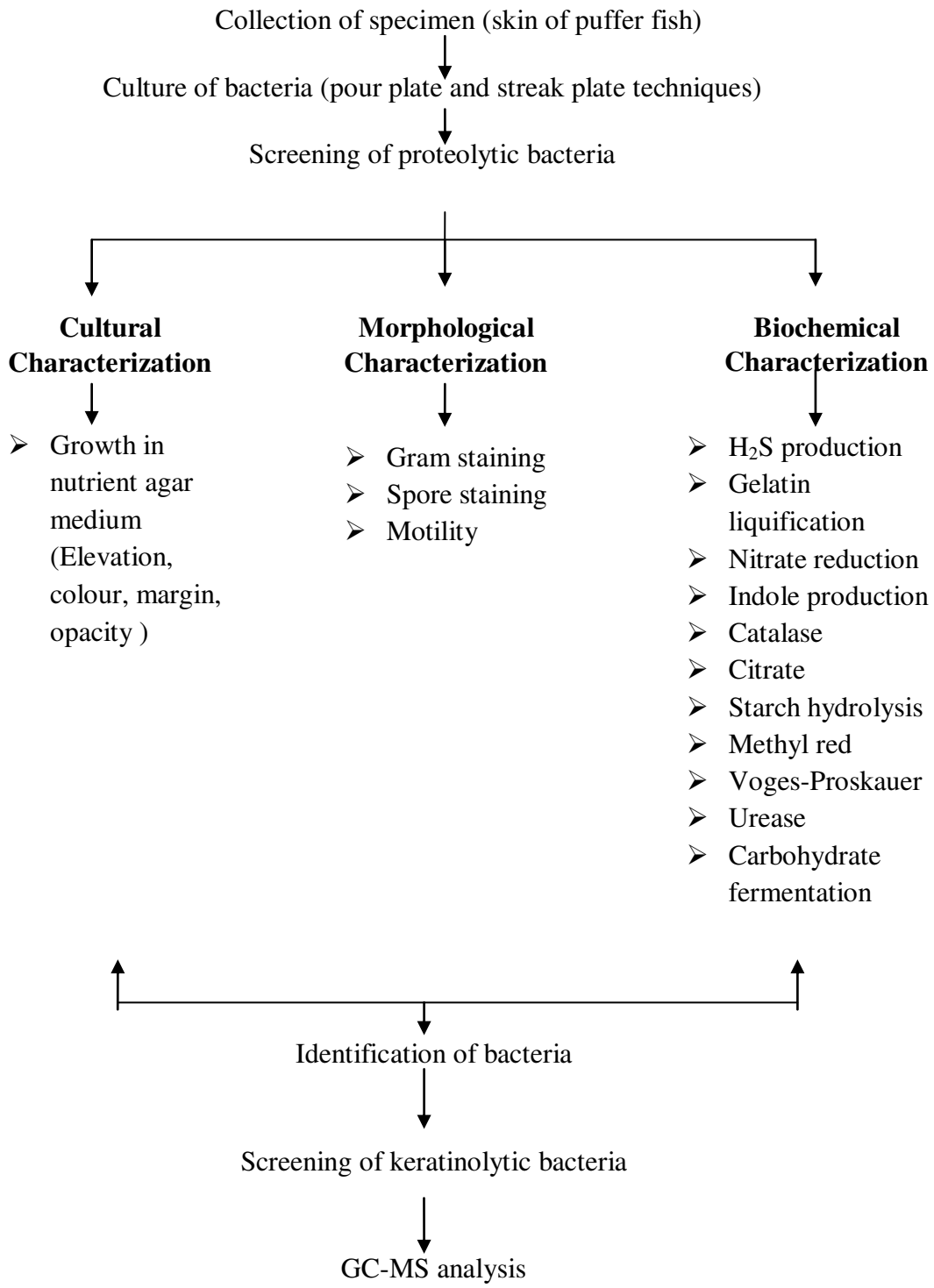
Optimization of keratinase production for feather degradation by *Bacillus subtilis*, Jundishapur was reported by Mousavi *et al*., (2013). Optimized production and characterization of a detergent-stable protease from *Lysinibacillus fusiformis* C250R was reported by Mechri *et al*., (2017). Tang *et al*., (2004) reported the purification and characterization of an alkaline protease used in tannery industry from *Bacillus licheniformis.*

Screening and characterization of keratinolytic bacteria from puffer fish skin waste has been reported by Raden Lukas Martindro Satrio Ari Wibowo *et al*., (2017). Raden Lukas Martindro Satrio Ari Wibowo *et al*., (2020) investigated the characteristics and optimal condition of the keratinase production from three bacillus strains isolated from fish market waste.

MATERIALS AND METHOD

EXPERIMENTAL DESIGN

Screening and characterization of keratinolytic bacteria isolated from the skin of Puffer fish *Arothron hispidus*



4. MATERIALS AND METHOD

4.1. Collection of Specimen

The specimens of the Puffer fish were collected from the fish landing centre at fishing harbour Thoothukudi. Immediately after collection, the fresh samples were kept in an ice box and transported to the Laboratory and stored in a deep freezer at -20^{o}C until they are used.

4.2. Cultural Characterization

4.2.1. Serial dilution

Specimen of Puffer fish was thawed and the skin was incised. Few pieces of skin were homogenized with 5ml of sterile distilled water. Several serial dilution series were made by placing 1ml of the skin extract into the Erlenmeyer flask containing 99 ml of sterile distilled water to obtain stock solution. To make 10^{-1} dilution, exactly 1 ml of stock solution was placed in a test tube containing 9 ml of sterile distilled water. From the 10^{-1} dilution, 1 ml of solution is placed into the test tube containing 9 ml of sterile distilled water to obtain 10^{-2} dilution. The sample was therefore diluted from 10^{-1} to 10^{-6} for the fish sample.

4.2.2. Pour plate culture

2.8g of nutrient agar was dissolved in 100ml of distilled water. The medium was autoclaved at 121^{0}C for 15 minutes. 1ml of the diluted

Plate 1

Puffer fish *Arothron hispidus* (Muller, 1841)

Plate 2

Skin of puffer fish (*Arothron hispidus)*

solution (10^{-1} to 10^{-6}) was poured into the petridish, the agar medium was poured and rotated clockwise and anticlockwise direction for thorough mixing. Then the medium was allowed to solidify. All plates were incubated at 37°C for 24 hours.

4.2.3. Isolation of bacterial colonies

Isolated colonies were marked and numbered on the agar plates. The selected colonies were observed under the low-power stereomicroscope. The cultural characteristics of isolated colonies were observed. The colonies were collected with an inoculation loop and transferred to a sterile broth medium.

4.2.4. Subculture

The isolated colonies were sub cultured in the agar plates. Each bacterial colony were collected with an inoculation loop and streaked on the agar plate to grow a single species of bacteria.

4.2.5. Maintenance of pure culture

A pure culture consists of a nutrient medium in which a single species of bacteria grows. Pure cultures were required to study the properties of the bacteria, so the cultures were stored on an agar slants.

The agar slants were prepared in sterilized test tubes. The agar medium was allowed to cool in an inclined position. The medium was

hardened with a sloped surface and inoculated with a loop of bacteria. The cultures were kept upright at room temperature for two days and preserved in the refrigerator.

4.2.6. Broth culture

Broth medium

Nutrient broth - 1.3 g

Distilled water - 100 ml

5 ml of sterilized broth medium was taken in the sterilized test tube. The inoculation loop was flamed and allowed to cool for few minutes. The isolated colonies were transferred to the broth medium and incubated at room temperature.

4.3. Screening of proteolytic bacteria

The pure cultures were streaked on the skim milk agar plates and the plates were incubated at 37^{o}C for 48 hours. After incubation, the formations of a clear zone surrounding the bacterial growth were observed.

The bacterial strains showed positive results were used for morphological and biochemical characterization.

4.4. Morphological characterization

Gram stain morphology for initial identification of bacterial isolates

4.4.1. Smear preparation and fixation

A loopful of 18 hour bacterial culture was transferred to a clean glass slide. The drop is evenly spread over the glass slide, forming a thin film. The film was fixed by heating in a flame to make the cells adhered to the slide.

4.4.2. Gram staining

Gram stain is a differential stain that requires primary staining and counter staining. A smeared glass slide was flooded with crystal violet stain and allowed to stand for 5 minutes. The stain was drained and then washed gently with tap water. Thereafter, Gram's iodine solution was added and washed after five minutes and the slide was allowed to dry. 95% alcohol caused decolourisation. Finally the counter stain saffranin was added and air dried for two minutes. Then the slides were thoroughly rinsed and observed under oil immersion objective.

4.4.3. Spore staining

After smear preparation and heat fixation, the slide was flooded with 0.5% malachite green and kept for 5 minutes. Then the slide was rinsed gently in tap water. Then the counter stain saffranin was added and kept

for 30 seconds. The slide was washed again, allowed to dry and observed under oil immersion objective.

4.4.4. Observation of motility of bacteria

Motility of bacteria was observed followed by hanging drop method. A small amount of vaseline was applied on the corners of the clean coverslip and a loopful of 18 hour broth culture was transferred into the centre of the coverslip. A cavity slide was turned upside down over the coverslip, so that the drop of the culture was hanging in the centre of the cavity. The preparation was examined under the microscope.

4.5. Biochemical characterization

4.5.1. H_2S production

SIM medium

Beef extract	- 0.3 g
Peptone	- 3.0 g
Ferrous ammonium sulphate	- 0.02 g
Sodium thiosulphate	- 0.0025 g
Agar	- 0.3 g
Distilled water	- 100 ml

The medium was inoculated with the bacterial culture by means of stab inoculation and incubated for 24 hours at 37°C. The presence of H_2S is based on the metal formed. The black precipitate on the medium indicates the formation of hydrogen sulphide.

4.5.2. Gelatin liquification

Gelatin medium

Peptone	- 0.2 g
Beef extract	- 0.2 g
Gelatin	- 12.0 g
Distilled water	- 100 ml

The gelatin medium was inoculated with 24 hour broth culture and incubated for 24 hours at 30°C. After the incubation period, the tubes were placed in the refrigerator for one hour. The solid nature of the medium showed the negative result and the positive result was observed from the liquification of gelatin.

4.5.3. Nitrate reduction

Peptone broth

Peptone	- 1.0 g
Potassium nitrite	- 0.2 g

Distilled water - 100 ml

To a 24 hour culture in the peptone broth medium, two drops of sulphanilic acid and two drops of α-naphylamine solution were added. The presence of nitrate was indicated by a pink-red colour after the addition of reagents.

4.5.4. Indole Production

Peptone broth

Peptone - 1.0 g

Distilled water - 100 ml

Indole production was tested by inoculating a loopful of 24 hour broth culture in peptone broth medium. The medium was incubated at 37^{o}C for 48 hours. At the end of incubation period, 5 drops of Kovac's reagent was added directly to the tube. The presence of indole was noted by the formation of a pink to red colour in the reagent layer on top of the medium.

4.5.5. Catalase test

Two drops of hydrogen peroxide were added to the 24 hour culture broth. The immediate evolution of gaseous bubbles indicated the presence of catalase.

4.5.6. Citrate test

Simmon's Citrate agar

Agar	- 2.0 g
Sodium chloride	- 5.0 g
Sodium citrate	- 2.0 g
Ammonium dihydrogen phosphate	- 1.0 g
Dipotassium phosphate	- 1.0 g
Magnesium sulphate	- 1.0 g
Distilled water	- 100 ml

The above ingredients were dissolved and sterilized well. 15-20 ml of Simmon's agar was transferred into the sterilized petridish. After hardening of the medium, a loopful of 24 hour culture was streaked on the surface and incubated for 24 hours at 37^{o}C. A colour change from green to blue indicated the positive result.

4.5.7. Starch hydrolysis

Starch Agar

Soluble starch	- 0.2 g
Beef extract	- 0.3 g
Agar	- 1.5 g
Distilled water	- 100 ml

To prepare the starch medium, all the above ingredients were thoroughly dissolved and sterilized. About 15-20 ml of starch medium was poured into the sterilized petridish. After hardening of the medium; streak inoculation was made on its surface. Then the medium was kept for two days at room temperature. At the end of incubation period, two or three drops of Lugol's iodine solution were added on the surface of the medium. A clear zone outside the area of growth indicated the extent of starch hydrolysis.

4.5.8. Methyl red test

Peptone - 0.5 g

Glucose - 0.5 g

K_2HPO_4 - 0.5 g

Distilled water - 100 ml

The medium was inoculated with a loopful of organism and incubated for 2 days at room temperature. After the incubation period, 0.5 ml of methyl red indicator was added. Formation of red colour indicated the positive result. A negative test was indicated by the yellow colour.

4.5.9. Voges- Proskauer test

The substrate broth used for methyl red was also used for Voges-Proskauer test. MR-VP medium was inoculated with a loopful of organism

and incubated for 2 days. After the incubation period, 0.6 ml of 5% α-naphthol and 0.2 ml of 40% potassium hydroxide were added. The tubes were shaken well. A pink to red colour in the medium indicates positive result.

4.5.10. Urease Test

Urea - 2.0 g

Na_2HPO_4 - 0.95 g

KH_2PO_4 - 0.91 g

Yeast - 0.01 g

Phenol red - 0.001 g

Distilled water - 100 ml

The medium was prepared and sterilized well. The medium was inoculated with a loopful of organism and incubated at 37^{o}C for 2 days. The appearance of pink colour indicated the positive result. The pale yellow colour indicated the negative result.

4.5.11. Carbohydrate fermentation test

Peptone - 1.0 g

Phenol red - 0.001 g

Glucose - 0.5 g

Sodium chloride - 0.5 g

Distilled water - 100 ml

The above ingredients were dissolved and sterilized well. Durham tubes were inserted in the test tubes and it was fully filled with the broth. A loopful of 24 hour old bacterial culture was inoculated to the medium and tubes were incubated at 37°C for 24 hours. Acid reaction was indicated by the production of yellow colour in the medium. Gas production can be detected by the presence of small bubbles in the inverted Durham tubes.

4.6. Screening of keratinolytic bacteria

Feather minimal medium

NH_4Cl - 0.5 g

NaCl - 0.5 g

K_2HPO_4 - 0.3 g

KH_2PO_4 - 0.4 g

$MgCl_2$.6H2O - 0.1 g

Yeast extract - 0.1 g

2.5 ml of the bacterial inoculum was added in 100 ml of feather minimal medium and a feather of about 5cm in size was introduced in the medium. The conical flask was kept in the shaker with 150 rpm at 5°C, 30°C and 40°C for seven days.

Plate 3

Conical flask containing feather minimal medium with bacterial isolates

After incubation the most effective feather degrading strain was selected for GC-MS analysis. The culture fluid was subjected to GC-MS analysis.

4.6. GC-MS analysis

GC-MS analysis was carried out by following the method of Hema *et al*., (2010). GC-MS method is a direct and fast analytical approach for identification of chemical compounds. The importance of the study is due to the biological activity of these compounds. Analysis was performed by using a GC, Varian CP 3800 and MS, Saturn 2200 (VF 5 ms 30 X 0.25 system) equipped with Elite-1, fused silica capillary column composed of 5% phenylarylene-95% Dimethyl poly siloxane. The system comprising a COMBIPAL auto sampler set under the following conditions: helium was used as carrier gas at a constant flow of 1ml/min and an injection volume of 1µl EI was employed (split ratio of 1:10) injector temperature 250^{o}C; the oven temperature was programmed from 100-2700^{o}C at the rate of 50^{o}C; total GC running time was 63 minutes. Interpretation on mass spectrum of GC-MS was done by using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown components was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the test materials were ascertained.

RESULTS

5. RESULTS

5.1. Isolation of proteolytic bacteria

Totally ten strains were isolated from the skin of puffer fish (Plate 4). The isolated colonies were subcultured using nutrient agar medium (Plate 5). All isolates were subjected to primary screening on skim milk agar plate for proteolytic activity. Seven of ten bacterial isolates produced clear zones, which supported the degradation and utilization of casein (skim milk) by the respective isolates (Plate 6).

5.2. Cultural characteristics of isolated bacteria

Cultural characteristics of bacteria included the appearance of growth of cells of bacteria that developed on nutrient agar.

Elevation of growth of colonies on nutrient agar was raised in all seven strains. The shape of the colony was circular in S_1 and S_3; irregular in S_2, S_6 and S_7; rhizoid in S_4 and filamentous in S_5.

The type of margin was entire in strains S_1, S_3 and S_6. It was undulate in S_2 and S_7. The margin was rhizoid in strain 4 and filiform in strain 5.

The colour of the colony was yellow in strain 1. The strain 2 and 7 appeared dull white in colour. The white colour was noted in S_3, S_5 and S_6. Dull yellow colour was seen in strain 4.

Plate 4

Nutrient agar plates showing bacterial colonies developed from the skin of Puffer fish *Arothron hispidus*

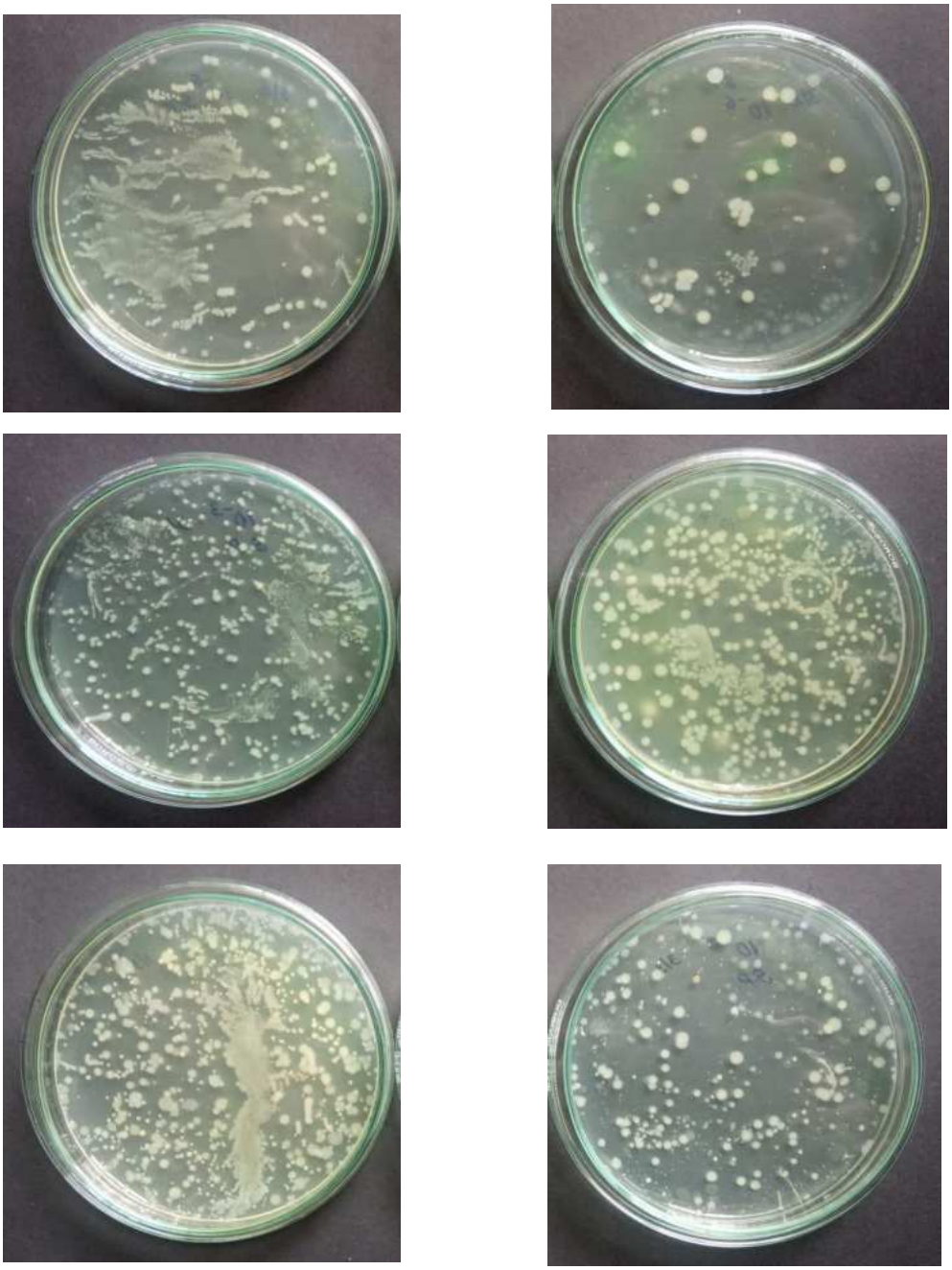


Plate 5

Nutrient agar plates showing sub culture

Strain 1

Strain 2

Strain 3

Strain 4

Strain 5

Strain 6

Strain 7

Plate 6

Skim milk agar plates showing proteolytic activity of bacterial isolates

Strain 1

Strain 2

Strain 3

Strain 4

Strain 5

Strain 6

Strain 7

The opacity of the colony was opaque in all strains except S_2, which was translucent (Table 1).

5.3. Morphological characteristics of isolated bacteria

Morphological characteristics including staining reaction represent one of the major properties of bacteria. These characteristics were determined by examination of appropriately prepared specimens under microscope.

Strains S_2, S_3, S_4 and S_7 were Gram positive while other strains were Gram negative. The strain S_1 and S_2 were long rods. The strain S_3, S_4, S_5, S_6 and S_7 were short rods (Plate 7). All the bacterial isolates were motile except strain 6. Spore staining indicated that all isolates were spore forming bacteria (Plate 8, Table 2).

5.4. Biochemical characteristics of isolated bacteria

5.4.1. H_2S production

After incubation, blackening of the medium was noted in the strains S_1, S_3, S_4, S_5, S_6 and S_7 indicating the production of hydrogen sulphide. The other strain S_2 showed negative result (Plate 9).

Table 1

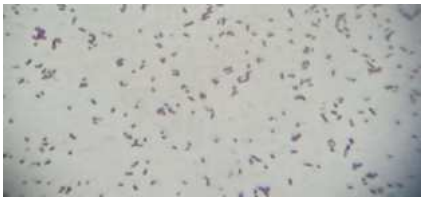
Cultural characteristics of isolated bacteria

S.No.	Colony Appearance				
	Shape	**Elevation**	**Margin**	**Colour**	**Opacity**
S_1	Circular	Raised	Entire	Yellow	Opaque
S_2	Irregular	Raised	Undulate	Dull White	Translucent
S_3	Circular	Raised	Entire	White	Opaque
S_4	Rhizoid	Raised	Rhizoid	Dull Yellow	Opaque
S_5	Filamentous	Raised	Filiform	White	Opaque
S_6	Irregular	Raised	Entire	White	Opaque
S_7	Irregular	Raised	Undulate	Dull White	Opaque

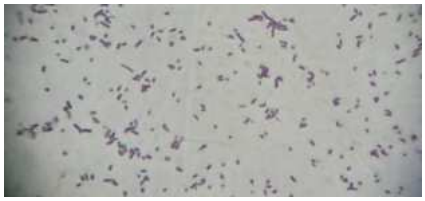
Plate 7

Gram staining of bacterial isolates

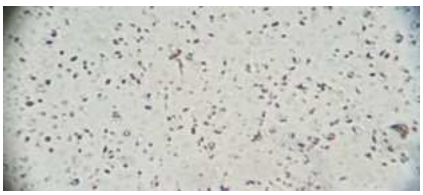
Strain 1



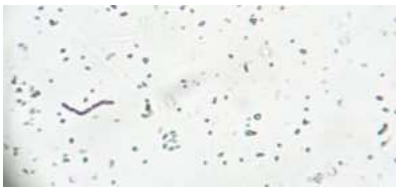
Strain 2



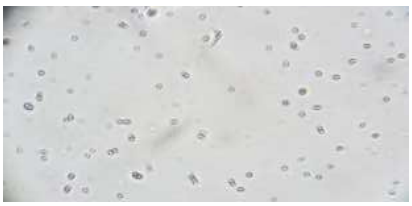
Strain 3



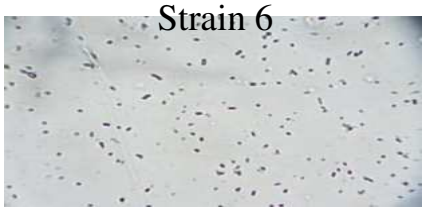
Strain 4



Strain 5



Strain 6



Strain 7

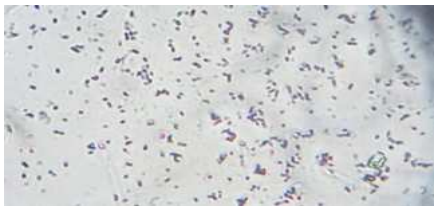
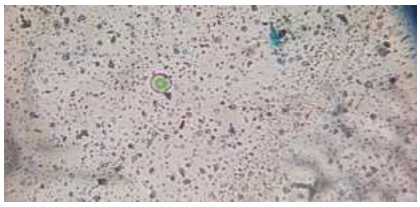


Plate 8

Spore staining of bacterial isolates

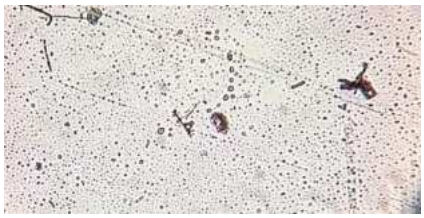
Strain 1

Strain 2

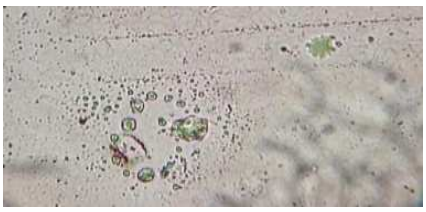


Strain 3

Strain 4



Strain 5



Strain 6

Strain 7

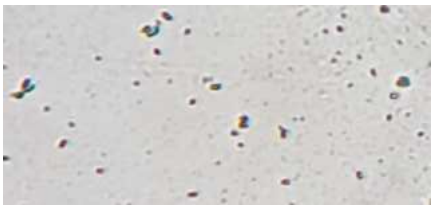


Table 2

Morphological characteristics of isolated bacteria

Bacterial number	Shape	Motility	Gram staining	Spore staining
S_1	Long rods	+	-ve	+
S_2	Long rods	+	+ve	+
S_3	Short rods	+	+ve	+
S_4	Short rods	+	+ve	+
S_5	Short rods	+	-ve	+
S_6	Rods	-	-ve	+
S_7	Short rods	+	+ve	+

Plate 9

Test tubes of SIM medium with bacterial isolates showing hydrogen sulphide production

Plate 10

Test tubes of gelatin medium with bacterial isolates showing the production of gelatinase enzyme

5.4.2. Gelatin liquification

After the incubation of the inoculated gelatinase test medium, the tubes were kept in the refrigerator for an hour. The strains S_1, S_3, S_4, S_5, S_6 and S_7 and S_6, the medium turned to liquid indicating the hydrolysis of gelatin. The strain S_2 showed negative result (Plate 10).

5.4.3. Nitrate reduction test

While adding few drops of sulphanilic acid and α-naphthylamine solution to the culture in the broth medium, of positive test was observed in strain S_4. No colour change was observed in the strains S_1, S_2, S_3, S_5, S_6 and S_7 (Plate 11).

5.4.4. Indole production

After the incubation of medium with bacterial isolates, 5 drops of Kovac's reagent was added. Formation of red colour ring on top of the medium was formed in the strains $S_5$and S_7. The other strains were not able to produce indole (Plate 12).

5.4.5. Catalase test

When a few drops of hydrogen peroxide were added to 24 hour old broth culture, gaseous bubbles were immediately seen as a white froth in all the strains except S_4 (Plate 13).

Plate 11

Nitrate reduction test showing the development of red colour in Nitrate broth

Plate 12

Test tubes with bacterial isolates showing the production of indole

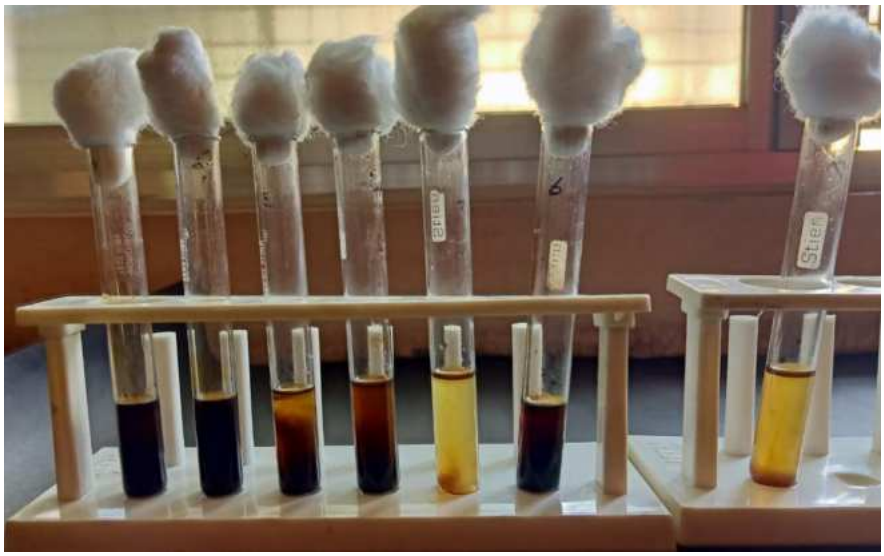


Plate 13

Test tubes with bacterial isolates showing the presence of catalase enzyme

5.4.6. Citrate test

All strains were able to produce citrate as a carbon source (Plate 14).

5.4.7. Starch Hydrolysis

By flooding the surface of the starch agar plates with Lugol's iodine solution, except S_2 and S_5, all the strains hydrolyzed starch completely (Plate 15).

5.4.8. Methyl Red Test

After incubation of MR-VP culture medium with bacterial isolates, methyl red solution was added, a distinct red colour was observed in the strains S_1, S_2, S_3, S_4 and S_7. Yellow colour was noted in the strains S_5 and S_6 (Plate 16).

5.4.9. Voges-Proskauer Test

On addition of 5% α-naphthol and 40% Potassium hydroxide to the MR-VP broth culture tubes, formation of red colour indicated the positive result. Positive result was noted in the strains S_5 and S_6. The remaining strains showed negative result (Plate 17).

5.4.10. Urease Test

All bacterial strains were unable to produce urease enzyme.

Plate 14

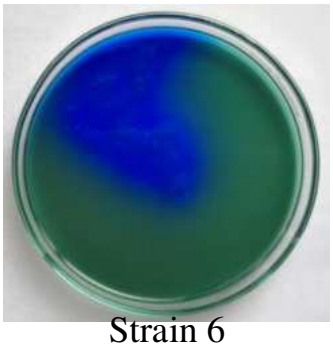
Citrate agar plates with bacterial isolates showing citrate utilization

Strain 1

Strain 2

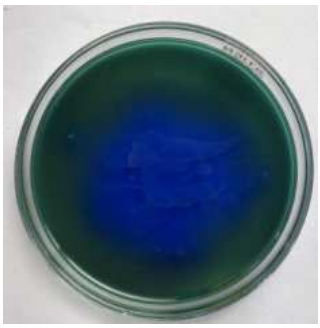
Strain 3

Strain 4



Strain 5

Strain 6



Strain 7

Plate 15

Starch agar plates with bacterial isolates showing starch hydrolysis

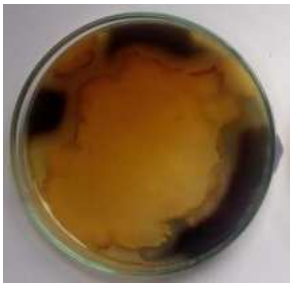
Strain 1

Strain 2

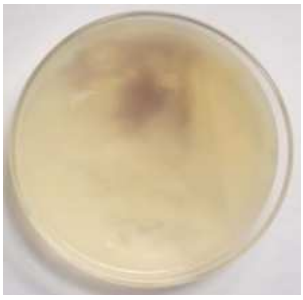
Strain 3

Strain 4

Strain 5



Strain 6



Strain 7

Plate 16

MR-VP medium with bacterial isolates showing production of acid

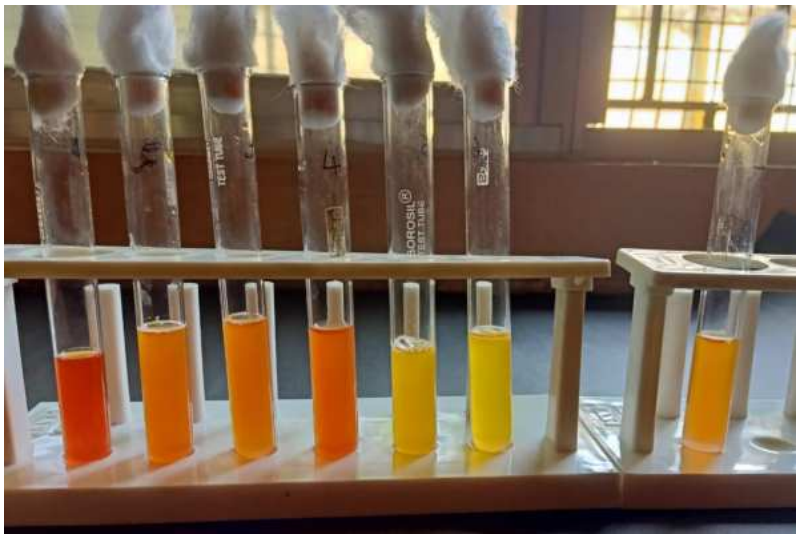
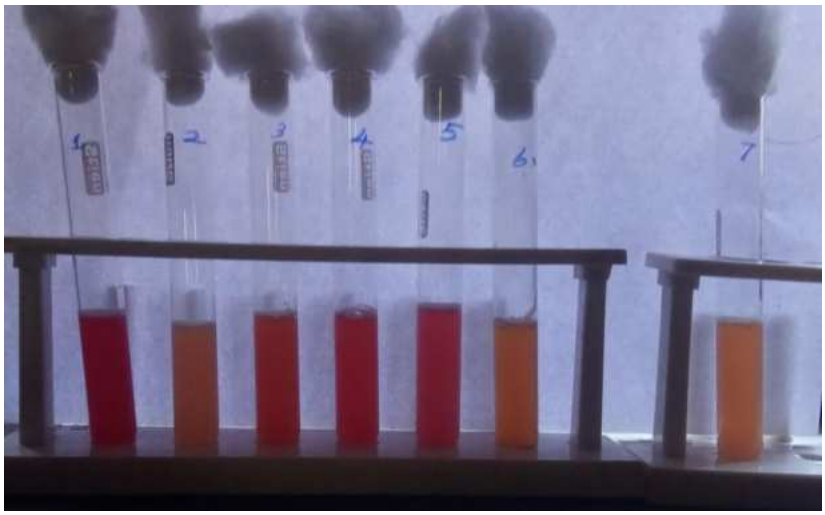


Plate 17

Voges-Proskauer test showing development of red colour in MR-VP medium



5.4.11. Carbohydrate Fermentation

The glucose medium was inoculated with bacterial strains and incubated for 24 hour. All bacterial strains were involved in fermentative metabolism indicating the production of yellow colour. The strains S_5 and S_6 produced gas (Plate 18).

Identification of bacterial isolates

Seven strains of bacterial associated with proteolytic activity were isolated from skin of puffer fish *Arothron hispidus* and identified upto genera level by comparing the results with Bergey's Manual of Determinative Bacteriology (1975).

S_1 -*Achromobacter* sp

S_2-*Escherichia coli*

S_3 -*Bacillus* sp

S_4- *Bacillus* sp

S_5-*Stenotrophomonas* sp

S_6 -*Klebsiella* sp

S_7-*Bacillus* sp

5.5. Keratinolytic activity

Keratinolytic activity of the isolates was virtually monitored during growth in feather minimal medium. *Achromobacter* sp and *Bacillus* sp

Plate 18

Test tubes of glucose medium with bacterial isolates showing fermentation of glucose with production of gas

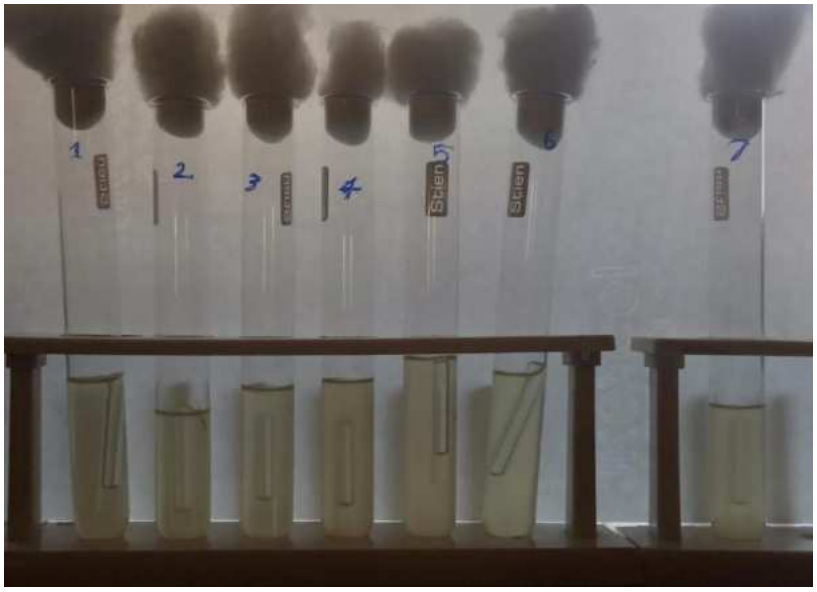


Plate 19

Feather minimal medium with bacterial isolates showing the degradation of feather

Table 3

Biochemical characteristics of isolated bacteria

S.No.	Name of the test	S_1	S_2	S_3	S_4	S_5	S_6	S_7
1.	H_2S production	+	-	+	+	+	+	+
2.	Gelatin liquification	+	-	+	+	+	+	+
3.	Nitrate reduction	-	-	-	+	-	-	-
4.	Indole production	-	-	-	-	+	-	+
5.	Catalase	+	+	+	-	+	+	+
6.	Citrate	+	+	+	+	+	+	+
7.	Starch hydrolysis	+	-	+	+	-	+	+
8.	Methyl red	+	+	+	+	-	-	+
9.	Voges-Proskauer	-	-	-	-	+	+	-
10.	Urease	-	-	-	-	-	-	-
11.	Carbohydrate fermentation	+	+	+	+	+	+	+
	Gas production	-	-	-	-	+	+	-

showed keratinolytic activity by degrading the feather both at 30^{o}C and 40^{o}C (Plate 19).

5.6. GC-MS analysis

GC-MS analysis was carried out both in the control and culture fluid of *Achromobacter* sp. GC-MS analysis revealed the presence of 16 compounds that were not present in the control. They are Tetradecane, 4-methyl- (RT-13.875), Tridecane, 3-methyl- (RT-13.875), Decane, 5-propyl- (RT-13.875), Disulfide, di-tert-dodecyl (RT-14.275),10-Methylicosane(RT-14.275),2,21-Dimethyldocosane (RT-14.275), 7-n-Propyltridecane (RT-15.752), 3,5-Dimethyldodecane (RT-15.752), Diisobutyl phthalate (RT-16.230), Diisohexyl phthalate (RT-16.230),Diundecyl phthalate(RT-16.230), 1-Chloroeicosane (RT-16.486), 1,22-Dibromodocosane (RT-16.486), 1,32-Dibromodotriacontane (RT-18.008),1-Bromo-11-iodoundecane (RT-18.008) and 1-Eicosanol, 2-hexadecyl-(RT-18.008) (Table 4,Figure 1-16).

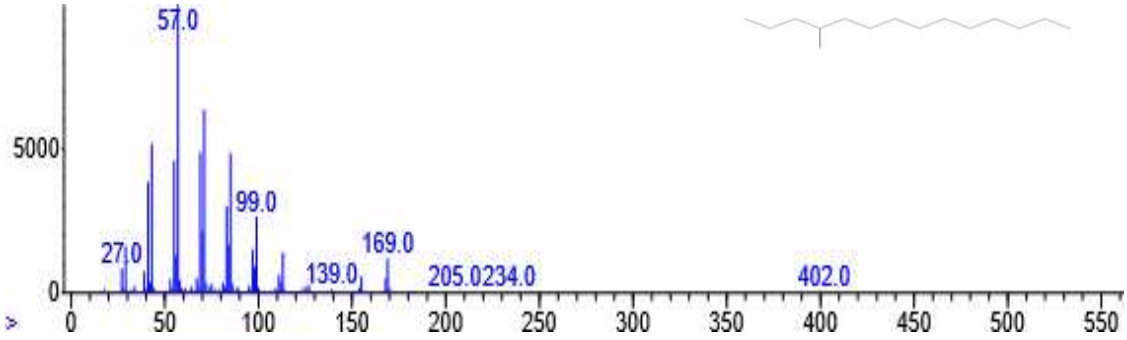


Figure: 1 Molecular Formula: $C_{15}H_{32}$

Name: tetradecane, 4-methyl- Molecular weight: 212.41

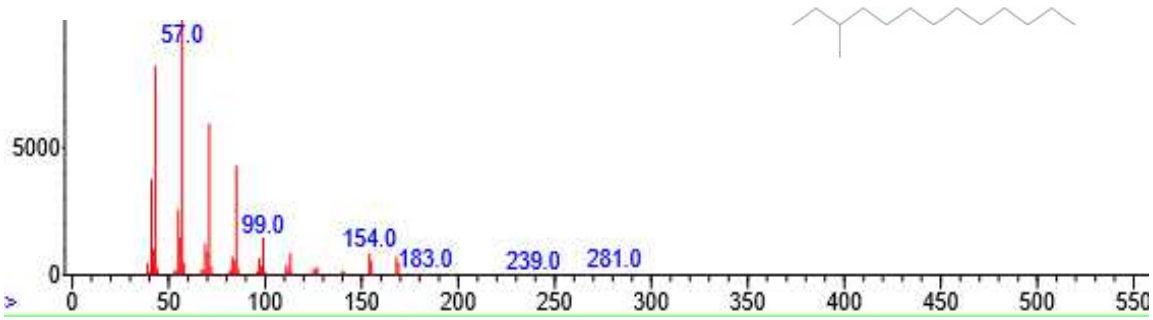


Figure: 2 Molecular Formula: $C_{14}H_{30}$

Name: Tridecane, 3-methyl- Molecular weight: 198.39

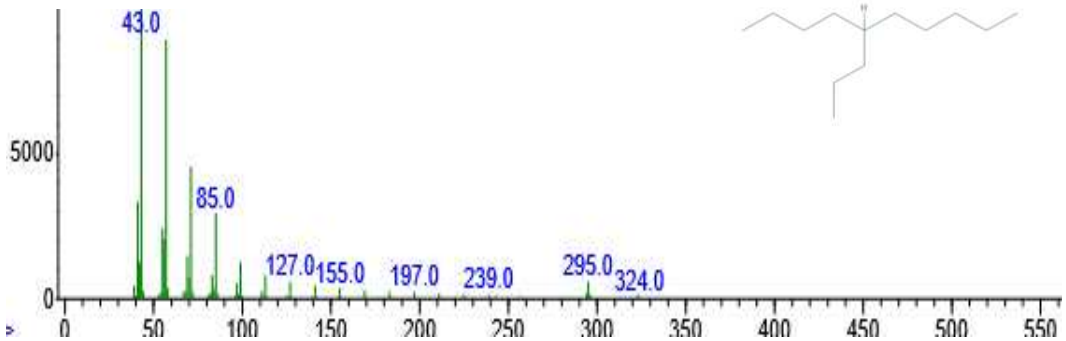


Figure: 3 Molecular Formula: $C_{13}H_{28}$

Name : Decane, 5-propyl- Molecular weight: 184.36

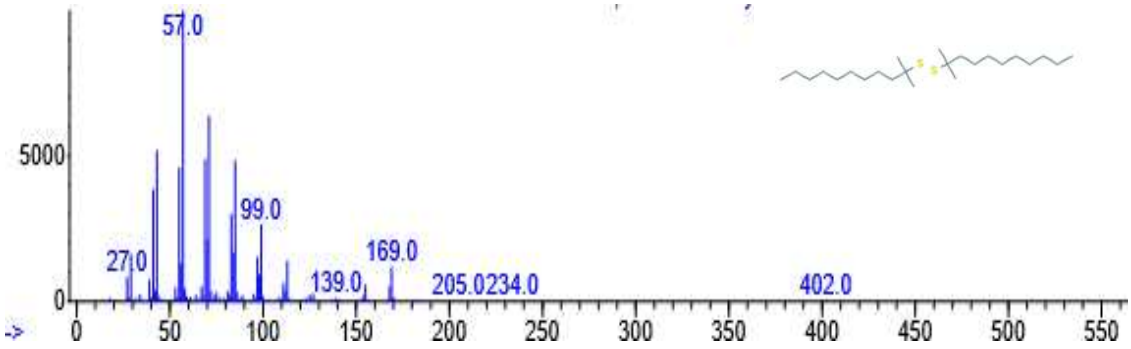


Figure: 4 Molecular Formula: $C_{24}H_{50}S_2$

Name:Disulfide, di-tert-dodecyl Molecular weight: 402.8

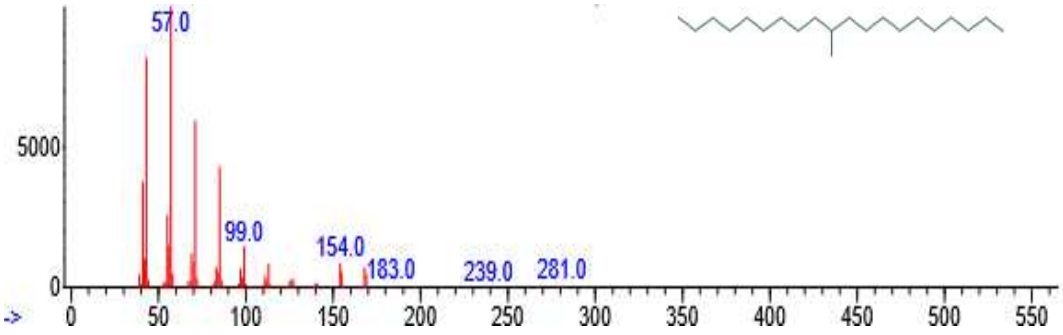


Figure: 5 Molecular Formula : $C_{21}H_{44}$

Name : 10-Methylicosane Molecular weight: 296.6

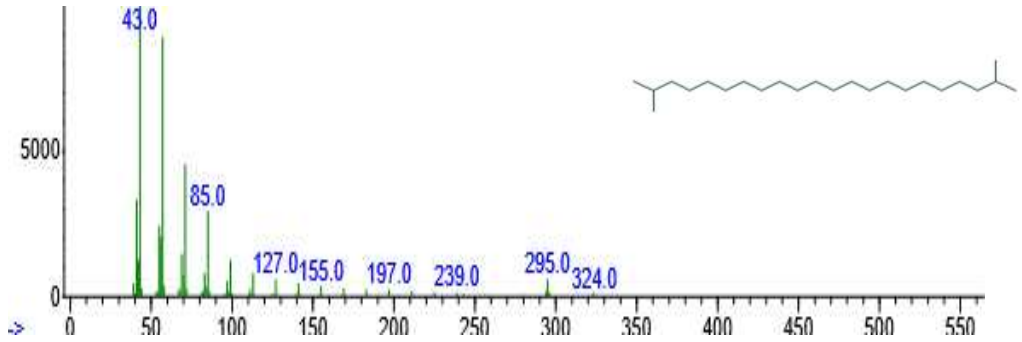


Figure: 6 Molecular Formula : $C_{24}H_{50}$

Name: 2,21-Dimethyldocosane Molecular weight: 338.7

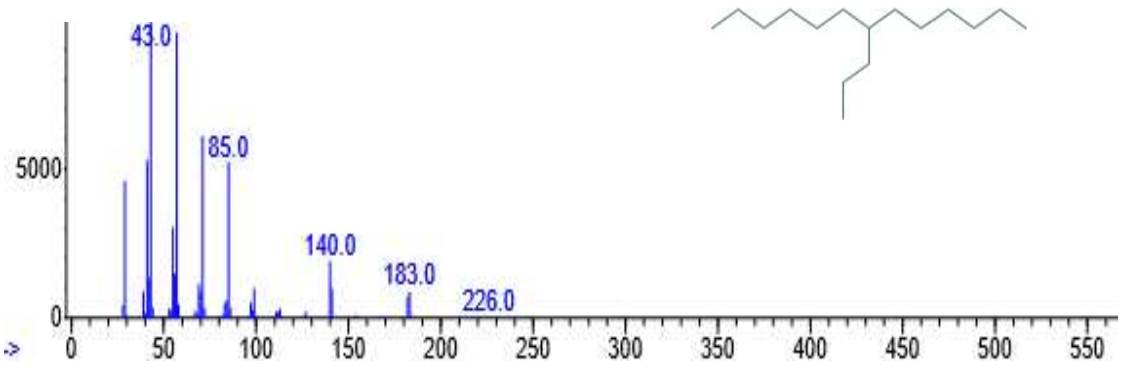


Figure: 7 Molecular Formula : $C_{16}H_{34}$

Name : 7-n-Propyltridecane Molecular weight: 226.44

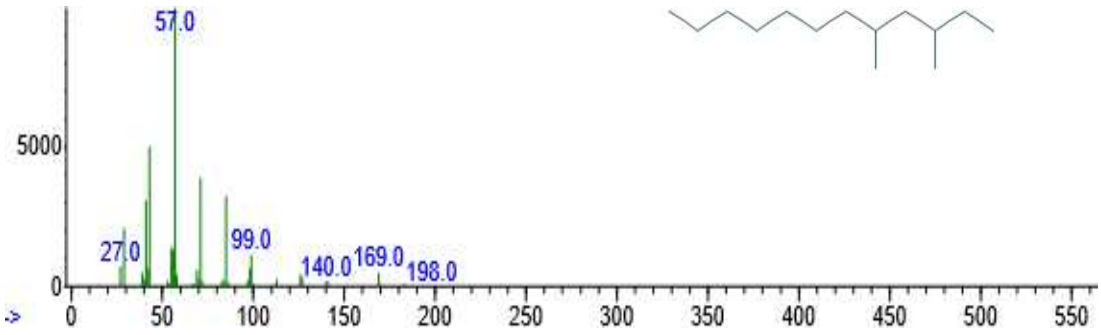


Figure: 8 Molecular Formula : $C_{14}H_{30}$

Name : 3,5-Dimethyldodecane Molecular weight: 198.39

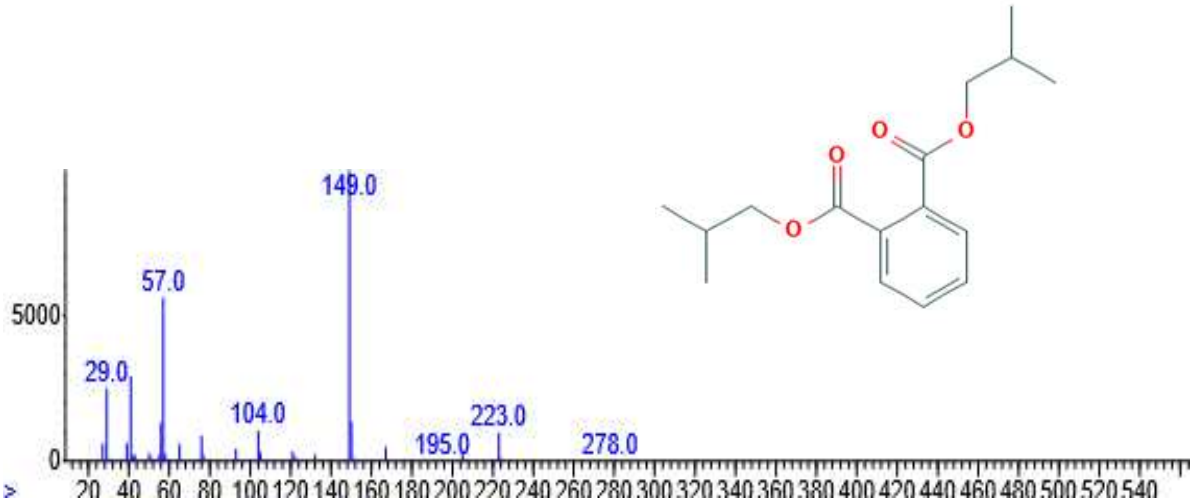


Figure: 9 Molecular Formula : $C_{16}H_{22}O_4$

Name : Diisobutyl phthalate Molecular weight: 278.34

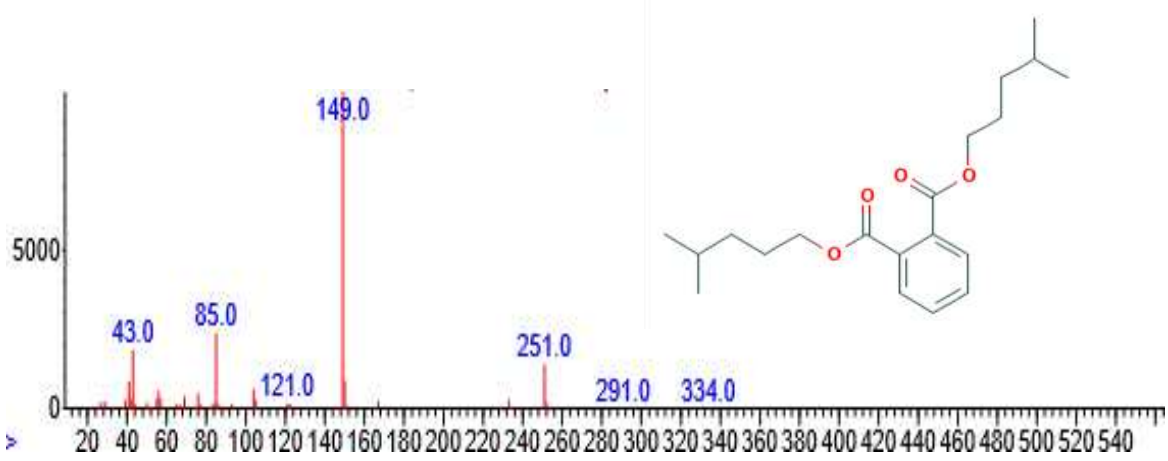


Figure: 10 Molecular Formula : $C_{20}H_{30}O_4$

Name : Diisohexyl phthalate Molecular weight: 334.4

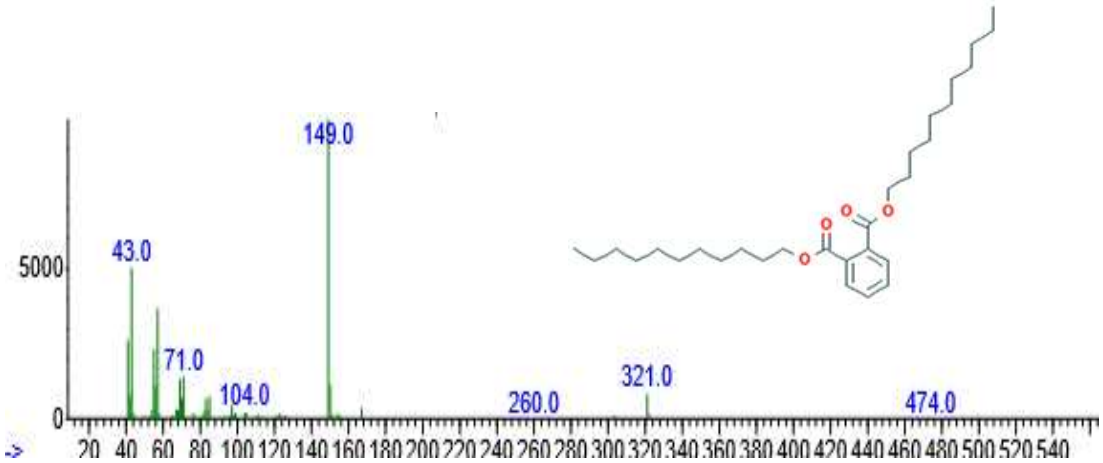


Figure: 11 Molecular Formula : $C_{30}H_{50}O_4$

Name : Diundecyl phthalate Molecular weight: 474.7

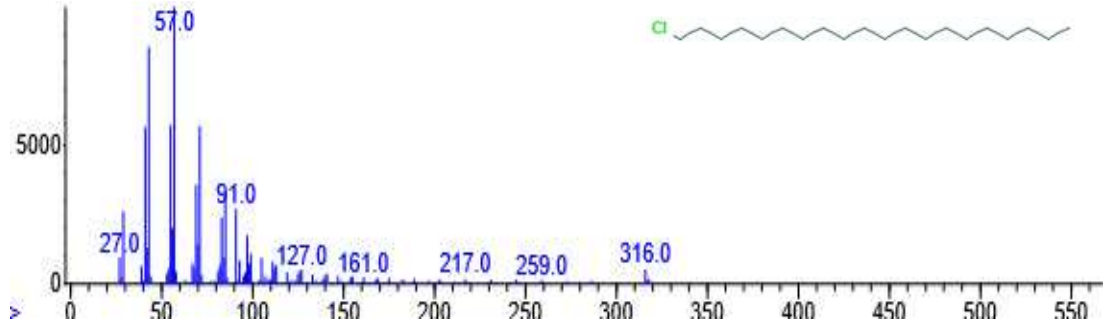


Figure: 12 Molecular Formula : $C_{20}H_{41}Cl$

Name : 1-Chloroeicosane Molecular weight: 317

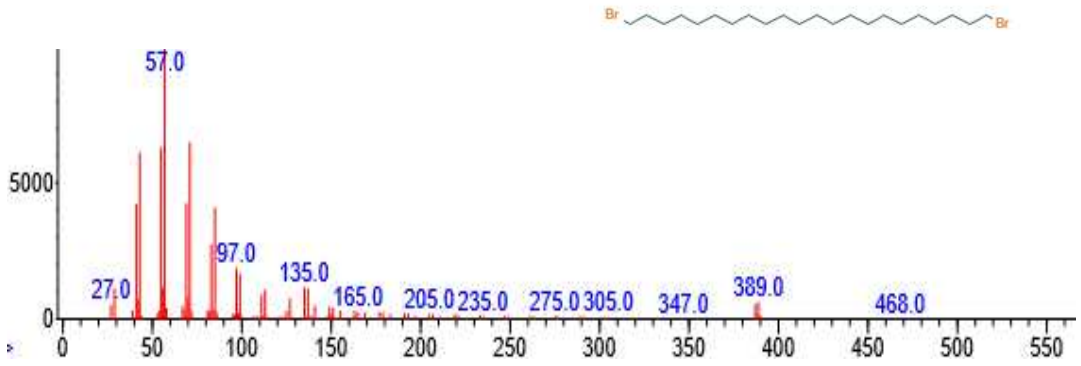


Figure: 13 Molecular Formula : $C_{22}H_{44}Br_2$

Name : 1,22-Dibromodocosane Molecular weight: 468.4

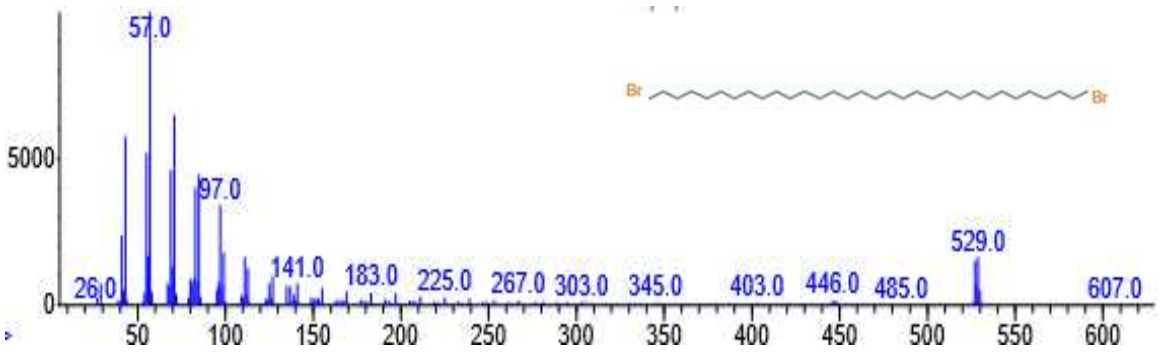


Figure: 14 Molecular Formula : $C_{32}H_{64}Br_2$

Name:1,32-Dibromodotriacontane Molecular weight: 608.7

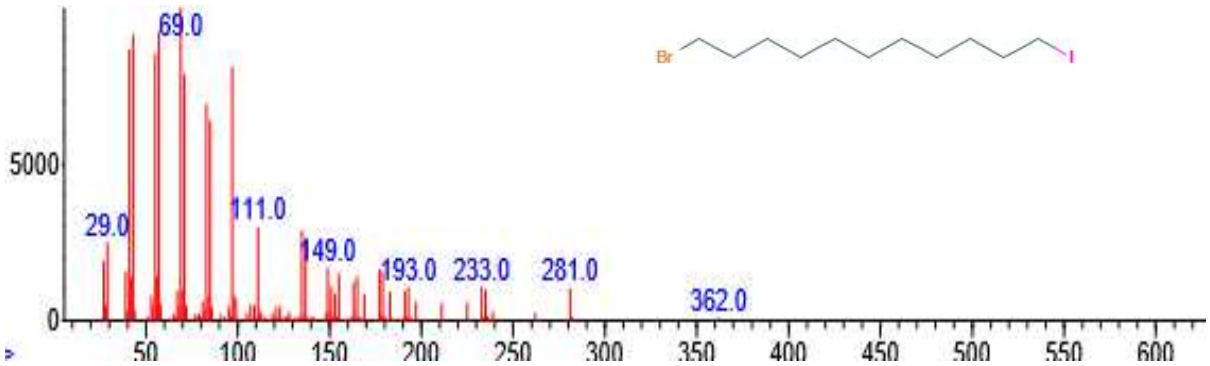


Figure: 15 Molecular Formula : $C_{11}H_{22}BrI$

Name : 1-Bromo-11-iodoundecane Molecular weight: 361.1

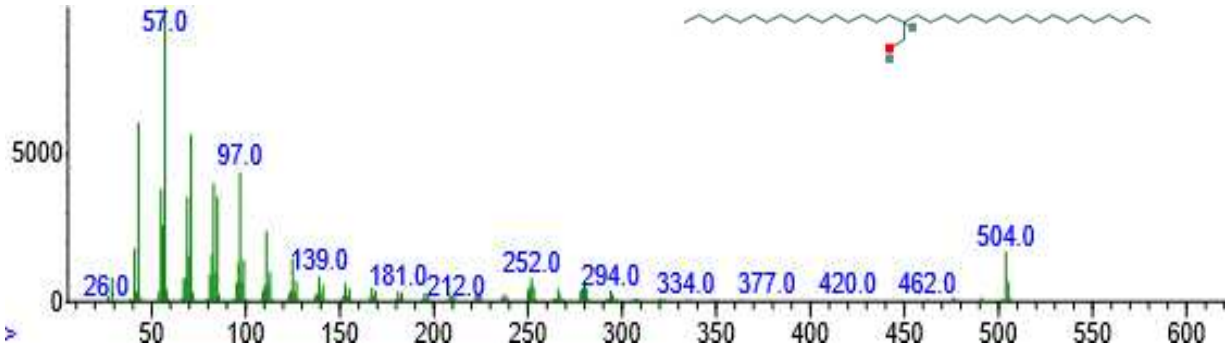


Figure: 16 Molecular Formula : $C_{36}H_{74}O$

Name: 1-Eicosanol, 2-hexadecyl- Molecular weight: 523

Table 4

List of compounds identified in the culture fluid of *Achromobacter* sp

S. No	RT	COMPOUND NAME	MOLECULAR FORMULA	MOLECULAR WEIGHT (g/mol)	PEAK AREA	ACTIVITY
1.	13.875	Tetradecane, 4-methyl-	$C_{15}H_{32}$	212.41	1.56	Antibacterial agent Antioxidant Antidiabetic Antitumor Antifungal Antimicrobial agent Cytotoxic agent Muscle relaxants Anti inflammatory agent
2.	13.875	Tridecane, 3-methyl-	$C_{14}H_{30}$	198.39	1.56	Antimicrobial agent Cytotoxic agent Antioxidant Antifungal Antagonistic agent Allelopathic agent
3.	13.875	Decane, 5-propyl-	$C_{13}H_{28}$	184.36	1.56	Antibacterial agent Anticancer agent Antimicrobial agent Antioxidant Antiviral agent

4.	14.275	Disulfide, di-tert-dodecyl	$C_{24}H_{50}S_2$	402.8	1.60	Lubricant Oxidizing agent Cytotoxic agent Catalytic agent Abortifacient activity
5.	14.275	10-Methylicosane	$C_{21}H_{44}$	296.6	1.60	Antipyretic agent Antimicrobial agent Antibacterial agent Antitumor Antifungal Cytotoxic agent
6.	14.275	2,21-Dimethyldocosane	$C_{24}H_{50}$	338.7	1.60	Antiasthmatics Urine acidifiers Antimicrobial agent Anti inflammatory agent Antioxidant Antitumor Antagonistic Keratinolytic agent
7.	15.752	7-n-Propyltridecane	$C_{16}H_{34}$	226.44	3.51	Antibacterial Antifungal Antmicrobial antioxidant
8.	15.752	3,5-Dimethyldodecane	$C_{14}H_{30}$	198.39	3.51	Antifeeding agent Antimicrobial Antifungal Anti inflammatory agent

						Aphrodisiac activity
9.	16.230	Diisobutyl phthalate	$C_{16}H_{22}O_4$	278.34	3.39	Diuretic agent Anti fouling agent Antimicrobial agent Cytotoxicity Antiproliferative activity
10.	16.230	Diisohexyl phthalate	$C_{20}H_{30}O_4$	334.4	3.39	Antiandrogenic activity Antioxidant Estrogenic agent Cytostatic activity
11.	16.230	Diundecyl phthalate	$C_{30}H_{50}O_4$	474.7	3.39	Tumour promoting agent Antibacterial agent Antifungal Inhibitory agent Estrogenic agent
12.	16.486	1-Chloroeicosane	$C_{20}H_{41}Cl$	317	2.11	Anti-inflammatory Analgesic Antipyretic agent Antifungal
13.	16.486	1,22-Dibromodocosane	$C_{22}H_{44}Br_2$	468.4	2.11	Antimicrobial Antioxidant Antilipoxygenase activity Cytotoxic agent
14.	18.008	1,32-Dibromodotriacontane	$C_{32}H_{64}Br_2$	608.7	1.83	Anxiolytics Anti cancer

						Antimicrobial Antibacterial Antioxidant Allelopathic activity
15.	18.008	1-Bromo-11-iodoundecane	$C_{11}H_{22}BrI$	361.1	1.83	Anthelmintics Antioxidant Antimicrobial Anticancer activity Anti – inflammatory
16.	18.008	1-Eicosanol, 2-hexadecyl-	$C_{36}H_{74}O$	523	1.83	Anti diabetics Antiproliferatives Antioxidants Anti tumour

DISCUSSION

6. DISCUSSION

Keratinolytic enzymes are widespread in nature and are produced by a group of microorganisms isolated from the skin of puffer fish. Seven bacterial strains isolated from puffer fish skin exhibited caseinolytic activity. Among the seven, two owned keratinolytic activity and ability to degrade feather at 30^{o}C and 40^{o}C after the incubation period of seven days.

Skim milk contains casein, a milk protein which will be degraded by proteolytic microorganisms into dissolved nitrogen compounds so the colony will be surrounded by a clear zone. It showed that these microbes have proteolytic activity (Fardiaz, 1992). Based on this test, there were only seven isolates that had the ability to degrade casein isolates while the other three did not.

Seven of ten isolates that showed protease activity were later corroborated by the skim milk agar and they were used for further research. Sivakumar *et al*., (2012) confirmed that the zone formed around colonies was due to the formation of the casein enzyme. It was considered as a positive result.

The caseinolytic ability of bacteria could be used to select the initial keratinolytic bacteria because most keratinolytic bacteria that derived from nature also had a good caseinolytic activity. The results obtained showed that the strains *Achromobacter* sp, *Bacillus* sp, *Escherichia* sp,

Stenotrophomonas sp and *Klebsiella* sp were able to degrade the casein because casein is the main protein in milk. Benson (2010) stated that the media became clear due to the caseinase exoenzymes produced by bacteria.

Based on the methods of Gupta and Ramnani (2006), the chosen casein agar media was related to the most reported keratinase enzyme derived from nature. Brandelli *et al*., (2009) stated that the hydrolysis ability of casein depended on the species and environment of the bacterial isolation place. Furthermore, in order to ensure the ability of bacteria in hydrolyzing protein, a protease activity test was conducted on 7 isolates using qualitative (the formation of the clear zone) and quantitative (enzymes activity) methods (Wardani, 2012).

Protease is also called peptidase or proteinase. It is a hydrolase-class enzyme that will breakdown proteins into simpler molecules such as short oligopeptides or amino acids, with hydrolysis reaction on the peptide bond. Proteolytic bacteria are the bacteria that are able to produce extracellular protease enzyme. The enzyme breaks protein that is produced in the cells and releases it out of the cell.

Numerous bacteria, actinomyces and Filamentous fungi, including dermatophytic species, have been described as keratin decomposers. The dominant group of microorganisms capable of keratinases biosynthesis is

bacteria of the genus Bacillus: among others, *B. subtilis, B. pumilus, B. cereus, B. coagulans, B. licheniformis* or *B. megatherium*. Degradation of keratin proteins can also be conducted by a number of other Gram-positive bacteria like *Lysobacter, Nesternokia, Kocuria, Microbacterium*, and some Gram-negative bacteria, e.g. *Vibrio, Xanthomonas, Stenotrophomonas and Chryseobacterium.* Similar abilities were found among microorganism's thermophilic and extremophilic, representatives by types: *Fervidobacterium, Termoanaerobacter, Nesternokia, Bacillus* (Nam *et al*., 2002; Gupta and Ramnani 2006; Brandelli *et al*., 2015).

Some keratinolytic microorganisms have been reported, including several species of fungi such as *Microsporum* (Essien *et al*., 2009), *Trichophyton* (Anbu *et al*., 2006), *Streptomyces* (Szabo *et al*., 2000; Tatineni *et al*., 2008) and *Actinomycetes* (Young and Smith., 1975; Bockle *et al*., 1995). Recently, keratinolytic activity was also reported for coccus that was rod shaped gram positive.

On the basis of extent of feather degradation, it was confirmed that *Achromobacter* sp and *Bacillus* sp were keratinolytic. These two strains from the skin of puffer fish are responsible for the degradation of feather keratin. The occurrence of feather degrading bacteria from the genera *Bacillus, Pseudomonas, Staphylococcus, Streptococcus,*

Stenotrophomonas and Escherichia are most frequent (Shawkey *et al*., 2005; Sivakumar and Ravindran, 2015)

Feather protein has been showed to be an excellent source of metabolizable protein (Klemersrud *et al.,* 1998) and that microbial keratinases enhance the digestibility of feather keratin (Lee *et al*., 1991; Young and Smith 1975). GC-MS analysis of culture fluid of *Achromobacter* sp showed the occurrence of 16 compounds that were not found in the control. It was concluded that these compounds may be produced during the digestion of feather keratin.

SUMMARY

7. SUMMARY

Seven groups of bacteria isolated from the skin of puffer fish *Arothron hispidus* showed proteolytic activity. Cultural, morphological and biochemical characteristics were analyzed to identify the bacteria.

Different colours like yellow, dull yellow, white and dull white were seen in bacterial colonies. Rhizoid, entire, filiform and undulate types of margins were noted in bacterial colonies while the elevation of all colonies was raised.

Gram staining showed that *Achromobacter* sp and *Bacillus* sp were Gram positive bacteria while *Escherichia coli, Stenotrophomonas* sp *and Klebsiella* sp were Gram negative.

All bacterial isolates were motile except *Klebsiella* sp. Spore staining showed that all bacterial isolates were able to form spores.

Biochemical tests showed that *Achromobacter* sp*, Bacillus* sp, *Escherichia coli, Stenotrophomonas* sp *and Klebsiella* sp produced acid indicating fermentative metabolism. Except *Escherichia coli*, others viz, *Achromobacter* sp*, Bacillus* sp*, Stenotrophomonas* sp *and Klebsiella* sp produced hydrogen sulphide.

All bacterial strains were able to liquefy gelatin except *Escherichia coli*. *Bacillus* sp showed nitrate reduction while others showed negative

result. *Bacillus* sp and *Stenotrophomonas* sp showed indole production while other strains showed negative result. All bacterial strains were able to produce citrate as carbon source and unable to produce urease enzyme. Except *Escherichia coli* and *Stenotrophomonas* sp, others viz, *Achromobacter* sp, *Bacillus* sp and *Klebsiella* sp were able to hydrolyse starch.

Achromobacter sp, *Bacillus* sp and *Escherichia coli* showed positive result to methyl red test. , *Stenotrophomonas* sp *and Klebsiella* sp showed positive result to Voges-Proskauer test.

On various cultural, morphological and biochemical characters, seven groups of proteolytic bacteria were identified. They are *Achromobacter* sp, *Bacillus* sp, *Escherichia coli, Stenotrophomonas* sp *and Klebsiella* sp.

Achromobacter sp and *Bacillus* sp showed keratinolytic activity in feather meal medium by degrading the feather both at 30^{o}C and 40^{o}C.

GC-MS analysis was performed both in control and cultural fluid of *Achromobacter* sp. The cultural fluid of *Achromobacter* sp showed the occurrence of 16 compounds which are not found in the control. So this analysis revealed that the 16 compounds were produced during the decomposition of keratin by the keratinolytic bacteria, *Achromobacter* sp.

CONCLUSION AND SUGGESTIONS

8. CONCLUSION AND SUGGESTIONS

Keratin is the most abundant protein in epithelial cells and forms major components of skin, hair, nail, feather and wool. Utilization of keratinolytic enzyme in leather making is a promising application. In the leather industry, keratinases support the dehairing process, allowing to partially replace lime-sulfide treatment. This study aims to discover bacteria with the ability to degrade keratin of puffer fish *Arothron hispidus,* which is helpful for an ecofriendly tanning process. This study will help the researchers to overcome environmental pollution resulting from tanning process, which have not been widely studied by other researchers. The enzyme produced by the bacteria isolated from the puffer fish, can be used as a substitute for chemicals in tanning process. Further, species level identification of the bacterial strains which produced keratinase could provide an innovative solution in supporting the cleaner production of the tannery.

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9. BIBLIOGRAPHY

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PLANT GROWTH PROMOTING HIZOSPHERIC BACTERIA- ISOLATION AND CHARACTERIZATION

Dissertation submitted to

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By

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(Re-accredited with A^+ Grade by NAAC)

THOOTHUKUDI

APRIL – 2021

CERTIFICATE

This is to certify that this dissertation entitled **PLANT GROWTH PROMOTING RHIZOSPHERIC BACTERIA-ISOLATION AND CHARACTERIZATION** submitted by **S. PONN SHIVA SHANKARI, Reg. No. 19APZO05 to St. Mary's College (Autonomous), Thoothukudi,** affiliated to Manonmaniam Sundaranar University in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2020-2021 under my guidance and supervision. It is further certified that the dissertation or any part of this has not been submitted elsewhere for any other degree.

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DECLARATION

I do hereby declare that this thesis entitled **'PLANT GROWTH PROMOTING RHIZOSPHERIC BACTERIA-ISOLATION AND CHARACTERIZATION'** submitted by me for the award of the degree of Master of Science in Zoology is the result of my original and independent research work carried out under the guidance of **Dr. Sri Priya, M.Sc., PhD.,** Assistant Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi, and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date: 15.04.2021

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1. INTRODUCTION

Agriculture ensures survival of the human race by producing the necessary food. But in the recent years the burden of exploding human population is shouldered by agriculture. Hence developing newer and faster techniques in agriculture to cope up with the ever growing population has become mandatory. In fact, the world's population is estimated to be around 7 billion people and had reached 8 billion by 2020 (Glick, 2012).Therefore, it is urgent to considerably increase the agricultural production to reply to the strong food demand to reduce the risk of malnutrition and poverty.

Therefore, the new cereal varieties with high yield are being developed. In addition, agrochemicals such as chemical fertilizers, herbicides, fungicides, and pesticides are currently improperly and over used to increase crop yield. The direct result of the use of these agrochemicals is the contamination of the groundwater and environmental products by heavy metalswhich is known to cause public health problems like increased occurrence of cancer (Koo and Cho, 2009).

In addition to medical damage, other consequences in agricultural areas such as the interruption of the natural cycle of ecological nutrients and the destruction of biological communities in soil are often reported(Karuppiah and Rajaram, 2011). Regarding the harm caused by excessive use of agrochemicals, other methods of

research are being discovered in the world. Among the explored paths, the use of plant growth microorganisms that are present in the rhizosphere are considered to be a potent environmental friendly strategy for improving agricultural yield.

Soil microorganisms promote material cycling and energy flow on the ecosystem. They play important roles as both producers and decomposers in the ecosystem (Wu *et al.,* 2013; Zhang and Yu, 1990). In addition, soil microorganisms perform processes such as oxidation, nitrogen fixation, nitrification and ammoniation in the soil to promote the decomposition of soil organic matter and nutrient conversion.

Soil microorganisms are widely distributed in the plant rhizosphere and are most specialized in a dynamically changing environment. The soil layer which is influenced by the plant root is called rhizosphere. In 1904, the German scientist Lorenz Hiltner first proposed the concept of the "rhizosphere" which refers to the soil around the root system. In the rhizosphere, plant root activity alters the physical and chemical properties of the soil, providing a special ecological environment for interaction between plant and soil microorganisms (Compant *et al.,* 2010; Kloepper *et al.,* 1980; Liu, 2005).

Since the concept of the "rhizosphere" was proposed, there have been increasing number of studies on the plant rhizosphere, mainly involving the

physiological structure of the root system, rhizosphere soil nutrients, rhizosphere soil enzyme activities and rhizosphere soil microorganisms, as well as the connection between them (Li, 2002). Rhizosphere has been broadly subdivided into the following three zones (Pinton *et al,* 2001)

1. Endorhizosphere: that consists of the root tissue including the endodermis and cortical layers.

2. Rhizoplane: is the root surface where soil particles and microbes adhere. It consists of epidermis, cortex and mucilaginous polysaccharide layer.

3. Ectorhizosphere: that consists of soil immediately adjacent to the root.

Rhizospheric bacteria which play an important role in plant growth promotion and termed as PGPRs. The PGPR is a group of bacteria capable of colonizing actively plant roots system and improving their growth and yield. Rhizosphere bacteria can promote plant growth, increase plant biomass, promote the absorption and utilization of soil nutrients by plants, improve the micro ecological environment of the rhizosphere soil, and inhibit pathogenic bacteria (Vessey, 2003).They not only promote plant growth but also help in sustainable agricultural development and protecting the environment. PGPR can degrade pollutants in soil, improve soil fertility, control pests and diseases, and reduce the environment pollution and soil compaction caused by the use of pesticides and fertilizers (Zhang *et al.,* 2013).

PGPRs are a heterogeneous group of bacteria in the rhizosphere, on root surfaces and in association with roots, including a number of bacterial species associated with the plant rhizosphere belonging to genera *Azospirillum, Alcaligenes, Acinetobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Rhizobium and Serratia* are able to exert a beneficial effect on plant growth.There are a large number of active microorganisms, such as fungi, bacteria and actinomycetes, in the rhizosphere soil, approximately 28-48 times higher than in non-rhizosphere soil.

The plant growth promoting (PGP) effect of the PGPR is mostly explained by the release of metabolites directly stimulating growth. Several mechanisms have been postulated to explain how PGPR benefits the host plant. These include, the ability to produce plant growth regulators or phytohormones such as Indole acetic acid (IAA) cytokinins and gibberellins (Glick, 1995; Marques *et al.,* 2010), enhancing asymbiotic N_2 fixation (Sahin *et al., 2004*; Khan, 2005), Solubilizing inorganic phosphate and mineralization of organic phosphate and other nutrients (Glick, 1995; Jeon *et al.,* 2003).

Bacteria colonize the plant tissue and promote plant growth through mechanism such as IAA, phosphate solubilization and production or supplying necessary vitamins to plants for their growth and development (Ryan *et al.,* 2008;

Ma *et al.,* 2011). Rhizosphere bacteria can act as biocontrol agents against pathogenic microorganisms and help in nitrogen fixation (Weyens *et al.,* 2009).

The use of biocontrol bacteria isolated from the rhizosphere may present an alternative for plant disease prevention (Compant *et al.,* 2005; Fernando *et al.,* 2006; Fatima *et al.,* 2009). In crop protection, integrated pest management involves the application of different bacteria alone or in combination with other antagonistic agents. One of the plant growth promoting mechanisms of rhizobacteria is the antagonism against phytopathogenic microorganisms due to the production of antimicrobial metabolites like siderophores, antibiotics, cyanides, fungal cell wall degrading enzymes and gaseous products including ammonia (Idris *et al.,* 2007; Lugtenberg and Kamilova, 2009).

Increasing crop yield through the use of PGPR as microbial inoculants is now the method of choice by most people because of increased demand for food and sustainable environment (Arora, 2000). Hence, the focus of this study is to isolate, identify PGPR bacterial isolates and to determine their suitability as plant growth promoting rhizobacteria to prepare bioinoculant which are environmentally friendly and economically wise to maintain sustainable crop productivity and production without harming the environment, human and animal health.

2. OBJECTIVES

The main objectives of this study are

1. Isolation of plant growth promoting bacteria (PGPR) from rhizosphere of *Avicennia marina.*

2. To identify and characterize the isolated bacterial strains by biochemical analysis.

3. To determine the plant growth promoting activity of the bacterial strains by biochemical assays.

4. To assesses the PGP potency of the isolates by plant assays.

3. REVIEW OF LITERATURE

The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root. Complex gradient of substrate availability, water potential, and redox state distinguish this habitat from bulk soil, and constrains the distribution and the activity of the tremendously diverse rhizosphere microbiota (Kumar *et al.,* 2013). Bacteria that can improve plant growth through various mechanisms have been known for decades and have been introduced into soil, on seeds or roots to improve plant growth and health Raaijmakers *et al.,* 2002).

Bacteria are the most abundant microbes in the rhizosphere and hence they are bound to influence the plant in a significant manner. Up to 15 % of the total root surface may be covered by a variety of bacterial strains (van Loon, 2007). The most common genera of bacteria that have been reported in the rhizosphere are *Pseudomonas, Bacillus, Arthrobacter, Rhizobia, Agrobacterium, Alcaligenes, Azotobacter, Mycobacterium, Flavobacter, Cellulomonas* and *Micrococcus.* Predominant bacterial strains in the rhizosphere includes gram-negative, rod shaped, non-sporulating bacteria belonging to the groups proteobacteria and actinobacteria (Atlas and Bartha, 1993; Teixeira *et al.,* 2010) of which *Pseudomonas* are the most abundant. This may be attributed to the efficiency of gram- negative bacteria to

utilize the root exudates and hence they are stimulated by rhizo deposition while the gram- positive bacteria are rather inhibited (Steer and Harris, 2000).

Root exudates include amino acids, organic acids, carbohydrates, sugars, vitamins, mucilage and proteins. The exudates launch signals and attract the microbes towards root by stimulating biological and physical interactions between roots and soil microorganisms (Brevic, 2012). Consequently, the rhizosphere supports large and active microbial populations capable of exerting positive, neutral, or negative effects on plant growth (Reddy, 2013). According to Raynaud and Nunan (2014) the vast majority of soil organisms in the rhizosphere were bacteria, with densities as high as 108 cells per gram of bulk soil which depends on biotic conditions like soil pH, temperature and moisture.

Plant growth promoting rhizobacteria (PGPR) are natural microflora of soil that is able to colonize plant roots and stimulate plant growth when applied to roots and other propagules (Egamberdieva *et al.,* 2010). The relationship of PGPR with the host plants may be of two types and PGPR can be divided into two groups: symbiotic bacteria and free-living rhizobacteria (Khan, 2005). Several workers also designated PGPR two groups according to their residing sites: iPGPR (i.e., symbiotic bacteria), which live inside the host cells, produce nodules, and are localized inside the nodule; and ePGPR (i.e., free-living rhizobacteria), which do not produce nodules, but still can prompt plant growth (Gray and Smith, 2005). The best-known

PGPR are Rhizobia, which produce nodules in leguminous plants and provide nitrogen to them (Hayat *et al.,* 2010).

PGPR can prevent the deleterious effects of phytopathogenic organisms on the environment. The mechanisms by which PGPR can influence plant growth may differ from species to species as well as from strain to strain. It may promote growth directly by producing phytohormones, increase the phosphorous uptake by solubilization of inorganic phosphates, by fixing the atmospheric N2, increasing the availability to plants and ammonia production were reported as best known mechanisms of plant growth promotion (Podile and Kishore, 2006; Zhang *et al.,* 2012; Singh *et al.,* 2013; Kumar *et al.,* 2013). Production of siderophores and secretion of different antifungal compounds to inhibit the phytopathogens are considered as indirect methods of plant growth promotion.

The second important division of beneficial bacteria in the rhizosphere are those referred to as plant growth promoting bacteria (PGPB), which promote growth via production of phytohormones and improvement of plant nutrition status (Bai et al., 2002). Because of these properties, the co-inoculation of these PGPB with the symbiotic rhizobia is currently becoming a valuable technique in the development of sustainable agriculture. Among the major groups of plant growth promoting bacteria, the most widely studied and efficient group include *Azosprillum sp*.

(Bertrand *et al.,* 2001), Pseudomonas spp. (Amy *et al.,* 2002) and *Bacillus* sp. (Bai *et al.,* 2002).

Plant hormones play important role in plant growth and development. Several stages such as cell elongation, cell division, tissue differentiation, and apical dominance are controlled by the plant hormones, especially auxins and cytokinins. Auxins and cytokinins can be synthesized by both the plants and the microorganisms. Auxin, indole-3-acetic acid (IAA), is an important phytohormone produced by a number of PGPR, and treatment with auxin-producing rhizobacteria increased the 8 plant growth (Vessey 2003; Erturk *et al.,* 2008). The auxin type phytohormone known as indole-3-acetic acid (IAA) is the main type of phytohormone produced by plant growth promoting bacteria (Patten and Glick 1996; Gonzalez and Bashan 2000; Patten and Glick, 2002).

IAA production by bacteria can vary among different species and strains, and it is also influenced by factors as culture condition, growth stage and substrate availability (Mutluru and Konada, 2007). The bacteria synthesize IAA generally through two pathways- *Rhizobium, Bradyrhizobium,* and *Azospirillum* synthesize IAA via the Indole-3-pyruvic acid (IPyA) pathway (Costacurta and Vanderleyden, 1995; Patten and Glick, 1996; Burdman *et al.,* 2000).

On the other hand, the indole-3-acetamide (IAM) pathway is used by some pathogenic bacteria such as *Pseudomonas syringae*, *Agrobacterium tumefaciens*, and *Erwinia herbicola* to synthesize IAA (Dobbelaere *et al.,* 2003). Among PGPR species, *Azospirillum* is one of the best studied IAA producers (Dobbelaere *et al.,* 1999). Other IAA producing bacteria belonging to *Aeromonas* (Halda-Alija, 2003), *Azotobacter* (Zahir *et al.,* 2000), *Bacillus* (Swain *et al.,* 2007), *Burkholderia* (Halda-Alija 2003), *Enterobacter* (Shoebitz *et al.,* 2009), *Pseudomonas* (Hariprasad and Niranjana 2009) and *Rhizobium* (Ghosh *et al.,* 2008) have been isolated from different rhizosphere soils.

Culture filtrates of plant growth-promoting rhizobacteria (PGPR) *Bacillus amyloliquefaciens* (FZB24, FZB42 and FZB45) and *Bacillus* subtilis FZB37 which are reported to produce IAA, have a strong growth promoting activity. During the bioassays, seedling segment elongation and coleoptiles bending performed with diluted *Bacillus* culture filtrates demonstrated that length growth of maize seedlings was significantly enhanced. *Bacillus amyloliquefaciens* FZB42 exhibited the highest enhancement on plant growth comparable with concentrations of 10–6 to 10–7 mol/l IAA (Idris *et al.,* 2007).

Reetha *et al.,* (2014) reported the effect of IAA producing *Pseudomonas fluorescens* and *Bacillus subtilis* on the growth of onion. Both the isolates are isolated from the rhizosphere of onion and analyses for in vitro indole acetic acid. In

the quantitative measurements, the highest value of IAA production was obtained by *P. fluorescens* followed by *B. subtilis,* as they produced (15.38 ± 0.537) and (12.67 ± 0.325) respectively. Both bacteria demonstrated increase in root length, shoot length, root and shoot fresh and dry weight, on bacteria.

Generally, rhizobacterial IAA interferes with the many plant developmental processes because the endogenous pool of plant IAA may be altered by the acquisition of IAA that has been secreted by soil bacteria (Glick, 2012; Ahemad and Kirbet, 2014). Evidently, IAA also acts as a reciprocal signalling molecule affecting gene expression in several microorganisms. Consequently, IAA plays a very important role in rhizobacteria-plant interactions (Spaepen and Vanderleyden 2011).

Phosphate solubilizing bacteria (PSB), which are very common in soil, can be used to overcome the situation. Microorganisms offer a biological rescue system capable of transforming insoluble P to soluble monobasic and dibasic ions and may also solubilize inorganic phosphate (Kumar and Narula, 1999). Most of the P is insoluble making it unavailable to plants. Even in P rich soil very little amount is readily available to plants. The plants are able to absorb their own mono and dibasic phosphate, but organic or insoluble forms of P need to be mineralized or solubilized (Ramaekers *et al.,* 2010).

Most of the insoluble P forms are present as aluminum and iron phosphates in acid soils (Mullen, 2005), and calcium phosphates in alkaline soils (Goldstein and Krishnaraj, 2007). The mechanism by which PSB solubilize P, involves process of acidification, chelation, exchange reactions, and production of gluconic acid (Rodriguez *et al.,* 2004; Chung *et al.,* 2005; Hameeda *et al.,* 2008).

Members belonging to the genera *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aerobacter, Flavobacterium* and *Erwinia* have the ability to solubilize insoluble inorganic phosphate (mineral phosphate) compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyl apatite and rock phosphate (Goldstein, 1986; Rodríguez and Fraga, 1999; Rodríguez *et al.,* 2006). Organic P can constitute between 30 and 50% of the total P of the soil, a high proportion of it corresponding to phytate (Borie *et al.,* 1989; Turner *et al.,* 2003). According to Richardson (2001) phytate is the major component of organic phosphate in the soil.

Chakraborty *et al.,* (2006) reported phosphate solubilization by *Bacillus megaterium* and thereby promoting the growth of tea plants. Phosphate solubilization by the tested organisms was accompanied with pH reduction of the culture medium. In another study, Orhan *et al.,* (2006) reported that plant growth promoting effects of two *Bacillus* strains OSU-142 (N-fixing) and M3 (N-fixing and phosphate solubilizing) were tested alone or in combinations of organically grown

primo cane fruiting raspberry (cv. Heritage) plants and a significant increase in yield (33.9 and 74.9%), cane length (13.6 and 15.0%), number of cluster per cane (25.4 and 28.7%), and number of berries per cane (25.1 and 36.0%) were observed when compared with that of the control.

Hameeda *et al.,* (2006) reported that plant biomass increased with *Serratia marcescens* EB 67 and *Pseudomonas* sp CDB 20 35 under both glasshouse and field conditions. And also, seed treatment with EB 67 and CDB 35 increased the grain yield of field-grown maize by 85 and 64% compared with the uninoculate control. In an experiment Elkoca *et al.,* (2008) showed that the controlled environment and in the field trials, single and dual N-fixing B. subtilis (OSU-142) and P-solubilizing B. megaterium (M-3) inoculations significantly increased height, shoot, root and nodule dry weight, N%, chlorophyll content, pod number, seed yield, total biomass yield, and seed protein content in chickpea compared with the control treatment, equal to or higher than N, P, and NP treatments.

Genetic manipulation of phosphate-solubilizing bacteria is another way to enhance their ability for plant growth improvement (Rodríguez and Fraga, 1999; Rodríguez *et al.,* 2006). The approach may include cloning gene (s) involved in both mineral and organic phosphate solubilization, followed by their expression in selected rhizobacterial strains (Rodríguez *et al.,* 2006).

Nitrogen is an essentially required nutrient for plant development and growth. It is one of the principal plant nutrients, and its low availability due to the high losses by emission or leaching is a limiting factor in agricultural ecosystems, hence bacteria with ability to make atmospheric N available for plants play an important role. The production of chemical fertilizers is a highly energy intensive process using large amounts of fossil energy. Use of excessive amount of manures to achieve high yields has created environmental problems and degradation in natural resources (Sahin *et al.,* 2004).

During the past couple of decades, the use of PGPR for sustainable and environment friendly agriculture has been increased tremendously in various parts of 24 the world (Figueiredo *et al.,* 2008). Increasing and extending the role of bio-fertilizing with PGPR would reduce the need for chemical fertilizers and decrease their adverse environmental effects.

An important feature of these plant growth-promoting bacteria is their ability to colonize roots and promote plant growth (Sharma *et al.,* 2003; Patten and Glick, 2002). The potential of rhizosphere colonization by PGPB is very crucial for what is known as soil biofertilization (Villacieros *et al.,* 2003). The term 'biofertilizer', though misleading is a widely used term to describe bacterial inoculants. It refers to preparation of microorganisms that may be a partial or complete substitute for chemical fertilization like rhizobial inoculants (Bashan

1998). Improving plant growth by biofertilization is a crucial mechanism by which iron acquisition in most agricultural crops is achieved.

Normally the total iron in the soil is by far much higher than most crops require. However, the concentration of free Fe+3 in most soils is far below that required for optimum growth (10-9 and 10-4M Fe+3) in the soil solution (Masahla *et al.,* 2000). In the decades before, many studies have indicated that the production of siderophores by plant growth promoting bacteria, particularly by the biocontrol *Pseudomonas* spp. increases plant iron acquisition (Masahla *et al.,* 2000). The high binding affinity and specificity for iron facilitates the transport of iron into the bacterial cells. Plants make use of this ferric-siderophore complex in their systems through the action of enzymes like ferric reductase (Sharma *et al.,* 2003). Under iron-limiting conditions PGPR produce low-molecular-weight compounds called siderophores to competitively acquire ferric ion (Whipps, 2001). Siderophore producing PGPR can prevent the proliferation of pathogenic microorganisms by sequestering Fe3+ in the rhizosphere (Siddiqui, 2006). Fe depletion in the rhizosphere does not affect the plant, as the low Fe concentrations occur at microsites of high microbial activity during establishment of the pathogen.

Another important aspect of biofertilization is that it accounts for approximately 60 % of the nitrogen supply to crops worldwide. This is achieved both by the symbiotic and free-living nitrogen fixers. To date the genes involved in

nitrogen fixation and nitrogen assimilation have been described for Azosprillum (Bloemberg and Lugtenberg, 2001).

Phytopathogens have a great impact on crop yields. They can reduce the performance of plant and crop quality. Plant-growth promoting rhizobacteria (PGPRs) have been used as good biocontrol agents against soil borne pathogens. Potential biocontrol agents produce antibiotics, siderophore that chelate iron, making it unavailable to pathogens; the ability to synthesize anti-fungal metabolites that cause disease suppression and production of fungal cell wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens; the ability to successfully compete with pathogens for nutrients or specific niches on the root increase yield of plants. The biological control that results from PGPR are reported to be caused by several mechanisms such as competition, antibiosis, and induced systemic resistance (Kloepper *et al.,* 1980)**.**

Production of lytic enzymes is one of the most important mechanisms that PGPR use for the control of plant pathogens and thus indirectly promoting the growth of plants. These microbially synthesized enzymatic compounds include defence enzymes, such as chitinase, β-1, 3-glucanase, peroxidase, protease and lipase (Bashan and de-Bashan, 2005; Karthikeyan *et al.,* 2006)**.**

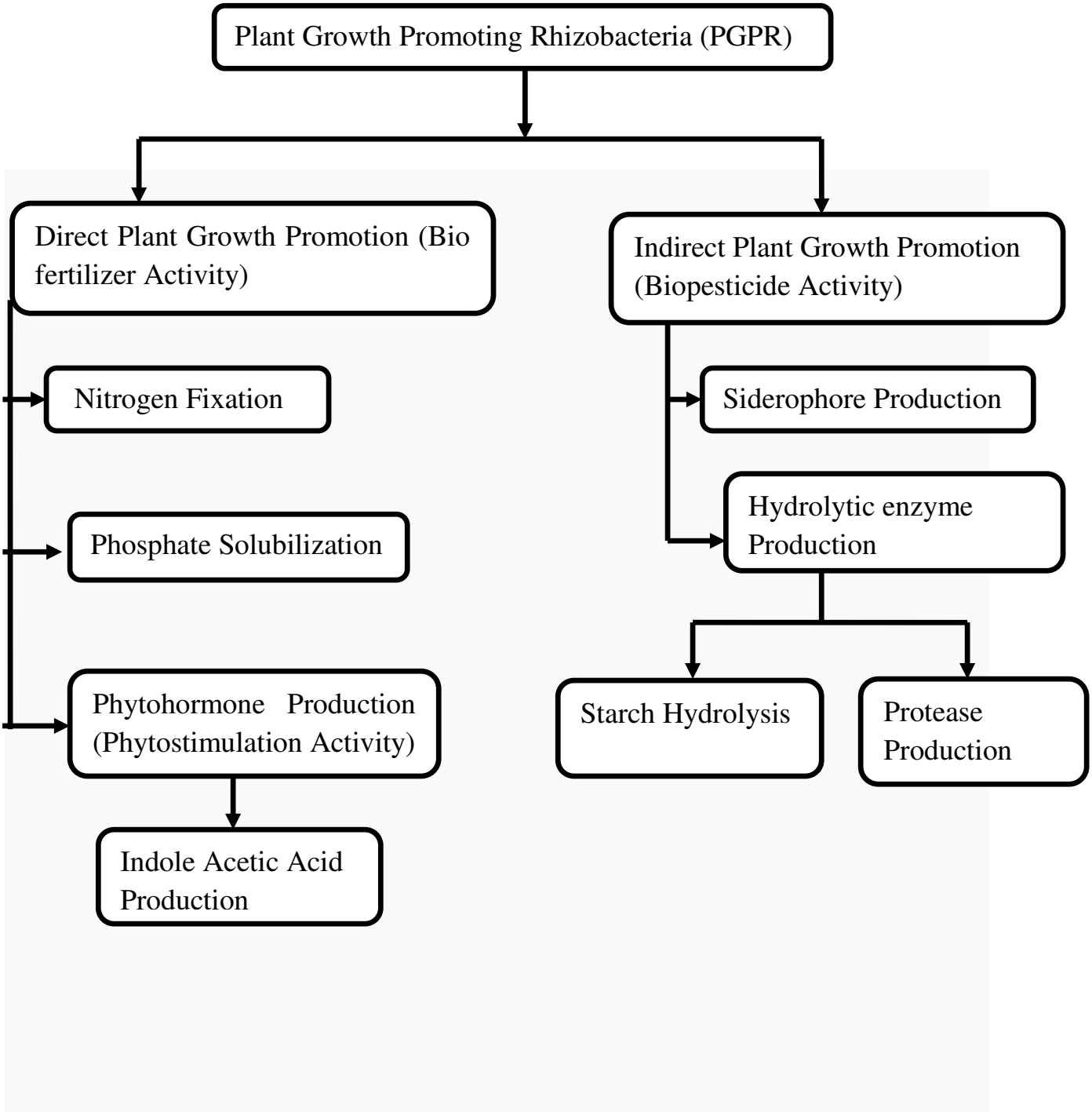
Some rhizobacteria are capable of producing HCN (hydrogen cyanide, also known as cyanide (Rezzonico *et al.,* 2007). It is a volatile, secondary metabolite that suppresses the development of microorganisms and that also affects negatively the growth and development of plants (Siddiqui *et al.,* 2006). Cyanide is toxic to plants capable of disrupting enzyme activity involved in major metabolic processes, its role as a biocontrol substance is overwhelming (Voisard *et al.,* 1989; Devi *et al.,* 2007).

Hydrogen cyanide (HCN) among cyano genic compounds effectively blocks the cytochromeoxidase pathway and is highly toxic to all aerobic microorganisms at very low concentrations. However, the microbes, which produce the compound, mainly *Pseudomonas*, are reported to be resistant (Bashan and de-Bashan, 2005). HCN is formed from glycine through the action of HCN synthesize enzyme, which is a membrane-bound flavoenzyme that oxidizes glycine, producing HCN and CO2. HCN does not appear to have a role in primary metabolism and is generally considered a secondary metabolite (Blumerand Haas, 2000).

HCN production was more common trait of *Pseudomonas* (88.89%) and *Bacillus* (50%) (Ahmad *et al.,* 2010). Chandra *et al.,* (2007) reported that the rhizosphere competent *Mesorhizobium loti* MP6 also produced HCN under normal growth conditions and enhanced the growth of Indian mustard (*Brassica campestris*). Wani *et al.,* (2007) tested the rhizospheric isolates for HCN producing ability in vitro to find that most of the isolates produced HCN and helped in the plant

growth. The psychrotolerant bacterium *Pseudomonas fragi* CS11RH1 (MTCC 8984), was reported to produce hydrogen cyanide (HCN) and the seed inoculation with the isolate significantly increased the percentage germination, rate of germination, plant biomass and nutrient uptake of wheat seedlings (Selvakumar *et al.,* 2009). Pathma *et al.,* (2011) reported that, fluorescent pseudomonas are capable of biological control phytopathogens by the production of HCN. Various studies attribute a disease protective effect to HCN, e.g. in the suppression of "root-knot" and black rot in tomato and tobacco root caused by the nematodes *Meloidogyne javanica* and *Thielaviopsis basicola,* respectively (Voisard *et al.,* 1989; Siddiqui *et al.,* 2006). The plant growth promoting activity of microorganisms is represented as a flow chart in Fig. 3.1

Fig. 3.1 Schematic diagram showing the plant growth promoting activity of bacteria



4. MATERIALS AND METHODOLOGY

4.1 Collection of sample:

Soil and root sample were collected aseptically in sterile plastic bags from the rhizosphere of mangrove trees (*Avicenia marina*) near Harbour beach in Thoothukudi.

The study area was 2 km distance from the outlet area of Tuticorin Thermal power station (TTPS). It is located between at the latitude of 08°46'593" and N 78°09'335E longitude between TTPS and Tuticorin Fishing harbour (Figure 1). The study area is bordered with Mangrove plants (*Avicenia marina*) and small salt pans close to it.

4.2 Characterization of culture:

4.2.1 Isolation of Bacteria from Serial dilution:

Rhizospheric soil samples were separated from roots of mangrove tree by brushing gently in a petridish. 1 gram of rhizospheric soil was taken into a test tube containing 10 ml of distilled water. The soil sample was serially diluted and spread plated to obtain single colony isolates

4.2.2 Plating of culture:

The nutrient agar was dissolved in 100 ml of distilled water. The medium was autoclave at 121^0C for 15 minutes. The medium was poured into petridish and allowed to solidify. After harden of the agar medium, 1 ml of the diluted solution is

added (10^{-1} and 10^{-9}) and rotate it in a clockwise and anti-clockwise direction for 2 seconds. Plates were incubated at 37^0C for 24 hours.

4.2.3 Isolation of pure bacterial colonies:

Isolated colonies were marked and numbered on the agar plates. Single colonies were picked and quadrant streaked to obtain single pure colonies. Pure cultures were stored at 4^0 C for further study.

4.2.4 Characterization of isolates:

Morphological characteristics of the colony of each isolate was explored in appropriate and specific media. All the isolates were streaked on petriplates containing suitable media. After three days of incubation, morphological characteristics of colonies such as shape, size, colour, pigmentation, gram staining and endospore staining were recorded.

4.2.5 Seed inoculation with bacterial strain:

Vigna radiate seeds were surface sterilized in 70% ethanol for 2 minutes and 1.2% sodium hypochloride for 10 minutes and rinsed ten times in sterile tap water. Then the seeds were treated with the bacterial suspension at the concentration strain of 10^{-3}, 10^{-4} and 10^{-5} CFU $ml^{-1}$for 1 hour under sterilized condition.

4.2.6 Seed germination assay:

Culture for seed germination assay was prepared by inoculation of single colony into nutrient broth and grown over night in an orbital shaker (150 rpm) for 24 hours. Seeds of green gram (*Vigna radiata*) were treated with culture (1 OD-A_{600}) by shaking the seeds in it for 1 hour. After 1 hour, the seeds are planted in the pots with garden soil. Seed treated with sterile distilled water served as control. Experiments were performed in a completely randomized block design and growth parameters were measures after 3 days.

4.3. Staining of isolates:

The isolates were stained and viewed under light microscope

4.3.1Gram staining:

Gram staining was performed for all colonies isolated according to standard procedure. A smear of bacterial cells was prepared on a clean glass slide by gentle

heat fixation. Heat fixed smear filled with crystal violet solution for one minute. The smear was washed with distilled water and then gram iodine was added. Smears were washed with 95% decolourisation and cleaned with water. Finally safranin was used as counter stains for 60-80 sec and washed with water. Then observed under a microscope.

4.3.2. Endospore staining:

After preparing the bacterial smear and heat fixing, the slide was treated with 0.5% malachite green and kept for 5 minutes. Then the slide was rinsed gently in tap water. Then the counter stain safranin was added and kept for 30 seconds. The slide was washed again allowed to dry and observed under oil immersion objective.

4.4 Biochemical characterization:

Isolates were analysed for biochemical characters such as production of Hydrogen sulphide, Gelatin liquification, Nitrate reduction, Citrate production and Catalase production.

4.4.1 Production of Hydrogen sulphide (H_2S):

The medium was inoculated with the bacterial culture by penetration method of inoculation and incubated for 24 hours at 37^0 C. The presence of H_2S is based on

the metal formed. The black precipitate on the medium indicates the formation of hydrogen sulphide.

4.4.2 Gelatin liquefaction:

The gelatin medium was inoculated with the culture and incubated for 24 hours at 30^0C. After the incubation period, the tubes were placed in the refrigerator for one hour. Liquefaction of the media represents a positive result.

4.4.3 Nitrate reduction test:

To a 24 hours culture in the peptone broth medium, two drops of sulfanic acid and two drops of α-naphylamine solution were added. The presence of nitrate was indicated by the pinkish red colour after the addition of reagents.

4.4.4 Citrate test:

About15-20 ml of Simmon's citrate agar was transferred to the sterilized petridish. After the solidification of the medium, the culture was streaked and incubated for 24 hours at 37^0C. A colour change of the media from green to blue indicates a positive result (Rashmi *et al.,* 2017).

4.4.5 Catalase test:

Two drops of hydrogen peroxide was added to the 24 hours broth culture. The immediate evolution of the gas bubbles indicates the production of catalase enzyme by the isolates and hence considered catalase positive (Hadioetomo 1990).

4.5 In vitro screening of Bacterial isolates for their plant growth promoting traits:

4.5.1 Production of Ammonia:

Bacterial isolates were screened for the production of ammonia in peptone water as described by Ajaykumar (2012). Freshly grown cultures were inoculated in a peptone broth and incubated for 48 hours. Nessler's reagent was added in each test tube. Formation of yellow to dark yellow or orange colour indicates a positive result for ammonia production.

4.5.2 Production of Indole Acetic Acid (IAA):

Indole acetic acid production was tested by inoculating a loopful of 24-hour broth culture in peptone broth medium. The medium was incubated at 37^0C for 48 hours. At the end of incubation period, 3 drops of kovac's reagent was added directly to the tube. The presence of indole was indicates by the formation of pink to red colour in the reagent layer on the top of the medium (Rashmi *et al.,* 2017).

4.5.3 Nitrogen fixation assay:

The isolated bacterial strain was grown in nitrogen free medium (Burk's medium). 100 ml of the medium was prepared by dissolving 0.02gm $MgSo_4$, 0.08gm K_2HPO_4, 0.02gm $K_2H_2PO_4$, 0.013gm $CaSo_4$, 0.00145gm $FeCl_3$, 0.0000253gm Na_2MaO_4, 2gm sucrose, and 1.5gm Agar. The medium was prepared and poured into sterilized plate. After hardening the bacterial strain was streak on its surface of medium. Plates were incubated at 30^0C for 24 hours. The isolates that were able to grow after incubation indicated the potential to fix atmospheric nitrogen.

4.5.4 Phosphate solubilization:

Bacterial isolates was screened invitro for their phosphate solubilizing activity using potato dextrose rose Bengal agar. The cultures were streaked on the agar. Plates are incubated at 30^0C for 24 hours. The growth of the bacterial colony indicates a positive result for phosphate solubilization (Martin, 1950).

4.6 Invitro screening of bacterial isolates for their plant biocontrol properties:

4.6.1 Hydrogen cyanide production:

Bacterial isolates were screened for HCN production by inoculating the culture in nutrient broth containing 4.4gm of glycine per litre and incubated for 24 hours. Whatmann filter paper was cut into small pieces soaked in 2% sodium

carbonate and 0.5% picric acid solution and then, inserted into the broth medium. After 30 minutes the colour change of the filter paper from deep orange to reddish brown indicated the production of HCN.

4.6.2 Screening for Hydrolytic enzyme production:

Bacterial isolates were screened for their hydrolytic enzyme production like protease and amylase.

4.6.2.1 Protease production activity:

Bacterial isolates were screened for the ability to produce proteolytic enzymes in skim milk agar (or) SM medium. The medium was poured into a sterilized petridish and the isolate bacterial strain was streaked on the surface of the medium. Formation of a clear zone is indicative of protease production.

4.6.2.2 Starch hydrolysis activity:

About 20 ml of starch medium was poured into the sterilized petridish. The isolate was streaked and incubated for two days at room temperature. At the end of incubation period, two or three drops of lugol's iodine solution were added on the surface of the medium. A clear zone around the area of growth indicates starch hydrolysis activity of the isolate.

Table 1 Morphology of the isolates

Isolates	Colour	Shape	Margin	Elevation	Opacity
S1	Dull white	Circular	Entire	Raised	Opaque
S2	Dull white	Filamentous	Filiform	Raised	Opaque
S3	yellow	Rhizoid	Lobate	Convex	Opaque
S4	Dull white	Circular	Entire	Raised	Transparent
S5	Dull white	Irregular	Entire	Convex	Opaque
S6	Dull white	Circular	Entire	Convex	Opaque
S7	Dull white	Circular	Undulate	Convex	Opaque
S8	Dull white	Irregular	Entire	Convex	Opaque
S9	Dull white	Circular	Entire	Raised	Opaque
S10	Dull white	Circular	Entire	Convex	Opaque

Table 6 Plant growth activity of the isolates

Isolates	Shoot		Root	
	Length (cm)	Weight (gm)	Length (cm)	Weight in (gm)
Control	1	0.247	2.3	0.027
S3	21.5	0.232	4.6	0.042
S4	23.5	0.381	3.5	0.040
S5	27	0.324	4.1	0.026

Table 2 Morphological characteristics of PGPR isolates

Isolates code	Gram staining	Endospore staining
S1	Negative	Present
S2	Negative	Absent
S3	Negative	Present
S4	Negative	Present
S5	Negative	Present
S6	Negative	Present
S7	Negative	Present
S8	Negative	Present
S9	Negative	Present
S10	Negative	Present

Table 3 Biochemical characterization of bacterial isolates

Isolates	Hydrogen Sulphide	Gelatin Liquification	Nitrate reduction	Citrate test	Catalase test
S1	+++	++	+++	-	-
S2	-	++	++	-	++
S3	-	+++	+	+++	+
S4	+++	-	++	+++	-
S5	-	++	+	++	+++
S6	+	+++	+	++	++
S7	+	+	+	-	-
S8	+	++	+++	++	++
S9	-	++	+	-	_
S10	-	-	++	+	+

Note; - = no production, + =weak production, ++ = medium production, +++ = high production

Table 4 Plant growth promoting traits of the isolates

Isolates	Ammonia Production	IAA Production	Nitrogen fixation	Phosphate Solubilization
S1	+	+	-	-
S2	+	++	+	-
S3	-	+++	++	+++
S4	+++	+++	+	++
S5	++	+++	+	+
S6	+	+	+	+
S7	+	+	+	-
S8	-	+	+	++
S9	++	+	-	-
S10	-	++	-	+++

Note; - = no production, + =weak production, ++ = medium production, +++ = high production

Table 5 Biocontrol properties of the isolates

Isolates	Hydrolytic enzyme production		HCN Production
	Protease Production	Starch Hydrolysis	
S1	-	+++	+++
S2	+	++	++
S3	+++	+	+
S4	-	-	-
S5	+++	++	+++
S6	+	+++	+
S7	-	+++	+
S8	-	+++	-
S9	++	++	+
S10	+	-	+

Note; - = no production, + =weak production, ++ = medium production, +++ = high production

5. RESULT

5.1 Isolation of bacteria:

Ten bacterial strains were isolated from the rhizospheric soil sample from mangrove plants in the study area mentioned (Figure 1). Pure culture of the 10 isolates were obtained and named as S1, S2, S3, S4, S5, S6, S7, S8, S9, and S10. The pure cultures were stored at 4^0 C for further study.

5.2 Morphological characteristization of isolated bacteria:

5.2.1. Morphological Characterization

The 10 bacterial isolates were characterized for the colony morphology such as shape, colour, margin, elevation and opacity and also characterized for cellular morphology using light microscope (Table 1).

5.2.2 Microscopical observatio:

The major properties of bacteria can be analysed by microscopical observations. These include Gram reaction and production of endospores. Gram staining and endospore staining was done on all the isolates. All the bacterial were gram negative strain. The strains S1, S4, S6, S7, S9, and S10 were Circular. The strain S2 appeared as filamentous, while S-3 rhizoid in shape and S5 and S8 were

irregular (Table 1, Figure 3). All the bacterial strain produced endospores except the strain S2 (Table 2, Figure 4).

5.3 Biochemical Analysis of isolate bacteria:

Biochemical test was performed for isolates and results are tabulated in Table 3. In hydrogen sulfide production test, the bacterial strain S1, S4, S9, S10 showed production of precipitates which indicate H_2S production and S2, S3, S5, S6, S7, S8 showed negative result (Table 4, Figure 5).

Gelatin liquification test is done to identify strains that produce extracellular proteolytic enzyme (Gelatinase). In Gelatin liquification test, strain S4, S10 did not liquefy gelatin and the bacteria strain S1, S2, S3, S5, S6, S7, S8 and S9 showed positive result, showing that they produce gelatinase enzyme (Table 4, Figure 6).

Nitrate reduction test is done to identify bacterial strains that reduce nitrate to nitrite. The strains, S1, S2, S3, S4, S5, S8, S9, and S10 showed positive result by formation of a reddish pink colour (Table 4, Figure 7).

Citrate test is done to identify the bacterial strains that can utilize citrate. In citrate test, S3, S4, S5, S6, S8, S10 bacterial strain produced blue colour on sodium citrate medium, which shows that the strains can utilize citrate. The strains S1, S2, S7 and S9 strain showed negative result (Table 4, Figure 8).

Catalase test is done to identify strains that produce the catalase enzyme. In Catalase test, S1, S4 and S7 did not produce air bubbles which indicate that they do not produce the catalase enzyme. The strains S2, S3, S5, S6, S8, S9, S10 are positive for catalase test (Table 4, Figure 9).

5.4 Analysis of bacterial isolated for their plant growth promoting traits:

The result of bacteria isolates for plant growth promoting traits were tabulated in Table 4

5.4.1 Production of Ammonia:

Ammonia production by microbes is an important aspect of plant growth promoting trait of bacteria. Qualitative analysis of ammonia production was studied. The strains S1, S2, S4, S5, S6, S8 and S9 produced ammonia whereas the strains S3, S7 and S10 showed negative results (Figure 10) without changing a colour.

5.4.2 Production of Indole acetic acid:

IAA production was found to be a common trait in all isolates. All isolates were positive for IAA production that shows pink to red colour in the top layer (Figure 11).

5.4.3 Ability of nitrogen fixation:

Nitrogen fixing bacteria have the unique ability to grow on Berku's medium. Based on the result all strains except S3, S4, S8 grew on Berku's medium which indicates that they have the ability to fix nitrogen (Figure 12).

5.4.4 Production of phosphate:

Phosphate solubilization ability of bacteria can be detected by culturing the isolates on potato dextrose rose Bengal agar plate method (Figure 13). Growth on this medium confirms their phosphate solubilization activity. The strains S3, S4, S5, S6, S8 and S10 grew on the potato dextrose Rose Bengal Agar, which shows that they can solubilize phosphate.

5.5Analysis of bacterial isolated for their plant biocontrol properties:

The result of bacterial isolates for their plant biocontrol properties are tabulated in Table 5.

5.5.1 Screening for hydrogen cyanide (HCN) production:

Hydrogen cyanide production of the bacterial strains indicates its biocontrol activity. The test for HCN production showed that except the strain S4 all other strains showed a positive colour change of the filter paper from deep orange to reddish brown, which indicated that all the strains except S4 are good biocontrol

agents. The bacterial strains S1, S2, S3, S5, S6, S7, S8, S9, S10 indicated the production of HCN (Figure 14)

5.5.2 Hydrolytic enzyme production:

In protease test, the strain S2, S3, S5, S6, S9, S10 showed a zone of clearance around the growth area, which indicates positive result. The bacterial strains S1, S4, S7, S8 did not produce hydrolytic enzyme production (Figure 15).

In starch hydrolysis test, the strain S4 and S10 did not show clear zone outside the growth area, which indicated that they do not have the ability to hydrolyze starch. A zone of clearance around the growth of the strains S1, S2, S3, S5, S6, S7, S8, S9 indicates that they have the ability to hydrolyze starch (Figure 16).

5.6 Seed germination Assay:

The application of PGPR strain should promote shoot and root growth. In this study, application of bacterial strain supported higher germination rate and other growth parameters. The shoot (height and weight), and root (height and weight) parameters was compared between the control and treated seeds and are tabulated (Table 6, figure17).

In all the pots where *Vigna radiata* seeds treated with bacteria were sown, there was a significant increase in growth rate when compared to control untreated

seeds. The highest germination percentage was observed in pots where the strain S5 was used for seed treatment. The higher plant growth promoting activity of the strain can be correlated with its higher IAA and phosphate solubilization activity in the biochemical assays.

6. DISCUSSION

PGPR are free living soil bacteria that aggressively colonize the plant roots and when applied to the seeds they enhance the growth and yield of the plants. The use of novel PGP bacteria as biofertilizers, biopesticides and phytostimulator in agricultural sectors to improve crop yield, quality and maintaining the soil fertility is advisable. The present study is carried out to characterize the potential of rhizobacteria to support the growth of plants by its direct and indirect mechanisms.

The exact mechanism by which PGPR stimulate plant growth is not clearly known, although several mechanisms such as production of phytohormones, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved in plant growth promotion. There are many papers related to the advantages and screening of PGPR from crop plants but few on *Avicenia marina.* In this study, about 10 rhizobacterial strains were isolated from *Avicenia marina* (Mangrove Plant) rhizosphere samples and screened for different plant growth promoting traits and biocontrol properties. A total of 10 bacterial colonies showed diverse morphological characteristics as indicated from variation in shape, colour, margin elevation and opacity. On the basis of their gram reaction all of the isolates were found to be gram negative.

Ammonia production is an important characteristic of PGPR, which influence plants growth (Yadav *et al.,* 2010). In *P.fuscovaginae* and *K.oxytoca* 4.5% of the

isolates were able to produce ammonia and enhance plant growth. The presence of ammonia producing PGP bacteria is an indication for ammonification process which takes place in the rhizosphere than non rhizosphere soil. In this study, the bacterial isolates S4, S5 and S9 showed high production of ammonia which attributes to its plant growth promoting activity.

IAA is one of the most important phytohormone and function as important signal molecules in the regulation of plant development. It has been reported that IAA production by PGPR can vary among different species and strains and also is influenced by culture conditions, growth stage and substrate availability (Mirza *et al.,* 2001). Higher level of IAA production in PGPR has been recorded by other research workers earlier (Xie, H. 1996). In our study, all the isolated were positive for IAA production.

Nitrogen fixing ability is an important criterion for the selection of potential PGPR. In this study, the PGP bacterial colonies S3, S4 and S5 isolated from *Avicenia marina* plant rhizosphere grew well on N-free agar medium, which confirmed their potential for fixing atmospheric nitrogen on such media. Our result was supported by the findings of Naher (2009) who characterized a few N-fixing bacteria by acetylene reduction assay (ARA).

The capability of rhizobacteria to solubilize insoluble phosphate has been of interest to agriculture microbiologist as it can enhance the availability of phosphorous for the plant to improve plant growth and yield (Liu, 2016). It has been reported that higher concentration of phosphate solubilizing bacteria are commonly found in the rhizosphere as compared to bulk soil (Dasgupta, 2015). The use of phosphate solubilizing PGPR as inoculants is one of the alternative biotechnological solutions in sustainable agriculture to meet thee phosphate demands of plant. In this study the isolates S3, S4, S5 and S10 isolates were able to solubilize phosphate at higher rates when compared to other isolates. All of the above identified PGP bacterial strains were found to be the most efficient phosphate solubilizers, which have a great role in increasing crops productivity and production without contaminating the environment and affecting human health.

HCN production by rhizobacteria has been postulated to play an important role in the biological control of pathogens (Voisard, 1989). In this study, 80% of the bacterial isolates were positive for HCN production which acts as an inducer of plant resistance. Several factors have been reported to influence the rate of HCN production. HCN secreted by *P.fluorescent* strain CHAO has been demonstrated to stimulate root hair formation and suppress back root rot caused by Thielonopsis basicola in tobacco plant. Indeed the hydrogen cyanide is part of powerful antifungal

compounds produced by PGPR and involved in pathogens biological control (Haas *et al.,* 2005).

Hydrolytic enzymes act as agents for prevention of plant diseases by causing lysis of pathogenic microbes in the close vicinity of the plant as they secrete increased level of cell wall lytic enzymes like chitinase, amylase and proteases (Tsegaye, 2015). In this study, the production of proteases activity in S3 and S5 is high when compared to other bacterial strain of starch hydrolysis. PGPR that synthesize one or more of these lytic enzymes has been found to have biocontrol ability against a range of plant pathogenic fungi and bacteria and enhance crop yield.

In this present study, the isolated bacterial strain S5 was found to be most efficient PGPR which solubilised phosphate, produced IAA, produced ammonia, produced HCN, produced catalase and showed hydrolytic enzymes activity. The strain can be further used as a biofertilizer and a bioconrol agent. Such type of study is necessary as it advocates that use of PGPR as inoculants or biofertilizers is an efficient approach to replace chemical fertilizer.

7. SUMMARY

- Ten bacterial strains were isolated from rhizospheric soil and characterized for cellular morphology using light microscope. All the bacteria were a gram negative. They colony morphology was diverse - circular, filamentous, rhizoid and some are irregular too.
- Biochemical tests for H_2S production, catalase test, nitrate reduction, gelatin test were performed, to identify the bacterial strains.
- For analysis of direct growth promoting activity of the strains, following tests were performed- production of NH_3, production of IAA , nitrogen fixation, phosphate solubilization. Many of the strains isolated were positive for the above mentioned tests.
- Indirect (or) plant biocontrol properties of PGPR were also tested by checking for production of HCN and hydrolytic enzymes. Most of the strains isolated exhibited indirect plant growth promoting activities.
- To confirm the PGPR activity, plant assay was done by inoculating *Vigna radiate* seeds with three PGPR strains isolated (S3, S4, and S5),and plant growth parameters were compared between treated and untreated seeds. The seeds treated with bacteria showed significant higher growth rate (maximum in S5) than control.

8. CONCLUSION AND SUGGESTIONS

The research met our objective in identifying plant growth promoting bacterial isolates from the rhizosphere of *Avicennia marina.* The study provided promising resultsworth exploring in further studies to develop bio-fertilizer with good ability to solubilize phosphate, production of hydrolytic enzyme and production of IAA. Isolates with good plant growth promoting ability was characterized and the best efficient strains were identified from among all the isolated strains. The potential of the strain for application in plant growth promoting activity was investigated in detail. One strain (S5), showed a very good growth promoting and biocontrol activity.

Suggestions for future

The identification of the bacterial genera has to be done by 16sr DNA sequencing. In future the strain S5 can be taken for further analysis. The identified strains can be made as biofomulation and taken for field application which would be beneficial from crop improvement and crop production.

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Figure 1: Isolation of pure cultures of the bacterial strains

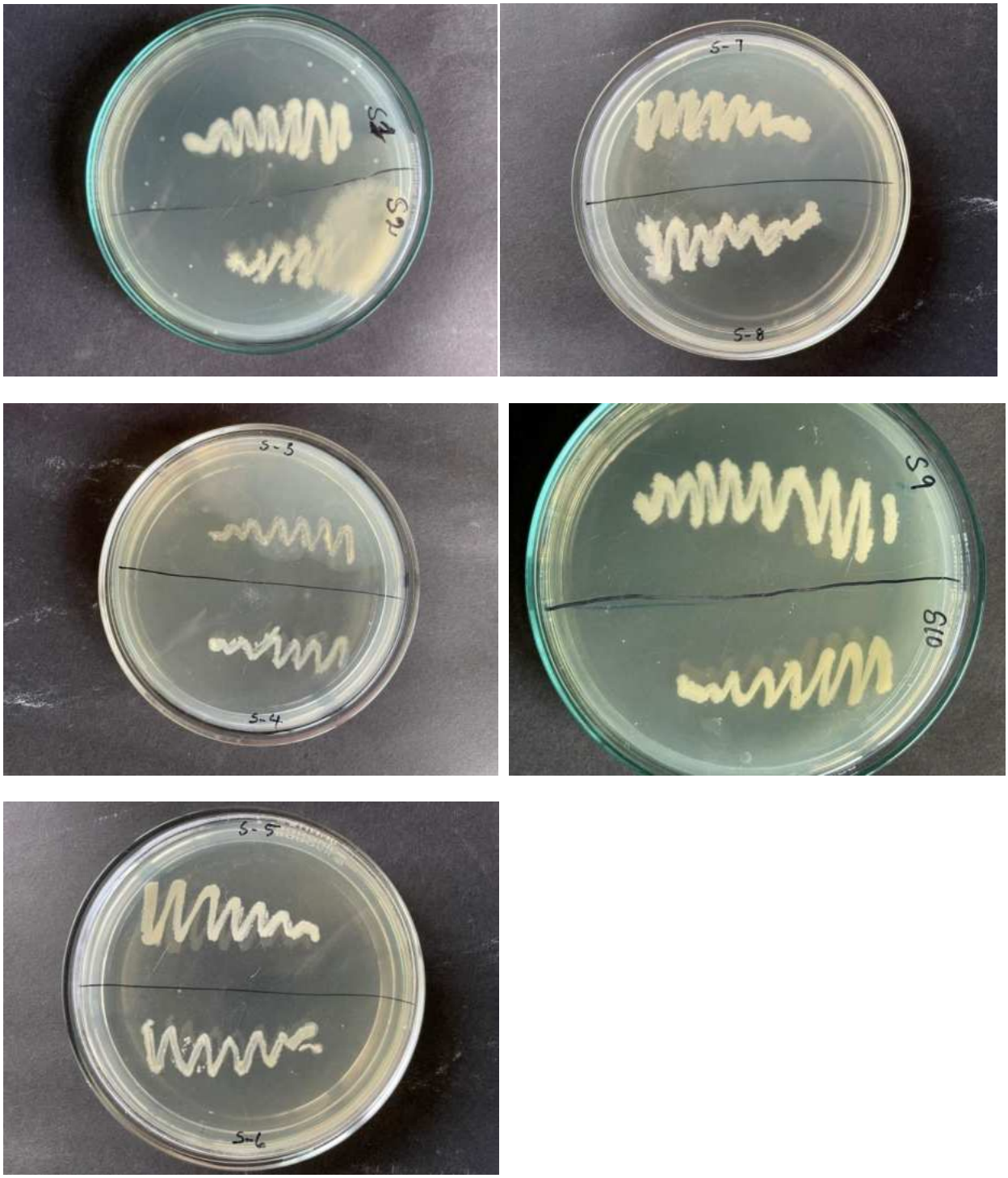
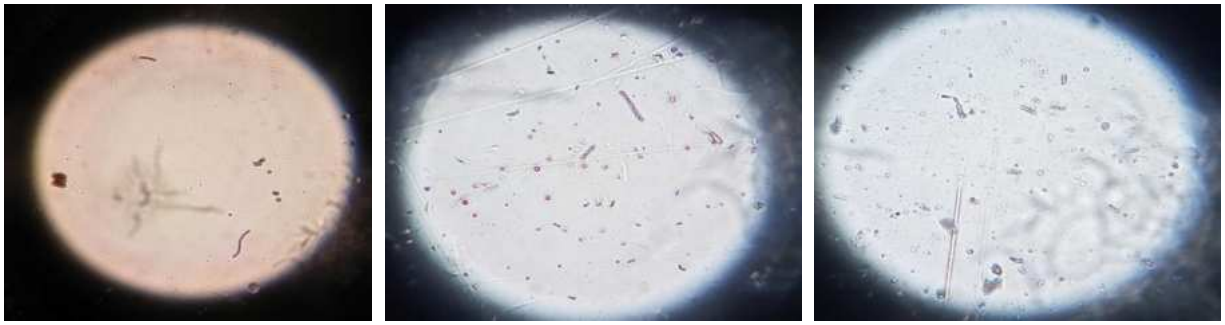


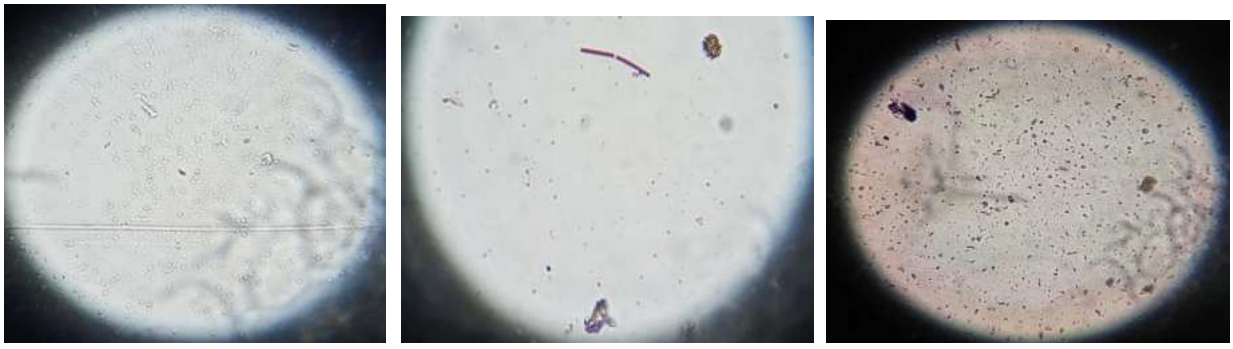
Figure 2: Gram staining of isolated bacterial strains

Strain 1 Strain 2 Strain 3

Strain 4 Strain 5 Strain 6



Strain 7 Strain 8 Strain 9



Strain 10

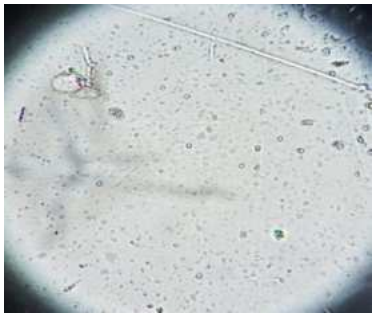
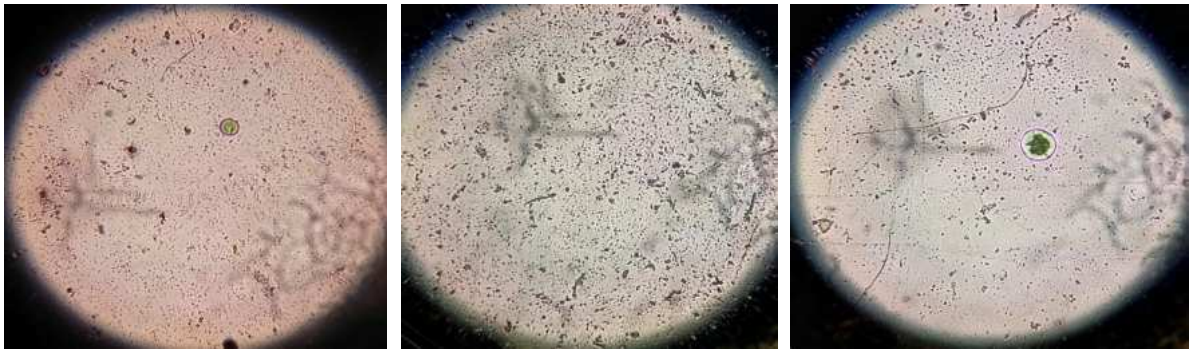


Figure 3– Endospore staining of isolated bacterial strains

Strain 1 Strain 2 Strain 3



Strain 4 Strain 5 Strain 6

Strain 7 Strain 8 Strain 9

Strain 10

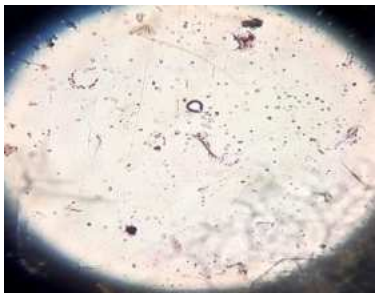


Figure 4- Hydrogen sulphide production of the bacterial strains

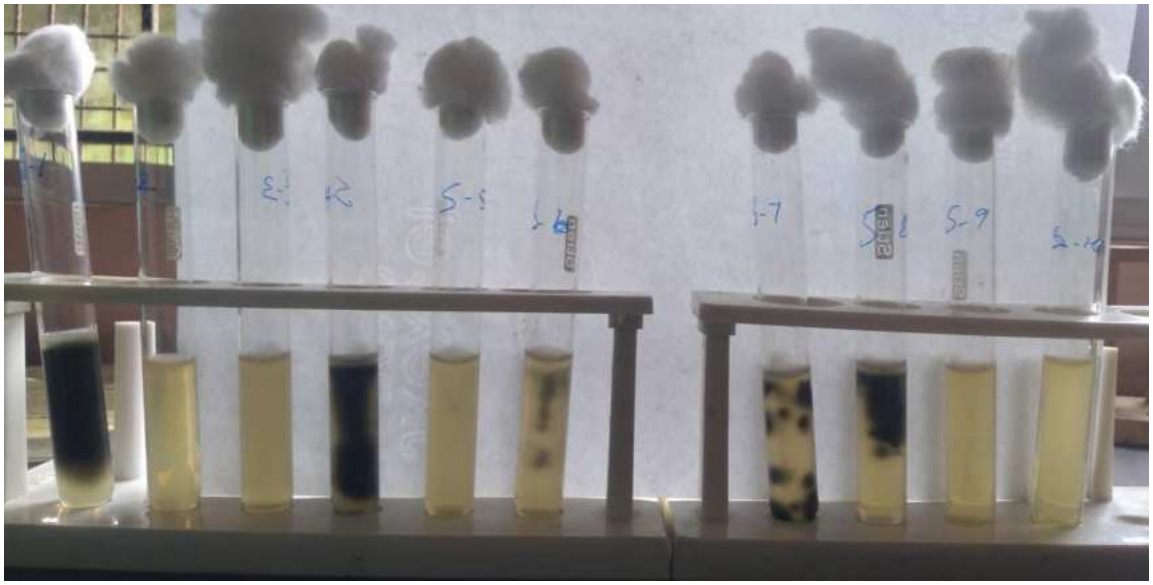


Figure 5- Gelatin liquification test for the bacterial isolates k

Figure 6- Nitrate reduction test for the bacterial isolates.

Figure 7- Citrate tests of the bacterial strains

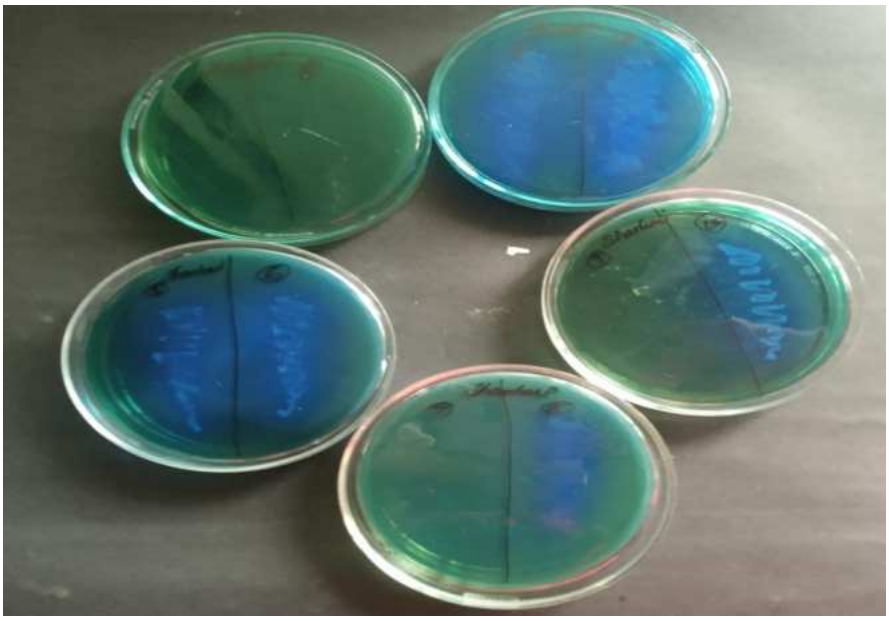


Figure 8- Catalase test for the isolated bacterial strains

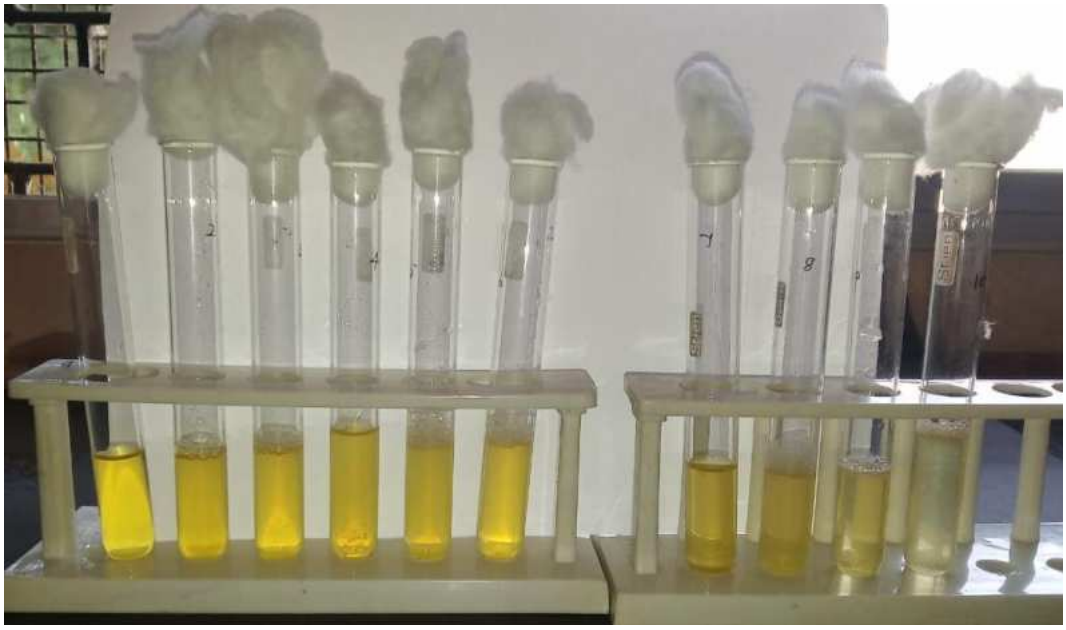


Figure 9- Ammonia production of the bacterial isolates

Figure 10- IAA production of the bacterial isolates.

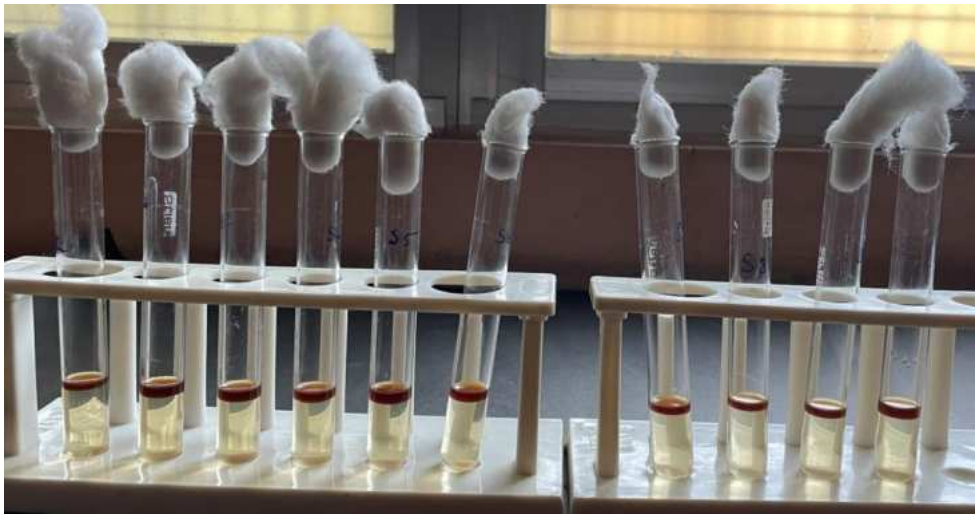
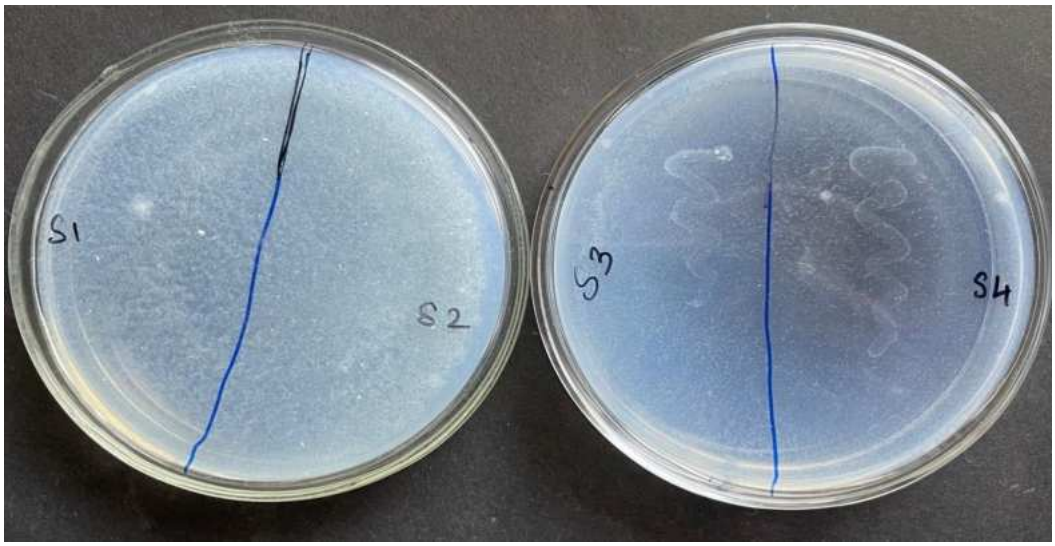


Figure 11- Nitrogen fixation test for the bacterial strains



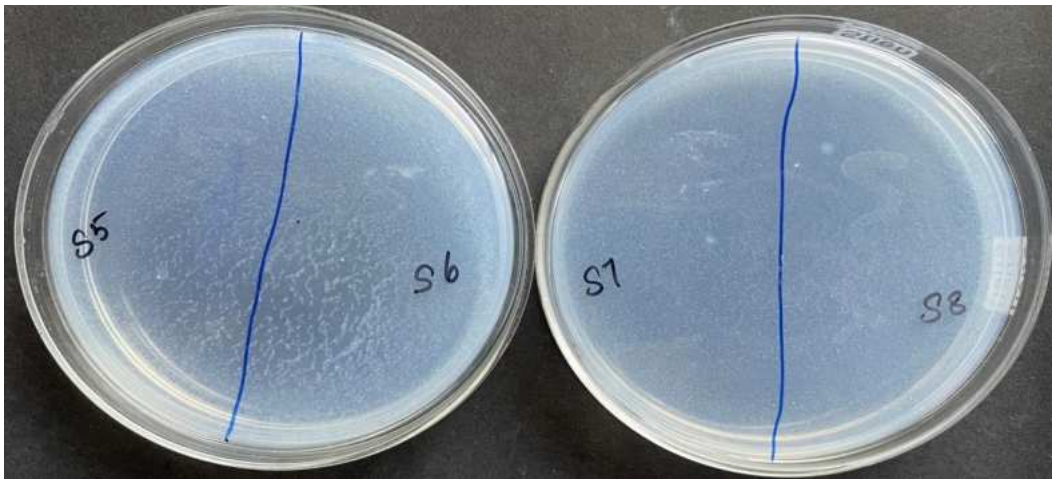


Figure 12- Phosphate solubilization of the bacterial strains

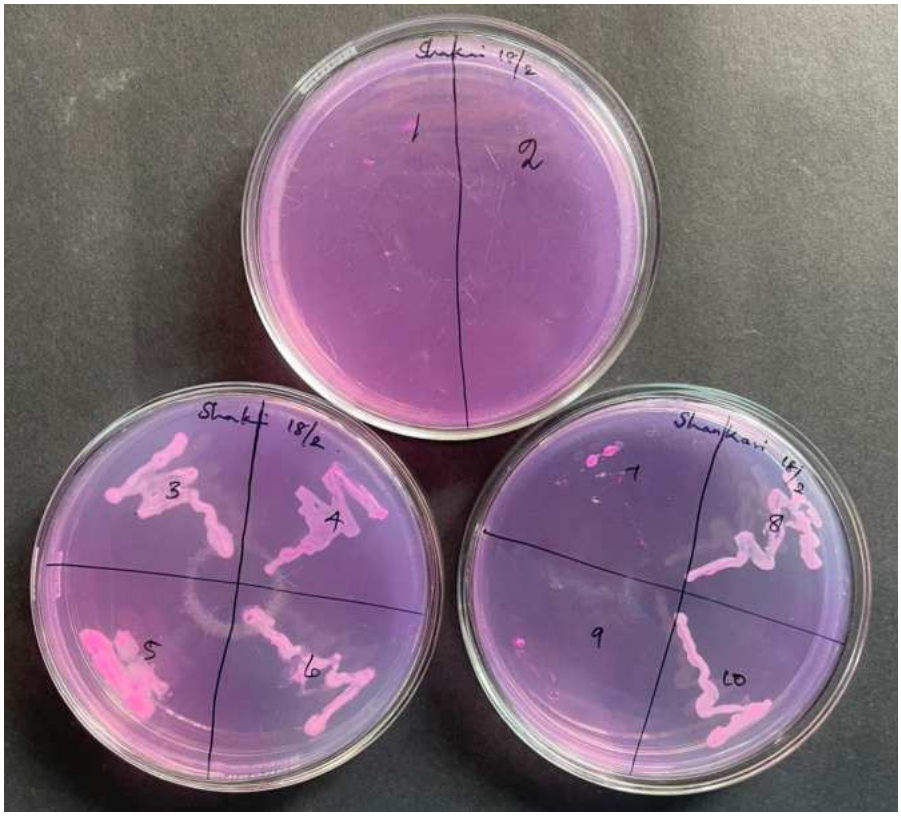


Figure 13 – Hydrogen cyanide production of the bacterial strains

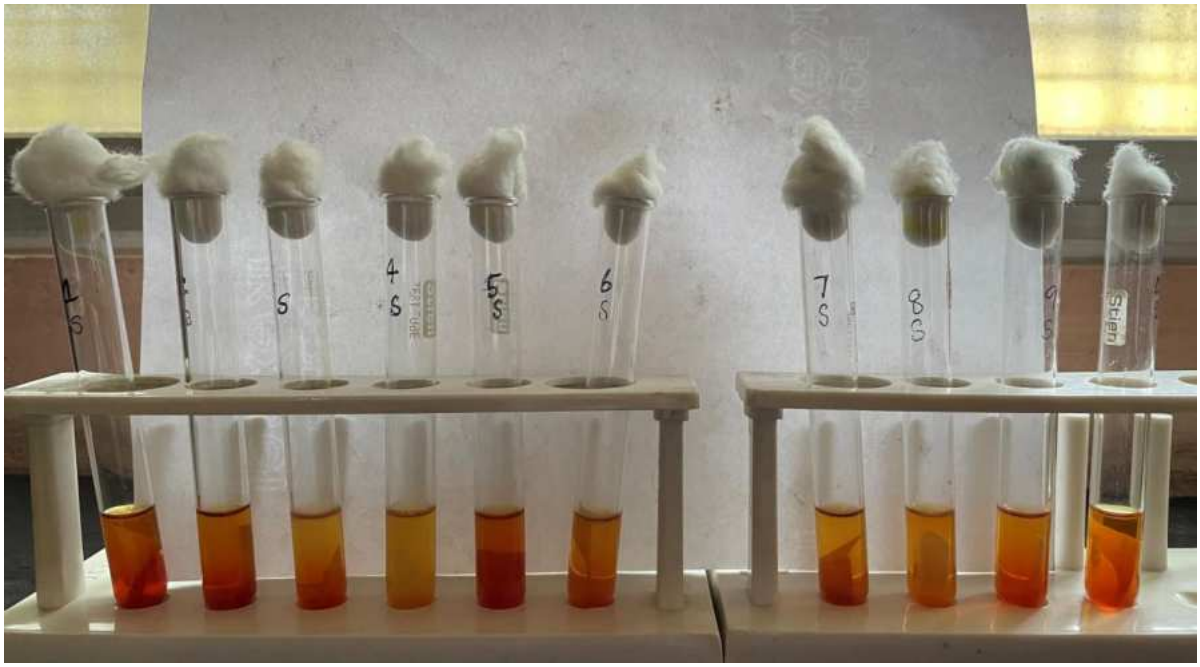


Figure 14- Protease production test of the bacterial strains

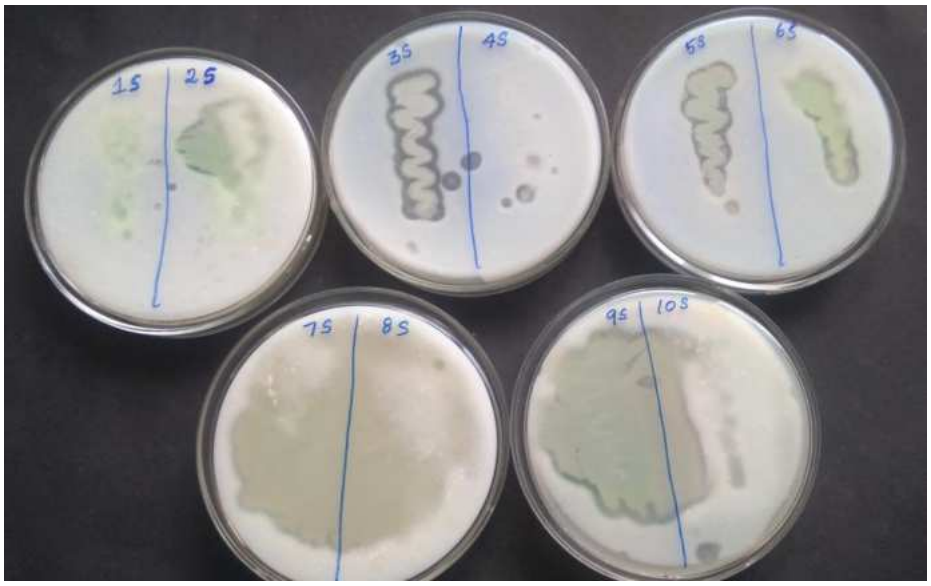


Figure 15- Starch hydrolysis test for the bacterial isolates

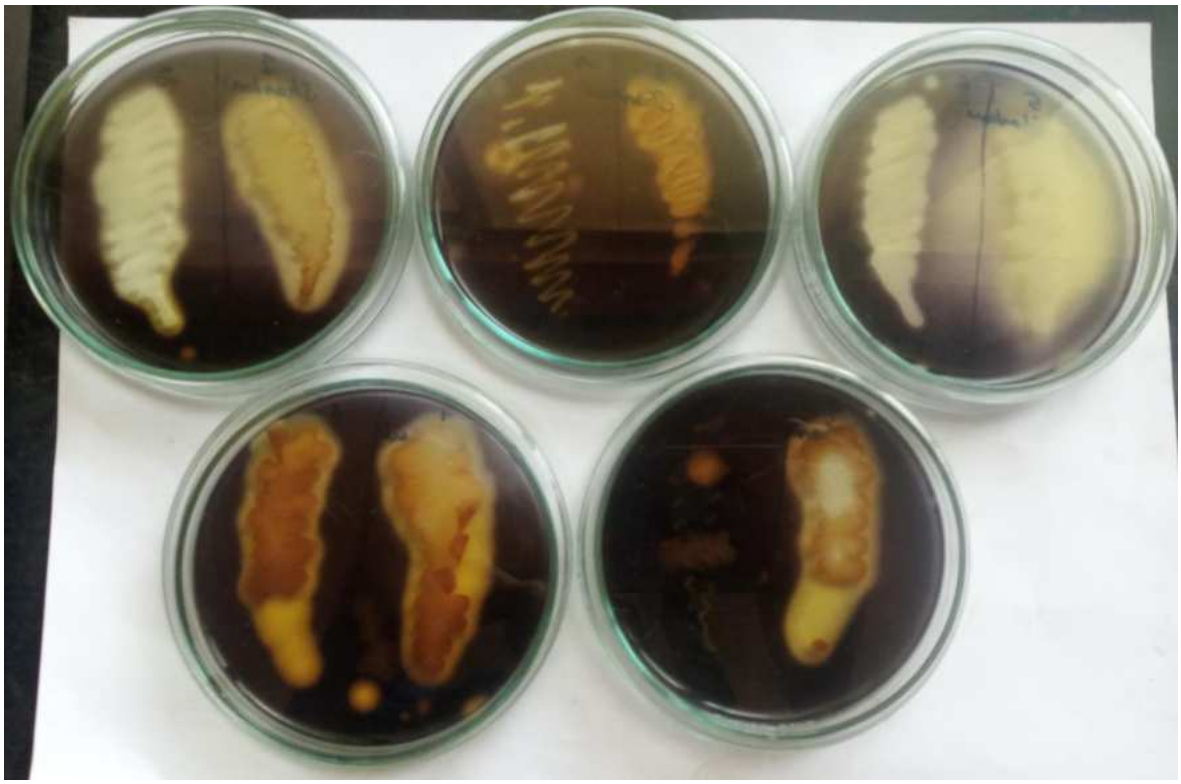


Figure 16: *Vigna radiata* plants treated with the bacterial isolates

COMPARATIVE STUDY ON THE NUTRITIONAL COMPOSITION AND ACCUMULATION OF HEAVY METALS IN GIANT TIGER PRAWN (*Penaeus monodon)* AND GREEN TIGER SHRIMP (*Penaeus semisulcatus)* IN NEW HARBOUR COAST, THOOTHUKUDI, GULF OF MANNAR

Dissertation submitted to

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By

S. REVATHI

Reg. No: 19APZO06

DEPARTMENT OF ZOOLOGY

ST. MARY'S COLLEGE (AUTONOMOUS)

(Re-accredited with A^+ Grade by NAAC)

THOOTHUKUDI

APRIL – 2021

CERTIFICATE

This is to certify that this dissertation entitled **"COMPARATIVE STUDY ON THE NUTRITIONAL COMPOSITION AND ACCUMULATION OF HEAVY METALS IN GIANT TIGER PRAWN (*Penaeus monodon*) AND GREEN TIGER SHRIMP (*Penaeus semisulcatus*) IN NEW HARBOUR COAST, THOOTHUKUDI, GULF OF MANNAR"** submitted by **S.REVATHI, Reg.No.19APZO06** to St. Mary's College (Autonomous), Thoothukudi, affiliated to Manonmaniam Sundaranar University in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2020-2021 under my guidance and supervision. It is further certified that the dissertation or any part of this has not been submitted elsewhere for any other degree.

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Examiner

DECLARATION

I do hereby declare that this thesis entitled **"COMPARATIVE STUDY ON THE NUTRITIONAL COMPOSITION AND ACCUMULATION OF HEAVY METALS IN GIANT TIGER PRAWN (*Penaeus monodon*) AND GREEN TIGER SHRIMP (*Penaeus semisulcatus*) IN NEW HARBOUR COAST, THOOTHUKUDI, GULF OF MANNAR"** submitted by me for the award of the degree of Master of Science in Zoology is the result of my original and independent research work carried out under the guidance of **Dr. Mrs. S.R.T. SHERLY CROSS M.Sc., B.ED., M.Phil., PhD.,** Assistant Professor, Department of Zoology, St.Mary's College (Autonomous), Thoothukudi, and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date 15.04.2021

S. Revathi
Signature of Candidate

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INTRODUCTION

1. INTRODUCTION

Prawn, a crustacean which is commercially known as shrimp due to its exquisite taste is supposed to be a cherished delicacy through the world. There are different varieties of prawns, inhabiting the sea and fresh water bodies like lakes, rivers and estuaries but the bulk production comes from the sea.

Shrimp is an excellent source of protein and it is one of the most popular species of every nations traditional meat, superior to meat and poultry, having high quality of body composition including proteins, fats and amino acids etc. which are the indicators of the existence of good physical and biomedical conditions they are highly nutritive due to the presence of highly essential omega-3and omega-3 and omega-6, fatty acids (oksuz *et al.,* 2009 and Hulya turan *et al.,*2011)

The proximate composition and biochemical constituents were analysed in the muscle tissue of Penaeid prawns selected in the present investigation namely *Peaneus monodon, Penaeus indicus, Penaeus semisulcatus, Litopenaeus vannamei, Metapenaeus monoceros* and *M. dobsoni.* The crude protein, carbohydrate, lipid, moisture, ash, free amino acids, free

fatty acids including Saturated, Mono-Unsaturated and Poly-Unsaturate Fatty acids quantified in the muscle tissue of Penaeid Prawns clearly demonstrates the nutritional status of edible Portion of prawns.

Shrimp are the group of popular sea foods found worldwide, belonging to the Decopoda of the crustacean. As the world population is increasing, the per captia consumption of sea foods is also increasing rapidly due to its nutritional superiority than all other sources of food. The global market for shrimps and prawns is ever on the increase largely because of high consumption rate (Gillett, 2008)

Shrimp are group of popular sea food found worldwide, belonging to the order Decapoda of the class crustacean there are about 8,500 species of decapods (Hulya *et al.,* 2011)including 2,000 shrimp species found and approximately 300 species are of commercial importance .

The nutritional properties of fishes and shellfish are valuable food stuffs for human health (Usydus 2011) Fish and shellfish as a whole has a lot of food potential and can therefore provide relief from malnutrition, especially in the country like Bangaladesh. It provides superior quality protein to that of meat, milk, and eggs. (Haruna 2003).

The nutritive requirements of *penaeus japonicas* have been manifested by the introduction of refined test diets by Kanazawa *et al.,* (1970) and Deshimaru (1974) and the prawn has been shown to necessitate adequate levels of proteins, lipids, carbohydrates, minerals and vitamins as to other aquatic animals.

The nutritive values of edible marine organisms depend upon their biochemical composition, such as protein, amino acids, lipid, fatty acids, carbohydrate, vitamins and minerals. Protein is essential for the substances of life and accordingly exists in the largest quantity of all nutrients as a component of the human body. (Sudhakar M *et al.,* 2009).

The *Penaeus monodon* and *Macrobrachium rosenbergii* are available in our country having high market value. However, consumers do not know the exact nutritive value of these species that play a significant role in our body. In addition, several studies have outlined various differences in nutritional composition between cultured and wild fish and shellfish, where not cultured are deemed of lesser quality. Zhou L *et al.,* (2014)

Several studies are available on the nutrient values of shrimps. Nandanan.S (2017) Therefore, the objective of the present work was to evaluate the proximate composition of basic biochemical constituents, such as total

protein, lipids, carbohydrates, water content and protein electrophoresis as well as amino acids and fatty acids analysis in the edible muscles in both sexes of green tiger prawn, *P. semisulcatus* India.

The nutritive values of crustaceans depend upon their biochemical composition, such as protein, amino acids, lipids, fatty acids, carbohydrates, vitamins and minerals. Among the Seafood, Prawns and Shrimps contribute about 20-22% by volume of the world Seafood market. FAO. World Aquaculture and Fisheries Statistics. FAO Publication (2014) due, to their nutritious nature, apart from the supply of good quality proteins, lipids, they also contain several dietary minerals such as Calcium, Iron etc., which are beneficial and essential and play an important role in maintenance of Physiological and Biochemical activities in human beings.

Heavy metal is used widely in modern industries. Heavy metals including essential and non- essential elements have a particular significance in ecotoxicology since they are highly persistent, and all have the potential to be toxic living organisms. (Gipson and Basker 1979).

The continuous utilization of the shrimp is, however, a function of their heavy metal concentration as well as the health and ecologicalrisk they pose to human population and aquatic ecosystems. (Abubakar *et al.,* 2015).

In the marine environment, heavy metal is potentially accumulated in sediments and marine organisms and is subsequently transferred to the next tropic level of the food chain (Pourang et al., 2005).

Crustaceans have been used successfully as biological indication of coastal pollution and in the assessment of the influence of heavy metals on the marine environment. Marine crustacean, including shrimp can be employed as bio indicator to assess the marine environment, since they can accumulated heavy metals and other pollutants. (Darmono Denton 1990;Kress et al., 1998, Mantellata et al., 1999).

Heavy metals contamination of the environment which has been recognized as serious pollution problems is capable of exerting considerable biological effects even at low levels because of their pervasiveness and persistence nature (Singh and Chandel, 2006).

Accumulated heavy metals in the tissues of aquatic animals become toxic when accumulation reaches a substantially high level (Yildirim *et al.,* 2009).

According to Qasimet et al., (1988), while Mn, Cu, Fe, and Zn are considered as essential micronutrients, mercury, cadmium and lead are not required for any important biological functions of organisms. As such, these

heavy metals, if found in abnormal concentrations in salinity, lead to thyroid, liver damage and other harmful effects in consumes. (Munoz *et al.,* 2005).

Ganesan and kannan (1995) showed higher concentration of Fe and Mn in the sea water, sediment and algae in the vicinity of Tuticorin. Port. Palanisamy and Rajendran (2000) indicated high concentration of Cd, and Pb in the bottom waters than the surface waters off Tuticorin. Satyanarayanan and Murty (1990) showed relatively higher concentration of trace metals and nutrients concentration of trace metals and nutrients in low saline inshore waters, but lower with high salinity in the offshore waters of Bay of Bengal. Baskaran et al., (2002) observed relatively higher concentration of Fe, Cu, Zn, and Al in the fly ash dumping area than in the deeper waters off Tuticorin.

The aim of our research is to compare the nutritional quality of the selected shrimp species (*Penaeus monodon, Penaeus semisulcatus*) at New Harbour coast, Thoothukudi and assess the accumulation of heavy metal in their body tissues to indicate that they are a healthy choice of feed for human consumption.

OBJECTIVES

2. OBJECTIVES

- ✓ To analysis the physic-chemical parameters of the sea water in New Harbour Coast, Thoothukudi, Gulf of Mannar.
- ✓ To evaluate the proximal values of selected bioindicators such as *Penaeus monodon* and *Penaeus semisulcatus*.
- ✓ To determine the concentration of heavy metals such as, copper, Lead, Zinc, Magnesium in the *Penaeus monodon*, *Penaues semisulcatus* from New Harbour Coast, Thoothukudi, Gulf of Mannar.
- ✓ To analysis the ecological characters of *Penaeus monodon*, *Penaeus semisulcatus* from New Harbour Coast, Thoothukudi, Gulf of Mannar.

REVIEW OF LITERATURE

3. REVIEW OF LITERATURE

De Grave & Fransen (2011) studied the Cardeorum catalogus of the recent species of the Dendrobranchite, Stenopodidean, procardidean and Cardidean Shrimps. Radhakrishnan *et al.,* (2012) was reported the prawn fauna (Crustacean: Decopoda) of India an announced checklist of the penoid, Sergestoid, Stenopodid and Caridea Prawns.

Naylor *et al.,* Minchin (2001, 2007) observed the economic importance of many Crustaceans has led to their deliberation introduction into several of many countries for commercial farming.

Ruiz *et al.,* (2000) observed that a number of characteristics have contributed to the success of these animals, including their small size, especially in the juvenile stages, and their exoskeleton which protects from damage during transportation and any other potential sources of physical injury. Chanda & Bhattacharya (2002) reported that *Melicertus similis,* an new species of prawn Decapoda:Penedae from Indian coast.

Kannan D.k Jagadeesan *et al.,* (2014) explained the maturation and spawning of commercially important penaoid Shrimp *Penaeus monodon.* Motoh (1981) studied on the fisheries biology the Giant Tiger Prawn, *Penaeus monodon* in Philpipines.

Abulude *et al., (*2006) observed prawns contain good amount of organic and inorganic constituents. The main constituents are protein, carbohydrate and lipid. In addition to that prawns also contain a significant proportion of minerals (Ca, P, Mg, Mn and Cl) and vitamins (A, C and D).

Devi *et al.,* (2010), Studied the discharge from chemical, paper, pharmaceutical and food product based industries is considered as chief source metals such as Pb, Cr, Cu, Zn, Ni, Hg and Cd into the water sources .

Sandoval *et al.,* (2014) explained the spatial distribution and abundance of the Giant Tiger Prawn, *Penaeus monodon* (Fabricus 1789) in the Gulf of Urba (Caribbean), Colombia, South America.

Grave & Anker (2008) studied the Global diversity of Shrimp's m Crustacean Decopod : Caridea in freshwater.

Jayachandran (2002) studied palaemonoid prawns: Biodiversity, taxonomy, biology and management.

Jayachandran (2005) studied the biodiversity Palaemonidae Prawns from India seas (in Hindi).

Jayachandran *et al.,* (2010) studied the Indian Palaemonidae Prawns Decopods Crustaceans its taxonomic status, research challengers and the conservations needed.

Kathirvel *et al.,* (2007) studied the Indian Penaeid Shrimps their biodiversity and economical values.

Nandhakumar *et al.,* (2007) studied the observation on the Prawn fishery of Sakthikulangara in the high of monsoon trawling ban.

Bat., L Bilgin, *et al.,* (2001) reported the individual and combined effects of copper and lead on the marine Shrimp Palaemon Adspersces Rathke, 1837 (Decapoda: palaemonidae).

Yilmaz, (2007) identified the influences of sex and seasons on levels of heavy metals in tissue of Green Tiger Shrimp (*Penaeus semisulcatus,* 1844).

Huang *et al.,* (2008) identified the histopathological and biochemical evidence of hepatopancreatic toxicity caused by cadmium and Zinc in the white Shrimp, *Liptopenaeus vannamei* Chemos.

Cogun *et al.,* (2005) reported the seasonal variations and tissue distribution of heavy metals in Shrimp and Fish species from the Yumurtalik coast of Iskenderum Gulf.

Pouring *et al.,* (2005) studied the distribution of heavy metals in *Penaeus semisulcatus* from Perisin Gulf and possible role of metallothioneon in their redistribution during storage.

Celik and Yanar *et al.,* (2004) identified the seasonal changes in total carotenoid contents of Wild Marine Mediterranean.

Mantle analysed the nutrition, physiology and metabolism in Crustacean Book Science Publishers.

Ashraj, Vosylience Jankaite, Farombi *et al.,* (2005 to 2007) reported that the heavy metals contamination may have devasting effect on the ecological balance of the recipient environmental and a diversity of aquatic organisms.

Sallam *et al.,* (2006) reported the biochemical composition and heavy metal accumulation in some commercial Crustaceans from the Mediterranean Coast.

Shahira banu *et al*., (2016) evaluated the nutritional status of Penaeid Prawns through proximal composition studies.

Rosa and Nunes (2003) studied the biochemical composition of deep sea Decapods Crustacean with two different benthic life strategies of the portruguese South Coast of Deep Sea.

Tajul and yang *et al.,* (2013) analysed the biochemical and texture property changes during molting process of Giant Tiger Prawns, *Paenaeus monodon.*

Abdul Salam (2013) reported the evaluation of nutritive quality of commercially cultured Indian White Shrimp, *penaeus indicus.*

Tsape *et al.,* (2010) analysed the comparative of the fatty acids and steroid profile of widely consumed Mediterranean Crustacean species.

Yanar and Celick. (2006) identified the seasonal amino acids profiles and minerals contents of Green Tiger Shrimp *(Penaeus semisulcatus* De haan, 1844) and speckled Shrimp (*Metapenaeus Monoceros* Fabricus, 1789) from the Eastern Mediterranean.

Oksuz, *et al.,* (2009) studied the comparative study on proximate mineral and fatty acids composition of Deep Sea Water Rose Shrimp *(Parapenaeus longirostris*, and Red Shrimp.

STUDY AREA

4. STUDYAREA

NEW HARBOUR COAST, THOOTHUKUDI, GULF OF MANNAR

Tuticorin is located on the central region of Indian Coast of Gulf of Mannar. Along the Tuticorin Coast, New Harbour landing centre has been selected for the present study. The Harbour of Tuticorin is well known as a pearl diving centre. The port was used for export of salt, cotton, yarn, senna leaves, dry fishes, and other goods to neighbouring countries and for import of coal, pulses and grains. Tuticorin is a port town situated in the Gulf of Mannar about 125km. About 36,000 species of flora and fauna exist in the region covered with mangroves, sandy shores, sea grass beds that are conductive for turtle nesting.

Harbour is one of the 13 major ports of India. It was declared a major port on 11 july 1974.It is second largest port in Tamil Nadu and fourth largest container terminal of India.

The major industries are fishing, shipping, agricultural, power and chemical industries. The fishing harbour is one of the oldest and largest of Tamil Nadu. In addition to this there are several private power plants Coastal Energen, sterlite Industries. Heavy water board plant, Nila sea foods. Tuticorin

Alkali Chemicals are some small scale and large scale industries in the city. Air pollution is the major environmental challenge faced by Tuticorin while water and noise pollution are major issues.

Tuticorin has the second highest Human Development index in Tamil Nadu next to Chennai. Tuticorin an "Emerging Energy and Industrial hub of South India".

MATERIALS AND METHODS

5. MATERIALS AND METHODS

The quality of marine water was evaluated by the determination of the following physicochemical parameters:

PARAMATERS	METHOD OF ANALYSIS
Temperature	Thermometer
pH	Ph meter
Salinity	Hand refractometer
DO	Winkler's method
BOD	BOD analyser bottle
Alkalinity	Spectrophotometer
Ammonia	Spectrophotometer
Phosphate	Spectrophotometer
Silicate	Spectrophotometer

Proximal composition of studied shrimps was estimated by following method:

Estimation of protein:

The protein content of fresh tissue was estimated using following method of lowry et al., (1951).

10mg fresh tissue was taken and homogenized with 5 ml or 10% Trichloroacetic acid in a homogenizer. The homogenates were centrifuged for 10 minutes at 3000 rpm. The supernatant was disclared. The precipitate was dissolved thoroughly in 5ml of 0.1n sodium hydroxide solution and kept in a water bath at 60^0 c and 70^0 c for 10 minutes. From the solution 0.5ml was pipette out and poured in a clean dry test tube.

To this 4 ml of copper solution was added. It was mixed well by lateral shaking and kept in room temperature for 10 minutes. To this 0.4ml of folin phenol reagent was added.

The test tube was shaken well for uniform mixing and kept in room temperature for another 30 minutes. The blue colour appeared at 640nm against a reagent blank in UV spectrophotometer. The standard curve obtained using Bovine serum albumin expressed the protein content in mg/g.

$$\% \text{ of protein} = \frac{\text{Standard value x OD value}}{\text{Weight of tissues}} \times 100$$

Estimation of carbohydrate:

Carbohydrate was estimated by anthrone method. 10 mg of fresh tissue sample was homogenized in 2 ml of 10% TCA and 8 ml of distilled water. The homogenate was centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and measured and it was used for the analysis of carbohydrate. 1ml of the supernatant was taken in a test tube and to this 5ml of anthrone reagent was added and mixed well. The test tube containing the mixture was kept in boiling water bath for 10 minutes. The test tubes were then cooled at room temperature and optical density was measured in a spectrophotometer at 620 nm with the use of standard graph. The amount of carbohydrate present in the sample was converted for one gram.

$$\% \text{ of carbohydrate} = \frac{\text{Standard value x OD value}}{\text{Weight of the tissue}} \times 100$$

Estimation of Lipid:

Lipid was estimated following the method of Bragdon (1951). A known weight about 10mg fresh tissue was grouped with a few ml of chloroform solution and was centrifuged at 3000 rpm for 15 minutes. The supernatant was evaporated to dryness. The 3ml of 2% potassium dichromate in

concentrated sulphuric acid was added, which was followed by 3ml of distilled water. The developed colour was read in spectrophotometer using filter 640nm against a reagent blank. The standard curve was obtained by using cholesterol and the lipid was expressed in mg/g.

$$\% \text{ of lipid} = \frac{\text{Standard value x OD value}}{\text{Weight of the tissue}} \text{ x100}$$

Heavy metal analysis:-

Biota samples were collected from south east coast of New Harbour. They are thoroughly washed with Milli-Q water to prevent the soil and other dirts. Whole tissue was oven dried at 110^0 C, powdered with pestle and mortar and taken for heavy metal analysis. Exact 2.5 g powdered sample was diluted with 10ml of nitric acid – Perchloric acid (10:4) Uv room temperature over night and allow to cool to room temperature. The digested sample was filtered and made up to 50ml with distilled water and the sample metal content

were analyzed using a Flame Atomic Absorption Spectrophotometer (AASELI CO SD 194)The concentrate was expressed as (mg. kg-1) wet weight of tissue (Kingston and Jassie 1988).

TABLES AND FIGURES

Table 1: Physico -Chemical Parameters of Water Sample from New Harbour Coast, Thoothukudi. (2020-2021)

S.No	Physico- Chemical Parameters	Water Sample
1	Temperature (Ċ)	28.9
2	PH	8.32
3	DO (mg/l)	3.38
4	BOD (mg/l)	8.46
5	Salinity (PPt)	35
6	Alkalinity (mg/l)	82
7	Ammonia (µg/l)	0.497
8	Silicate (µg/l)	6.48
9	Phosphate (µg/l)	0.923

Table 2: Ecological Characters Of *Penaeus* Sp in New Harbour Coast. Thoothukudi (2020-2021).

Scientific name	**English Name**	**Feeding habit**	**Biotype Complex**	**Length (mm)**	**Weight (g)**
Penaeus Monodon	Giant Tiger Prawn	Omnivorous	Benthic	270 mm	260 g
Penaeus semiscultus	Green Tiger shrimp	Carnivorous	Benthic	180 mm	130 g

Table 3: Proximal Composition (%) of *Penaeus* in New Harbour Coast, Thoothukudi. (2020-2021)

S.No	Species	Protein	Lipid	carbohydrates
1	*Penaeus monodon*	8.98	6.38	2.76
2	*Penaeus semisulcatus*	9.56	6.65	3.30

FIGURE: 1

PROXIMAL COMPOSITION (%) of *PENAEUS* SP IN NEW HARBOUR COAST, THOOTHUKUDI (2020-2021)

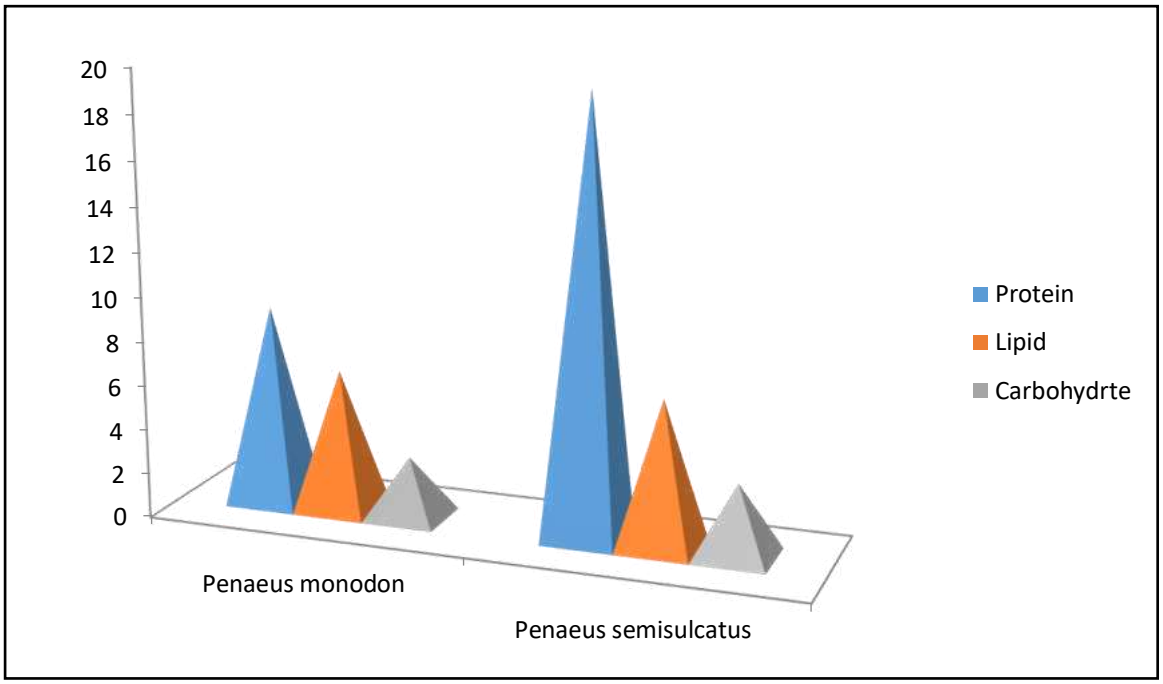
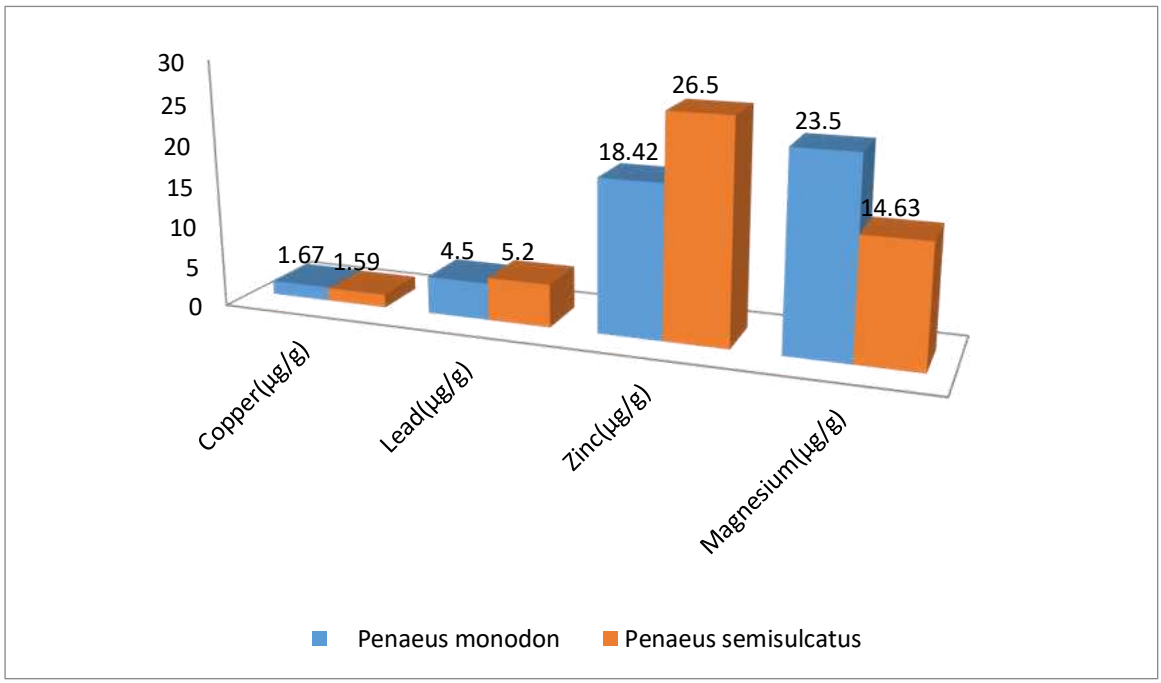


Table 4; Accumulation of heavy metals in *penaeus* sp µg/g in New Harbour Coast, Thoothukudi. (2020-2021)

S.No	parameter	*Penaeus monodon*	*Penaeus semisulcatus*
1	Copper(µg/g)	1.67	1.59
2	Lead(µg/g)	4.5	5.2
3	Zinc(µg/g)	18.42	26.50
4	Magnesium(mg/g)	23.5	14.63

FIGURE: 2

ACCUMULATION OF HEAVY METALS IN *PENAEUS* µg/g IN NEW HARBOUR COAST, THOOTHUKUDI. (2020-2021)



Penaeus monodon

Penaeus semisulcatus

RESULTS

6. RESULTS

The bio chemical composition of the shrimp muscle tissue on dry weight basis is shown in (table 3) (figure 1).

Protein content:

The protein content of *penaeus monodon* was 6.38 % (Table 3). The protein content of *Penaeus semisulcatus* was 19.56 % (Table 3).

Lipid content:

The lipid content of *Penaeus monodon* was 8.96 % (Table 3). The lipid content of *Penaeus semisulcatus* 6.65 % (Table 3).

Carbohydrate content:

The carbohydrate content in *Penaeus monodon* was 2.76 % (Table 3). The carbohydrate content of *Penaeus semisulcatus* was 3.30 % (Table 3). The Zinc is more high value in the prawns.

Heavy Metals:

The results of quantitative analysis of heavy metals copper, zinc, lead, magnesium are present in the Table 4.

Copper content:

The copper content of the *penaeus monodon* was 1.67 % (Table 4). The copper

content of the *Penaeus semisulcatus* was 1.59 % (Table 4).

Lead content:

The lead content of the *Penaeus monodon* was 0.13 % (Table 4). The lead content of the *Penaeus semisulcatus* was o.21 % (Table 4).

Zinc content:

The zinc content of the *Penaeus monodon* was 18.42 % (Table 4). The zinc content of the *Penaeus semisulcatus* was 26.50 % (Table 4).

Magnesium content:

The magnesium content of the *Penaeus monodon* was 23.5 % (Table 4). The magnesium content of the *Penaeus semisulcatus* was 14.63 % (Table 4).

Water Parameters:

During the study period, the temperature was 28.9 Ċ, pH was 8.32 and Dissolved oxygen was 3.38 mg/l, BOD is 8.46 mg/l, salinity was 35 PPt, Alkalinity is 82 mg/l, Ammonia was 0.497 mg/l, silicate was 6.48 mg/l, Phosphate was 0.923mg/l.

Ecological characteristics of (length and weight) of the shrimp were measured and tabulated (Table2).

DISCUSSION

7. DISCUSSION

The nutritive values of edible marine organisms depend upon their biochemical composition, such as protein, amino acids, lipid, fatty acids, carbohydrate, vitamins and minerals. Animal protein is vital in the diet because of the various functions and performs. Among sources of animal protein, seafood and related sources are important dietary sources of proteins with high biological values. Backer G *et al.,* (2003).

Protein is essential for the sustenance of life and accordingly exists in the largest quantity of all nutrients as a component of the human body.(Sudhakar M, Manivannan K and Soundrapandian P (2009) . Protein is essential for normal function, growth and maintenance of body. Among the seafood, prawns and shrimps contribute about 20-22% by volume of the world seafood market (FAO, 2014). Due to their nutritious nature, apart from the supply of good quality proteins, lipids, they also contain several dietary minerals such as calcium and iron, which are beneficial and vital and play a chief role in the maintenance of physiological and biochemical activities in human beings. Therefore prawns and shrimps are considered to be most popular species as it is a part of almost. Every nation's traditional meal rich in protein and minerals.

Protein has been reported to be the most vital biomolecule in crustaceans, from eggs to adulthood and is conspicuously dominant in young phases (Varadharajan and Soundarapandian, 2014). From the present results the protein content of *Penaeus semisulcatus* was 19.56% (Table 3). Had the highest protein content than *penaeus monodon* was 6.38% (Table 3).

Penaeus semisulcatus occurs soft-sediment and coastal habitats with early life stages preferring the sheltered productive habitats of sea (RA sid anam, Edorardo Mostards 2012) *P.monodon are* nearally found in sandy estuaries and mangroves and upon adulthood they move to deeper water (0-110m) and live on muddy and rocky bottom (FAO-FIRA,2010) The high amount of protein content recorded for *Penaeus semisulcatus* (19.56%) species in this study may be attributed to their high protein dietary intake which included algae, diatoms, crustaceans, molluscs and partly digested fishes (Osibona, 2005).

Lipids are important in the structural and biological functioning of the cells. In crustaceans, not only do lipids function as the main organic reserve and source of metabolic energy but are also indispensable in maintaining cellular integrity. Generally, lipids act as major food reserve together with protein and may fluctuate periodically due to environmental variable like

temperature (Varadharajan and Soundarapandian, 2014). Lipid of shrimp contains mostly polyunsaturated fatty acid (EAs). Thease EEAs available in shrimp provide healthbenefits for human eg eye retina and brain development and function. (ConnerW Neuriner M Reisbick S (1992). The high lipid content was in *Penaeus monodon* 8.96% (Table 3). The lipid content of Penaeus *semisulcatus was* 6.65% (Table 3).The high lipid content in *P.monodon* can be attributed to is omnivorous feeding habit (Abdel-Salam H and Hamdi A (2011).

Carbohydrates are a group of organic compounds that includes sugars, starches and fiber, which is a major source of energy for animals. The present study showed that carbohydrate contents in the edible muscle in *Penaeus monodon* was 2.76% (Table 3). The carbohydrate content of *Penaeus semisulcatus* was 3.30% (Table 3). The variations in the carbohydrate level in edible tissues of different marine crustacean organisms were reported by (Devi *et al.,* Hareesh K and Srinivasulu R (2015)) They mentioned that, this variation in carbohydrate contents may be attributed to the variation of various factors which change carbohydrates percentage in edible muscles of crustacean animal, such as starvation, rest, exercise, gonad development and other physiological stages.

Biochemical analysis revealed that *P. semisulcatus* have the best nutritive value. The nutritional analysis of muscle of both species indicate the presence of high amount of protein, moderate content of lipids, low level of carbohydrates.

Heavy metal pollutions are particularly hazardous contaminants in food and the environment. In general, they are not biodegradable and have long biological half lives. According to the World Health Organization (World Health Organization 1995) heavy metals must be controlled in food sources in order to assure public safety. Excessive concentration of food heavy metals is associated with the ethiology of a number of diseases, especially cardiovascular, renal, neurological, and bone diseases (Chailapakul *et al*., 2008). A major reason to monitor levels of toxic metals in foods follows from the fact that contamination of the general environment has increased

It is known that some shrimp and crab may provide useful means of monitoring such elemental concentration levels and their impact on the aquatic environment. In 2005 BU-Olayan and Thomas showed higher trace metal levels in benthic molluscs and annelids of Kuwait Bay.

The Cu, Pb, Zn,and Mg levels were determined in the whole body of the Giant tiger prawn *P.monodon* and Green tiger shrimp (Table 2). There are remarkable differences in the Giant Tiger Prawn and Green tiger shrimp heavy metal concentrations. The order of the heavy metal concentrations in Giant tiger prawn *P.monodon* was found: Mg>Zn>Pb>Cu, while in whole body of Green tiger shrimp *P.semisulcatus* was Zn>Mg>Pb>Cu.

Pourang et al. (2005) examined Green tiger shrimp heavy metal distribution, in the North Western (near the Bushehr Province Coastline) part of the Persian Gulf. In his study highest mean of Zn concentration (43.39 ppm/fresh weight) was found in hepatopancreas of prawn.. Also, the Zn levels of the exoskeleton and muscle were 8.56 and 8.98 ppm wet weight, respectively. In the current study, the level of the Zn/dry weight of whole body of *Penaeus semisulcatus* was 26.50 ug/g and *P. monodon* was (18.42 ug/g). The presence of Zn in both shrimps of New Harbour Coast is within the permissible limit by WHO (100 mg/g).

Cu is an essential trace metal for animal metabolism but at high levels is a very toxic substance to aquatic life (Bryan et al., 1983). The main sources of Cu in the coastal waters are antifouling paints (Goldberg, 1975) and

this metal entered into the water body through industrial effluents containing

CuSO4 used in metal plating and fishing operations (Goldberg, 1975; Mithra and Chowdhury, 1993). Despite the existence of a number of detoxifying and storage systems for Cu, it is the most toxic metal after mercury and silver, to a wide spectrum of marine life, hence its value in antifouling preparations (Clark, 1997). In the present geographical locale, the major source of Cu is the antifouling paints used for conditioning fishing vessels and trawlers apart from industrial discharges. Levels of Copper in shrimps from the New Harbour Coast ranged from1.67ug\g in *P.monodon* and1.59ug\g in *P. semisulcatus.* is far below the normal permissible range, i.e. 120 ppm as recommended for the crustacean tissue (Franklin, 1987). According to the Seafood Standards, maximum allowable limits for shrimps for Cu is 20 ppm (The Seafood Standards 2003) also indicated Cu accumulation of shrimp species in western sector of Indian Sundarbans relatively higher than seafood standards bench mark. *et al.,* 2009)

Pb is a cumulative poison and potent enzyme inhibitor as it incorporates into enzyme structure. (Pb is a neurotoxin that cause behavioral deficits in aquatic organisms and can cause decreases in survival, growth rates and metabolism (Burger *et al.,* 2000). There is often little accumulation of Pb in marine and

freshwater species. Consequently lead is not a threat to fisheries resources except at extreme pollution (Clark, 1997). The most toxic of the heavy metals is Pb, which finds its way in new harbour coastal water is through the discharge of industrial waste waters, painting of boats and ships Thermal power plant, Spic, importing and exporting of coals in the Harbour Antifouling paints used to prevent growth of marine organisms at the bottom of the boats and trawlers also contain lead as an important component. These paints are designed to constantly leach toxic metals into the water to kill organisms that may attach to bottom of the boats, which ultimately is transported to the sediment and aquatic compartments. When compared with the recommended value of World health Organization (WHO, 1989) We found that Pb concentration of *P.monodon* was 4.5 ug/g and *P.semisulcatus* was 5.2 ug\g, which are above the permissible limit in context to consumption of prawn (2 ppm for Pb). The values of Pb in shrimp samples were compared with maximum allowable limits of the Seafood Standards and also with available literatures. In the present study, Pb contents in *P.monodon* (4.5ug/g) and *P. semisulctus* (5.2ug/g) obtained from New Harbour coast was found to be above the threshold values.

Aquatic animals can get the essential nutrients calcium and magnesium from their environment and their food. The amount of magnesium is found to be higher *P.monodon* (23.5mg/g) and *P. semisulctus* (14.63ug/g). The

species by nature are rich in magnesium The pollution of the environment has caused the species to accumulate more and more of magnesium that the level has increased considerably.

CONCLUSION AND SUGGESTIONS

8. CONCLUSION AND SUGGESTIONS

Fishes are the major source of food utilized by mankind in spectrum. Life cannot exist without adequate nourishment. Human adequate food for growth and development and to lead an active healthy life. Intake of sea food is one of the nutritionally superior natural protein sources accessible to man.

Consumption of fish and other marine products has always been a factor in the economy and nutrition of the coastal inhabitants. Fish is a potential source of protein, essential amino acids and good fatty acids.

The long term assessment of heavy metal level in edible fish is important. Tuticorin is the main coastal and industrial city present in Gulf of Mannar region.

There are plenty of reports available about the heavy metals contamination in Gulf of Mannar in particular to water and biological samples.

From this study it was inferred that the sea water has prominent physio chemical characteristics.

The chemical composition of both shrimp varies from one individual to another depending on age, sex, environment and season. *Penaeus semisulcatus* belong to a high protein category as it contains 19.25% of protein. American

journal of clinical nutrition, eating prawns is part of a healthy diet, and because they are common throughout the world, there are healthy prawn dishes within almost every style or type of cuisine.

The present study reported, the heavy metal accumulation in two *Penaeus* species tissues. The high accumulation of heavy metals in *Penaeus* is its feeding habits and their aquatic environment. The present study contributed to our knowledge, the level of concentration of metals copper, zinc, lead, magnesium in Giant Tiger Prawn and Green Tiger Shrimp.

SUGGESTIONS

- The waste water such as domestic sewage and industrial effluents can be partially treated before discharge in to the sea.
- Awareness should be created among the local people by the government about Shrimps.
- To educate people about danger related to metal bioaccumulation in food organisms.
- Provide quality conservations and research project opportunities for public aquarium.

SUMMARY

9. SUMMARY

- New Harbour Coast, Tuticorin was selected as the study area.
- *Penaeus species Penaeus monodon* and *Penaeus semisulcatus* are selected as the study species.
- Physic- chemical parameters of the study area was found to be prominent.
- The proximal analysis shows the high nutritive value in the muscles of *Penaeus monodon* and *Penaeus semisulcatus*.
- The accumulation of metal levels indicates that long term consumption of these shrimps may lead to potential risks to human in future.
- Regular monitoring of marine resources is essential to improve the quality of sea food against contamination.

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PHARMACOLOGICAL ANALYSIS OF THE TISSUE & INK FROM SEPIA BREVIMANA

Dissertation submitted to

ST. MARY'S COLLEGE (AUTONOMOUS) THOOTHUKUDI

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in partial fulfilment for the award of the degree of

MASTER OF SCIENCE IN ZOOLOGY

by

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(Re- Accredited with 'A^{+}' Grade by NAAC)

THOOTHUKUDI-628001

APRIL-2021

CERTIFICATE

This is to certify that this dissertation entitled, **PHARMACOLOGICAL ANALYSIS OF THE TISSUE & INK FROM SEPIA BREVIMANA** . is a record of original research work done by **A.SAFAHATH FATHIMA** under my supervision, and submitted in partial fulfillment for the degree of Master of Science in Zoology. This dissertation has not formed the basis of any other degree.

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DECLARATION

I do hereby declare that this dissertation entitled, "**PHARMACAOLOGICAL ANALYSIS OF THE TISSUE & INK RROM SEPIA BREVIMANA**" submitted by me for the award of the degree of **Master of Science in Zoology**, is the result of my original independent research work carried out under the guidance of **Dr. Jemma Hermelin Jesy Diaz, M.Sc., B.Ed., M.Phil., Ph.D.,** Assistant Professor, Department of Zoology, St. Mary's college (Autonomous), Thoothukudi, and it has not been submitted elsewhere for the award of any other degree.

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Date: 15.04.2021

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INTRODUCTION

1.INTRODUCTION

Marine environment constitutes about 70% of the earth's total surface. The marine includes the shorelines, with mud flats, rocky and sandy shores, tide pools, barrier islands, estuaries, salt marshes, and mangrove forests making up the shores lines segment. Marine ecosystems support a great diversity of life and variety of habitats. Many species rely on marine ecosystem for both food and shelter from predators. Marine organisms are known for their high medicinal values (Guadalupe- Mirosolva Suarez-Jimenez *et al*., 2012).

Mollusks are invertebrates; the second largest phylum next to Arthropod consisting of around 100,000 species all over the world in almost all types of habitats. Most of the molluscs are highly delicious seafood because of their nutritive value, next to fin fishes and crustaceans. Among the molluscs some animals exhibited pharmacological activities or other properties which are useful in the biomedical arena.

Scientific interest for cephalopods is increased over the last century because of their value as experimental animals for biomedical and behavioral research (Hochner, 2008; Castellanos-Martinez and Gestal, 2013), their position in the world marked as a major fishery resource (Boyle

and Redhouse, 2005; Castellanos-Martinez and Gestal, 2013). Cephalopods contain low level of fat, rich in protein containing all the essential amino acids, polyunsaturated fatty acids (PUFAs), Cal, Vitamins, and many other nutrients (Venugopal 2005). Regular seafood consumption is associated with beneficial health effects.

Cephalopods are famous for their defences, from their fast jetting escape movements to changes in colouration that can be cryptic, disruptive or startling to arm autotomy to toxin venom and to inking (Hanlon and messenger, 1996; Norman, 2007). Cephalopods ink produced at the end of the cuttlefish maturation process is a suspension of melanin granules in a various colorless medium. Ink gland cells of the digestive tract in the mantle cavity degenerate and shed their contents into the ink sac, acting as a reservoir of the exhausted material. Ejection of dark ink from the sac is a defensive means cuttlefish employed to avoid dangers and risks.Background researchers showed that ink is an intermixture, beside large amounts of melanin; the ink contains proteins, lipids, glycosaminoglycans and various minerals, etc. The main components are melanin and protein polysaccharides complex (Palumbo *et al*., 2003).Chemically defended species contain an array of bioactive molecules (Kicklighter *et al*., 2003). Identification of bioactive compounds from defended organism is often a research focus.

Oxygen is essential element to the life of most biological systems. Oxidation, the transmission of electron from an atom, is the main part of aerobic life and metabolism of living organisms (Pieta, 2000; Shinde *et al*., 2006).Oxygen is the final acceptor of electron transmission system, which produces energy from ATP in the body.Antioxidants are natural molecules, which prevent the formation of uncontrolled free radicals, harmful action of the free radicals by enzymatic and non-enzymatic mechanisms. Antioxidants are natural or synthetic origin (Chaudiere and Ferrari-Iliou, 1999; Sukandar and Adnyana, 2015). Synthetic antioxidants, because of their carcinogenic effects, are less essential for consumptions.

Chelation therapy is the administration of chelating agents to remove heavymetalsfromthebody (URL:http://en.wikipedia.org/wiki/Chelationtherapy). Disruption of iron ions homeostasis may lead to oxidative stress, a state where increased formation of reactive oxygen species (ROS) overwhelms body antioxidant protection and subsequently induces DNA damage, lipid peroxidation, protein modification and other effects, all symptomatic for numerous diseases, involving cancer, cardiovascular disease, diabetes, atherosclerosis, neurological disorders (Alzheimer's disease, Parkinson's disease), chronic inflammation and others (Rogus,*et al*.,1993).

Chronic inflammatory processes cause a significant change in iron metabolism with a drop in serum iron and a redistribution of iron to the activated reticuloendothelial system. Fe (iron) overload appears to selectively worsen joint inflammation whilst nutritional Fe deficiency has the converse effect. Inflammatory synovial fluid contains Fe in a form capable of generating the hydroxyl radical. The effect of synovial iron on the progression of rheumatoid disease has been reported by(Blake *et al*., 1984). This may be correlated to metal chelation in patients with synovial iron. It has been suggested that iron may play an important part in acute and chronic phases of the arthritic inflammatory process. Iron may catalyse free radical production in the joints, leading to lipid peroxidation and membrane disruption. An abnormal accumulation of iron may promote an infiltration of lymphocytes and macrophages into the synovium of affected joints (Potter *etal*., 1984).

Natural antioxidant can play a major important role in the health of mankind because of having antivirus, anti-inflammatory, anticancer, antitumour and liver protection properties(Atlia Godze *et al*., 2015; Gupta Pooja *et al*., 2015; Vishwakarma Pratima *et al*., 2016 and Bekta Ersan *et al*., 2016).Current research in free radicals has confirmed that food items rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancer

and neurodegenerative diseases, including parkinson's and alzheimer's diseases, as well as inflammation and problems cause by cell and cutaneous aging (Li *et al* .,2007).

In the search of new antioxidants, exploration of aquatic habitats has led to the discovery that marine plants and invertebrates also contain antioxidants. Among the invertebrates, the discovered bioactive compounds in molluscs were identified essentially as peptides, depispeptides, sterols, sesquiterpenes, macrolides, prosta glandins and fatty acid derivatives and alkaloids presented antioxidant activities (Balcazar *et al*., 2006).

Diabetes mellitus is a group of metabolic disorders characterized by a chronic hyperglycemic condition resulting from defects insulin secretion, insulin action or both.There are two main types of diabetes mellitus: Type 1 diabetes, also called insulin dependent diabetes mellitus (IDDM), is caused by lack of insulin secretion by beta cells of the pancreas.Type 2 diabetes, also called non-insulin dependent diabetes mellitus (NIDDM), is caused by decreased sensitivity of target tissues to insulin.In both types of diabetes mellitus, metabolism of all the main food stuffs is altered. The basic effect of insulin lack or insulin resistance on glucose metabolism is to prevent the efficient uptake and utilization of glucose by most cells of the body, except those of the brain (Guyton and Hall, 2006).The prevalence of diabetes is

increasing rapidly worldwide and the World Health Organization (2003) has predicted that by 2030 the number of adults with diabetes would have almost doubled worldwide, from 177 million in 2000 to 370 million in 2025.

Amylase are a class of enzymes that hydrolyze starch to yield low molecular weight dextrins and sugars and they play important role in the digestion of carbohydrates. Inhibition of α-amylase along with α-glucosidase can significantly reduce the post-prandial increase of blood glucose and can be important strategy in the control of blood glucose level in the type-2 diabetic patients. Hence, pancreatic amylase and gastric glucoamylasee are the major therapeutic targets for the type-2 diabetes mellitus.(Jayaraj *et al*., 2013).The most widespread applications of alpha amylase are in the starch industry, which is used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups. The supermolecules conversion of all starch includes gelatinization, that involves the dissolution of starch granules, thereby forming a viscous suspension; phase change, that involves partial chemical reaction and loss in the body; and saccharation, involving the assembly of aldohexose and disaccharide.

Inhibition of α-amylase enzyme that plays a role in digestion of starch and glycogen, is considered a strategy for the treatment of disorders in carbohydrate uptake such as diabetes and obesity as well as dental caries and

periodontal diseases.(Sales *et al*., 2012). Due to the gastro intestinal side effects exhibited by oral anti-hyperglycemic agents, searching for new amylase inhibitors became essential in treatment of diabetes.

Inflammation is the reaction of the living tissues to injury; it comprises systematic response (involving nervous and hormonal adjustments and proliferation of the lymphoreticular system); and local response (pain, redness, warmth and swelling). The three important aspects of inflammation that render themselves readily to measurement are erythema (local vasodilation), edema (increased capillary permeability) and formation of granulation tissue (Satoskar *et al*., 2010). Inflammation is the most common reason for physician consulatation in most developed countries. It is a major symptom in many medical conditions and can interfere with a person's quality of life and general functioning.

Cyclooxygenase (COX) , is an integral membrane glycoprotein officially known as prostaglandin –endoperoxide synthase (PTGS) that is responsible for formation of inflammation. COX-I is constitutively expressed in all tissues. While COX-2 is increasingly expressed during inflammatory molecules such as Carregeenan (Cater, 2000).

Nonsteroidal anti-inflammatory drugs (NSAIDS) are of huge therapeutic benefit in the treatment of various types of inflammatory conditions. The target for these drugs is COX, a rate limiting enzyme involved in the conversion of arachidonic acid into inflammatory prostaglandins (Van Ryn *et al*., 2000). Although NSAIDs inhibit COX and suggested as anti-inflammatory agents, serious adverse effects limit their use. Increasing the intake of omega-3 fatty acids while decreasing the omega-6 fatty acids in the diet has led to a decrease of nom-steroidal anti-inflammatory agents in patients with rheumatoid arthritis.

Proteases are ubiquitous in all living cells. As soon as cells are disrupted, proteases are released and can quickly degrade any protein. This can drastically reduce the yield of protein during isolation and purification. Contaminating proteases can be inhibited by protease inhibitors, thereby protecting the protein of intrest from degradation.Protease inhibitors (Pls) are proteins or peptides capable of inhibiting the catalytic activity of proteolytic enzymes. They are widely distributed in nature and can be found in all kingdoms of cellular life and also in viral genomes. (Lingaraju*et al*., 2008). Deficiencies or alterations in the regulation of these enzymes underline several pathological conditions, such as cancer, arthritis, neurodegenerative and cardiovascular diseases. (Kennedy, 1998).

REVIEW OF

LITERATURE

2. REVIEW OF LITERATURE

Marine organisms are having very rich source of food, medicine and energy.They have also proven to be a rich source of structurally diverse bioactive compounds with the valuable pharmaceutical and biomedical application (Shanmugam, and Mody, 2000). There are 66,535 species widely distributed throughout the world and have many representation in the marine and estuarine ecosystem. Marine environment comprises of many organisms which are known to posses bioactive components as a common means of self – defense or for the protection of eggs and embryos (Peruru *et al*., 2012).

Mollusks not only exhibit the antimicrobial activity, they constitute many classes of bioactive compounds which include antitumour, antileukemic, antiviral activities, anti-inflammatory and antioxidant activities have been reported worldwide (Rajaganapathy *et al*., 2000; Benkendroff *et al*., 2011). Antioxidant compounds are playing an important role to trap free radical and reduce the risk of chronic disease (cancer and heart disease) act as a health – protecting factor (Kamala *et al*., 2013; Sivaperumal *et al.,* 2013; Sivaperumal *et al.,* 2015). Antioxidants in biological systema have multiple roles, including defending against oxidative damage and participating in the major signaling pathways of cells.

One major function of antioxidants in cells is to prevent damage caused by the action of reactive oxygen species (Kamala *et al.,* 2013; Sivaperumal *et al*., 2015).

The muscles of molluscs species providing resistance capacity to various kind of oxyradicals (Regoli *et al*., 2000; Regoli *et al.,* 2002) and it has dietary antioxidants such as phenolic content (Fang *et al* ., 2002, Gorinstein *et al* ., 2002, Singh *et al*., 2002 and Vinson *et al.,* 2001). These antioxidant molecules are present in various molluscs species to prevent cell damage from oxidation reaction to consumers (Nagash *et al.,* 2010). Rajapakse *et al*., (2005) evaluated the ant oxidative effects (*in-vitro*) of giant squid muscle peptides on free radical- mediated system. Mendisn *et al*., (2005) investigated the jumbo squid (*Dosidius gigas*) for their antioxidant effects. Lin *et al*., (2005) studied the antioxidant effects of melanins extracted from various tisssues of animals.

The antioxidant activity of methanolic extract of *Squid brevimana* and *Sepiellainermis* was carried out by Illamparithi *et al.,* (2011). Ponsamy *et al.,* (2016) evaluated the antioxidant activity from tissue extracts of cephalopods *Sepiapharaonis, S.intermis* and *Octopus vulgaris* around Madrs Atomic Power Station, Kalokam Coast. In the cuttlefish *Sepia officinalis*, NO plays a key role in the defense system, regulating the metabolism of the

ink gland and the activity of chromatophores (Palumbo *et al.,* 2000; Fiore *et al*., 2004; Mattiello *et al*., 2010).

In the presence of antioxidants like flavonoids, the ω-3PUFA's in cuttlefish liver oil inhibit free radical generation and thus improve the antioxidant defence system in rats. (Joseph *et al*., 2006). Chen *et al*., (2007) studied on the free radical scavengering activities of melanin from squid ink. Lei *et al*., (2007) evaluated antioxidant ability of *Sepia officinalis* in hyperlipidemia rats and radio-protective effect of cuttlefish ink on hemopoietic injury. In-vitro antioxidant activity of solvent extracts of *Loligo duvauceli* and *Donaxstrateus* from Indian water was studied by Nagash *et al., (*2010). Wang *et al.,* (2010b) reported Sepia ink extract on protection from oxidative damage of cardiac muscle and brain tissue in mice. Effort of squid ink on growth performance, immune functions and antioxidant ability of broiler chickens was studied by Liu *etal., (*2011).

Barwin *et al*., (2012) extracted, characterized and studied the *invitro* antioxidant potential of chitosan and sulfated chitosan from the cuttlebone of *Sepiaaculate* collected from Cuddalore coast. Subhapradha *et al.,* (2013) prepared the phosphorylated chitosan from the gladius of *Sepioteuthis lessoniana* from Thondi coast and studied its antioxidant activity. Fahmy and Soliman (2013) investigated the *in vitro* antioxidant, analgesic and

cytotoxic activities of *S.officinalis* ink and *Coelatura aegyptica* extracts. Vate and Benjakul *et al*., (2013) studied the antixidative activity of melanin free ink from splendid squid (*Loligo formosana)*. Xin *et al*., (2013) prepared the water soluble melanin from squid ink using ultrasound –assisted degradation and observed its anti-oxidant activity. Ensibi cherif *et al*., (2015) evaluated the *in-vitro* antioxidant activity of *Aplysia depilans* ink collected from Bizirte channel. *In vitro* antioxidant , antimutagenic and antiproliferative activities of collagen hydrolysates of jumbo suid (*Dosidicus gigar*) by products was reported by Brauer *et al*., (2015). Evaluation of antioxidant capacity and free radical scavengering activities of pepsin extract of cuttlefish (*Sepia pharaonis*) from Persian Gulf was studied by Amir Siahpoosh (2015). Nalinanon *et al*., (2016) studied the collagen hydrolysate isolated from the skin of cuttlefish (*Sepia pharaonis*) with antioxidant activities.

The squid processing units produces ink sac as processing waste. Squid processing discard can be utilized to obtain the valuable component. Processing unit can used ink as natural antioxidant in many food products to retard the lipid oxidation and shelf-life of the food can also be enhanced (Arun Kumar , *et al.,* 2020).

Sohair*et al* .,2013 studied the antioxidant activities in the ink extract of *Sepia officinalis*(IE) and *Coelatura aegyptiaca* extract (CE). The antioxidant activities of both extracts were evaluated using 1, 1- diphenyl-2-picrylhydrazyl (DPPH) scavenging and lipid peroxidation assays.

Three different types of melanin free ink (MFI) extracts such as pure squid ink with 5 times dilution and squid ink with 10 times dilutions from *Loligo sp* showed the antioxidant activity. (Agustini *et al*., 2018).The water soluble melanin fractions obtained from squid *Ommastrphes bartrami* under alkaline conditions with molecular weight above 10kDa exhibited in vitro antioxidant activity(Guo *et al*., 2014). In vitro antioxidant activities were evaluated based on reducing power, metal chelation, ABTS, hydroxyl radical and superoxide anion radical scavengering assays.(Balasubramanian *et al*.,2016).

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion. Food habits and genetic factors are responsible for diabetes. A study revealed that urbanization of rural India has doubled the rate of diabetes. (Mitra *et al*., 2007).The estimated worldwide prevalence of diabetes among adults in 2010 was 285 million (6.4%) and this value is predicted to rise to around 439 million (7.7%) by 2030. (Shaw *et al*., 2010). Clinical studies on different species of animals

have shown that consuming less food (caloric restrictions) reduces the risk of diabetes and heart disease. Current treatment for Type 2 diabetes remains inadequate, prevention is preferable.(Mitra *et al*., 2008).

One therapeutic approach for treating Type2 diabetes is to decrease postprandial hyperglycemia. Modern medicines such as biguanides, sulfonylureas and thiozolidinediones are available for the treatment of diabetes. However, they also have undesired effects associated with therir uses. (Fowler *et al*., 2007). The number of individuals diagnosed with type 2 diabetes mellitus, which is caused by insulin resistance and /or abnormal insulin secretion, is increasing worldwide, creating a strong demand for the development of more effective anti-diabetic drugs.

Antidiabetic and anti-inflammatory activities of ethyl acetate-methanol extracts of cephalopods namely, *Amphioctopus marginatus, Urothesis duvauceli, Sepia pharaonis, Sepiella inermis,* and *Cistopus indicus* were evaluated. (Kajal Chakraborthy,*et al*; 2017). The solvent extracts derived from the members of the order Octopoda demonstrated fairly good α-amylase inhibitory activity by proton nuclear resonance spectroscopy that supported in the i*nvitro* anti-diabetic and anti-inflamatory results.

Hydrolysis of dietary starch are the major source of glucose in the blood α-amylase and α-glucosidase being the key enzymes involved in starch break down and intestinal absorption, respectively. It is believed that inhibition of these enzymes can significantly decrease the postprandial increase of blood glucose level after a mixed carbohydrate diet and therefore, it can be an important strategy in the management of hyperglycemia linked to type II diabetes. (Shetty *et al*., 2008).

The anti hyperglycemic activity of the crude extract of *Scapharca ineuivalvis* (Bruguire, 1789) was studied in rat models (Tiwari *et al*., 2008). Ravi *et al*.,(2012) studied the α-amylase inhibitory activity in the methanol and acetone tissue extract of two marine gastropods *H. pugilinus* and *N. didyma.* Results indicated that the mollusc extract possessed anti-diabetic activity. Both acetone and methanol extracts of the two gastropods exhibited the alpha amylase inhibition in the *in-vitro* condition.

The α-Glucosidase and α- amylase are the major enzymes present in the intestinal lumen that hydrolyze disaccahrides into monosachharides thereby resulting in their absorption into the blood vascular system.(Ravi, *et al*., 2013). In vitro anti-diabetic activities of ethyl acetate –methanol (EtOAc- MeOH) extracts of the cephalopods were evaluated using α-

glucosidase, α-amylase and DPP-4 enzyme inhibition assay.while the anti inflammatory potentials were assessed by the in vitro inhibition of COX-1 and COX-2 and 5-lipoxygenase (5-LOX) enzymes. In this activity ingestion of carbohydrate rich diet causes elevation in blood glucose level by the rapid absorption of carbohydrates in the intestine aided by the action of glycoside hydrolyses, which breaks dietary carbohydrates into absorble monosachharides (Berdanier *et al*., 2007).

Giji Sadhasivam, *et al*., (2013) studied the antiamylase properties of methanol and acetone extracts of nudibranchs, *Bursatella leachii, Kalinga ornate* and *Aplysia* sp.Conventional antidiabetic drugs are effective, however, also with unavoidable side effects. (Atta- ur-rahman, *et al*., 2019).The current medications of diabetes mellitus focus on controlling and lowering blood glucose levels in the vessel to a normal level .(Ngan Tran, *et al*., 2020).

The protease inhibitors (PIs) from marine venomous animals, such as from sea anemone extracts and Conus venom as well their counterparts in marine venomous animal.(Caroline ,*et al*., 2013).

OBJECTIVES

3.OBJECTIVES

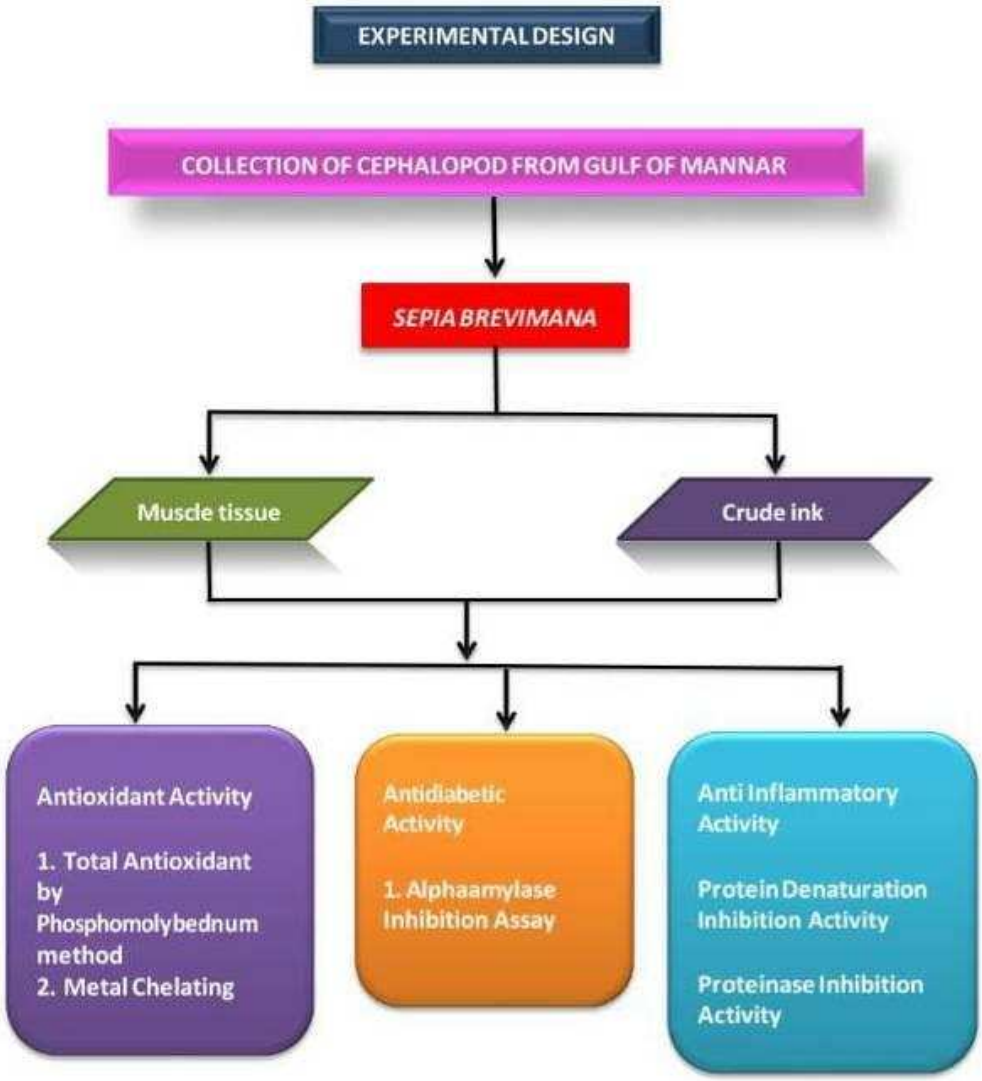
The present study has been carried out with the following objectives:

To investigate antioxidant activity in the ink and tissue extract of *Sepia brevimana* (Total antioxidant activity by Phosphomolybednum method, Metal chelating activity).

To find out the antidiabetic activity of the two extract by alpha amylase inhibition assay.

To evaluate the anti-inflammatory activity in ink and tissue.

EXPERIMENTAL DESIGN


EXPERIMENTAL DESIGN
COLLECTION OF CEPHALOPOD FROM GULF OF MANNAR
SEPIA BREVIMANA
Muscle tissue
Crude ink
Antioxidant Activity
1. Total Antioxidant by Phosphomolybednum method
2. Metal Chelating
Antidiabetic Activity
1. Alphaamylase Inhibition Assay
Anti Inflammatory Activity
Protein Denaturation Inhibition Activity
Proteinase Inhibition Activity

MATERIALS AND METHODS

5.MATERIALS AND METHODS

5.1. Description of the study area

The Gulf of Mannar is located between India and Srilanka (Long.78º8'' to79º 3º'' E and Lat 8º35'' to 9º 25'' N). It is a part of the southward extension of the Bay of Bengal and it meets in the Indian Ocean. This geographical area runs from Pamban Island including Rameshwaram to Cape Comarian along the Southeast coast of India to a distance of about 170 nautical miles. This coast maintains a rich biological diversity of flora and fauna largely due to diversified microhabitats such as mangroves, corals, seaweed beds, sea grasses; sandy, rocky and muddy shore etc.The faunal diversity is also well pronounced with reference to different molluscan groups. For the present study the animals were collected from the fishing trawlers operated for crabs and prawn from the Thoothukudi coastal region.

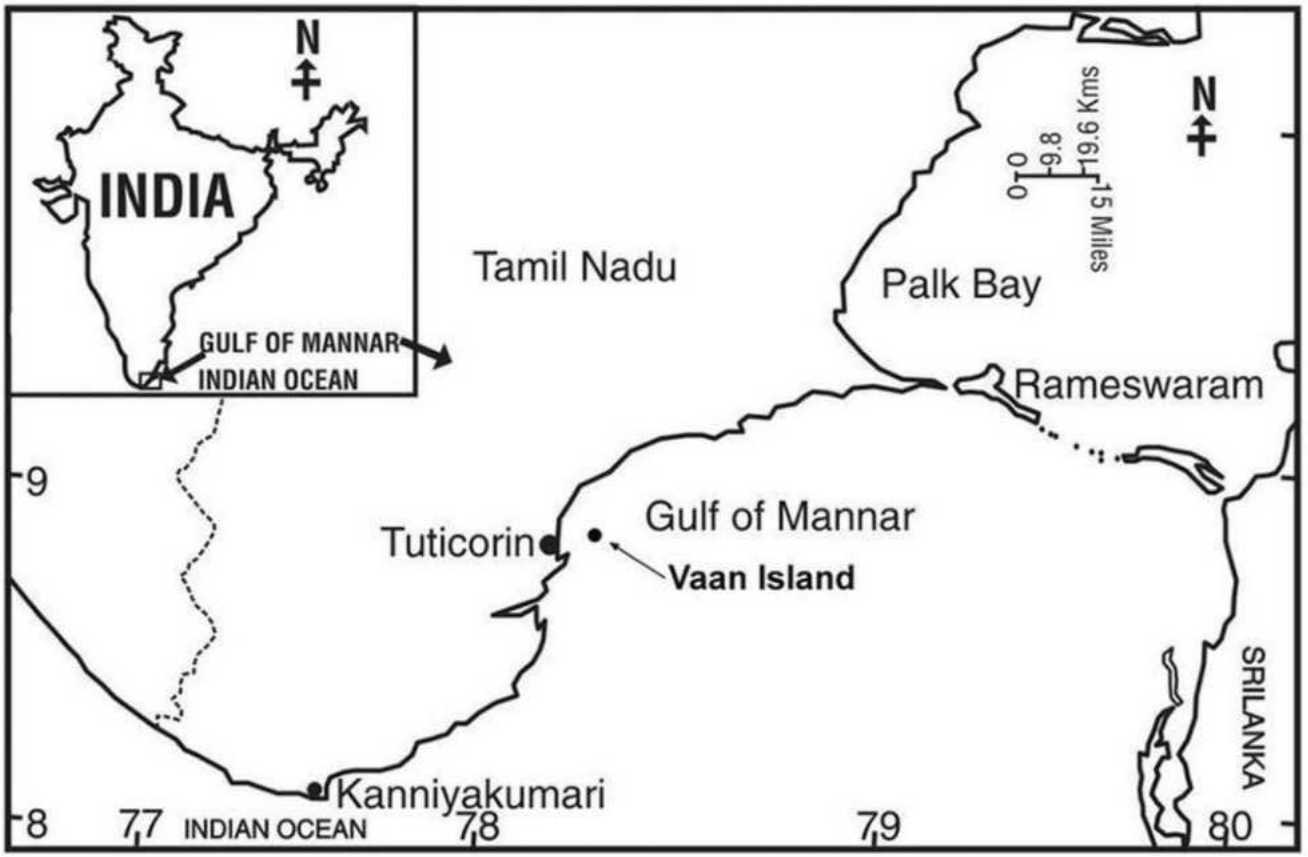


Figure : 1 Map showing Thoothukudi Shore , The Gulf of Mannar

5.2 Experimental Animal: *Sepia brvimana* (Steenstrup, 1875)

Systaematic position of *S. brevimana*

Phylum : Mollusca

Class : Cephalopoda

Sub class : Coleoidea

Order : Sepioidea

Family : Sepiidae

Genus : *Sepia*

Species : *S.brevimana*

The tentacular clubs are short with well-developed swimming and protective membranes along with 6-8 small sub equal suckers arranged in transverse rows. The cuttlebone is flat and distinctly acuminate anteriorly.The dorsal surface is rugose and a shallow medium groove is present in the striated area. The striae is '>' shaped with a median shallow groove broadening anteriorly. The inner core of the cuttlebone is white in colour. The spine is sharp and slightly curved.

The mantle is broadly ovate. The anterior margin is prominently projected in the mid dorsal plane in to a lobe, which is much acuminate. The ventral anterior margin is slightly emarginated

in the middle. The posterior end is also too much acuminate owing to the presence of a long and pointed spine at the extremity of the cuttlebone. The fins are narrows, start very nearer to the mantle opening and gradually widen posteriorly. The funnel is long and reaching the base of the ventral arms. The funnel valve is rounded at the anterior end. The head is as long as broad and eyes are prominent. The buccal membrane is seven lappetted. The oral arms are short, sub equal and less than half the length of mantle. The arm suckers are quadriserial and small in size. The arm order is usually 4,3,2,1. The dorsal arms are round and swimming membrane is slightly developed in the ventral arms.

The tentacular clubs are short and slender. It bears eight oblique rows of small suckers. There is no enlarged sucker present. The swimming and protective membranes are well developed. In males, the left hectocotylised are IV has three rows of normal suckers in the proximal end followed by 10 rows with minute sucker in the lower region and the corresponding upper region beset with ridges; the distal end of the arm has got normal suckers.

Plate: 1a. Dorsal view of *Sepia brevimana*

b. Ventral view of *Sepia brevimana*

Plate 2. a. Dissection showing inkgland of *Sepia brevimana*

b. Inkgland (Enlarged view)

5.3.Collection and preparation of extract

In the present study the animal was collected from Gulf of mannar,Thoothukudi coastal region (Long 78º8'' to 79º 30'' E and Lat 8º 35'' to 9º 25'' N) by trawl catch, brought to the laboratory, cleaned and washed with fresh sea water to remove all impurities. The abdomen of cephalopods was cut open and the ink glands and mantle tissue were carefully removed.

5.4.ANTIOXIDANT ACTIVITY

5.4.1.Total antioxidant activity by Phosphomolybednum method

Determination of Total antioxidant activity: The total antioxidant activity was evaluated by Phosphomolybednum method described by(Prieto *et al*., 1999)1.0 ml of the extract was mixed with 1.0 ml of the standard reagent solution (0.6M sulphuric acid, 28Mm sodium phosphate and 4Mm ammonium molybdate). The tubes were capped and incubated in a thermal block at 95ºc for 90 min.After cooling to room temperature, the absorbance was measured at 695nm against a reagent blank. The total antioxidant capacity was expressed as percentage

% Antioxidant activity= 100-[(Standard Absorbance –Sample Absorbance/Standard Absorbance)x100].

5.4.2.Metal chelating activity

The metal chelating ability of SPH for chelation of ferrous ion was assessed using the method of (Decker and Welch *et al.,* 1990). A 1 ml aliquot of SPH was mixed with3.7ml of distilled water then, 0.1ml of 2Mm $FeCl_2$ and 0.2ml of 5Mm ferrozine were added. The reaction mixture was held for 10 min at room temperature before reading the absorbance at 562nm using a UV-Vis spectrophotometer (V-530; Jasco). Distilled water and EDTA at 250 ppm were used as a control and a positive control, respectively. The metal chelating were used as a control and a positive control, respectively. The metal chelating activity was calculated as:

Metal chelating activity(%) =1 –(Abs_{sample}/ $Abs_{control}$) x100.

5.5.ANTIDIABETIC ACTIVITY

5.5.1.Alpha amylase inhibition assay

The α-amylase inhibition assay was performed using the 3,5-dinitrosalicyclic acid (DNSA) method. The sample was dissolved in minimum amount of 10% DMSO and was further dissolved in buffer ((Na_2HPO_4/NaH_2Po_4 (0.02M), NaCl (0.006 M) at Ph 6.9) to give concentrations ranging from 10 to 1000µg/ml. A volume of 200µl of α-amylase solution (2 units /ml) was mixed with 200µl of the extract and was

incubated for 10 min at 30ºc. Thereafter 200µl of the starch solution (1% in water (w/v) was added to each tube and incubated for 3 min. The reaction was terminated by the addition of 200µl DNSA reagent (12g of sodium potassium tartarate tetra hydrate in 8.0 ml of 2M NaOH and 20 ml of DNSA reagent (12 g of sodium potassium tartarate tetra hydrate in 8.0 ml of 2M NaOH and 20 ml of DNSA reagent (12 g of sodium potassium tartarate tetra hydrate in 8.0 ml of 2M NaOH and 20 ml of 96Mm OF 3,5- dinitrosalicyclic acid solution) was oiled for 10 min in a water bath at 85-90ºc. The mixture was cooled to ambient temperature and was diluted with5 ml of distilled water, and the absorbance was measured at 540 nm using a UV-Visible spectrophotometer. The blank with 100% enzyme activity was prepared by replacing the plant extract with 200µl of buffer. A blank reaction was similarly prepared using the plant extract at each concentration in the absence of the enzyme solution. The α-amylase inhibitory activity was expressed as percent inhibition and was calculated using the equation given below:

% Inhibition =[Absorbance Control-Absorbance Test)/ Absorbance control]x100.

5.6.ANTI INFLAMMATORY ACTIVITY

5.6.1.Protein denaturation inhibition activity

Protein denaturation inhibition assay was done according to the method described by(Gambhire *et al*.,2009). The reaction mixture (5ml) consisted of 0.2 ml of 1% bovine albumin, 4.77 ml of phosphate buffered saline (PBS, pH 6.4), and 0.02 ml of extract, and the mixture was mixed, and was incubated in a water bath (37ºc) for 15 min, and then the reaction mixture was heated at 70ºc for 5 min. After cooling, the Bradford reagent added and absorbance was measured at 660 nm using a UV/VIS spectrometer. Phosphate buffer solution was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula:

% inhibition= [Absorbance Test/Absorbance control] x100.

5.6.2.Proteinase inhibition assay

Proteinase inhibition assay was done according to the method described by(Gambrine *et al*.,2009). The reaction mixture (5 ml) consisted of 0.2 ml of 1% bovine albumin, 4.78 ml of phosphate buffered saline (PBS, Ph 6.4), and 0.02 ml of extract, and 0, 05 ml of trypsin was mixed, and was incubated in a water bath (37ºc) for 15 min, After cooling, the Bradford reagent added and absorbance was measured at 660nm using a UV/VIS spectrometer.

Phosphate buffer solution was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula:

% Inhibition=[Absorbance Test/ Absorbance control]x100.

RESULTS

6.RESULTS

6.1.ANTIOXIDANT ACTIVITY

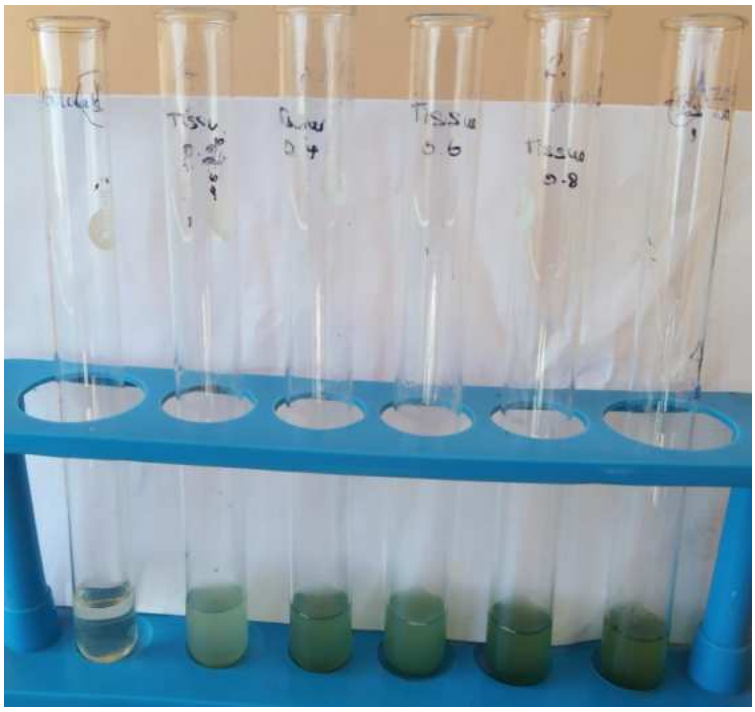
The antioxidant activities of cephalopod (*Sepia brevimana*) has been evaluated by two different methods namely total antioxidant and metal chelation inhibition activity at five different concentration (200µg/ml,400µg/ml, 600µg/ml, 800µg/ml and 1000µg/ml).

6.1.1. Total Antioxidant activity by Phosphomolybednum method

The tissue of *S.brevimana* showed inhibition activity ranging from 55% to 60%. At a concentration of 200µg/ml the tissue shows 57% of inhibition and at 1000 µg/ml activity increased to 60%. Activity increased as the concentration of tissue increased from 200 µg/ml to1000 µg/ml.

The ink of *S.brevimana* exhibits good antioxidant activity with the inhibition of 43% at 200µg/ml concentration, 44% at 400µg/ml concentration, 46% at 600µg/ml concentration, 47% at 800µg/ml concentration, 56% at 1000µg/ml concentration. Highest activity was observed at 1000µg/ml and the lowest activity was noted at a concentration of 200µg/ml. The total antioxidant activity is directly proportional to the concentration of the ink. The percentage of inhibition was compared with the control Ascorbic acid.

Plate:3.a.Total antioxidantby Phosphomolybednum method of tissue sample



b.Total antioxidant by Phosphomolybednum method of Ink sample

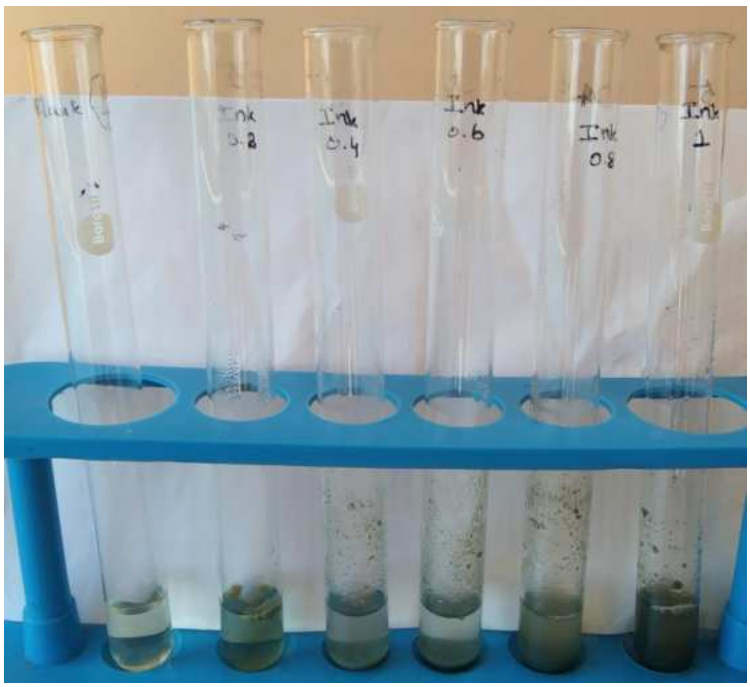
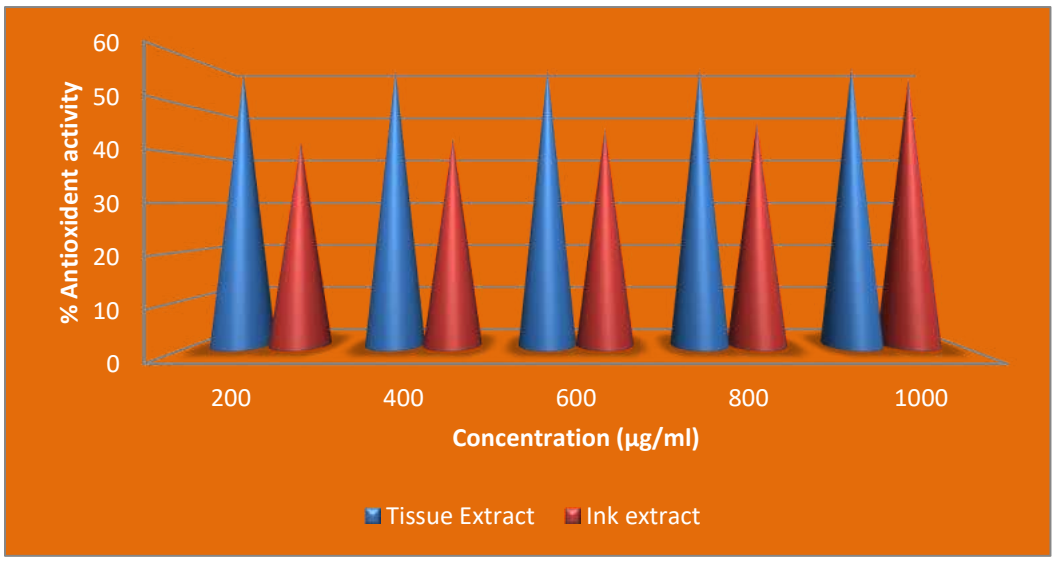


Table:1.a.Total antioxidant activity of tissue & ink extract in *S. brevimana*

Concentration (µg/ml)	Antioxidant(%) of (Tissue extract)	Antioxidant (%) of (Ink extract)
200	57.28	42.93
400	58.04	43.70
600	58.15	45.87
800	58.26	46.96
1000	58.59	56.20

Figure:2:a.Total antioxidant activity of tissue & ink extract in *S.brevimana*

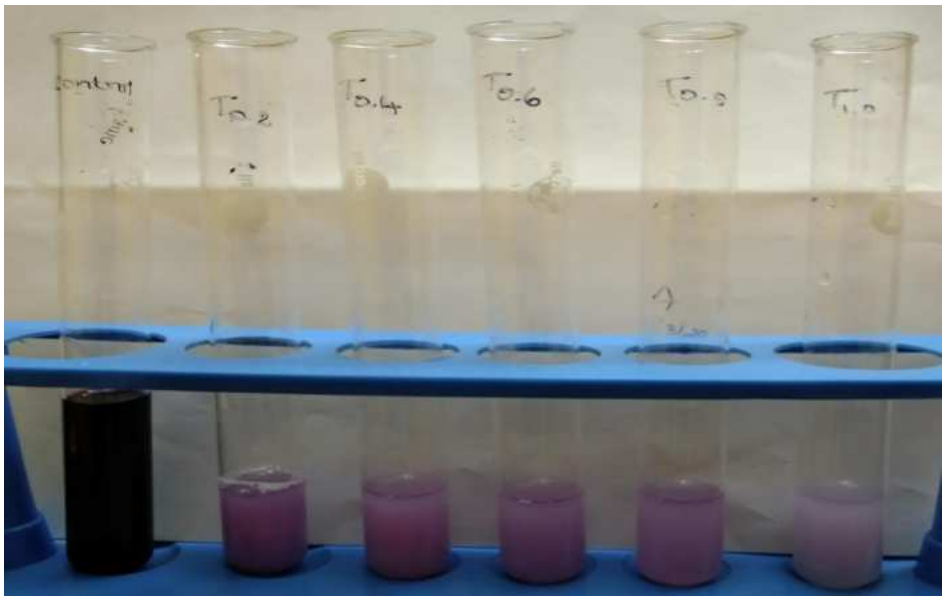


6.1.2. METAL CHELATING ACTIVITY

The metal chelation activity of the tissue extract of *S.brevimana* was measured at five different concentrations (200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml, 1000µg/ml). The highest activity of 96% was noted at a concentration 1000µg/ml and the lowest activity of 57% at a concentration 200µg/ml.

The ink of *S.brevimana*exhibits good Metal chelation activity with the inhibition of 8% at 200µg/ml, 10% at 400µg/ml, 11% at 600µg/ml, 13% at 800µg/ml and 39% of inhibition at 1000µg/ml concentration. Highest activity was observed at concentration of 1000µg/ml and the lowest activity was noted at a concentration of 200µg/ml. As concentration of the extract increased activity also increased. Comparing the two extract ink and tissue, tissue extract shows more activity.

Plate:4.a.Metal chelating activityof tissue sample



b.Metal chelating of Ink sample

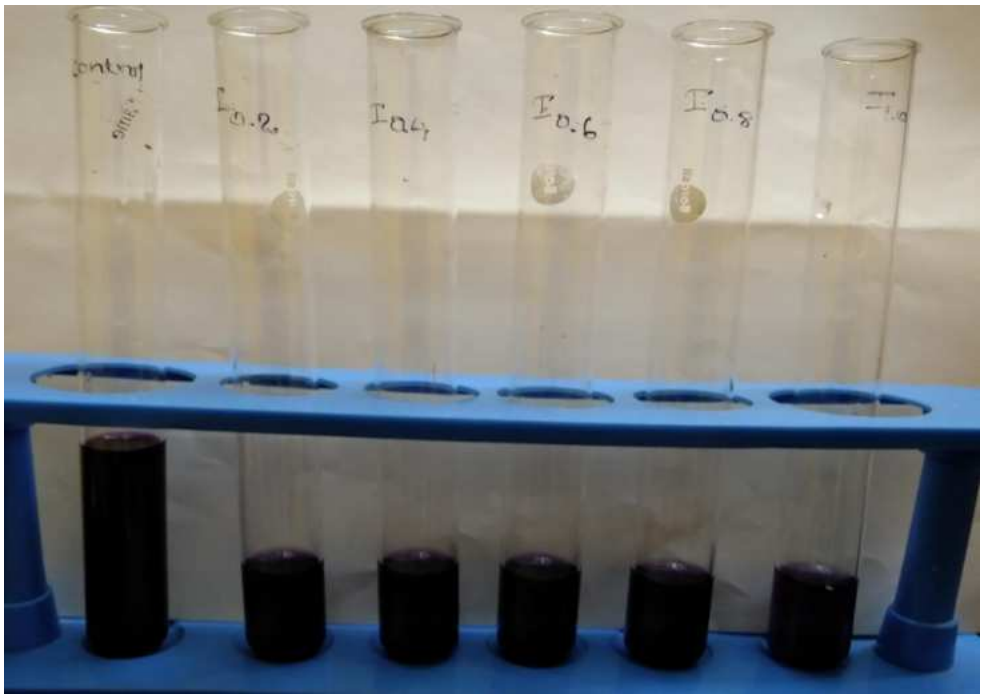
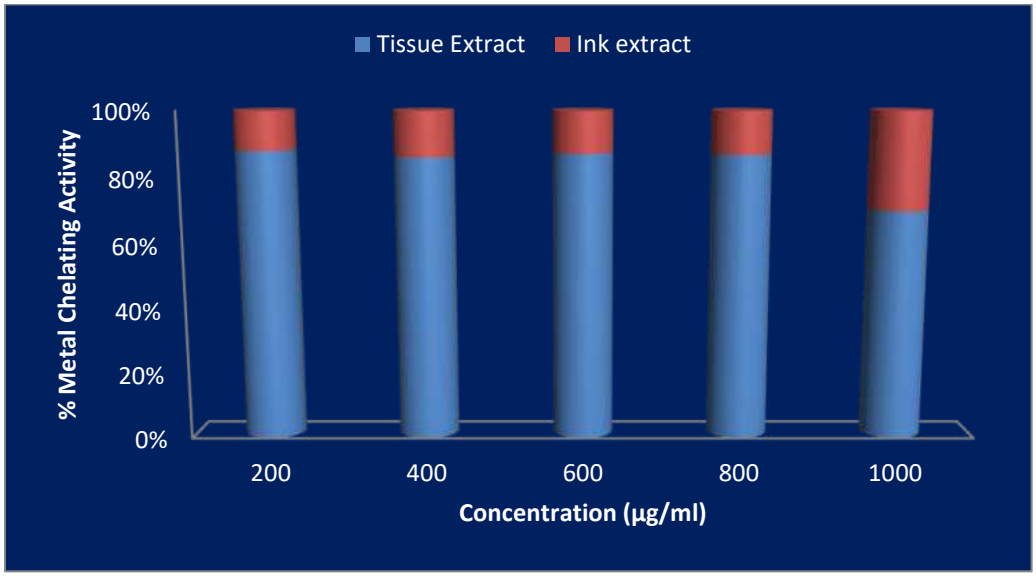


Table:1:b.Metal chelating activity of tissue & ink extract in *S. brevimana*

Concentration (µg/ml)	Metal chelating(%) of (Tissue extract)	Metal chelating(%) of (Ink extract)
200	56.92	7.69
400	64.10	10.26
600	73.03	10.67
800	89.74	13.44
1000	92.56	39.49

Figure:2.b.Metal chelating activity of tissue & ink extract in *S.brevimana*



7.1.ANTIDIABETIC ACTIVITY

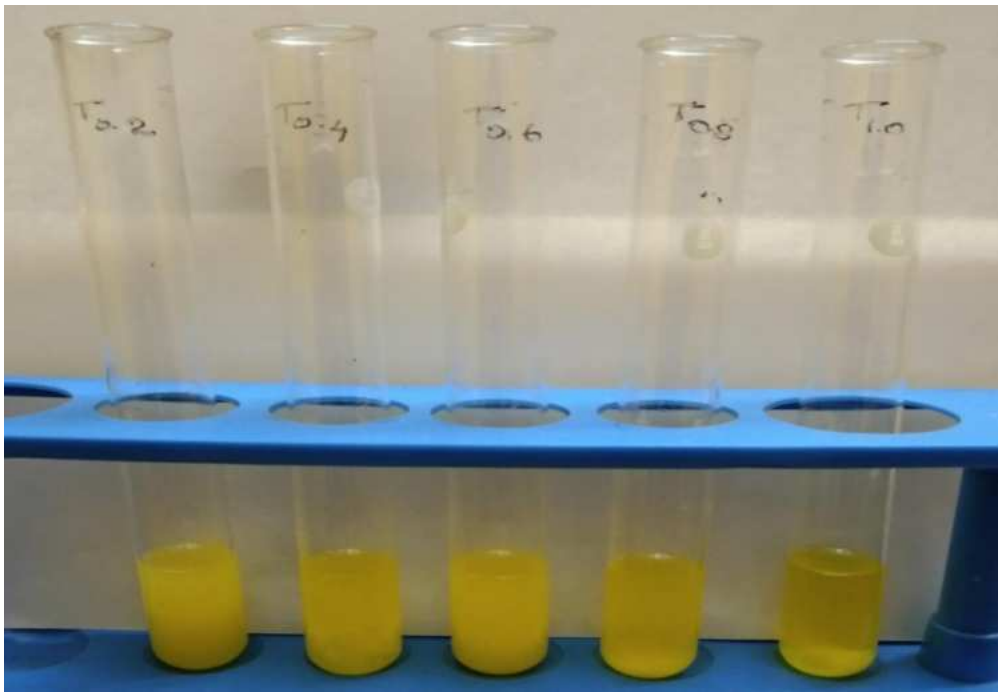
7.1.1.ALPHA AMYLASE INHIBITION ASSAY

The antidiabetic activity of cephalopod (*S.brevimana*) has been evaluated by Alpha amylase inhibition assay method at five different concentrations (200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml, 1000µg/ml).

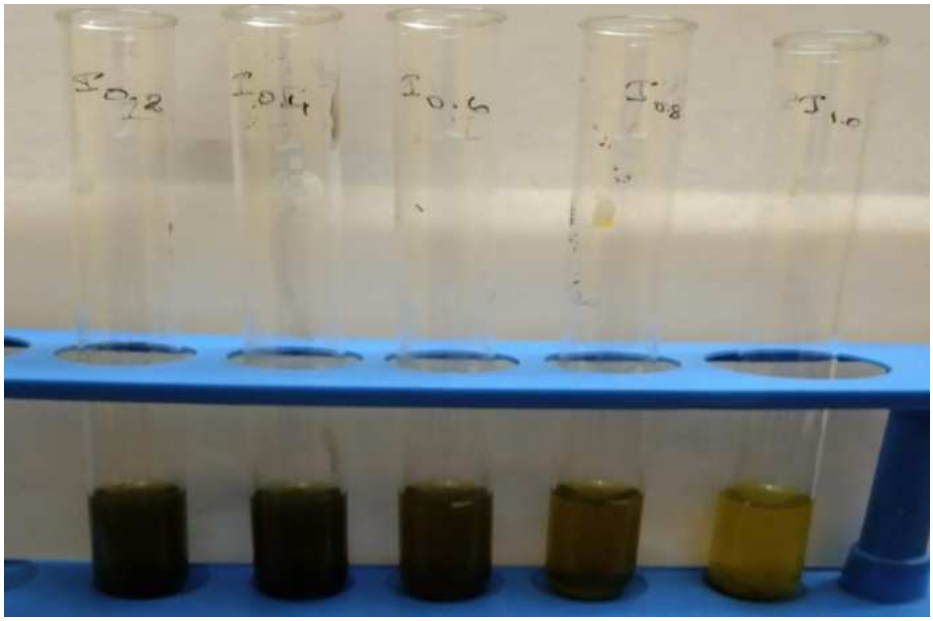
The tissue of *S.brevimana*showed inhibition activity ranging from 35% to 90%. At a concentration of 200µg/ml the tissue showed 39% of inhibition. Alpha amylase inhibition assay increased as the concentration of tissues increased and the activity raised to 89% at 1000µg/ml concentration.

The ink of *S.brevimana* exhibits good antidiabetic activity with the inhibition 18% at 200µg/ml concentration, 41% at 400µg/ml concentration, 53% at 600µg/ml concentration, 76% at 800µg/ml concentration and 84% of inhibition at 1000µg/ml concentration. Highest activity was observed at 1000µg/ml and the lowest activity was noted at a concentration of 200µg/ml. The inhibition activity is directly proportional to the concentration of the ink. The percentage of inhibition was compared with the control.

Plate:5.a.Alpha amylase inhibition assay of tissue sample



b. Alpha amylase inhibition assay of ink sample



8.1.ANTIINFLAMMATORY ACTIVITY

8.1.1.Protein Denaturation inhibition activity

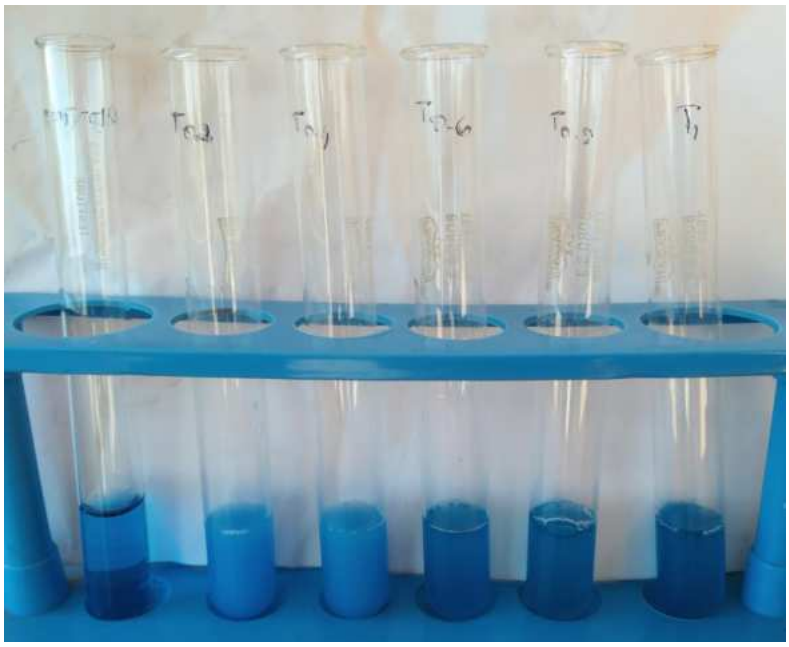
The protein denaturation inhibition activity of *S.brevimana* tissue was shown in Fig:4.a.Protein denaturation inhibition activity was measured at five different concentrations (200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml,1000µg/ml). The highest antioxidant activity (10%) was noted at a concentration 1000µg/ml and the lowest activity of 9% at a concentration 200µg/ml.

The ink of *S.brevimana*exhibits good antioxidant activity with the inhibition of 0.4% at 200µg/ml, 4% at 400µg/ml, 6% at 600µg/ml, 8% at 800µg/ml, 9% at 1000µg/ml. Highest concentration activity was observed at a concentration of 1000µg/ml and the lowest activity was noted at a concentration of 200µg/ml. The results showed that the extract exhibited dose depend protein denaturation inhibition activity.

8.1.2.PROTEINASE INHIBITION ACTIVITY

The proteinases inhibition activity *of S.brevimana*tissue was measured at five different concentrations (200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml, 1000µg/ml). The highest inhibition activity (33%) was noted at a

Plate:6.a. Protein denaturation inhibition activity of tissue sample



b. Protein denaturation inhibition activity of ink sample

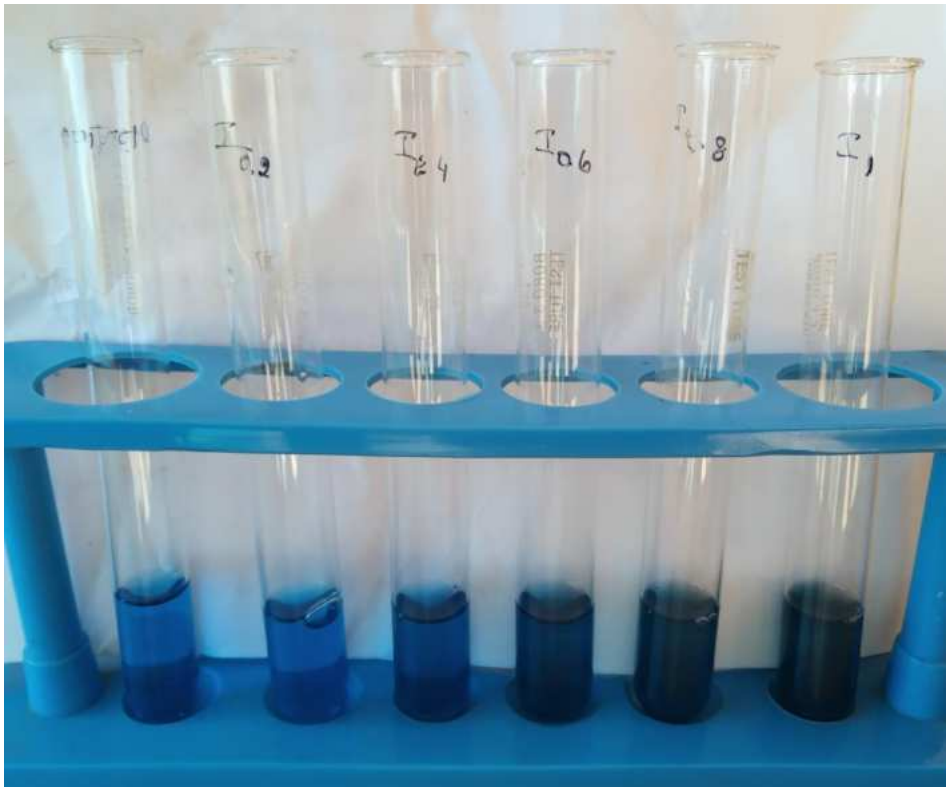


Table:3a.;Protein denaturation inhibition activity of tissue & ink extract in*S.brevimana*

Concentration (µg/ml)	Protein denaturation(%) of tissue extract	Protein denaturation(%) of ink extract
200	8.88	0.44
400	9.11	3.55
600	9.33	6
800	10	8.22
1000	10.44	9.11

Figure: 4.a. Protein denaturation inhibition activity of tissue & ink extract in*S. brevimana*

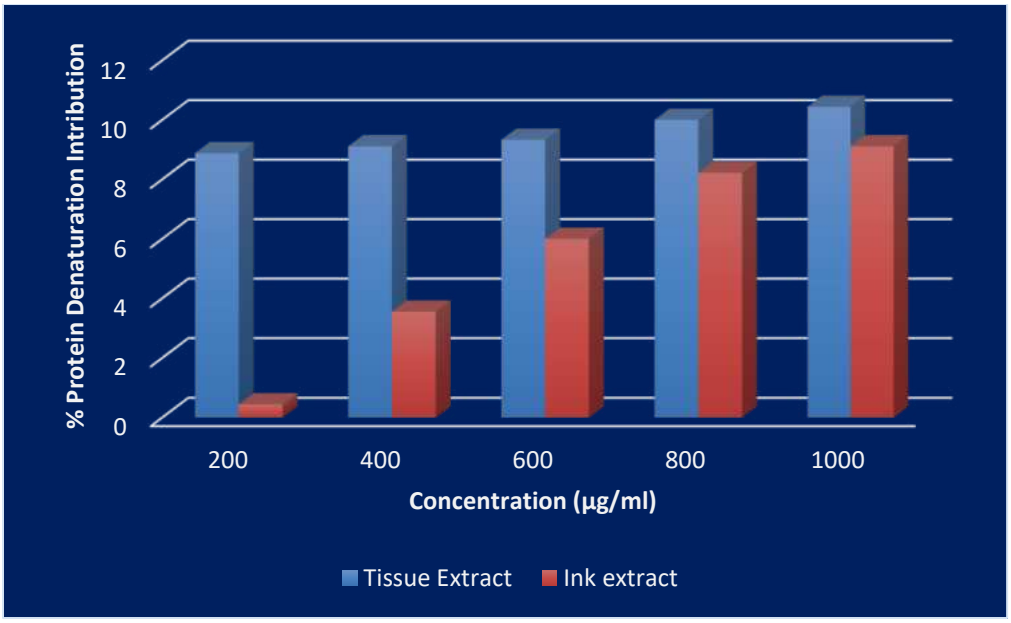
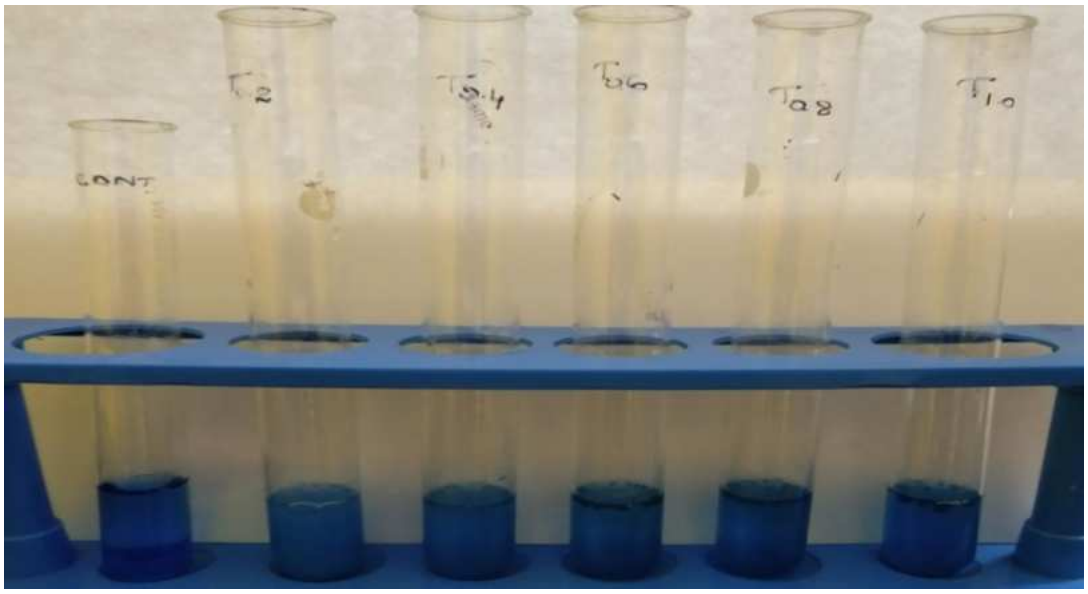


Plate:7.a. Proteinase inhibition assay of tissue sample



b. Proteinase inhibition assay of ink sample

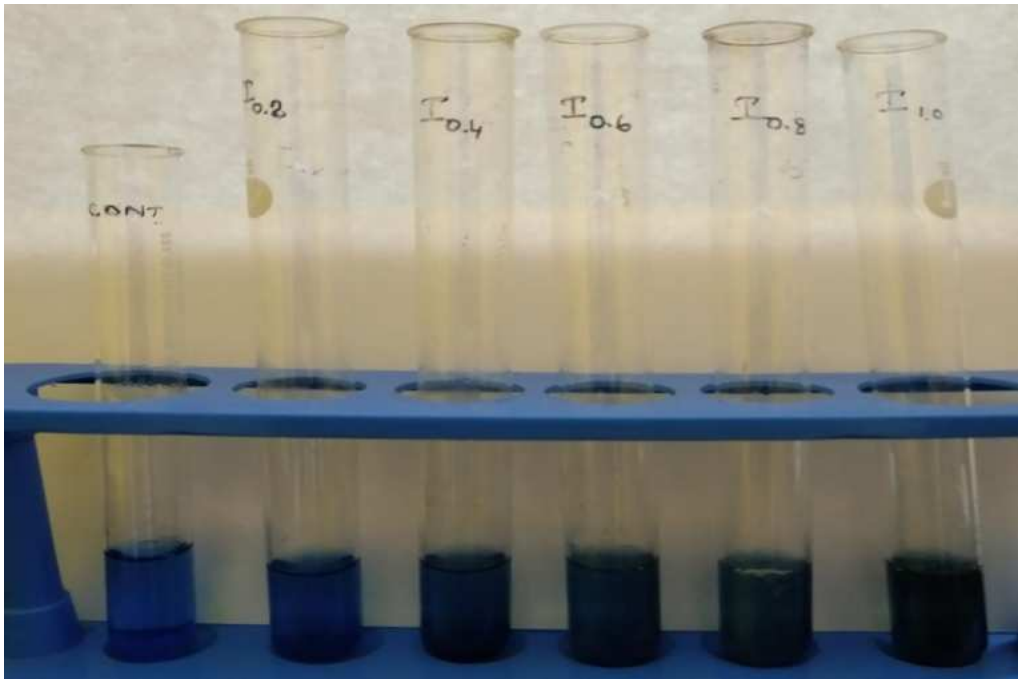
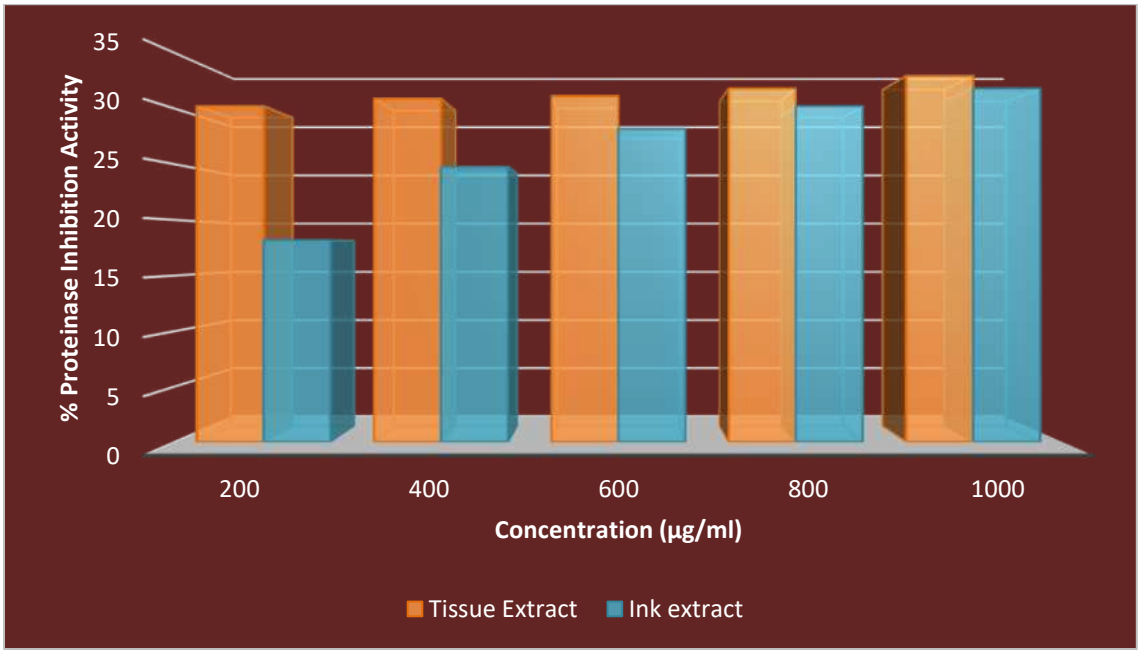


Table:3:b.Proteinase inhibition assay of tissue& ink extract in*S.brevimana*

Concentration (µg/ml)	Proteinase(%) of (Tissue extract)	Proteinase(%) of (Ink extract)
200	30.23	18.18
400	30.91	24.77
600	31.14	28.18
800	31.82	30.23
1000	32.95	31.82

Figure:4:b. Proteinase inhibition assay of tissue & ink extract in*S.brevimana*



concentration of 1000µg/ml and low at activity of 30% at a concentration of 200µg/ml.

The ink of *S.brevimana*exhibit good activity with the inhibition of 18% at 200µg/ml, 25% at 400µg/ml, 28% of 600µg/ml, 30% of 300µg/ml and 32% of inhibition of 1000µg/ml concentration.Highest activity was observed at concentration of 1000µg/ml and lowest activity was noted at a concentration of 200µg/ml.Tissue extract of *S.brevimana* shows better inhibition activity than the ink extract so tissue extract can be used as an anti-inflammatory agent.

DISSCUSION

7. DISSCUSION

In the present study antioxidant activities of cephalopod (*Sepia brevimana*) has been evaluated by two different methods namely total antioxidant and metal chelation inhibition activity. The tissue of *S.brevimana*showed inhibition activity ranging from 55% to 60%. At a concentration of 200 µg/ml the tissue shows 57% of inhibition and at 1000 µg/ml activity increased to 60%. Activity increased as the concentration of tissue increased from 200 µg/ml to 1000 µg/ml.

This results supports the previous work done by IIIamparthi*et al*., (2011) who studied the antioxidant activity (DDPH) of methanolic extract of squid, *Sepiabrevimana* and *Sepiellainermis* at different concentration (20,40,60,80, and 100µg/ml). The tissue of *S.brevimana* showed inhibition activity ranging from 55% to 60%. At a concentration of 200 µg/ml the tissue shows 57% of inhibition and at 1000 µg/ml activity increased to 60%. Activity increased as the concentration of tissue increased from 200 µg/ml to 1000 µg/ml. Similarly Ponnusamy*et al.,* (2016) who reported the highest scavengering activity at 100µg/ml concentration from *S.pharaonis, S.intermis* and *Octopus vulgaris* (81.25,73.31 and 54.47% respectively by DPPH assay.

The results of the present study shows that the highest activity in the ink extract of *Sepiabrevimana* was observed at 1000µg/ml and the lowest activity was noted at a concentration of 200µg/ml. The total antioxidant activity is directly proportional to the concentration of the ink. This study corroborated with the study of EnsibiCherif*et al*., (2015). He studied the antioxidant activity of *Aplysiadepilans* ink extract using DPPH scavenging assay. The results revealed that the strongest antioxidant activity of the ink. Collagen hydrolysates of fins and arms of Jumbo squid (*Dosidicugigas*) are capable of acting as antioxidant scavenging radicals (Surez-Jimenz*et al.,* 2015). This studies coincides with the results of the present study.

Ponnusamy*et al.,* (2016) studied the nitric oxide free radical scavengering activity in the methanolic extract and the results showed that the methanolic extract of cephalopods had scavenging ability 84.02%, 74.31% and 51.65% respectively at 100µg/ml concentration. This is in accordance with present work.

Chelating agents are able to capture ferrous ion before ferrozine, thus hindering the formation of ferrozine -Fe2+. Ferrozine can quantitatively form complexes with Fe2+. In the presence of chelating agents, the complex formation is disrupted, resulting in a decrease in the red colour of the complex. Measurement of colour reduction therefore allows estimating the

metal chelating activity of the coexisting chelator. In the present investigation the ink of*S.brevimana*exhibits good Metal chelation activity with the inhibition of 8% at 200µg/ml, 10% at 400µg/ml, 11% at 600µg/ml, 13% at 800µg/ml and 39% of inhibition at 1000µg/ml concentration.

A similar finding was also obtained byNaveen Kumar Vate and SoottawatBenjakul,(2013) who investigated the antioxidant activity and metal chelating activity of melanin-free ink(MFI) from splendid squid (*Loligoformosana*). Squid ink had metal chelating activity of 4.0 ± 1.2µmol EE/g protein. Some compounds in MFI could chelate prooxidative metals, thereby lowering orretarding the initiation of lipid oxidation process. The capacity of antioxidant forchelating metals is strongly dependent on the number of hydroxylic groups inortho-position (Maqsood and Benjakul 2010). Squid ink was reported to function asantioxidant in hyperlipidemia rats and broil chicken (Lei *et al.* 2007; Liu *et al*.2011).SimilarlayGayathri*et al*., 2017 evaluated antioxidant activity of ampullariidae (*Pilavirens*) whole tissue extract at various concentrations (20-250µg/ml). The results of gastropods methanolic tissue extracts exhibited significant total antioxidant activity, DPPH, Hydroxyl radical scavenging activity, total reducing power, chelating ability on ferrous ions activity which predicted as 67.09%, 74.83%, 60.21% and 59.89%. respectively.

The metal chelating capacity is important since it reduces the concentration of transition metals that may act as catalysts to generate the first few radicals to initiate the radical-mediated oxidative chain reactions in biological and food systems.

Gajendra*et al.,* (2020) studied the antioxidant activity in the melanin free ink of *Loligoduvauceli*by Diphenyl-1- picrylhydrazyl free radical scavenging activity (DPPH FRSA), Ferric reducing antioxidant power (FRAP) activity. The result indicates that the MFI which can be obtained from the suid ink sac, a waste from squid processing unit can be used as natural antioxidant in many food products to retard the lipid oxidation and shelf- life of the food can also be enhanced.

Results of DPPH assay showed that the water extraction of squid ink powder has the highest 94.87±4.87% followed by ethanol 67.57±7.55% and hexane extract 2.10±1.18%.FRAP assay result presented the same trend with water extraction had the highest value of 929.67±2.31 µmol Fe (II) /g of sample extract, followed by ethanol extract 201.00±26.29 µmol Fe (II) per gram sample and hexane 79.67±12.66 µmol Fe (II) /g of sample extract. Each of value obtained showed the difference of each extraction solvent used. The results also indicate that all extracts of squid ink are able to act as a reducing agent which provides electron for stability.(Fatimah *et al*.,2018).

Ingestion of carbohydraterich diet causes elevation in blood glucose level by the rapid absorption of carbohydrates in the intestine aided by the action of glycoside hydrolases, which breaks dietary carbohydrates into absorbable monosaccharides.(Berdanier*et al.*, 2007).Oral anti-hyperglycaemic agents such as acarbose and voglibose are used in treating the non-insulin-dependent DM but they cause side effects such as abdominal distension, bloating, flatulence and diarrhoea. These are all caused by the excessive inhibition of pancreatic α-amylase, leading to bacterial fermentation of undigested carbohydrate in the colon (Jaiyesimi*et al.* 2009).Due to the gastro intestinal side effects, searching for new amylase inhibitors became essential in treatment of diabetes.Thus, the use of glycosidase inhibitor, such as α-glucosidase and α-amylase inhibitors, would be a prospective therapeutic agent for the effective management of diabetes.Hence in the present study, the potency of the ink extract and tissue extract were tested for α amylase inhibition activity.

In the present study α- amylase inhibitory activity was carried out in the ink and tissue extract of *Sepiabrevimana*.Tissue extract showed the maximum inhibition of 89% at 1000µg/ml, and minimum of 39% at 200 µg/ml. Percentage of inhibition increases with the increasing concentration of the extract. Similar to the present study (KajalChakrabort*yet al.,*

2016)evaluated the effectiveness of ethyl acetate-methanol extracts of cephalopods namely, *Amphioctopusmarginatus, Urothethisduvauceli, Sepia pharaonis, Sepiellainermis, and Cistopusindicus*as an anti-diabetic agent.

The α-amylase inhibitory activities of *C. indicus, S.inermis, and U. duvauceli* were recorded to be significantly greater (IC90 ~ 1.7 mg/mL) when compared with other cephalopods (IC90 1.9 – 2.5 mg/mL; $p < 0.05$).*A. marginatus* displayed least α-amylase inhibitory activity (IC90 2.50 mg/mL) because of greater IC90 value than the other cephalopod species (IC90 lesser than 2 mg/ mL).

Pancreatic α-amylase is a key enzyme in the digestive system and catalyses the initial step in hydrolysis of starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose, and a number of small molecular weight α-(l–6) and α-(1–4) oligoglucans. These are then acted on α-glucosidases and further degraded to glucose, which on absorption enters the bloodstream. (Berdanier*et al*., 2007)**.** Degradation of this dietary starch proceeds rapidly and leads to elevated post-prandial hyperglycemia (PPHG). Retardation of starch digestion by inhibition of enzymes, such as α-amylase, plays a key role in the control of diabetes. Inhibitors of pancreatic α-amylase delay carbohydrate digestion causing a reduction in the rate of glucose

absorption and lowering the post-prandial serum glucose levels.(Sadhasivam*et al*., 2013).

The solvent extracts derived from the members of the order Octopoda demonstrated fairly good α-amylase inhibitory activity (IC90 ≤ 2.5 mg/mL), and in which, *C. indicus* displayed highest anti-α-amylase property (IC90 1.69 mg/mL). (Sadhasivam*et al*., 2013) explained α-amylase inhibitory properties of the methanolic extract of three marine mollusks,namely *Aplysiasp,*(93.0mg/mL), *Bursatellaleachii*(50.0mg/mL)*,* and *Kalingaornate* (0.1mg/mL). (Abirami*et al*., 2011) also observed moderate α-amylase inhibitory activity by the purple fluid of the marine gastropod mollusk*Dolabellaauricularia*. An α-amylase inhibition of 72% was observed by (Ravi *et al.,* 2012) for the methanolic extract of two marine molluscs *Hemifususpugilinus* and *Naticadidyma*. Reports of (Tiwari *et al*., 2008) confirmed the anti-glycemic activities of the crude extracts of bivalve mollusks in animal model. It is significant to note that the cephalopod species, particularly *C. indicus* displayed potential anti-diabetic activities as determined by in vitro α-amylase/α-glucosidase inhibition assays. More importantly, the anti-diabetic activities of the mollusks belonging to the class cephalopoda were found to be greater than other classes of molluscs

(Gastropoda and Bivalvia) reported in the literature. These findings concord with our results.

One of the therapeutic approaches for decreasing postprandial hyperglycemia is to prevent absorption of glucose by the inhibition of carbohydrate hydrolysing enzymes such as α-glucosidase and α-amylase. α-amylase, plays a key role in the control of diabetes. Inhibitors of pancreatic α-amylase delay carbohydrate digestion causing a reduction in the rate of glucose absorption and lowering the post-prandial serum glucose levels. The results of α-amylase inhibition activityof *Sepiabrevimana*extract proves that it can be used as an anti-diabetic agent.

The protein denaturation inhibition activity of *S.brevimana*inkwas measured at five different concentrations (200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml, 1000µg/ml).Highest concentration activity was observed at a concentration of 1000µg/ml and the lowest activity was noted at a concentration of 200µg/ml.(Sri Kumaran*et al*., 2021) studied the protein denaturation and anti-proteinase bioassay for *in vitro* assessment of anti-inflammatory activityin the ethanolic extraction of *L. vulgaris* ink. The ink was It was effective in protein denaturation at different concentrations 50, 100, 150, 200 µg/mL. 68.9% inhibition of protein denaturation by the

squid ink extract indicated that it has very good in vitro anti-inflammatory properties.

The proteinases inhibition activity *of S.brevimana*tissue as well as ink was measured. Highest activity was observed at concentration of 1000µg/ml and lowest activity was noted at a concentration of 200µg/ml. Tissue extract of *S.brevimana* shows better inhibition activity than the ink extract. But in contrast to the present study (SriKumaran*et al*., 2021) studied the antiproteinase ability of ethanolic extraction of *L.vulgaris* at different concentration. The extracts of *L. vulgaris* have shown 67.4 % inhibition activity at 200µg/ml concentration.

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a rich source of proteinase which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors. (Oyedepo and Femurewa , 1995).

SUMMARY

8. SUMMARY

The oceans are probably the earth's most valuable natural resource providing food mainly as fish and shellfish. Because of its phenomenal biodiversity, the marine world is a rich source for many biologically active compounds. The result suggest that the tissue and ink of cephalopod (*S.brevimana*) showed appreciable antioxidant activity, antidiabetic activity, anti-inflammatory activity.

Antioxidants in biological systems have multiple roles, including defending against oxidative damage and participating in the major signaling pathways of cells. Total antioxidant activity of *S.brevimana* showed inhibition activity ranging from 50% to 60% of (Tissue) 40% to 60% (ink). The highest inhibition activity was observed at (1000µg/ml) concentration. Comparing the two sample the tissue extract showed maximum inhibition activity.

Metal chelating activity is the administration on chelating agents to remove heavy metals from the body. The highest chelating activity of 93% was observed at a concentration of 1000µg/ml and the lowest activity of 57% at a concentration of 200µg/ml in tissue extract. Ink of *S.brevimana* exhibits good chelating activity with the percentage of inhibition of 8% at

200µg/ml, 10% at 400µ/ml, 11% at 600µg/ml, 13% at 800µ/ml, 39% at 1000µg/ml concentration. The metal chelating activity is directly proportional to the concentration.

Diabetes mellitus is group of metabolic disorders characterized by a chronic hyperglycemic condition results from defects hyperglycemic condition results from defects insulin secretion, insulin action or both. In *S.brevimana* a highest α- amylase activity of 89% at a concentration of 1000µg/ml and the lowest activity of 39% at 200µg/ml in tissue extract. Ink of *S. brevimana* exhibits a good amylase activity with the percentage inhibition (18% at 200µg/ml, 41% at 400µg/ml , 53% at 600µg/ml, 76% at 800µg/ml, 84% at 1000µg/ml) concentration. Comparing with two sample tissue extract have a maximum amylase activity.

Inflammation is part of the minimum response against infection and has been implicated in a broad range of diseases, including diabetes, cancer, hypertension and atherosclerosis. The maximum denaturation inhibition activity of 10% was attained at 1000µg/ml and minimum inhibition activity of 91% was observed at 200µg/ml concentration. The percentage of proteinase inhibition activity ranged from 30%-40%. The percentage of protease inhibition activity of tissue extract increased with increasing concentration. The percentage of proteinase inhibition activity of ink ranged

from 11%-30%. The maximum protection of 32% was attained at 1000µg/ml concentration and the minimum protection of 18% was observed at 200µg/ml concentration. Tissue extract of *S. brevimana* shows better inhibition activity than the ink extract so tissue extract can be used as anti-inflammatory agent.

The results of the present study showed that the ink and tissue extracts showed potential pharmacological activities which indicates the presence of potent bioactive substance in them and correct understanding and utilization may lead to its use an drug.

CONCLUSION

9.CONCLUSION

Cephalopods are considerably important as a food resource as well as in scientific investigations. There is an ever continuous and urgent need to discover new compounds with diverse chemical structures and novel mechanisms of action due to the alarming increase that has been witnessed in the incidence of both new and emerging infectious disesases. The present study recommends the use of tissue and ink as a valuable biopharmaceutical values and to pair them with acceses to reliable safe drugs globally.

Nature bioactive substances have the least quantum of side effects when compared to synthetic products. Since the ink of cephalopods is available abundantly as waste materials , if more attention is given on the isolation and characterization of its bioactive compounds, it may pave the way for the development of new drugs from cephalopod waste.. A novel therapeutic compound from this marine source would definitely aid in the control and emergence of drug resistant strains.

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SYNTHESIS OF ZINC OXIDE AND SILVER NANOPARTICLES FROM ASCIDIANS

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CERTIFICATE

This is to certify that this dissertation entitled, **"SYNTHESIS OF ZINC OXIDE AND SILVER NANOPARTICLES FROM ASCIDIANS"** submitted by **S. SUBBU LAKSHMI, Reg. No. 19APZO08** to St. Mary's College (Autonomous), Thoothukudi, affiliated to Manonmaniam Sundaranar University, Tirunelveli in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2020 - 2021 under my guidance and supervision. It is further certified that this dissertation or any part of this has not been submitted elsewhere for any other degree.

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EXAMINER

DECLARATION

I do hereby declare that this thesis entitled, "**SYNTHESIS OF ZINC OXIDE AND SILVER NANOPARTICLES FROM ASCIDIANS**" submitted by me for the award of the degree of Master of Science in Zoology is the result of my original independent research work carried out under the guidance of **Dr. M. Paripooranaselvi M.Sc., M.Phil., B.Ed., Ph.D., SET.**, Assistant Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date: 15.04.2021

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CONTENTS

LIST OF ABBREVIATIONS

nm	-	Nano-Meter
ZnONPs	-	Zinc Oxide Nanoparticles
XRD	-	x- Ray Diffraction
XPS	-	X-Ray Photoelectron Spectroscopy
UV-Vis	-	Ultra Violet Visible Spectroscopy
TiO_2	-	Titanium Oxide
TEM	-	Transmission Electron Microscopy
SPR	-	Surface Plasmon Resonance
Si	-	Silicon
SEM	-	Scanning Electron Microscopy
rpm	-	Rotation Per Minute
PBS	-	Phosphate Buffered Saline
mV	-	Mili volt
min	-	Minutes
MIC	-	Minimum Inhibitory Concentration
KBr	-	Potassium Bromide
ITO	-	Indium Tin Oxide
Hrs	-	Hours
Ga	-	Gallium
FTIR	-	Fourier Transform Infra Red Spectroscope
FESEM	-	Field Emission Scanning Electron Microscope
EDAX	-	Energy Dispersive X-Ray Analysis
CuO	-	Copper Oxide
Al	-	Aluminium
$AgNO_3$	-	Silver Nitrate
Ag_NPs	-	Silver Nanoparticles
µL	-	Micro Liter

1. Introduction

The field of nanotechnology is one of the most active research areas in modern materials science. Nanoparticles exhibit new or improved properties based on specific characteristics such as size, distribution and morphology. There have been impressive developments in the field of nanotechnology in the recent past years, with numerous methodologies developed to synthesize nanoparticles of particular shape and size depending on specific requirements. New applications of nanoparticles and nanomaterials are increasing rapidly. Nanotechnology can be termed as the synthesis, characterization, exploration and application of nanosized (1-100nm) materials for the development of science. It deals with the materials whose structures exhibit significantly novel and improved physical, chemical, and biological properties, phenomena and functionality due to their nano scaled size. Because of their size, nanoparticles have a larger surface area than macro-sized materials. The intrinsic properties of metal nanoparticles are mainly determined by size, shape, composition, crystallinity and morphology. Nanoparticles, because of their small size, have distinct properties compared to the bulk form of the same material, thus offering many new developments in the fields of biosensors, biomedicine and bio nanotechnology. Nanotechnology is also being utilized in medicine for diagnosis, therapeutic drug delivery and the development of treatments for many diseases and disorders. Nanotechnology

is an enormously powerful technology, which holds a huge promise for the design and development of many types of novel products with its potential medical applications on early disease detection, treatment and prevention.

Due to swift industrialization and urbanization our environment is undergoing huge smash up and a large amount of perilous and superfluous chemical, gases or substances are released, and so now it is our need to learn about the secrets that are present in the nature and its products which leads to the growth of advancements in the synthesis processes of nanoparticles. Nanotechnology applications are highly suitable for biological molecules, because of their exclusive properties. The biological molecules undergo highly controlled assembly for making them suitable for the metal nanoparticle synthesis which found to be reliable and ecofriendly. The synthesis of metal and semiconductor nanoparticles is a vast area of research due to its potential applications which was implemented in the development of novel technologies. The field of nanotechnology is one of the upcoming areas of research in the modern field of material science. Nanoparticle show completely new or improved properties, such as size, distribution and morphology of the particles etc. Novel applications of nanoparticles and nanomaterials are emerging rapidly on various fields (Kaviya *et al*., 2011).

Metal nanoparticles have a high specific surface area and a high fraction of surface atoms. Because of the unique physicochemical

characteristics of nanoparticles, including activity, optical properties, electronic properties, antibacterial properties, and magnetic propertiesthey are gaining the interest of scientist for their novel methods of synthesis (Catauro *et al*., 2005; Crabtree *et al*., 2003; Krolikowska *et al*., 2003; Zhao, 1998). Over the past few years, the synthesis of metal nanoparticles is an important topic of research in modern material science. Nano-crystalline silver particles have been found tremendous applications in the fields of high sensitivity biomolecular detection, diagnostics, antimicrobials, therapeutics, catalysis and micro-electronics. However, there is still need for economic commercially viable as well as environmentally clean synthesis route to synthesize the silver nanoparticles. Silver is well known for possessing an inhibitory effect toward many bacterial strains and microorganisms commonly present in medical and industrial processes (Jiang *et al*., 2004). In medicines, silver and silver nanoparticles have anexample application including skin ointments and creams containing silver to prevent infection of burns and open wounds (Duran *et al*., 2005), medical devices and implants prepared with silver-impregnated polymers (RO, 1999). In textile industry, silver-embedded fabrics are now used in sporting equipment (Klaus *et al*., 1999).

Nanoparticles can be synthesized using various approaches including chemical, physical, and biological. Although chemical method of synthesis requires short period of time for synthesis of large number of nanoparticles,

this method requires capping agents for size stabilization of the nanoparticles. Chemicals used for nanoparticles synthesis and stabilization are toxic and lead to non-ecofriendly byproducts. The need for environmental non-toxic synthetic protocols for nanoparticles synthesis leads to the developing interest in biological approaches which are free from the use of toxic chemicals as byproducts.

Plants provide a better platform for nanoparticles synthesis as they are free from toxic chemicals as well as provide natural capping agents. Moreover, use of plant extracts also reduces the cost of microorganisms isolation and culture media enhancing the cost competitive feasibility over nanoparticles synthesis by microorganisms (Garima Singhal *et al*., 2011).

The smaller particles have higher antibacterial activities due to the equivalent silver mass content. With respect to the clinical applications of nanoparticle, microorganisms including diatoms, fungi, bacteria and yeast producing inorganic materials through biological synthesis either intra or extracellularly made nanoparticles more biocompatible (Guidelli *et al*., 2011).

ZINC OXIDE NANOPARTICLES: It usually appears as a white powder and is nearly insoluble in water. The powder is widely used as an additive for numerous materials and products including plastics, ceramics, glass, cement, rubber (e.g. car tyres), lubricants, paints, ointments, adhesives, sealants, pigments, foods (source of Zn nutrient), batteries, ferrites, fire retardants, etc.

ZnO is present in the Earth crust as a mineral zincite; however, most ZnO used commercially is produced synthetically. ZnO is nontoxic and is compatible with human skin making it a suitable additive for textiles and surfaces that come in contact with human body. The increase in surface area of nanoscale ZnO compared to bulk has the potential to improve the efficiency of the material function.

IMPORTANCE OF ZINC OXIDE NANOPARTICLES

- It is used in paints, cosmetics, sunscreens, plastic and rubber manufacturing, electronics and pharmaceuticals products etc.
- It is also potentially used to treat leukemia and carcinoma cancer cell
- It is also a strong antibacterial agent
- It is also used as drug carrier
- ZnO nanoparticles is also used in industrial sectors including environmental, synthetic textiles, food, packaging, medical care, healthcare, as well as construction and decoration.

SILVER NANOPARTICLES: Silver nanoparticles are one of the promising products in the nanotechnology industry. The development of consistent processes for the synthesis of silver nanomaterials is an important aspect of current nanotechnology research.

Silver nanoparticles can be synthesized by several physical, chemical and biological methods. However for the past few years, various rapid

chemical methods have been replaced by green synthesis because of avoiding toxicity of the process and increased quality.

IMPORTANCE OF SILVER NANOPARTICLES

- It is used for purification and quality management of air, biosensing, imaging, drug delivery system.
- Biologically synthesized silver nanoparticles have many applications like coatings for solar energy absorption and intercalation material for electrical batteries, as optical receptors, as catalysts in chemical reactions, for biolabelling, and as antimicrobials.
- Though silver nanoparticles are cytotoxic but they have tremendous applications in the field of high sensitivity bimolecular detection and diagnostics, antimicrobials and therapeutics, catalysis and micro-electronics.
- It has some potential application like diagnostic biomedical optical imaging, biological implants (like heart valves) and medical application like wound dressings, contraceptive devices, surgical instruments and bone prostheses.
- Many major consumer goods manufacturers already are producing household items that utilize the antibacterial properties of silver nanoparticles. These products include nano silver lined refrigerators, air conditioners and washing machines.

APPLICATION OF NANOPARTICLES: Once materials are prepared in the form of very small particles, they change significantly their physical and chemical properties. In fact in nano-dimension, percentage of surface molecule compare to bulk molecule is high and this enhances the activity of the particle in nano dimension and therefore, the normal properties of the particle like heat treatment, mass transfer, catalytic activity, etc are all increases. But compare to non-metal nanoparticles, metal nanoparticles have more industrial application. Nanoparticles offer many new developments in the field of biosensors, biomedicine and bio nanotechnology-specifically in the areas-

- Drug delivery
- As medical diagnostic tools,
- As a cancer treatment agent (Gold nanoparticles).

Ascidians are dominant organisms in many marine communities, having a wide geographic distribution. This ecological success is because of their ability to synthesize secondary metabolites, which possess an important defensive role against predation. They have increasingly become the target of natural products research. A natural product is a chemical compound or substance produced by a living organism - found in nature that usually has a pharmacological or biological activity for use in pharmaceutical drug discovery and drug designing. Research typically focuses on sessile organisms or slow moving animals because of their inherent need for chemical defences.

Many marine sedentary organisms produce components with unique structural pattern, for their chemical defence which do not occur in terrestrial plants. Sponges, bryozoans and tunicates are important source of new active principles for drug development.

Due to physical and chemical conditions of the marine environment, almost every class of marine organism exhibits variety of molecules with unique structural features, which are not found in terrestrial natural products. Organisms with no apparent physical defence, like sessile organisms, are believed to have evolved chemical defences to protect themselves.

Marine organisms have been reported to be a rich source of biologically active compounds, especially ascidians which are most prominent sources of new compounds. The majority of metabolites reported from ascidians are derived from amino acids and it is an important source in drug discovery. They are considered as a nuisance as they grow on all underwater marine structures and are usually thrown away. Such discards may have a wealth of natural products. Marine organisms, especially those that are a nuisance to the environment like biofoulers can be usede for synthesis of nanoparticles.

The marine environment is an excellent source of novel chemicals, not found in terrestrial sources. According to Davidson, 1993; Faulkner, 2002 and Blunt *et al*., 2006 marine organisms such as ascidians, sponges and soft corals containing symbiotic microorganisms are a rich source of bioactive

compounds. Dhorajiya *et al*., 2012 expressed that some of the compounds derived from marine organisms have antioxidant properties and anti-cancer activities, but they are largely unexplored.

Ismail *et al*., 2008 and Dellai *et al*., 2010 noted that since the few last decades, marine environment have been recognized to be a rich source of bioactive metabolites with varied biological and pharmacological activities. Chakraborty and Ghosh, 2010 suggested that bioactive peptides with novel structures have also been shown in ascidians. Synthesis of nanoparticles from is lacking. As ascidians are available along the Tuticorin coast an attempt has been made to synthesize nanoparticles.

2. Objectives

- Collection of ascidian *Phallusia nigra*
- Synthesis of ZnO nanoparticles from *Phallusia nigra*
- Synthesis of silver nanoparticles from *Phallusia nigra*
- Determining characterization of nanoparticles By Uv-Vis Spectrophotometer
- Studying chemical composition of the synthesized nanoparticles by using FTIR spectrometer

3. Review of literature

Nanoparticles and nanostructure are becoming a part in human medical application, including imaging or the delivery of therapeutic drugs to cell, tissues and organs. Drug loaded nanoparticles interact organ and tissues and are taken up by cells. Several studies have shown that the tissue, cell and even cell organelle distribution of drugs may be controlled and improved by their entrapment in colloidal nanomaterials, mainly of the micellar structure, such as nanocontainer.

The structure of zinc oxide (ZnO) surface has been computationally investigated using new atomistic potentials. The bulk termination is also subjected to high concentrations of dimer vacancies which corresponds to fractional occupations in the surface layers. Mechanical properties such as internal stress or adhesion are important in order to guarantee the patterning accuracy and durability for various types of commercial applications.

Zinc oxide is no stranger to scientific study. In the past 100 years, it has produced as a subject of thousands of research papers, dating back as early as 1935. For its potential of ultra violet absorbance, wide chemistry, piezoelectricity and luminescence at high temperatures, ZnO has entered into industry and now is one of the critical building blocks in todays modern society. It is found in paints, cosmetics, plastic and rubber manufacturing,

electronics and pharmaceuticals. More recently however, it has again gained large interest for its semiconducting properties.

Among this oxide nanoparticles, ZnO nanostructure material has gained much interest owing to its wide applications for various devices such as solar cells, varistors, transducers, transparent conducting electrodes, sensors, and catalysts. However, these properties of the pure bulk ZnO are not stable and cannot meet the increasing needs for the present applications. In order to modify the properties of the ZnO, this semiconductor material was usually doped with some dopants such as Al, Si, and Ga. For example, Al-doped ZnO increases its conductivity withoutimpairing the optical transmission, which is regarded as a potential alternative candidate for ITO materials (Zeng *et al*, 2003). Gas sensors based on ZnO had already been developed for detection and control of gases such as CO, H_2, H_2S, NH_3, etc. (Zhu *et al*, 2005). ZnO nanoparticles embedded in polymer matrices like soluble starch are a good example of functional nanostructures with potential for applications such as UV-protection ability in textiles and sunscreens and antibacterial finishes in medical textiles and inner wears. ZnO nanoparticles has successfully been dispersed inside a soluble starch matrix using a simple water-based technique (Joshi *et al*., 2004). The stabilization of these nanoparticles was achieved by the presence of soluble starch in the reaction medium. The average size of the ZnO nanoparticles was estimated to be 38 ± 3 nm using a TEM (Vigneshwaran, 2006).

Silver is widely used as a catalyst for the oxidation of methanol to formaldehyde and ethylene to ethylene oxide (Nagy *et al*., 1999). Colloidal silver is of particular interest because of distinctive properties, such as good conductivity, chemical stability, catalytic and antibacterial activity (Frattini *et al*., 2005). For example, silver colloids are useful substrates for surface enhanced spectroscopy, since it partly requires an electrically conducting surface (Tessier *et al*., 2000; Rosi, *et al*., 2005).

Material Scientists are conducting research to develop novel materials with better properties, more functionality and lower cost than the existing ones. Several physical, chemical and biological synthesis methods have been developed to enhance the performance of nanoparticles displaying improved properties with the aim to have a better control over the particle size, distribution and morphology (Shibata *et al*., 1998; Shankar, *et al*., 2003). Synthesis of nanoparticles to have a better control over particles size, distribution, morphology, purity, quantity and quality, by employing environment friendly economical processes has always been a challenge for the researchers (Hahn, 1997).

Chemical reduction is the most frequently applied method for the preparation of silver nanoparticles as stable, colloidal dispersions in water or organic solvents. Commonly used reductants are borohydride, citrate, ascorbate, and elemental hydrogen (Lee *et al*., 2003; Ahmadi, *et al*., 2003).

The reduction of silver ions (Ag+) in aqueous solution generally yields colloidal silver with particle diameters of several nanometers. When the colloidal particles are much smaller than the wavelength of visible light, the solutions have a yellowbrown color with an intense band in the 380–400 nm range and other less intense or smaller bands at longer wavelength in the absorption spectrum (Tessier *et al*., 2000; Rosi *et al*., 2005). This band is attributed to collective excitation of the electron gas in the particles, with a periodic change in electron density at the surface (Henglein, 1989;).

Previous studies showed that use of a strong reductant such as borohydride, resulted in small particles that were somewhat monodisperse, but the generation of larger particles wasdifficult to control (Creighton *et al*., 1994; Schneider *et al*., 1979). Use of a weaker reductant such as citrate, resulted in a slower reduction rate, but the size distribution was far from narrow (Shirtcliffe *et al*., 1999; Emory *et al*., 1997). Controlled synthesis of Ag NPs is based on a twostep reduction process (Schneider *et al*., 1979). In this technique a strong reducing agent is used to produce small Ag particles, which are enlarged in a secondary step by further reduction with a weaker reducing agent (Lee *et al*., 1982). Different studies reported the enlargement of particles in the secondary step from about 20–45 nm to 120–170 nm (Schneider *et al*., 1994; Schirtcliffe *et al*., 1999; Rivas *et al*., 2001). Moreover, the initial sol was not reproducible and specialized equipment was needed (Nickel *et al*., 2000). The synthesis of nanoparticles by chemical

reduction methods is therefore often performed in the presence of stabilizers in order to prevent unwanted agglomeration of the colloidal silver nanoparticle solution.

Nanoparticles have many different effects on human health relative to bulk material from which they are produced (Albrecht, 2006). Increase the biological activity of nanoparticles can be beneficial, deterimental or both. Many nanoparticles are small enough to have an access to skin, lungs, and brain (Koziara *et al*., 2003; Oberdorster *et al*., 2004). Exposure of metalcontaining nanoparticles to human lung epithelial cells generated reactive oxygen species, which lead to oxidative stress and damage of the cells (Limbach *et al*., 2007; Xi *et al*., 2006). A study on toxic effects of silver nanoparticles was done on zebrafish as a model due to its fast development and transparent body structure. The results show a deposition of particles on organs and severe developmental effects. The biocompatibility and toxicity of silver nanoparticles were exhibited by observing single silver nanoparticle inside embryos at each development stage. The types of abnormalities in zebra fish were strongly dependent on the dose of silver nanoparticles (Asharani, 2008).

Alt *et al.,* 2004 observed that the nano silver cement showed high-antibacterial activity against all strains, including methicillin-resistant *S. epidermidis* and methicillin-resistant *S. aureus*. Stampoulis *et al.,* 2009

observed that seed germination was unaffected by any of the treatments, but Cu nanoparticles reduced emerging root length by 77% and 64% relative to unamended controls and seeds exposed to bulk Cu powder, respectively. During a 15-day hydroponic trial, the biomass of plants exposed to multiwalled carbon nanotubes and Ag nanoparticles was reduced by 60% and 75%, respectively, as compared to control plants and corresponding bulk carbon and Ag powder solutions. Although bulk Cu powder reduced biomass by 69%, Cu nanoparticle exposure resulted in 90% reduction relative to control plants. Both Ag and Cu ion controls (1-1000 mg/L) and supernatant from centrifuged nanoparticle solutions (1000 mg/L) indicate that half the observed phytotoxicity is from the elemental nanoparticles themselves.

Farooqui *et al.,* 2010 studied that nanotechnology has the potential to increase yield of nutrient values and also plays a vital role in developing improved systems for monitoring ecological conditions and increasing the capacity of crops to absorb nutrients or pesticides. According to Kaviya *et al.,* 2011 *Citrus sinensis* peel extract acts as a reducing and a capping agent. The effect of temperature on the synthesis of silver nanoparticles was carried out at room temperature (25°C) and 60°C. The successful formation of silver nanoparticles has been confirmed by UV-vis, FTIR, XRD, EDAX, FESEM and TEM analysis and their antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa* (gram-negative), and *Staphylococcus aureus* (gram-positive) has been studied and the results suggested that the

synthesized AgNPs act as an effective antibacterial agent. Iravani *et al.,* 2011 observed that nanoparticles produced by plants are more stable and the rate of synthesis is faster than in the case of microorganisms. Moreover, the nanoparticles are more various in shape and size in comparison with those produced by other organisms. Ali *et al.,* 2011 observed that the extracellular biosynthesis of silver nanoparticles using marine cyanobacterium, *Oscillatoria willei* NTDM01 which reduces silver ions and stabilizes the silver nanoparticles by a secreted protein.

Thangaraju *et al.,* 2012 tested the antimicrobial activity against four different human pathogens such as *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli* and *Staphylococcus aureus*. Seshadri *et al.,* 2012 observed that the intracellular synthesis of silver nanoparticles by the highly silver-tolerant marine bacterium, *Idiomarin*a sp. Sahayaraj *et al.,* 2012 explained that the biosynthesized silver nanoparticles were characterized with UV-vis Spectroscopy, FTIR, XRD, SEM and TEM. The thallus extract as well as silver-based nanoparticles of marine alga, *Padina pavonica Linnaeus* were tested against two important pathogens of cotton. *Fusarium wilts* (*Fusarium oxysporum f species vasinfectum*) and bacterial leaf blight *(Xanthomonas campestris pv malvacearum*) are responsible for significant yield losses in cotton worldwide. The *Padina pavonica* based silver nanoparticles inhibited the growth of the test pathogens for *Fusarium oxysporum* and *Xanthomonas campestris*. Gopinath *et al.,* 2012 observed that

the antibacterial property of synthesized nanoparticles by Kirby–Bauer method with clinically isolated multi-drug resistant bacteria such as *Streptococcus pyogens*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus.* Nagarajan *et al.,* 2013 studied the preliminary screening of physicochemical parameters such as concentration of metals, concentration of seaweed extract, temperature, pH and reaction time revealed that one seaweed *Sargassum myriocystum* were able to synthesize zinc oxide nanoparticles. It was confirmed through the initial colour change of the reaction mixture and UV visible spectrophotometer. Kannan *et al.,* 2013 stated that the formation of silver nanoparticles by the reduction of the aqueous silver metal ions during exposure to the seaweed *Chaetomorpha linum* extract. It may be inferred that these biomolecules are responsible for capping and efficient stabilization. Since no synthetic reagents were used in this investigation, it is environmentally safe and have potential for application in biomedicine and agriculture. Mavani *et al.,* 2013 specified that the synthesis of silver nanoparticles has become possible using NaBH4 (Sodium tetrahydridoborate) as a reducing agent and using $AgNO_3$ as a reductant.

Priyadharshini *et al.,* 2014 observed that extracellular synthesis of metallic silver and zinc oxide nanoparticles using the extracts of macro-algae *Gracilaria edulis*and also examined its anticancer activity against human prostate cancer cell lines (PC3). The formation of silver nanoparticles and

zinc oxide nanoparticles in the reaction mixture was determined by ultraviolet-visible spectroscopy andsuggested that the synthesized ZnONPs showed an effective anticancer activity against PC3 cell lines than AgNPs. Makarov *et al.,* 2014 observed that, the synthesis of metal nanoparticles in plant extracts-*Brassica juncea and Medicago sativa*, despite obvious limitations, has a significant potential and a number of substantial advantages relative to traditional methods of nanoparticle synthesis. Azizi *et al.,* 2014 observed thatthe present contribution deals with one pot method for synthesis of zinc oxide nanoparticlesthrough green process using the brown marine macroalgae *Sargassum muticum* aqueous extract.

Chauhan *et al.,* 2015 observed that the biological synthesis of silver and zinc oxide nanoparticles is a novel and cost-effective approach over harmful chemical synthesis techniques. The metallic nanoparticles synthesized using *Pichia fermentans* JA2 possess potent inhibitory effect that offers valuable contribution to pharmaceutical associations. Logeswari *et al.,* 2015 stated that plants extracts from *Ocimum tenuiflorum*, *Solanum tricobatum*, *Syzygium cumini*, *Centella asiatica* and *Citrus sinensis* were used for the synthesis of silver nanoparticles from silver nitrate solution. Antimicrobial activity of the silver bio-nanoparticles was performed by well diffusion method against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*. The highest antimicrobial activity of silver nanoparticles synthesized by *Solanum tricobatum*, *Ocimum*

*tenuiflorum*extracts was found against *Staphylococcus aureus* (30 mm) and *E. coli* (30 mm) respectively.

Panneerselvam *et al*., 2015 described that the green microalgae and diatom could be employed for the green synthesis of nanoparticles and also suggested that the algal biomass can be produced using wastewater in order to treat various wastewaters. Kathiraven *et al*., 2015 stated that the synthesized AgNPs have shown the best antibacterial activity against human pathogens such as *Staphylococcus aureus* and *Proteus mirabilis*. Lekshmi *et al*., 2015 tested the antimicrobial activity of haemolymph and silver nanoparticles from haemolymph against human and fish pathogens. According to Hashemi *et al.,* 2015 Ag-NPs synthesis does not use any toxic reagents and thus has potential for use in biomedical and agricultural application. Anbuvannan *et al*., 2015 tested the photocatalytic performance of the ZnO against the methylene blue dye under UV radiation has significant photo degradation. The antibacterial activities of the prepared products analyzed against some selected human pathogens.

Anjum *et al.,* 2016 reported the biologically synthesized AgNPs have upsurge applications in various sectors such as electronics, clothing, food industry, paints, sunscreens, cosmetics, biosensing, medicines, drug delivery and medical devices. Broad-spectrum bioactivities of AgNPs indicate their potential to solve many microbial resistance problems. Ibraheem *et al.,* 2016

reported that the AgNPs significantly reduced the growth of both gram positive *Staphylococcus aureus, Bacillus subtillis,* and gram negative *Salmonella speciesEscherichia coli* in addition to the unicellular fungus *Candida albicans*. Ahmed *et al.,* 2016 observed that, plant based biological molecules undergo highly controlled assembly for making them suitable for the metal nanoparticle synthesis. Jyoti *et al.,* 2016 stated that, synthesizing silver nanoparticles by green chemistry route, and reported a facile bottom-up 'green' route for the synthesis of AgNPs using aqueous leaves extract of *Urtica dioica* (Linn.). AgNPs in combination with antibiotics have better antibacterial effectas compared with AgNPs alone and hence can be used in the treatment of infectious dis-eases caused by bacteria. AgNPs against *Serratia marcescens* proving the synergistic role of AgNPs and it may be used to augment the activities of antibiotics.

According to Kuppusamy *et al.,* 2016 the biological syntheses of nanoparticles are being carried out by different macro–microscopic organisms such as plant, bacteria, fungi, seaweeds and microalgae. The biosynthesized nanomaterials have been effectively controlling the various endemic diseases with less adverse effect. The plants are used successfully in the synthesis of various greener nanoparticles such as cobalt, copper, silver, gold, palladium, platinum, zinc oxide and magnetite. Also, the plant mediated nanoparticles are potential remedy for various diseases such as malaria, cancer, HIV, hepatitis and other acute diseases. Singh *et al.,* 2016 proved that the

biological synthesis of nanoparticles is increasingly regarded as a rapid, ecofriendly and easily scaled-up technology. Metal nanoparticles produced using microorganisms and plant extracts are stable and can be monodispersed by controlling synthetic parameters such as pH, temperature, incubation period and mixing ratio. Ahmed *et al*., 2016 observed that the silver nanoparticles showed antibacterial activities against both gram positive - *Staphylococcus aureus* and gram negative - *Escherichia coli* microorganisms and photoluminescence studies of synthesised silver nanoparticles were also evaluated. Benakashania *et al.,*2016 reported that the synthesis of silver nanoparticles by reducing the silver ions present in the solution of silver nitrate by the cell free aqueous extract of *Capparis spinosa* leaves. Thatoi *et al.,* 2016 observed that the synthesized NPs showed varied zone of inhibition (9–16 mm) against the tested microbial pathogens. The synthesized nanoparticles possess strong biological activities in terms of antioxidant, anti-inflammatory, antidiabetic and antibacterial, potentials which could be utilized in various biological applications by the cosmetic, food and biomedical industries.

Agarwal *et al.,* 2017 proved that potential applications like antibacterial, antifungal, anti diabetic, anti- inflammatory, wound healing, antioxidant and optic properties. Due to the large rate of toxic chemicals and extreme environment employed in the physical and chemical production of these NPs, green methods employing the use of plants, fungus, bacteria, and

algae have been adopted. This review is a comprehensive study of the synthesis and characterization methods used for the green synthesis of ZnO NPs using different biological sources. Swathy *et al.,* 2017 observed that that marine yeast isolates SAG1 and SAG2 both are potential marine yeast isolates which can synthesize both the silver and ZnO nanoparticles. Balaraj *et al.,* 2017 reported that the ZnONPs were synthesized biologically using *Streptomyces* species as reducing agent. The antimicrobial activity of the ZnONPs and CFS of isolated *Streptomyces species* against *Escherichia coli and Bacillus subtilis* was also evaluated. Inhibition as high as 12 mm against *Escheichia coli* was observed for ZnONPs at concentration of 100 g/ml. Kokabi *et al.,* 2017 observed that zinc oxide nanoparticles with an average diameter of 16.51 nm were successfully biosynthesized using the aqueous extract of the red seaweed *Hypnea musciformis*. The morphology, purity and quality of biosynthesized *Hypnea*-ZnONps were highly comparable with its commercial counterpart with less toxicity. The minimum inhibitory concentration and minimum bactericidal concentration values were evaluated and the potential ecotoxicity of *Hypnea*-ZnO NPs against *Artemia salina* was investigated in various concentrations (0, 1, 5, 10, 15, 25 µg/ml) and mortality rate in 24 hours was evaluated. The findings provide preliminary information for designing cost-effective, eco-friendly green synthesis methods for large-scale production of ZnONPs using marine macroalge.

Siddiqi *et al*., 2018 observed that Ag NPs are smaller than the micro-organisms, they diffuse into cell and rupture the cell wall which has been shown from SEM and TEM images of the suspension containing nanoparticles and pathogens. It has also been shown that smaller nanoparticles are more toxic than the bigger ones. Ag NPs are also used in packaging to prevent damage of food products by pathogens. The toxicity of Ag NPs is dependent on the size, concentration, pH of the medium and exposure time to pathogens. Umamaheswari *et al*., 2018 observed that the Zinc oxide nanoparticle is readily soluble in biological fluids and tends to aggregate easily under different physiological condition. But physicochemical properties of the nanoparticle have an impact in the bioavailability. Dimkpa *et al.,* 2018 reported that metal-based nanoparticles such as Ag, ZnO, CuO, TiO_2, and others possess unique properties that lend them to a wide array of uses. This means that during manufacture, use, or upon disuse, these nanoparticles can become constituents of the soil. Upon interaction with soil, nanoparticles affect soil processes, and in turn are affected by soil properties.

Abdulwahid *et al.,* 2019 observed that ZnO nanoparticles show high inhibition activity against *Fusarium oxysporum* and *Rhizoctonia solani*. *Cladophora glomerata* - mediated synthesis of ZnO nanoparticles shows rapid and eco-friendly silver ion reduction process. Therefore, this present study elucidates that algae-mediated Green synthesized Zinc Oxide nanoparticles have antifungal activity against phytopathogenic fungi, so it can

be developed as a novel medicine for human welfare in biomedical applications in the near future. Yusof *et al.,* 2019 observed that the biological synthesis of ZnO NPs by the microbes, the mechanisms of the biological synthesis, parameters for the optimization process and their potential application as an antimicrobial agent and feed supplement in the animal industry as well as their toxicological hazards on animals.

Li *et al.,* 2020 observed that the antimicrobial activity of ZnO nanostructures is size, shape, and concentration-dependent. In particular, green ZnO nanoparticles have the lowest MIC values against Gram-positive and Gram-negative bacterial strains compared with ZnO NPs prepared by hydrothermal/solvothermal, sol-gel and polyol techniques. This can be attributed to green ZnO NPs exhibiting high purity and possessing bioactive phyto compounds, such as flavonoids and polyphenols for bactericidal activity. Thakur *et al.,* 2020 observed that Ag-doped ZnO shows a lower limit of detection as compared to pure ZnO for p-nitrophenol sensing. Bhuyar *et al*., 2020 observed that marine alga *Padina* species could be an alternative source for the production of Ag nanoparticles and are efficient antimicrobial compounds against both gram-negative and gram-positive bacteria which can be a promising material against infectious bacteria. Bhattacharya *et al.,* 2020 observed that the antimicrobial activity of synthesized nanoparticles against gram positive as well as gram negative, pathogenic bacteria i.e. *Pseudomonas aeruginosa* and *Staphylococcus aureus* species respectively. Zone of

inhibition (ZOI) exhibited by *Pseudomonas aeruginosa* and *Staphylococcus aureus* for disc diffusion and well diffusion assay was around 10-22 mm and 9-12mm respectively. Sharma *et al.,* 2020 observed that zinc salts have been used as precursor and phytochemicals in the plant extract reduce the metal salt to lower oxidation state as well as stabilize the ZnO NPs. The morphological and physico-chemical properties of obtained NPs analyzed by various characterization techniques have been discoursed. Further, antimicrobial activity and potential photocatalytic application in terms of the degradation of dyes have also been reviewed in addition to the toxicity aspects of these NPs on human beings and animals.

Rauvolfia tetraphylla (L.) seed extract was used to synthesize dark brown colored silver (Ag) and white colored zinc oxide nanoparticles. Synthesized nanoparticles were characterized by different spectroscopic analysis (XRD, XPS, and SEM with EDAX). Characterization results confirmed the particle morphology and structure. The synthesized Ag and ZnO NPs were analyzed against two gram positive and three gram negative bacteria (Vinay *et al.,* 2021).

4. Materials and methods

4.1. COLLECTION OF ANIMAL MATERIAL

Samples of simple ascidian *Phallusia nigra* Savigny, 1816 were collected from the under surface of the barges of Tuticorin harbor. The samples were washed with sea water to remove sand, mud and overgrowing organisms at the site collection, and then transported to laboratory. Identification upto the species level was carried out based on the key to identification of Indian ascidians by Meenakshi, 1997.

4.1.1. SYSTEMATIC POSITION

Phallusia nigra belongs to

Phylum : Chordata

Subphylum : Urochordata

Class : Ascidiacea

Order : Enterogona

Suborder : Phlebobranchia

Family : Ascidiidae

Genus : *Phallusia*

Species : *Nigra*

4.1.2. ANIMAL MATERIAL

Ascidians commonly called ‘sea squirts’ are an interesting group of marine, sedentary organisms found to occur abundance in Tuticorin coast. *Phallusia nigra* is a simple ascidian with a thick leathery envelope (tunic) containing cellulose like material. The tunic encloses a sac-shaped body with

separate water entrance and exit tubes (siphons). It is sessile and filter feeding. It lives on plankton that it filters from seawater with a mucous net. An adult *Phallusia nigra* may be 10 cm long. The tunic is usually velvet black or dark brown, but may be grey in specimens that are younger or living in shaded areas. They occur in shallow sheltered waters attached to hard natural and artificial substrata. As they are hermaphrodites simple ascidians are broad cast spawners. Larvae show gregarious settlement. Some species are induced to settle and metamorphose by extracts of conspecific adult tissues. The larvae can swim for few hours before settlement on a substrate where they metamorphose into sessile adult form.

4.1.3. EXTERNAL ORGANIZATION

(Plate - 1) Individuals are oval or elongated, laterally compressed with the free edges thick and rounded. The size varies from 1.5 cm to 9.5 cm. Attachment is by the posterior end or by one third of the posterior left side. In a few specimens the posterior basal part had a long flat creeping process for attachment. The anterior end narrows to a terminal branchial siphon. Atrial siphon is one third from the anterior end on the dorsal surface directed anteriorly. There are 8-10 branchial and 6-8 atrial lobes with ocelli in between them. The lobes are rounded without any tentacular fringe. The whole anterior part of the body is curved dorsally which is characteritic of the species so that the two apertures are quite close together. Test is firm, smooth,

shiny and jet black in colour. Main test vessel leaves the body two-third distance from the anterior end and branches profusely.

4.2. PREPARATION OF POWDER

The specimens were dried under shade. The dried animals were homogenized to get a coarse powder. The dried powder of the tunicate *Phallusia nigra* was used.

4.3. Synthesis of ZnO nanoparticles

The ZnO nanoparticles were prepared by wet chemical method using zinc nitrate and sodium hydroxides precursors and soluble starch as stabilizing agent. Soluble starch (0.5%) was dissolved in 500 ml of distilled water and treated in microwave oven (Samsung, Model NoCE103VD) for complete solubilization. Zinc nitrate, 14.874 g (0.1 mol), was added in the above solution. Then the solution was kept under constant stirring at room temperature using magnetic stirrer (Tarson spinnot digital) for one hour. After complete dissolution of zinc nitrate, 300ml (0.2 mol), of sodium hydroxide solution was added under constant stirring, drop by drop touching the walls of the vessel. The reaction was allowed to proceed for 2 h after complete addition of sodium hydroxide. After the completion of reaction, the solution was allowed to settle for overnight and the supernatant solution was then discarded carefully. The remaining solution was centrifuged (Remi cooling centrifuge instrument, Model No-C30BL) at 10,000 × g for 10 min and the supernatant was discarded. Thus produced nanoparticles were washed three

times using distilled water. Washing was carried out to remove the byproducts and the excessive starch that were bound with the nanoparticles. After washing, the nanoparticles were dried at 80°C for overnight. During drying, complete conversion of Zn (OH) 2 into ZnO takes place.

4.4. Synthesis of silver nanoparticles

Weighing 25 g of dry powder of *Phallusia nigra* was mixed with 100 ml sterile distilled water and filtered through Whatman No.1 filter paper (pore size 0.45 μm) and was further filtered through 0.22 μm sized filters. The extract was stored at 40° C for further experiments.

The aqueous solution of 1mM silver nitrate (AgNO3) was prepared and used for the synthesis of silver nanoparticles. 10 ml of *Phallusia nigra* extract was added into 90 ml of aqueous solution of 1 mM silver nitrate for reduction into Ag+ ions and kept for incubation period of 15 hours at room temperature. Here the filtrate act as reducing and stabilizing agent for 1 mM of AgNO3.

4.5. CHARACTERIZATION OF SILVER NANOPARTICLES:

4.5.1. UV-Vis Analysis:

The ZnO and Ag nanoparticles were characterized in a Perkin-Elmer UV-VIS spectrophotometer, Lambda-19 to know the kinetic behavior of ZnO and Ag nanoparticles. The scanning range for the samples was 200-800 nm at a scan speed of 480 nm/min. The spectrophotometer was equipped with "UVWinlab" software to record and analyze data. Base line correction of the spectrophotometer was carried out by using a blank reference. The UV-Vis

absorption spectra of all the samples were recorded and numerical data were plotted in the “Origin 6.5”.

4.5.2. FTIR analysis:

The chemical composition of the synthesized ZnO and silver nanoparticles was studied by using FTIR spectrometer (perkin-Elmer LS-55-Luminescence spectrometer). The solutions were dried at 75^{o} C and the dried powders were characterized in the range 4000–400 cm-1 using KBr pellet method.

Plate - 1. External appearance of *Phallusia nigra* Savigny, 1816

5. Results

5.1. Characterization of ZnO and Ag nanoparticles:

5.1.1. UV-Vis Spectrophotometer Analysis:

ZnO and AgNPs from the extract of *Phallusia nigra* was synthesized successfully. The UV-Vis spectra of ZnO NP was shown in Figure 1. The absorption peak of the prepared nano ZnO was found at around 380 nm. The UV-Vis absorption spectra of the Ag NP were shown in Figure 2. Absorption spectra of Ag nanoparticles formed in the reaction media has absorbance maxima at 270 nm.

5.1.2. FTIR Analysis:

FTIR measurements were carried out to identify the biomolecules for capping and efficient stabilization of the metal nanoparticles synthesized. The FTIR spectrum of ZnO and silver nanoparticles were shown in Figure 3 and 4.

The spectra for ZnO nanoparticles of *Phallusia nigra* revealed the presence of prominent peaks at 3450, 2924, 1623, 1384, 1163, 1111, 674, 611 and 468 cm^{-1} corresponding to different functional groups.

The spectra for Ag nanoparticles of *Phallusia nigra* revealed the presence of prominent peaks at 3424, 2923, 2106,1627, 1422, 1384, 1120,

1021, 874, 675, 610, 513 and 466 and cm^{-1} corresponding to different functional groups.

Figure 1: UV-vis spectra for ZnO nanoparticles of *Phallusia nigra*

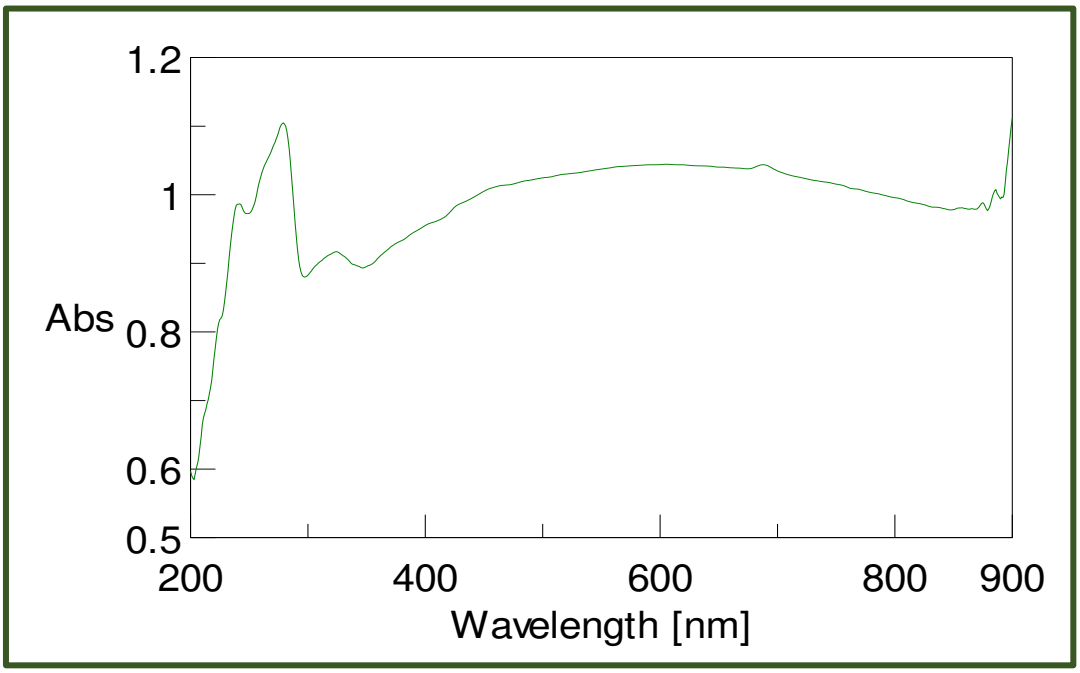


Figure 2: UV-vis spectra for Ag nanoparticles of *Phallusia nigra*

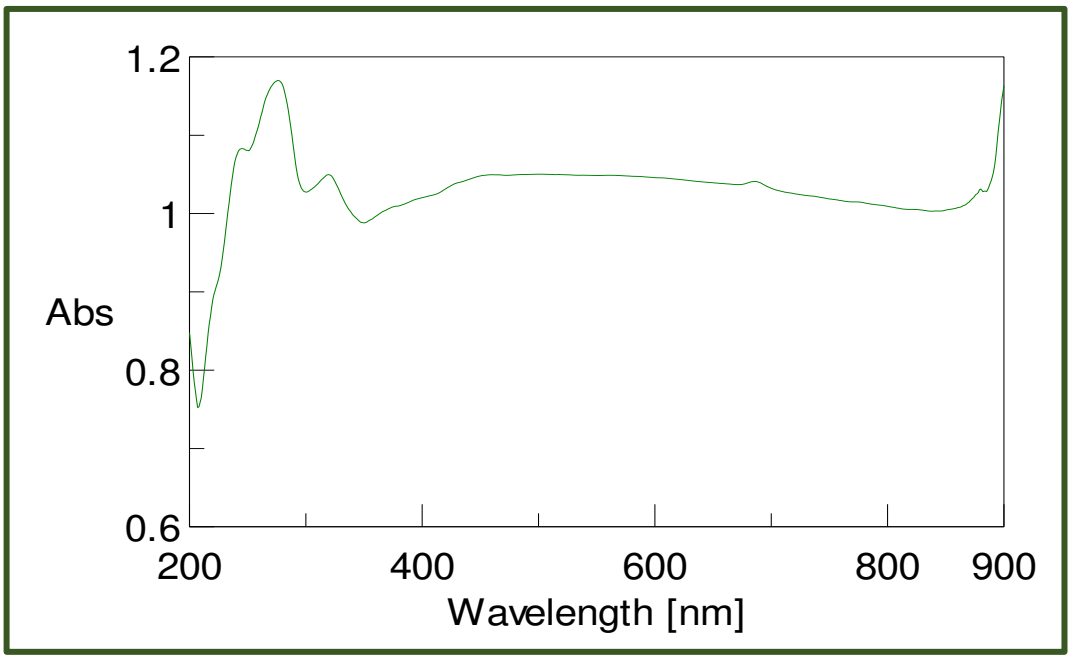


Figure 3: FTIR result for ZnO nanoparticles of *Phallusia nigra*

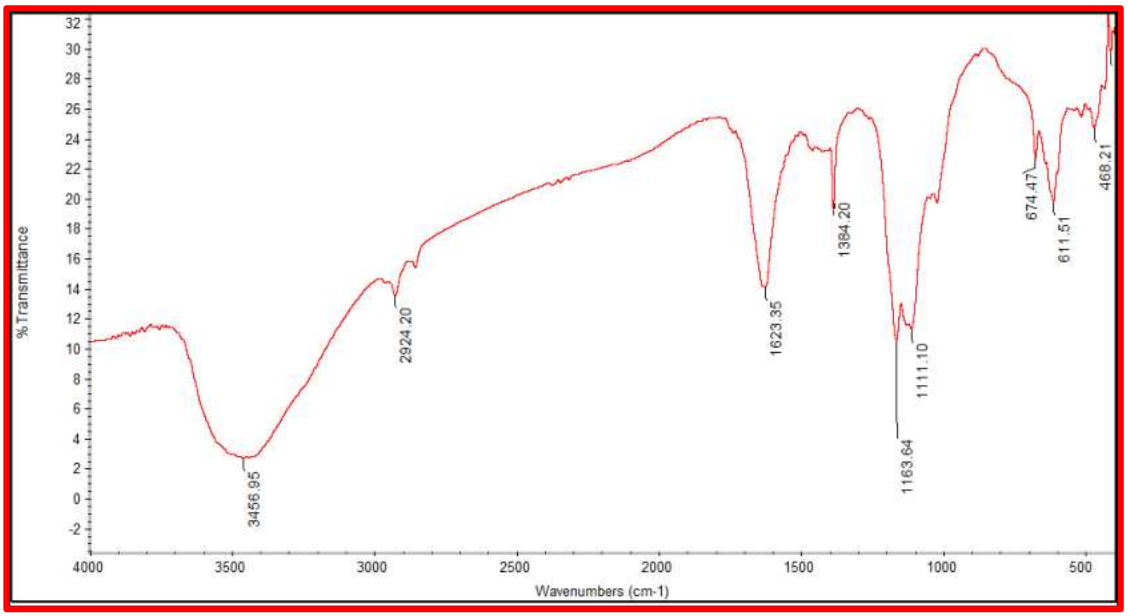
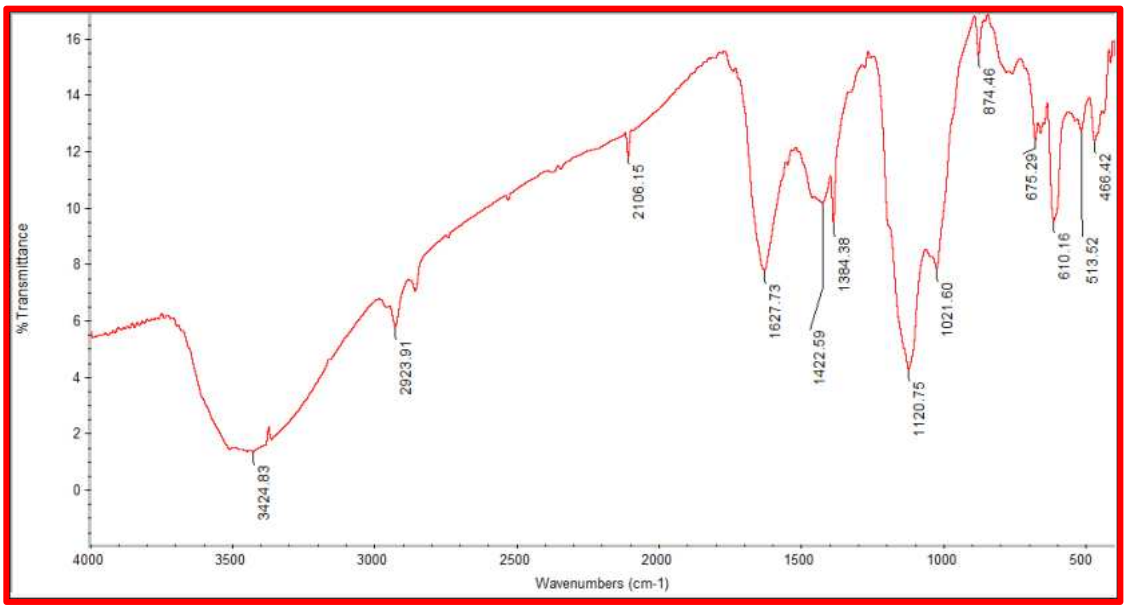


Figure 4: FTIR result for silver nanoparticles of *Phallusia nigra*



6. Discussion

Owing to the applicability of silver nanoparticles in wide sectors, its demand is increasing at an overwhelming rate which has resulted in increased production. Researchers are continuously developing newer methods for synthesis of highly monodispersed ZnO and silver nanoparticles which are efficient in terms of synthesis rate as well as energy usage. Biological methods have emerged as an alternative to the conventional methods for synthesis of nanoparticles. The present report was to test the efficacy of simple ascidian *Phallusia nigra* for the synthesis of ZnO and silver nanoparticle.

The synthesized nanoparticles were surrounded by proteins and metabolites such as terpenoids having functional groups from the analysis of FTIR studies we confirmed that the carbonyl groups from the amino acid residues and proteins has the stronger ability to bind metal indicating that the proteins could possibly from the metal nanoparticles (i.e.; capping of silver nanoparticles) to prevent agglomeration and thereby stabilize the medium. This suggests that the biological molecules could possibly perform dual functions of formation and stabilization of silver nanoparticles in the aqueous medium. Carbonyl groups proved that flavanones or terpenoids absorbed on the surface of metal nanoparticles. Flavanones or terpenoids could be adsorbed on the surface of metal nanoparticles, possibly by interaction

through carbonyl groups in the absence of other strong ligating agents in sufficient concentration. The presence of reducing sugars in the solution could be responsible for the reduction of metal ions and formation of the corresponding metal nanoparticles. It is also possible that the terpenoids play a role in reduction of metal ions by oxidation of aldehydic groups in the molecules to carboxylic acids.

7. Conclusion

The rapid biological synthesis of silver nanoparticles using *Phallusia nigra* extract provides environmental friendly, simple and efficient route for synthesis of nanoparticles. From the technological point of view these obtained silver nanoparticles have potential applications in the biomedical field and this simple procedure has several advantages such as cost-effectiveness, compatibility for medical and pharmaceutical applications as well as large scale commercial production. ZnO and Ag nanoparticles were synthesized successfully. The detail characterization of the nanoparticles was carried out using UV-Vis spectroscopy and FTIR studies.

8. Summary

Nanotechnology is an important field of modern research dealing with design, synthesis, and manipulation of particles structure ranging from approximately 1-100 nm in one dimension. Remarkable growth in this up-and-coming technology has opened novel fundamental and applied frontiers, including the synthesis of nanoscale materials and exploration or utilization of their exotic physicochemical and optoelectronic properties. Nanotechnology is rapidly gaining importance in a number of areas such as health care, cosmetics, food and feed, environmental health, mechanics, optics, biomedical sciences, chemical industries, electronics, space industries, drug-gene delivery, energy science, optoelectronics, catalysis, reorography, single electron transistors, light emitters, nonlinear optical devices, and photo electrochemical applications.

Samples of simple ascidian *Phallusia nigra* Savigny, 1816 were collected and identification was made upto the species level was carried out based on the key to identification of Indian ascidians by Meenakshi, 1997.The dried animals were homogenized to get a coarse powder. The dried powder of the tunicate *Phallusia nigra* was used. ZnO and $AgNO_3$nanoparticles were synthesized.

The ZnO and Ag nanoparticles were characterized in a Perkin-Elmer UV-VIS spectrophotometer. The chemical composition of the synthesized

ZnO nanoparticles and silver nanoparticles were studied by using FTIR spectrometer.

The absorption peak of the prepared nano ZnO was found at around 380 nm. The UV-Vis absorption spectra of the Ag NP were shown in Figure 2. Absorption spectra of Ag nanoparticles formed in the reaction media has absorbance maxima at 270 nm.

The spectra for ZnO nanoparticles of *Phallusia nigra* revealed the presence of prominent peaks at 3450, 2924, 1623, 1384, 1163, 1111, 674, 611 and 468 cm^{-1}. The spectra for Ag nanoparticles of *Phallusia nigra* revealed the presence of prominent peaks at 3424, 2923, 2106,1627, 1422, 1384, 1120, 1021, 874, 675, 610, 513 and 466 and cm^{-1} corresponding to different functional groups.

9. *Suggestion*

Anti-fungal, anti-bacterial, anti-viral and anti-cancerous activity of the ZnO and Ag nanoparticles may be investigated in details.

Various ascidian species can be used to synthesize Ag nanoparticles to find a more non-toxic and economical method of synthesis.

Detail mechanistic study of antibacterial function of nanoparticle may be elucidated.

Nanoparticles conjugate may be applied in various cancer cells to observe any anti-cancerous potential of the protein-NP conjugate.

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ISOLATION AND SCREENING OF PLANT GROWTH PROMOTING BACTERIA FROM COW DUNG

A dissertation submitted to

St. Mary's College (Autonomous), Thoothukudi

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in partial fulfilment for the award of the degree of

MASTER OF SCIENCE IN ZOOLOGY

By

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DEPARTMENT OF ZOOLOGY

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(Re- Accredited with 'A^{+}' Grade by NAAC)

THOOTHUKUDI- 628001

APRIL-2021

CERTIFICATION

CERTIFICATE

This is to certify that this dissertation entitled **"ISOLATION AND SCREENING OF PLANT GROWTH PROMOTING BACTERIA FROM COW DUNG "** submitted by **S. SUTHAPACKIYAM, Reg. No. 19APZO09 to St. Mary's College (Autonomous), Thoothukudi,** affiliated to Manonmaniam Sundaranar University in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2020-2021 under my guidance and supervision. It is further certified that the dissertation or any part of this has not been submitted elsewhere for any other degree.

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Examiner

DECLARATION

I do hereby declare that this thesis entitled "**ISOLATION AND SCREENING OF PLANT GROWTH PROMOTING BACTERIA FROM COW DUNG**" submitted by me for the award of the degree of Master of Science in Zoology is the result of my original and independent research work carried out under the guidance of Dr. Mrs. HerminPasangha, M.Sc., B.Ed., Ph.D., Associate Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi, and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date: 15.04.2021

S. Suthapackiyam

Signature of Candidate

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CONTENT

INTRODUCTION

Animals can play an important role in providing energy either in a negative way where keeping livestock contributes to deforestation, in large areas of woodland or in a positive way, such as by converting plant energy to useful work or by providing fertilizer that is used for fuel through fertilizer. Cakes or biofuels instead of charcoal, fuel wood, firewood, etc. (Kesavan and Swaminathan, 2008). Manure is a source of organic matter (Hutchison et al., 2005) because it stimulates plant growth (Muhammad and Amusa, 2003).

Cattle raising has become a traditional practice in India (Muhammad and Amusa, 2003). The gut of the cow contains several microorganisms (Ware et al., 1988), including *Lactobacillus plantarum*, *L. casei*, *L. acidophilus*, *Bacillus subtilis*, (Teo and Teoh, 2013) *Enterococcus diacetylactis*, *Bifido bacterium* and *Saccharomyces cerevisiae* (yeast) (Sawant et al., 2007).

Cow manure carries a wide variety of microorganisms that contain different species of bacteria (Nene: 1999, Kartikey: 2016) such as *Bacillus spp.* (Williams and Asher, 1996), *Corynebacterium spp*. and *Lactobacillus spp*., *Citrobacter*, *Enterobacter*, *Escherichia coli*, *Klebsiella spp*., *Pasteurella spp*. together with protozoa and yeast (Nene: 1999, Kartikey: 2016).

Cow manure is the surplus of plant material that does not contain water and microorganisms (Radha and Rao, 2014). It releases nutrients slowly and steadily and activates terrestrial microphone biology (Ayuso *et al*., 1996; Belay *et al*., 2001). Cow dung microflora contains a large number of *bacilli*, *lactobacilli* and *cocci* and some known and unknown fungi and branches (Muhammad and Amusa, 2003). Cow manure strengthens bile pigments, intestinal bacteria and mucus (Sawatdeenarunat *et al*., 2016). Resistance to cow dung in pests and diseases (Sharma and Singh, 2015). Dog manure bacteria are more abundant (Holman *et al*., 2016) and able to suppress conidial germination (Basak and Lee, 2001).

Cow dung increased the growth and yield of plants (Gudugi, 2013; Akande *et al*., 2006; Mehedi *et al*., 2011). Cow dung is a bioresource for sustainable development (Gupta *et al*., 2016). Cattle manure is a biodegradable fertilizer and widely studied as a transparent, viable and other fuel in the form of biofuel with methane values (Nene, 1999; Kartikey, 2016). Biofuels are produced by anaerobic digestion or transfer of biodegradable materials such as biofuels, fertilizers, sewage, municipal waste, green waste, plant and crop materials (NNFCC, 2013). Biofuels are also created by converting cow manure through anaerobic digestion to methane biofuels.

Global warming caused by the generation of energy from fossil fuels has accelerated the use of renewable fuels such as biofuels. Biofuels are one of the

most renewable and sustainable resources that significantly reduce greenhouse gas emissions compared to the release of waste gas into the atmosphere (Murphy *et al*., 2004).

Cow manure is high in about 3% nitrogen, 2% phosphorus and 1% potassium as organic matter and is rich in microbial diversity (Gupta *et al*., 2016). In India cow dung manure (FYM) is the most common organic fertilizer, containing 0.5 - 0.7% N, 0.3 - 0.9% P205 and 0.4 - 1.0% K, depending on the type of animal and the nature of nutrition (Chhonkar, 1995).

Chemical fertilizers provide essential plant nutrients such as nitrogen, phosphorus and potassium, but overuse of these fertilizers can have unexpected environmental effects (Shenoy *et al*., 2001; Adesemoye *et al*., 2009). Excessive use of chemical fertilizers leads to effects on ecological activities and biochemical properties in soil including loss of soil nutrients and acidification of the soil (Maghanga, *et al*., 2013; Yang *et al*., 2018). This soil acidification not only leads to deficiencies in NPK and Mg nutrition but also increases the content of heavy metals (Jin *et al*., 2005; Zhang *et al*., 2007; Ruan *et al*., 2012).

Organic farming not only affects the quality of food products, but also improves soil fertility (Maeder *et al*., 2002). More recently, a higher number of cultivated bacterial genes have been obtained from organic production prepared using fermentable cow manure (Giannattasio *et al*., 2013).

Soil is a mixture of various organisms and organic and mineral substances (Kabata-Pendias, 2004). Soil health is essential to maintain plant and animal productivity, strengthen water and air quality and promote plant and animal health (Doran and zeiss, 2000). Microbes can be considered as earthworms (Rajendhran and Gunasekaran, 2018) or reduce important organisms and also play an important role in the environment (Emperor *et al*., 2015). It contains a wide variety of microorganisms that include bacteria, yeast, fungi, algae and protozoa (Braga, *et al*., 2016). Microorganisms can live in an environment with humans and in real conditions such as hot springs, miles deep in the ocean, within rocks and in extreme cold temperatures (Hongmeri *et al*., 2005). Although the number of microorganisms varies in different places, it has been identified that the mass of carbon from these microorganisms can be trillions of tons (Eilers *et al*., 2012; Angelow, 2008; Brock *et al*., 1991).

Interaction between plants and bacteria occurs through symbiotic and endolytic processes with roots and surrounding soil (Bacon and Hinton, 2006). Plants are at the heart of agriculture. It provides the raw materials for our food and drink. It regulates the soil fertility. Plants are the first link in terrestrial and aquatic food webs. Plants are essential for maintaining a healthy environment and enrich our intellectual life process (NAP, 1992).

Bacterial-inducing plant growth is divided into two groups (Bashan and Holguin, 1998) i.e., the first groups have the ability to synthesize plant growth

content (Glick and Bashan, 1997; Lucy *et al*., 2004) and a second group is capable of reducing the effects of phytopathogenic microorganisms (Bashan and Holguin, 1998). Bacterial-inducing plant growth is the bacteria that live in or on plant roots and contribute to plant productivity through various plant growth stimulating activities such as effective plant nutrition, hormonal production and plant pathogen inhibition (Ji *et al*., 2019).

In plant growth products some naturally occurring chemicals in plant cigarettes (i.e. endogenously) have a regulatory role, rather than a nutritional role in growth and development. These fertilizers, which are usually active at very low concentrations (Klerk *et al*., 2008). Plants play an important role in water uptake and nutrition from the soil (Ross *et al*., 2005). Nutritional imbalance and soil physical pollution hinder the sustainable use of inorganic fertilizers in the tropics (Ewulo; Ojeniyi and Akanni, 2008).

Biological nitrogen fixation (BNF) is the process responsible for the reduction of N_2 to ammonia (NH_4) (Newton, 2000; Franche *et al*., 2009) and is played in diazotrophic microorganisms, to specific bacteria and archaea (Dixon and Kahn, 2004). Phosphorus (P) is an essential nutrient or key element of metabolic, biochemical and photosynthetic pathways for plants (Khan *et al*., 2009; Richardson and Simpson, 2011). Plants take in two soluble forms of phosphates: the monobasic (H_2PO^{4-}) and the dibasic (HPO^{42-}) (Glass, 1989).

Plant roots respond to environmental conditions through the secretion of a wide range of compounds, depending on nutrient status and soil conditions (Cai *et al*., 2012; Carvalhais *et al*., 2013).

Nitrogen enters a living organism through nitrogen fixation (Egamberdieva and Kucharova, 2008; Frank *et al*., 2012). These microbes may be symbiotic or free-living in nature (Reghuvaran *et al*., 2012).

Phosphate-solubilizing bacteria dissolve inorganic ground phosphates, such as $Ca_3(PO_4)_2$, $FePO_4$, and $AlPO_4$, through the production of organic acid, siderophores, and hydroxyl ion production in agricultural soils for plant growth stimulating bacteria (Jones, 1998; Chen *et al*., 2006; Rodríguez *et al*., 2006, Sharma *et al*., 2013).

This study aims to separate microorganisms from cow dung sample to identify the potential applications of cow dung in stimulant markers of plant growth and seed germination. It describes the ability of PGPR isolated from cow dung and the growth they induce in the experimental plant *Vigna radiata* in pots under sterile conditions so that further study will shed light on how these bacteria could be used as biofertilizers. These bacteria can be considered as promising candidates for application in sustainable agricultural management.

REVIW OF LITERATURE

Bloem *et al*. (1994) and Matson *et al*. (1997) compared the community of soil flora and fauna which is influenced directly or indirectly by management practices, e.g. cultivation and the use and application of organic and inorganic fertilizer. Droogers and Bouma (1996), Mader *et al*. (2002) and Girvan *et al*. (2004) in his report the organic farming leads to higher soil quality and more biological activity (microbial populations and microbial respiration rate) in soil than conventional farming. Bacillus spp. are used as a biocontrol agents (Latour *et al*., 1996; Bloemberg and Lugtenberg, 2001) in seed treatment programs against soilborne pathogens (Walker *et al*., 1998).

In this review, bacterial isolates refer to specific bacterial strain (PGPB or rhizobia) that can promote plant growth after inoculation. “Carrier” refers to the abiotic substrate (solid, liquid, or gel) that is used in the formulation process. “Formulation” is to the laboratory or industrial process of unifying the carrier with the bacterial strain. “Inoculant” refers to the final product of formulation containing a carrier and bacterial agent or consortium of microorganisms. “Quality control” refers to the process of measuring defined quality parameters of the inoculant. “Quality assurance” is the overall evaluation that quality control procedures and techniques are achieving what they intend to achieve. In legumes, the quality of the inoculant is defined as the number of viable and effective cells capable of nodulating plants and fixing nitrogen of the intended strain delivered

by the inoculant at point-of-sale. For PGPB, similar parameters apply, with higher emphasis on contaminant-free inoculants (Bashan 1998; Deaker *et al*. 2011).

Application of organic manures to enhanced counts of *Trichoderma* and *Aspergillus spp* studied by Bopiah and Bhat (1981). Soil is a most precious natural resource and contains the most diverse assemblages of living organisms. Indigenous microbial populations in soil are of fundamental importance for ecosystem functioning in both natural and managed agricultural soils (O'Donnell *et al*., 1994; Doran and Zeiss, 2000) because of their involvement in such key processes as soil structure formation, organic matter decomposition, nutrient cycling and toxin removal (Van Elsas, 1997; Doran and Zeiss, 2000).

Srikanth *et al*. (2000) and Patil *et al*. (2003) found significant reduction in soil bulk density by FYM application to the soil. Similar findings were also reported by Rajkannan *et al*. (2001), in his studies by using FYM and coir pith. Ravikumar and Krishnamoorthy (1980) compared the effects of organic and inorganic soil amendments on physical properties of black soil and opined that organic amendments, maize straw and FYM were superior to the inorganic fertilizers, improving the aggregate stability, hydraulic conductivity and available water content of the soil.

Raaijmakers *et al*. (2002) found the bacteria that can improve plant growth through various mechanisms have been known for decades and have been introduced into soil, on seeds or roots to improve plant growth and health. Claffin,

(1986) studied the Genus *Rhizobium*, an example of a growth-promoting organism, is the most widely known group. It has been successfully commercialized with many practical applications in agriculture by developing symbiosis with plant debris. Leslie, (1990) reported the fungi are widely distributed in host root tissues under field conditions and respond to stress in the plant by taking advantage of preferential growth conditions to incite diseases.

Liu *et al*., (1995), Leeman *et al*. (1995) and Nandakumar *et al.* (2001) found that different biotic and abiotic inducers are involved in induction of systemic induced resistance in plants against various pathogens. These include pathogens, chemical plant products and PGPR. Van Per *et al*. (1991) and Maurhoef *et al*. (1994) report the mechanism by which these inducing agents stimulate resistance is that they activate defence genes encoding chitinase, peroxidase, -1, 4-glucanase and enzymes involved in the synthesis of phytoalexins.

Another important division of beneficial bacteria in the rhizosphere are those referred to as plant growth promoting bacteria (PGPB), which promote growth via production of phytohormones and improvement of plant nutrition status (Bai *et al*., 2002). Because of these properties, the coinoculation of these PGPB with the symbiotic rhizobia is currently becoming a valuable technique in the development of sustainable agriculture. Among the major groups of plant growth promoting bacteria, the most widely studied and efficient group include

Azosprillum spp. (Bertrand *et al*., 2001), *Pseudomonas spp*. (Amy *et al*., 2002) and *Bacillus spp*. (Bai *et al*., 2002).

An important feature of these plant growth-promoting bacteria is their ability to colonize roots and promote plant growth (Sharma *et al*., 2003; Patten and Glick, 2002). The potential of rhizosphere colonization by PGPB is very crucial for what is known as soil biofertilization (Villacieros *et al*., 2003). The term 'biofertilizer', though misleading is a widely used term to describe bacterial inoculants. It refers to preparation of microorganisms that may be a partial or complete substitute for chemical fertilization like rhizobial inoculants (Bashan, 1998).

Improving plant growth by biofertilization is a crucial mechanism by which iron acquisition in most agricultural crops is achieved. Normally the total iron in the soil is by far much higher than most crops require. However, the concentration of free Fe^{+3} in most soils is far below that required for optimum growth (10^{-2} and 10^{-4}M Fe^{+3}) in the soil solution (Masahla *et al*., 2000). In the decades before, many studies have indicated that the production of siderophores by plant growth promoting bacteria, particularly by the biocontrol Pseudomonas spp. increases plant iron acquisition (Masahla *et al*., 2000).

Gaur (2002) studied microbial solubilization of inorganic phosphate compounds is of great economic importance in plant nutrition. Goldstein (2001) also reported bacteria from genera such as *Achromobacter, Agrobacterium*,

Bacillus, Enterobacter, Erwinia, Escherichia, Flavobacterium, Mycobacterium, *Pseudomonas* and *Serratia* are highly efficient in solubilizing unavailable complexed phosphate into available inorganic phosphate ion.

The high binding affinity and specificity for iron facilitates the transport of iron into the bacterial cells. Plants make use of this ferric-siderophore complex in their systems through the action of enzymes like ferric reductase (Sharma *et al*., 2003). Walter *et al* (1994) according to many reports, the possible role of plant growth promoting bacteria in iron uptake by plants in the rhizosphere is indicated by the fact that, under non sterile soil system plants show no iron deficiency symptoms in contrast to plants grown in sterile system.

Another important aspect of biofertilization is that it accounts for approximately 60 % of the nitrogen supply to crops worldwide. This is achieved both by the symbiotic and free-living nitrogen fixers. To date the genes involved in nitrogen fixation and nitrogen assimilation have been described for *Azosprillum* (Bloemberg and Lugtenberg, 2001).

A promising trend in the field of inoculation technology with plant growth promoting bacteria is, then finding that co-inoculation of growth promoting bacteria with other microorganisms increased growth and yield (Bashan, 1998). Mixed inoculations allow the bacteria to interact synergistically and provide nutrients, remove inhibitory products and enhance some beneficial aspects of

their growth of non-legume plants by directly affecting the metabolism of the plants (Bashan and Holguin, 1997).

Numerous studies stated that the yield and quality of plants products produced by organic farming (Chemura, 20014; Olivera *et al*., 2013; Luthria *et al.,* 2010).Nitrogen-fixing bacteria have frequently been reported as plant growth promoters (Requena *et al*., 1997; Gonzalez-Lopez *et al*., 2005)

Tang, (1994) the discovery of *Azosprillum* as PGPR, many other bacteria such as *Bacillus*, *Flavobacterium* and *Acetobacter* have been evaluated for their potential in plant growth promotion. The biocontrol agents, mainly *P. fluorescens* and *P. putida* are also regarded as agents of plant growth promotion.

Vining (1990) reported that the hydrogen cyanide is formed during the early stationary growth phase and does not take part in growth, energy storage or primary metabolism, but is generally considered to be a secondary metabolite that has an ecological role and confers a selective advantage on the producer strains. Ahmad *et al.* (2008) studied the production of HCN was a more common trait of *Pseudomonas* (88.89%). Bakker and Schippers (1987) found that cyanide occurs in solution as free cyanide, which includes the cyanide anion (CN-) and the non-dissociated HCN. Cyanide is a phytotoxic agent capable of inhibiting enzymes involved in major metabolic processes and is considered one of the typical features of deleterious rhizobacterial isolates. Devi *et al*. (2007) studied the applications of it in areas of biocontrol methods.

Kremer and Souissi (2001) describes some cyanogenic rhizobacteria that are typically host specific and associated with the roots of their host plants. Therefore, HCN produced in the rhizosphere of seedlings by selected rhizobacteria is a potential and environmentally compatible mechanism for biologically controlling weeds and minimizing deleterious effects on the growth of desired plants.

Jha and Saraf (2012) studied the plant growth was further improved maximally when the three were applied together. By virtue of their rapid colonization of the rhizosphere and stimulation of plant growth, there is currently considerable interest in exploiting these rhizosphere bacteria to improve crop production.

In the last decade, several reviews summarized the field of plant inoculation. Most concentrated on specific genera, such as *Rhizobia* (Catroux et al. 2001; Deaker *et al*. 2004; Herridge, 2007; Stephens and Rask, 2000), *Azospirillum* (Bashan *et al*. 2004; Bashan and de- Bashan, 2010), field performance of several PGPB (Rizvi *et al*. 2009), availability of various PGPBs and their modes of action (Andrews *et al*. 2003; Lodewyckx *et al*. 2002; Lucy *et al*. 2004; Vessey, 2003), reduction in the use of fertilizers by including inoculants (Adesemoye and Kloepper, 2009), and potential marketing (Mathre *et al*. 1999; Berg, 2009).

Although some reviews briefly mentioned formulations and practical aspects of inoculants, none of these recent reviews concentrated on that topic. The overall review clearly indicated that the practice of organic farming was highly useful for improving crop growth, yield, nutrient and postharvest qualities. The organic production of food grains, vegetables, fruits, pulses etc is well accepted by the consumers and the demand for the same is increasing day by day.

Almost all the organic produce is without pesticidal residue, non-hazardous and therefore becomes a potential commodity for export. In addition to this notable improvement in soil health was evident by addition of organic manures, which marks the first step to the novel way of sustainable development.

METHODS AND MATERIALS

3.1 REQUIREMENT MEDIUM

Nutrient Agar (NA), Nutrient Broth (NB), Pepton Broth (PB), Gelatin Medium, Starch Agar ,SM medium, SIM medium, Simmon's citrate medium, Burk's medium, and Potato dextrose rose Bengal agar

3.2 SAMPLE COLLECTION

Cow dung sample were collected from dairy farm. Cow dung was dried under sunlight and powdered.

3.3 ISOLATION OF BACTERIAL CULTURE

3.3.1 SERIAL DILUTION

One gram of cow dung sample mixed with 10ml of sterile distilled water. Fourteen test tubes were taken and each with 9ml of sterile distilled water. A sterile pipette was used to pipette out 1ml of mixed (Cow dung) sample. Then the sample was added to the first test tube 10^{-1} and dilution was repeated. From the dilution, (10^{-11} to 10^{-14}) 1ml was taken and plated.

3.3.2 POUR PLATE METHOD

The Nutrient agar was dissolved in 200ml of distilled water. The medium was autoclaved at 121ºC for 15 minutes. The medium was poured into the petriplates and allowed to solidify. One ml of each dilution (10^{-11}, 10^{-12}, 10^{-13}, and

10^{-14}) poured into the medium and plates were rotated clockwise and anticlockwise direction. These plates were incubated at 37ºC for overnight. After incubation colony characteristics were studied.

3.3.3 PURE CULTURE

Isolated colonies were marked and numbered on the agar plates. Typical bacterial colonies were observed over the streak in culture plates. The technique was repeated thrice and culture were made single colony type. For further study, the single colony were isolated and stored.

3.4 CHARACTERIZATION OF MICRO ORGANISMS

After isolation pure colonies of bacteria were identified and characterized on the basis of morphological analysis such as colour, size and shape. These colonies of bacteria were identified by the Gram's staining method

3.5 STERILIZATION OF SEEDS

Before seed inoculation, the seeds (*Vigna radiata*) were sterilized with ethanol (70%) for 3 minutes and 1.2% sodium hypochloride for 10 minutes and then 10 times washed with autoclaved distilled water (Pikovskaya, 1948).

3.6 SEED GERMINATION TEST:

The strain of SP3, SP4 and SP5 were inoculated in nutrient broth and kept in an orbital shaker (220nm and 150 rpm) for 24 hours. Seeds (*Vigna radiata*) were soaked into the bacterial suspension of SP3, SP4 and SP5 for 1 hour. After 1 hour, the seeds are planted in the pot containing red soil. Seed treated with sterile distilled water served as control. Experiments were performed in a completely randomized block design and growth parameters were measured after 3 days.

3.7 BIOCHEMICAL TEST

3.7.1 GRAM'S STAIN

A clean slide was taken and a smear of suspension was made with a loopful of sample. It was air dried and heat fixed. Crystal violet was poured and kept for about 30 seconds and rinsed with water. Gram's iodine was added and kept for 1 minute and washed with water. Then, the smear was washed with 95% alcohol or acetone for about 10-20 seconds and rinsed with water. Safranin was added for about 1 minute and washed with water. Air dried slide was observed under microscope.

3.7.2 ENDOSPORE STAINING

A clean slide was used to make a smear. Air dried and heat fixed smear was covered with a square of blotting paper or towel cut to fit the slide. The

blotting paper was saturated with malachite green stain solution and steam for 5 minutes. The slide was washed in tap water, counterstained with 0.5% safranin for 60 seconds, washed with tap water and allowed to air dry. The slide was examined under microscope (Harley, 2005).

3.7.3 CATALASE TEST

Catalase test method with (3% H_2O_2) H_2O_2 was adopted (Jay, 1992). The H_2O_2 test was carried out by pipeing 100µ of 30% H_2O_2 in the object's glass, taken by one bacterial isolate and then streaked on the object's glass, observing the formation of bubbles. If there are bubbles indicating positive catalase bacteria if there are no bubbles, including negative catalase bacteria (Hadioetomo, 1990).

3.7.4 SIMMON'S CITRATE TEST

About 100ml of Simmon's citrate agar medium was autoclaved at 121ºC for 15minutes and pour into Petriplates. Allowed to solidify, then the culture was streaked and incubated for 24 hours at 37ºC. A colour change from green to blue indicates a positive result.

3.7.5 INDOLE TEST

Indole production was tested by inoculating a loopful of 24 hour broth culture in peptone broth medium. The medium was incubated at 37ºC for 48 hours. At the end of incubation period, 3 drops of Kovac's reagent was added

directly to the tube. The presence of indole was indicated by the formation of pink to red colour ring in the reagent layer on the top of the medium.

3.7.6 STARCH HYDROLYSIS

About 100 ml of starch medium was poured into the sterilized Petriplates. After hardening of the medium, three isolated strains were streaked on its surface of the medium. Then the medium was kept for two days at room temperature. At the end of incubation period, two or three drops of Lugol's iodine solution were added on the surface of the medium. A clear zone outside the area of growth indicated the size of the starch hydrolysis.

3.7.7 NITRATE REDUCTION

To a 24 hours culture in the peptone broth medium, two drops of sulfanic acid and two drops of α-naphylamine solution were added. The presence of nitrate was indicated by the pinkish red colour after the addition of reagents.

3.7.8 GELATIN LIQUIFACTION

The gelatin medium was inoculated with bacteria and incubated for 24 hours at 30ºC. After the incubation period, the test tubes were placed in the refrigerator for one hour. Allowed to solidify the medium showed a negative result and a positive result was seen from the liquifaction of gelatin.

3.7.9 HYDROGEN SULPHIDE PRODUCTION

SIM Medium was inoculated with the bacterial culture by penetration method and incubated for 24 hours at 37º C. The presence of H_2S is based on the metal formed. The medium become black colour which indicates the formation of hydrogen sulphide.

3.7.10 HYDROLYTIC ENZYME PRODUCTION

3.7.10.1 PROTEASE TEST

Bacterial isolates were screened for the ability to produce proteolytic enzymes in skim milk agar (or) SM medium. The medium was poured into a sterilized Petriplates and the isolate bacterial strain was streaked on the surface of the medium. After 24 hours a clear zone is formed around the bacterial colonies.

3.7.11 PLANT GROWTH PROMOTORS

3.7.11.1 PHOSPHATE SOLUBILIZATION

Bacterial isolate was screened in Potato Dextrose Rose Bengal agar. The cultures were streaked on the sterile medium. Plates are incubated at 27±30ºC for 24 hours. The growth of the bacterial colony were indicated a positive result for phosphate solubilization.

3.7.11.2 NITROGEN FIXATION

The medium (Burk's) contained in 100ml $MgSO_4$ (0.02g), K_2HPO_4 (0.08g), KH_2PO_4 (0.02g), $CaSO_4$ (0.013g), $FeCl_2$ (0.00145g), Na_2MnO_4 (0.0000253), Sucrose (2g), Agar (1.5g). Plates were incubated at 30ºC for 24hrs. The isolates that were able to grow after incubation.

3.7.11.3 AMMONIA PRODUCTION

Bacteria isolates were screened for the production of ammonia in peptone water described by Ajaykumar (2012). Freshly grown cultures were inoculated in a peptone broth and incubated at 28ºC for 48 hours. Nessler's reagent was added in each test tube. Formation of brown to yellow colour indicates a positive result for ammonia production.

3.7.12 BIOCONTROL

3.7.12.1 PRODUCTION OF HYDROGEN CYANIDE

Nutrient broth was amended with 0.44g glycine/ml (Glickmann, Dessaux, 1995) and bacteria was inoculated. Then the inoculation suspension was incubated at 36ºC for 24 hours. A Whatman filter paper no.1 soaked in 2% sodium carbonate in 0.5% Picric acid solution and then the paper inserted into the nutrient broth. After 30 minutes the colour change of the filter paper from deep orange to reddish brown indicated the production of HCN.

4. RESULTS

4.1 COUNT OF BACTERIA

Enumeration of microbial count has been obtained as OD value which was read in a spectrophotometer. The results are shown in Table 1 and Figure 1

4.2 MORPHOLOGICAL FEATURES OF BACTERIAL ISOLATES

In the present study 10 bacterial species have been isolated from the cow dung samples. Only 8 isolates were selected for the study and remaining 2 were ignored for being similar in morphological features. Based on microscopic observations, the bacteria is isolated were identified as *Bacillus* (SP3), *Diplococcus* (SP4), *Streptococcus* (SP5), *Coccus* (SP6), *Bacillus* (SP7), *Bacillus* (SP8), *Bacillus* (SP9), and *Coccus* (SP10) (Figure 2). The 8 isolates mentioned in Table 2 shows the distinct morphological features of bacterial isolates. The features include characteristics of colony shape, colour, and margin, Gram staining, bacterial morphology, spore staining and motility. All isolated bacterial colonies and their microscopic views of Gram staining and spore staining are show in Figure3 & Figure 4.

4.3 EVALUATION OF SEED GERMINATION (*Vigna radiata*)

Germination percentage of *Vigna radiata* seeds grown in cow dung bacterial suspension SP3, SP4, and SP5 were selected, because these three strains showed positive results in phosphate solubilization and nitrogen fixation. The

highest germination percentage was observed in SP5 compared to others. The increase in germination percentage and in other growth parameters can be attributed to the combined effect of the nitrogen fixing and phosphate solubilizing microbes which were able to fix atmospheric nitrogen and solubilize P and also produced growth promoting substances. These organisms have been isolated from cow dung and this observation indicates the reason for promotion and enhancement of crop growth and yield when cow dung is applied. It is a proof that these symbiotic microbes present in cow dung benefit the soil fertility. Observation on growth parameters such as plant weight and height, root length and weight of (*Vigna radiata)* seed plant are shown in Table 3.

4.4 BIOCHEMICAL TEST

The biochemical test results were interpreted by the reaction that occur in the form of change in colour that was observed by naked eye. In catalase test, the strain of SP3, SP4, SP7, SP9, and SP10 produced the bubbles and were positive whereas SP5, SP6, & SP8 did not produced the bubbles and showed negative results (Figure 5). In case of Simmon's Citrate test, all the 8 strains were producing the blue colour which referred as positive (Figure 6).

In Indole test, the strain SP3, SP5, SP6, SP7, SP8, and SP9 showed the pink to red colour ring in the reagent layer on the top of the medium and SP4 & SP10 showed negative results (Figure 7). In Starch hydrolysis, the strain SP3,

SP4, SP5, SP8, and SP10 showed a clear zone outside of the growth area and SP6, SP7, & S9 showed negative results (Figure 8).

As Nitrate reduction was indicated by the formation of yellow to pinkish red colour. Here, SP3, SP5, SP6, SP7, SP8, SP9, SP10 produced positive result and SP4 showed negative results (Figure 9). The strain SP6, SP7, SP10 showed negative result for Gelatin Liquifaction when allowed to solidify but SP3, SP4, SP5, SP8, SP9 were positive (figure 10). In Hydrogen sulfide production test, the bacterial strain namely SP5, SP6, SP7 were producing black colour slant which indicates H_2S production and SP3, SP4, SP8, SP9, SP10 showed negative results (Figure11).

4.4.1 HYDROLYTIC ENZYME PRODUCTION

In protease test, the strain SP4, SP5, SP6, SP7, and SP9 showed a clear zone outside of the growth area and SP3, SP8, & SP10 gave negative result (Figure 12).

4.4.2 PLANT GROWTH PROMOTORS

In Phosphate solubilization, the bacterial strain SP7 presented negative result and these seven bacterial strains SP3, SP4, SP5, SP6, SP8, SP9, and SP10 were able to grow in the Potato Dextrose Rose Bengal Agar and they were identified as phosphate solubilizing microorganisms (Figure 13).

In Nitrogen fixation, the bacterial strain SP4 provided negative result and SP3, SP5, SP6, SP7, SP8, SP9, SP10 were able to grow in nitrogen-free medium and they were identified as nitrogen fixing bacteria (Figure 14).

In Ammonia production, the microbial strain SP7, SP8, SP10 produced negative result and SP3, SP4, SP5, SP6, SP9 showed the formation of brown to yellow colour which is referred as positive result (Figure 15).

4.4.3 BIOCONTROL

In hydrogen cyanide production test, the bacterial strain SP5, SP8 showed negative result and SP3, SP4, SP6, SP7, SP9, SP10 showed the colour change of the filter paper from deep orange to reddish brown indicating the production of HCN (Figure16).The results were depicted in Table 4 .

TABLE-1

BACTERIAL STRAIN	OD VALUE
SP3	0.625
SP4	1.002
SP5	1.331
SP6	1.585
SP7	1.302
SP8	1.189
SP9	1.240
SP10	1.709

FIGURE-1

BACTERIAL COUNT IN BROTH

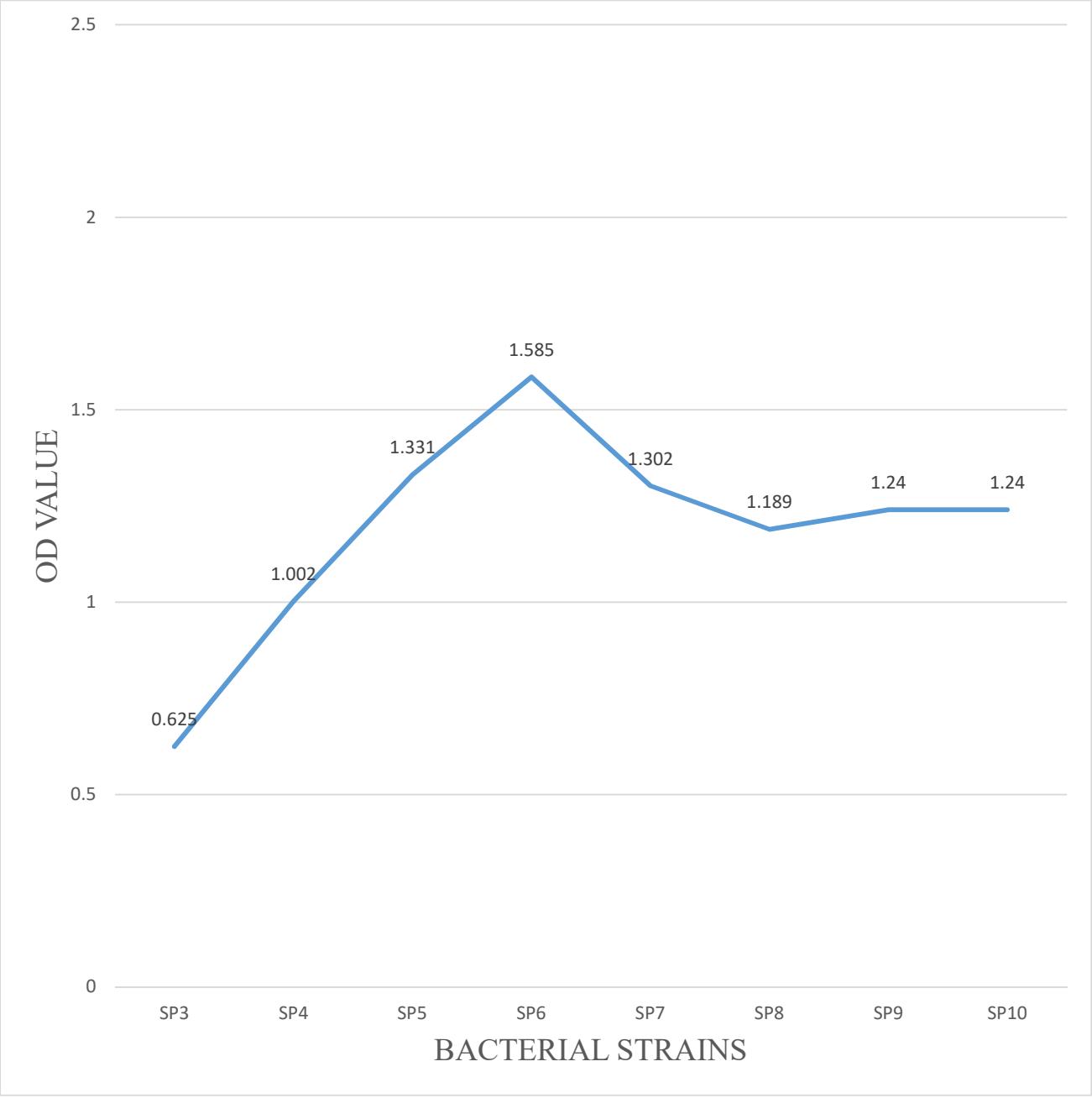
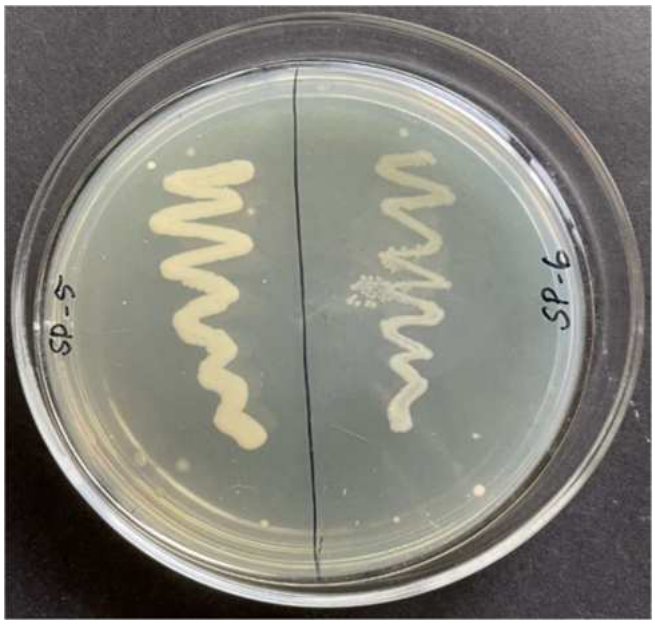


FIGURE-2

MORPHOLOGICAL FEATURES OF BACTERIAL STRAINS



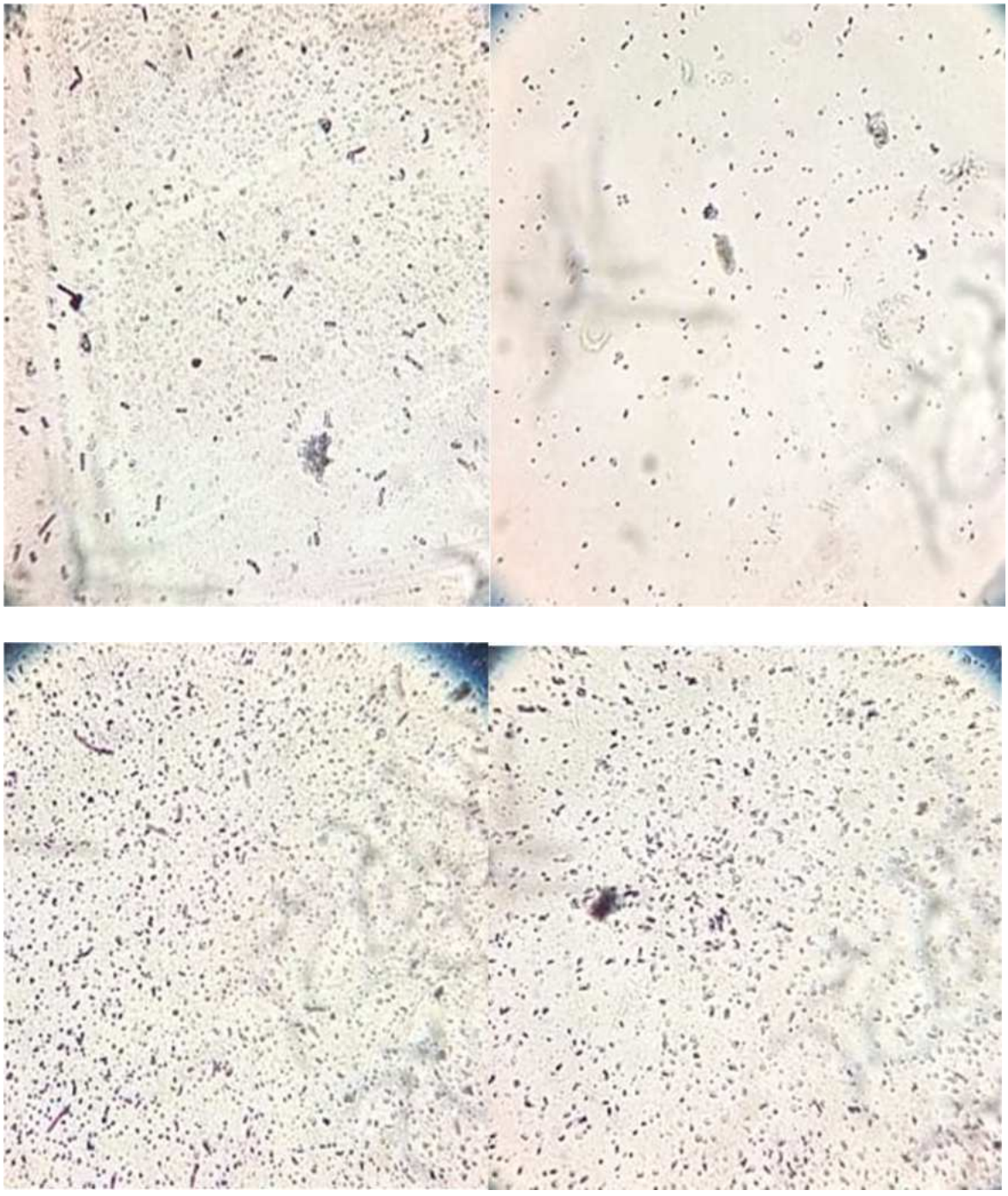

SP-7
SP-8


CO

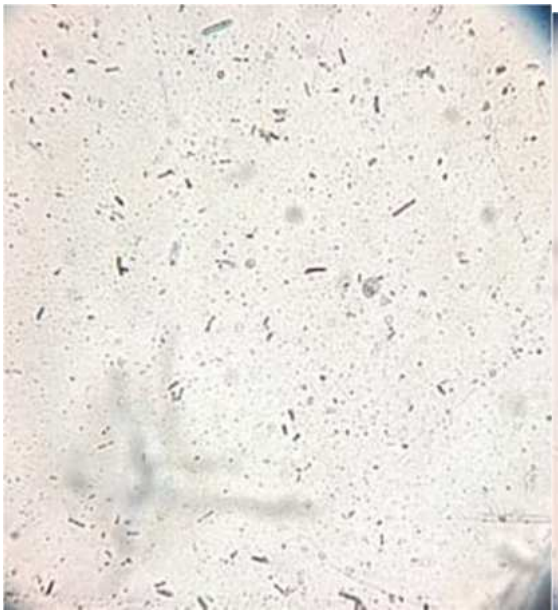
FIGURE- 3

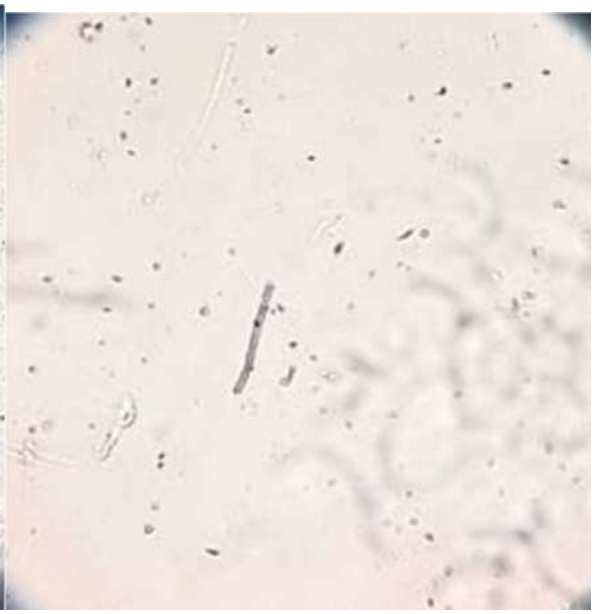
GRAM STAINING

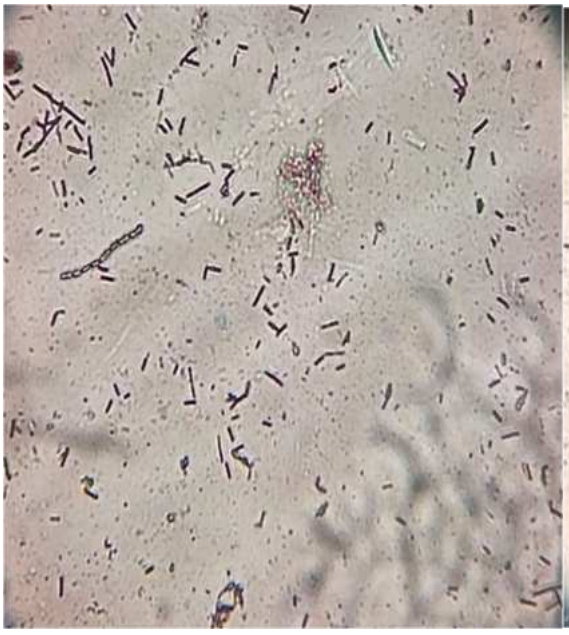
(A)



(B)







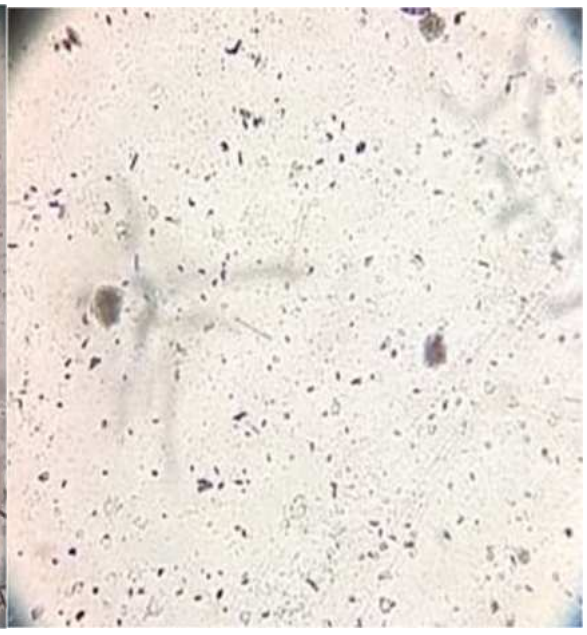
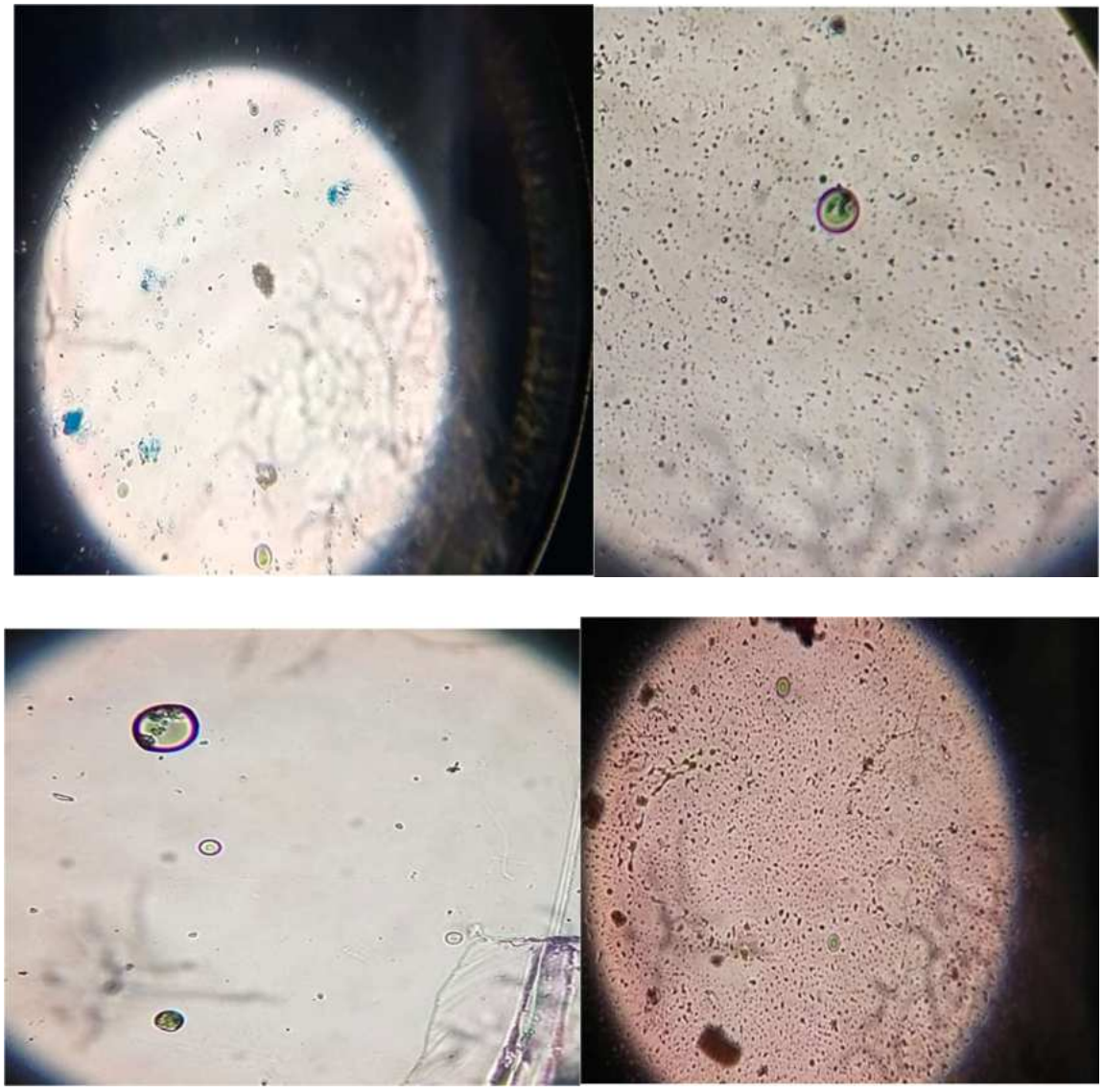


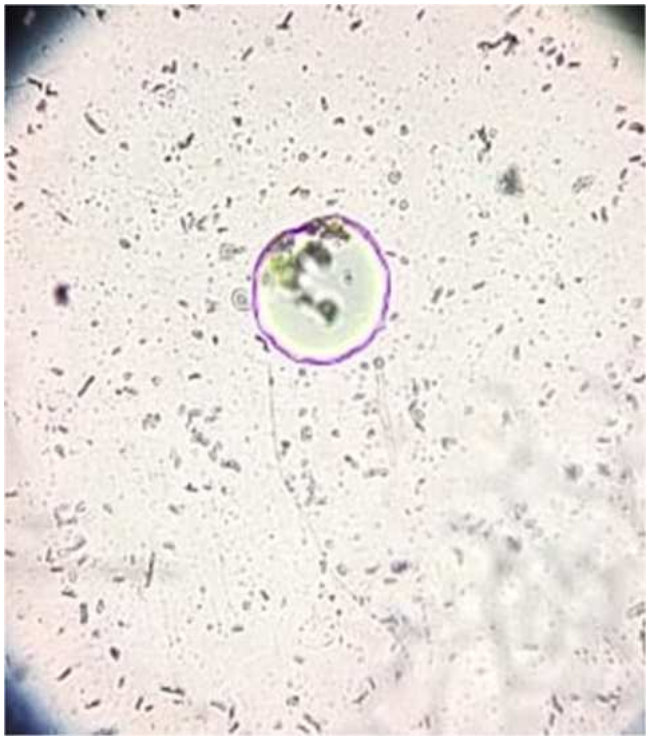
FIGURE-4

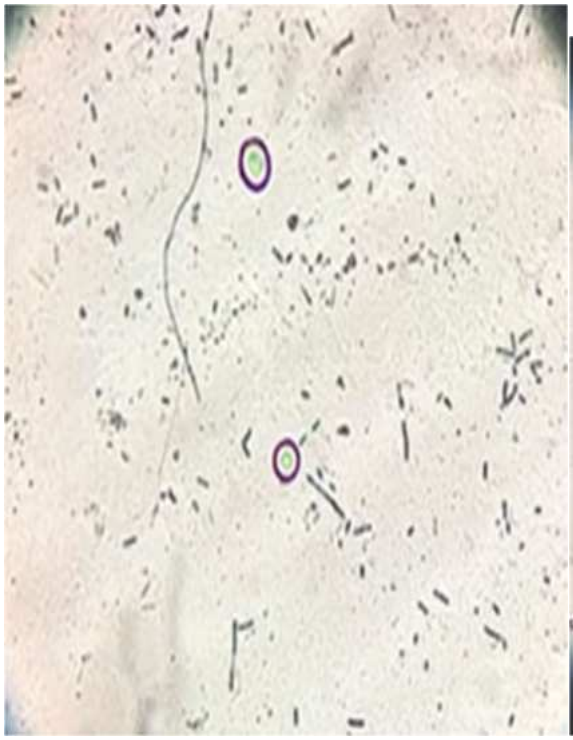
SPORE STAINING

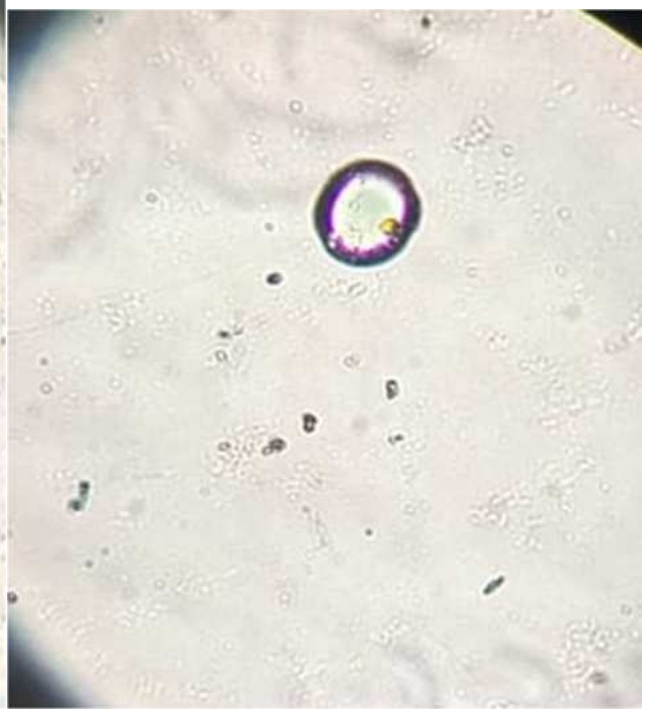
(A)



(B)







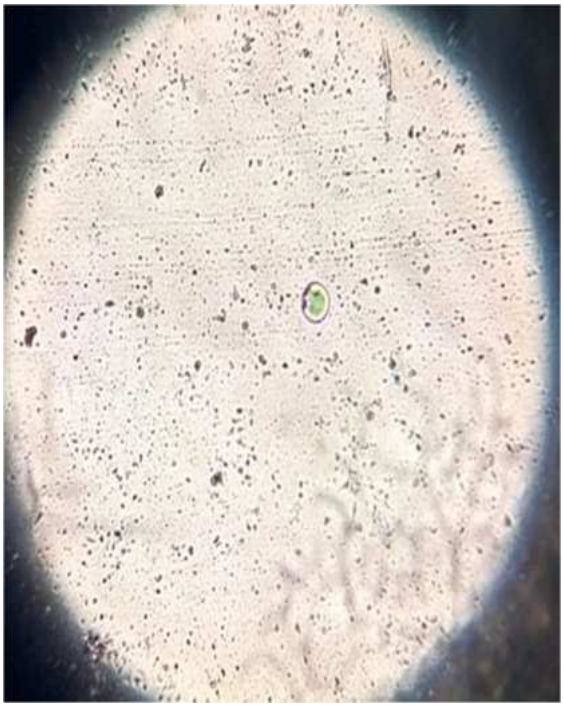


TABLE-2

MORPHOLOGICAL FEATURES IN BACTERIAL STRAINS

Colony characteristics	Shape	Elevation	Opatical property	Margin	Colour	Gram staining	Bacterial morphology	Spore staining	Motility
SP3	circular	Raised	Translucent	Entire	Dull white	negative	bacilli	positive	motile
SP4	circular	raised	opaque	entire	Dull white	positive	diplococci	positive	non motile
SP5	circular	convex	translucent	Entire	Dull white	negative	staphlococci	positive	non motile
SP6	filamentous	raised	opaque	Entire	Dull white	negative	cocci	positive	motile
SP7	filamentous	raised	Transparent	Filiform	Dull yellow	Positive	Bacilli	positive	non motile
SP8	filamentous	raised	Transparent	Filiform	Dull yellow	Positive	Bacilli	positive	motile
SP9	circular	raised	Translucent	Filiform	Dull white	Positive	Bacilli	positive	non motile
SP10	Irregular	raised	Translucent	Entire	Yellow	Negative	Cocci	positive	non motile

FIGURE-5

POT EXPERIMENT

(A)

(B) STRAIN SP3 GROWTH

(C) STRAIN SP4 GROWTH

(D) STRAIN SP5 GROWTH

(E) ROOT GROWTH IN THREE STRAIN

TABLE-3

GROWTH PARAMETERS IN POT EXPERIMENT

BACTERIAL STRAINS	PLANT HEIGHT (cm)	PLANT WEIGHT (gms)	ROOT HEIGHT (cm)	ROOT WEIGHT (gms)
CONTROL	9.0	0.247	1.0	0.027
SP3	10.5	0.250	3.5	0.077
SP4	11.0	0.383	3.0	0.053
SP5	14.0	0.414	3.0	0.068

TABLE-4

BIOCHEMICAL TEST

BIOCHEMICAL TEST	CATALASE	SIMMON'S CITRATE	INDOLE TEST	STRACH HYDROLYSIS	NITRATE REDACTION	GELATIN LIQUIFICATION	HYDROGEN SULPHIDE PRODUCTION	PROTEASE TEST	PHOSPHATE SOLUBILIZATION	NITROGEN FIXATION	AMMONIA PRODUCTION	HYDROGEN CYANIDE
SP3	+	+	+	+	+	+	-	-	+	+	+	+
SP4	+	+	-	+	-	+	-	+++	+	-	+	+
SP5	-	+	+	+	+	+	+	+++	+	++	+	-
SP6	-	+	+	-	+	-	++	+	+	++	+	+
SP7	+	+	+	-	+	-	+	+++	-	+++	-	+
SP8	-	+	+	+	+	+	-	-	+	+++	-	-
SP9	+	+	+	-	+	+	-	+	+	+++	+	+
SP10	+	+	-	+	+	-	-	-	+	+	-	+

FIGURE-6

CATALASE TEST

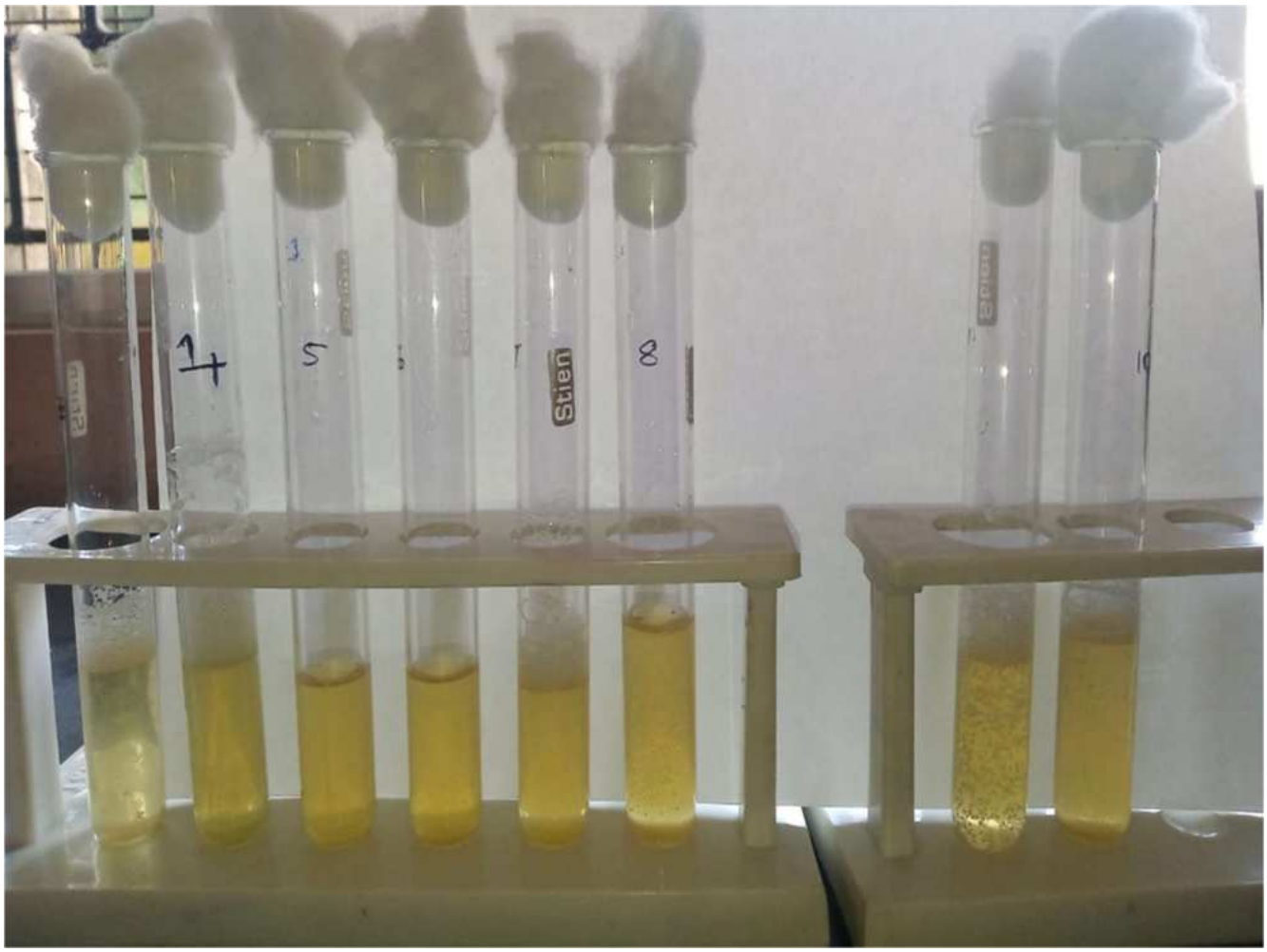


FIGURE-7

SIMMON'S CITRATE TEST

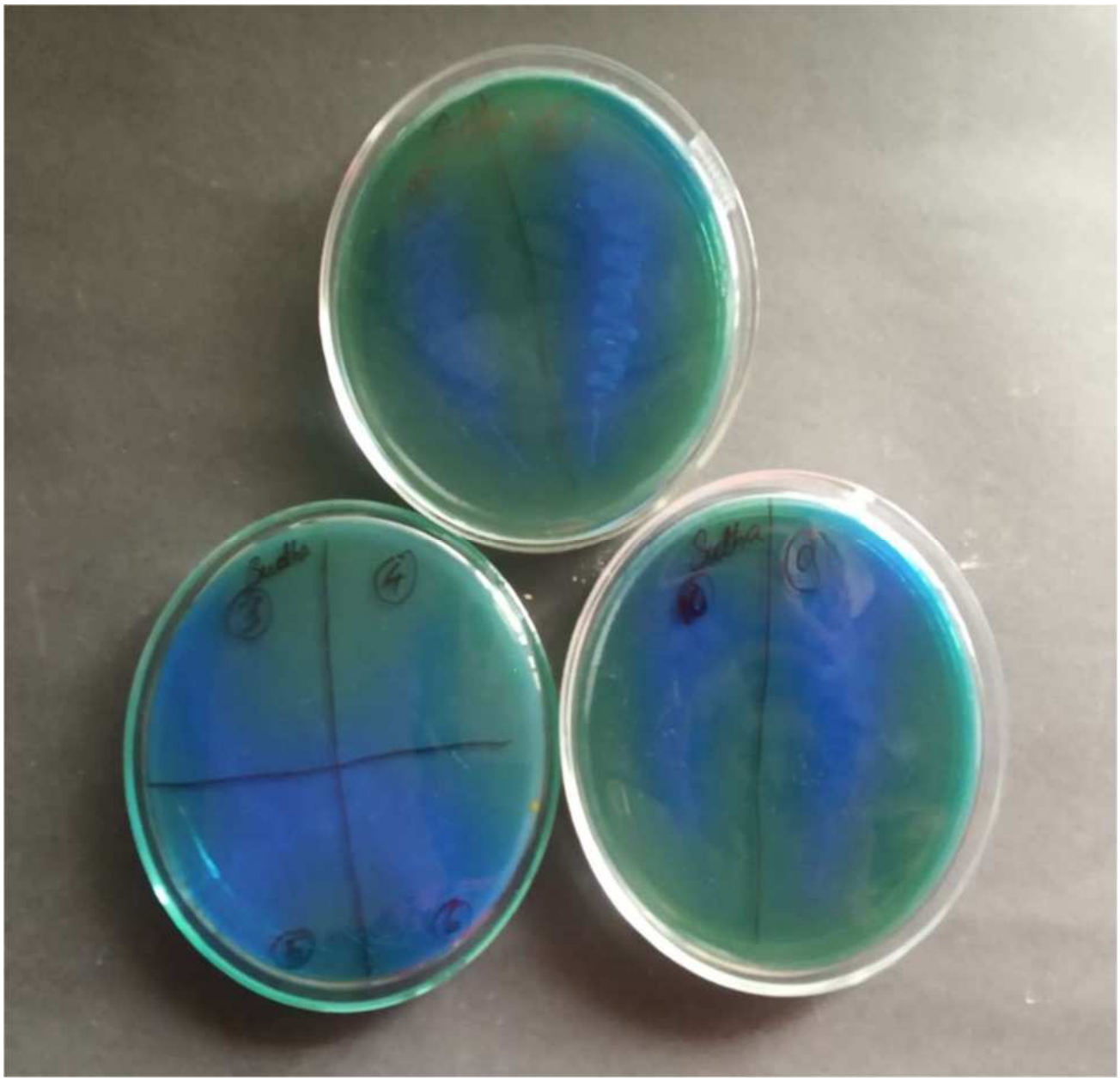


FIGURE-8

INDOLE TEST

FIGURE- 9

STRACH HYDROLYSIS TEST

FIGURE-10

NITRATE REDUCTION TEST

FIGURE- 11

HYDROGEN SULPHIDE PRODUCTION

FIGURE- 12

PROTEASE TEST

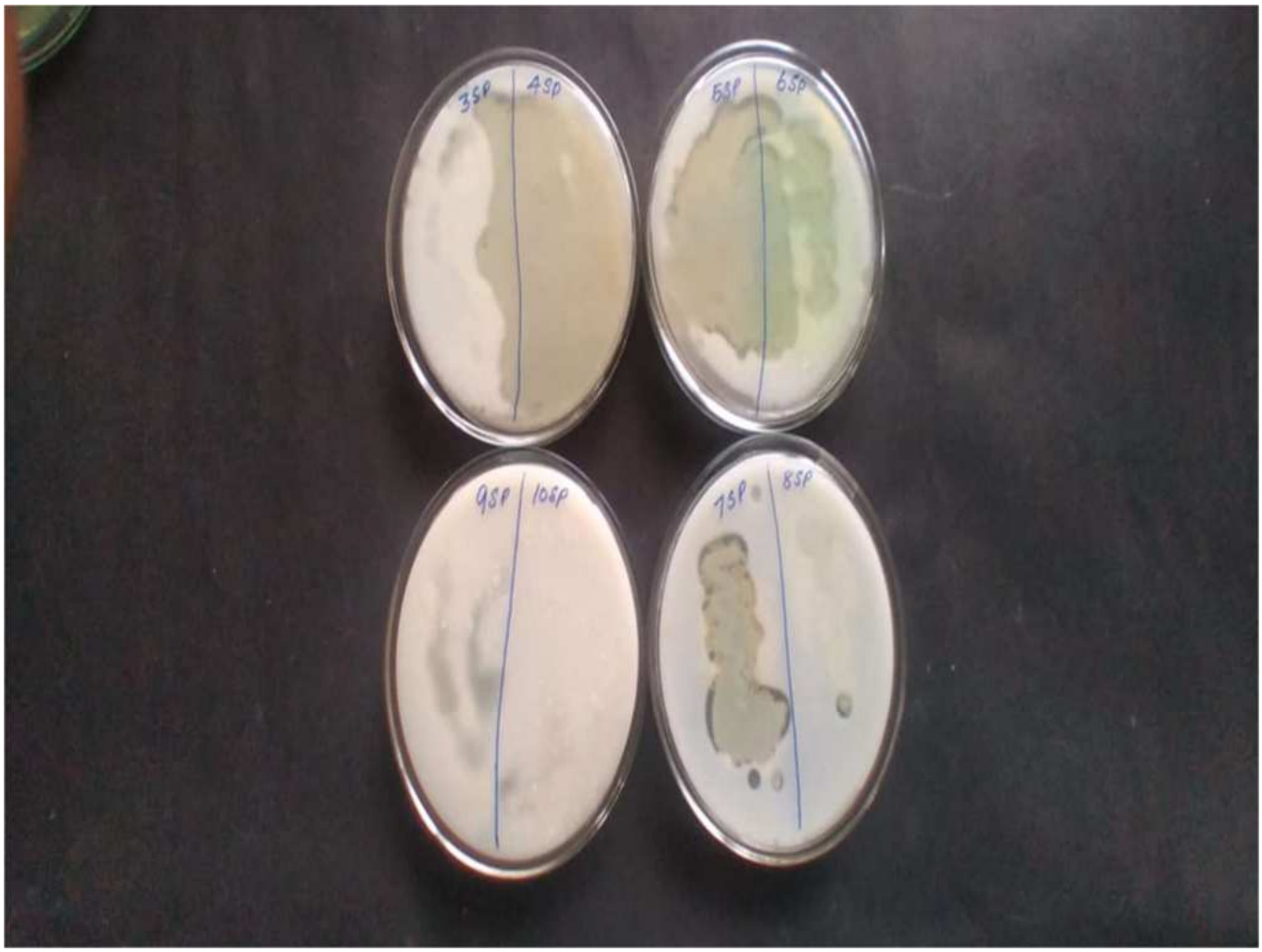


FIGURE- 13

PHOSPHATE SOLUBILIZATION TEST

FIGURE- 14

NITROGEN FIXATION TEST

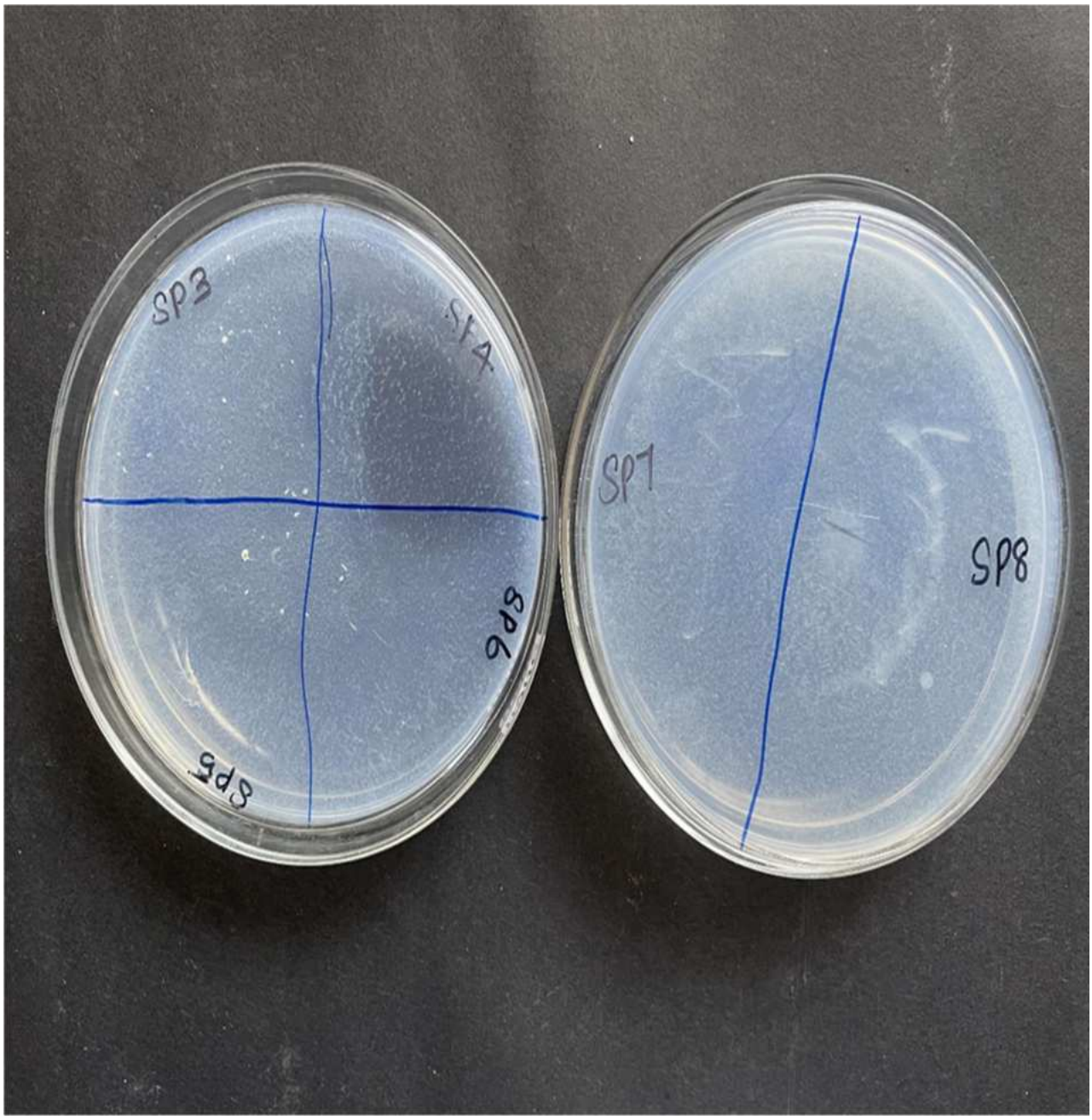
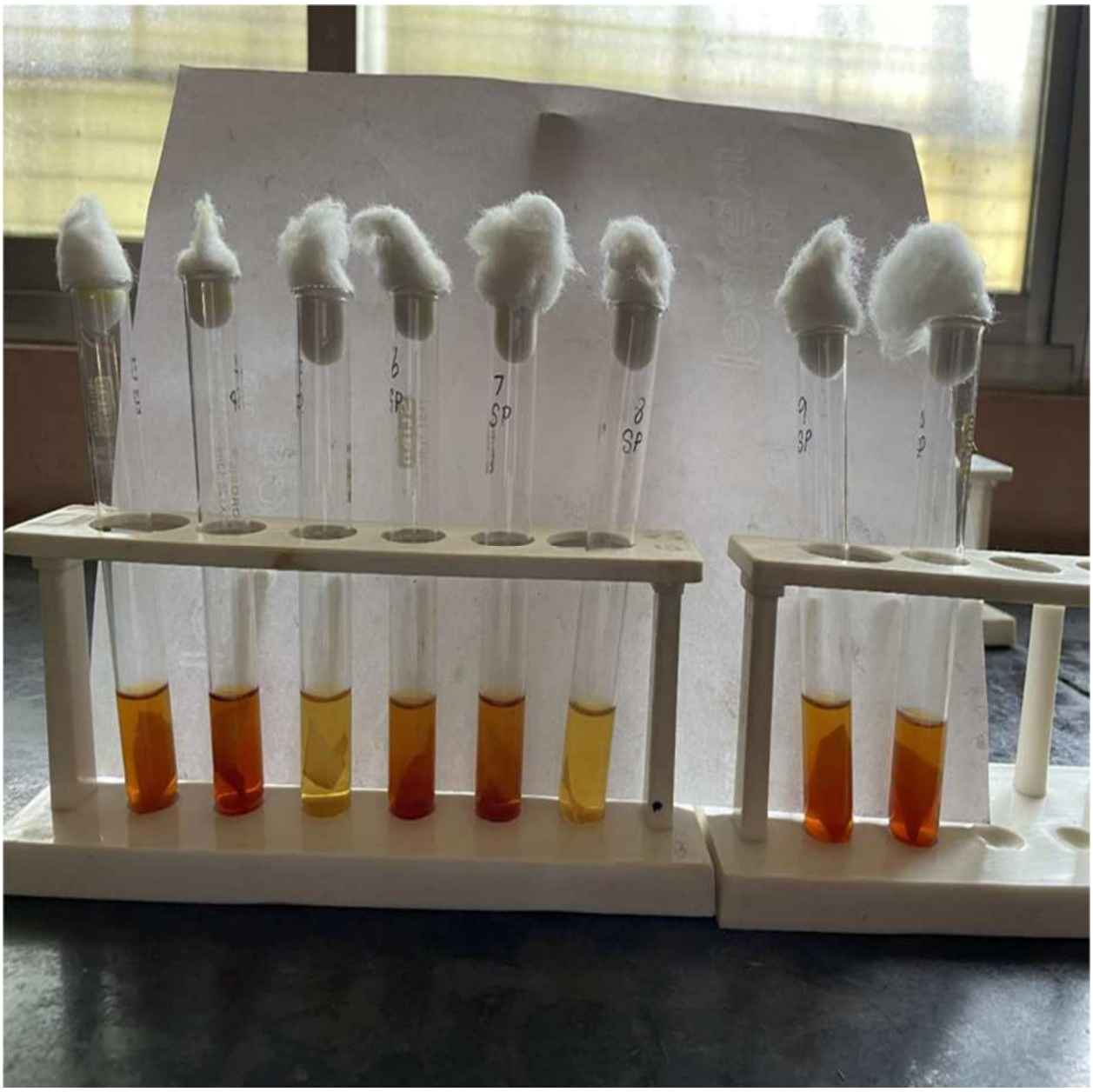


FIGURE-15

AMMONIA PRODUCTION

FIGURE-16

HYDROGEN CYANIDE PRODUCTION



DISCUSSION

Microorganisms produce colonies which were observed with the naked eye, and their cultural features were identified on nutrient-mediated plates after incubation. These morphological features have been studied in various forms such as colony shape, colony elevation, colony surface and colony colour. The collected samples of cow manure were counted for the microbial load of total bacteria. The highest number of bacterial population was displayed by SP10 strain with OD value at 1.709 cfu / ml and the lowest concentration was displayed in SP3 strain with OD value at 0.625 cfu / ml. The morphological studies of the isolated islands were confirmed by standard method of basal stain, Gram stain and endospore stain. Among these isolated layers, all the rays show the formation of an endospore. Similar work was carried out by Teo and Teoh (2011).

Significant biochemical reactions of nitrogen biological fixation occur primarily through symbiotic binding of microorganisms with legumes that convert atmospheric elemental nitrogen (N_2) to ammonia (NH_4). In this study, seven isolates, out of the 8 obtained from cow dung bacteria were able to reduce nitrate, an indirect measure of N_2 fixation. Even using a culture medium without N, other bacteria that did not secrete N_2 were isolated. The N required for their growth appears to have come from trace levels of N in the middle or from the bacterial compound based on the exchange of fixed carbon and nitrogen (Veal and Lynch, 1984).

Plant growth parameters such as plant height and weight, root height and weight measured by pot test. In this study, the SP5 strain showed an increase in plant height and SP3 showed an increase in root height and weight compared to control within 3 and 9 days after inoculation. Similar results were obtained by Spadden (2002) which was identified as having the ability to dissolve phosphate and also fix a large amount of nitrogen. The SVPR30 strain showed a significant increase in root and shoot parts compared to the controls within 15 and 30 days of blasting. Several strains of *Bacillus* and *Paenibacillus* expressed plant growth-promoting activity (PGP) and several of these strains were commercially developed as general plant growth promoters (Spadden Gardener, 2004).

Terry *et al*. (1996) found that the use of both *Azospirillum brasilense* and *Azotobacter chroocaccum* in combination with 30 kg N increased plant height, root length and fresh and dried weight of aerial parts in tomato. Plant height, gas thickness, and branch and leaf count were increased in *Ficus benjamina* by increasing the level of calcium superphosphate in combination with biofertilizers (Attia *et al*., 2004).

Fallik and Okon (1996) also observed greater shoot length, number of leaves, and number of branches, burning dry weight when *Selaria italica* and ornamental plants were inoculated with *Azospirillum spp* compared with control. Chezhiyan *et al*. (2003) described the maximum number of branches, number of

leaves, new weight and dry weight of roots of each plant in *Phyllanthus niritri*, with the application of *Azospirillum*, phosphobacteria and chicken manure.

Leaf, leaf area index: gas ratio and overall growth rate of *Stevia rebaiidiana* were found when treated with biofertilizers and FYM (Dube, 2011). Gadagi *et al*. (1999) reported that inoculation of *Azospirillum spp*. showed an increase in many parameters of plant growth, flower production and yield in *Gaillardia* and pepper. *A. brasilense* inoculation caused an increase in dry root weight, specific root length (root length per unit dry root weight) and total root length in soybeans (Molla *et at*., 2001).

Swain *et al*. (2007) showed in vitro IAA production by *B. subtilis* sequences (CM1 – CM5), CD isolates and with the application of *B. subtilis* and / or slurry CD culture inhibition on yam minisetts, the number of sprouts, roots and shoots increased, new rooting and burning stress and root ratio increased.

Protease is an enzyme that catalyzes proteolysis that breaks down proteins into their respective amino acids. In the present study five protease synthesizing isolates were recognized. Gupta *et al* (2002) documented that in addition, bacteria with protease activity have a number of applications mainly in the cleaning and food industries. Microbial proteases make up about 40% of enzyme sales worldwide.

Lekasi *et al*. (2005) also found that it is beneficial if the organic matter added to mineral soils to slowly release nutrients and the level of nutrient mining

increases as plant growth slows onwards. As the plant matures, good soil is expected to release enough nutrients for optimal plant growth.

In this study five bacterial strains are capable of producing ammonia. Similar results were obtained by Gómez-Brandón, Juárez, Zangerle, and Insam (2016) who reported a higher ammonium content in the digestion of the fertilizer. Elevated ammonium levels in the digestate were also attributed to mineralization of N compounds in the substrate (Gómez-Brandón *et al*., 2016).

It was studied that seven isolated spots were grown in the appropriate phosphate soluble medium by Gulati *et al*. (2010) show that *Acinetobacter* is an effective phosphate solubilizer.

In the present study six individuals were able to produce HCN. HCN production was a more common draw of *Pseudomonas* (88.89%) (Ahmad *et al*. 2008). Nevertheless, at present its applications in the fields of biocontrol modalities are growing (Devi *et al*. 2007). Thus, HCN extracted in the rhizosphere of seeds with selected *rhizobacteria* is a viable tool for biologically controlling weeds and minimizing harmful effects on the growth of plants cultivate (Kremer and Souissi, 2001).

CONCLUSION

In this study, bacteria isolated from cow dung has the potential to be used as plant growth stimulant. In-vitro experiments used for plant growth stimulation were HCN production, ammonia production, infectious nitrogen fixation, solubilization ability for phosphorous. Therefore, it is recommend that cow manure has the potential as biofertilizer which promotes the growth of plants. Inoculation is one of the most important sustainable practice in agriculture, as microorganisms establish connections with plants and stimulate plant growth through a number of beneficial properties.

The combination of different approaches with these bacteria, such as identification of plant growth stimulating traits, identification of bacterial rays, as well as assessments of seed inoculation in laboratory conditions and cultivation experiments in the pot, are part of the study for new agricultural crop technologies. Therefore, when this study shows a potential bacterial inoculant, suitable for reintroduction into the environment, many microbes such as *Azospirillum, Bacillus* and *Rhizobium* may be prime candidates. The discovery of beneficial bacteria is important for the development of new and effective inoculants for agriculture. There are also investments in technologies that can contribute to increase the efficiency of the inoculum and the survival rate of seed-borne bacteria, which are other essential features for successful circulation. Therefore, the introduction of beneficial bacteria into the soil tends to be less

aggressive and causes less impact on the environment than chemical fertilization, which makes it a sustainable agronomic practice and a way to reduce production costs. Thus, cow manure serves as a purifier of wastes in nature, a rich source of microflora that can be used as biofertilizer.

SUMMARY AND SUGGESTION

Cow manure has been used in agriculture for domestic and religious purposes since ancient times. Repeated application of chemical fertilizers in the same area for a few years results in the acidification of the soil content and loss of nutrients. Use of biofertilizers improves the fertility of the soil and reduces the acidity of the soil.

For the current study cow dung sample was taken from the Koottampuli dairy farm, Thoothukudi district. In this investigation plant growth promoting bacterial strains were isolated and screened. Out of eight bacterial isolates three were identified as phosphate solubilizing, nitrogen fixing and ammonia producing strains. Strain 3 and 5 were proved to be PGPR screened from cow dung.

Optimal use of microflora with high potential cattle manure can lead to better environmental sustainability. Cow manure improves soil accumulation, soil fertility, plant nutrition and also the growth of beneficial microbes. It improves soil ventilation and water retention. It eliminates diseases in plants and helps to contribute to plant growth, replenishes a number of microbes that include nitrogen fixatives, phosphate solubilizers, etc., and also provide macro and micronutrients to the crop plants. It also helps to improve the structural stability of the soil which helps to prevent soil erosion and ultimately increase the productivity of different crops.

Direct interactions between members of different microbial types often lead to the stimulation of key processes that are beneficial to plant growth and health.

This study provides baseline data for future studies in this area. These bacterial rays can be further studied to identify specific genes and enzymes that are responsible for plant growth stimulating cow dung. Thus the bacterial rays could enhance growth and development in plants, so that they can be utilized as biofertilizers is in a more efficient, economical and safer way.

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ANALYSIS OF HEAVY METALS AND BIOACTIVE COMPOUNDS OF THREE SPECIES *(Sardinella longiceps, Loligo vulgaris and Portunus pelagicus)* FROM THOOTHUKUDI COAST

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CERTIFICATE

This is to certify that this dissertation entitled **ANALYSIS OF HEAVY METALS AND BIOACTIVE COMPOUNDS OF THREE SPECIES (*Sardinella longiceps, Loligo vulgaris, Portunus pelagicus*) FROM THOOTHUKUDI COAST"** is a record of original research work done by I.THABITHAL under my supervision, and submitted in partial fulfilment for the degree of Master of Science in Zoology. This dissertation has not formed the basis of any other degree.

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DECLARATION

I hereby declare that the thesis entitled **"ANALYSIS OF HEAVY METALS AND BIOACTIVE COMPOUNDS OF THREE SPECIES FROM (*Sardinella longiceps, Loligo vulgaris, Portunus pelagicus*) THOOTUKUDI COAST"** submitted by me for the degree of Master of Science in Zoology is the result of my original and independent research work carried under the guidance of Dr. Sr. C. Shibana M.Sc., B.Ed., M. Phil., PhD. Assistant Professor of Zoology, St. Mary's College, Thoothukudi, and it has not been submitted for the reward of any degree, diploma, associateship, fellowship of any university or institution.

Place: Thoothukudi

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I. Thabithal.

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INTRODUCTION

1.INTRODUCTION

The coastal environment plays a vital role in nation's economy by virtue of the resource, productive habitats and rich biodiversity. India has a coastline of about 7,500 kms stretches along the Bay of Bengal, Indian Ocean and Arabian Sea. The coastline of Tamilnadu has a length of about 1076 kms constitutes about 15% of the total coastal length of India. In India, metal pollution is becoming a problem for local management especially in coastal waters and estuaries which are ultimately the repositories of pollutants from anthropogenic activities. Agricultural runoff and industrial effluents into aquatic system contaminate the ecosystem with mixtures of toxic or potentially toxic heavy metals. Due to structure and properties, many of these metals remain in the environment for prolonged periods and continue to pose environment problems and health risks. Coastal pollution due to chemicals is a global environmental problem. About 60% of the world Population live within 60K of coast line and use the coastline for their livelihood (Ives and Cardinale, 2004).

Pollution by heavy metals has been the most widely studied and documented topic because of the disastrous effect and diseases that are produced in man after consumption of contaminated food. The magnitude of the danger was first realized with the minamata disease in Japan where thousands of people suffered with mercury poisoning. Metals are natural components of the aquatic environment, but their levels have increased due to anthropogenic activities. The concentrations of heavy metals were found to rise in coastal ecosystem due to release of industrial waste, agricultural and mining activities in recent years

(Sankar *et al.,* 2006). Almost every industries utilizing water are potential sources of metal contamination.

Heavy metals such as Hg, Cu, Zn, Cd, Pb are discharged frequently through industrial and domestic effluents along the south east coast of India boarding Tamil Nadu state. (Brayan,1976).

Heavy metal pollution poses a serious threat to the environment because of their toxicity, persistence for several decades in the environment bioaccumulation and biomagnifications in the food chain (Gochfeld, 2003).

Continuous pollution of our waters, lagoons, estuaries and back water constitutes significant threat to aquatic flora and fauna, posing considerable set back to fishing for commercial purposes and ultimately constitutes adverse health hazards to humans. Contamination of aquatic ecosystem started long back but intensified during the last few decades, and the condition has become dreadful in India (Girija, 2007; Rajkumar *et al.,* 2011a)

The deposition of acids also causes an increased level of metals in the soil, and contributes to increased level of metals in surface waters (Borg, 1987).

As nutritionally beneficial as crustaceans and molluscs are potential sources of health risks to man due to accumulation of toxic heavy metals (HMS) such as lead (Pb) and cadmium (Cd). At a certain trace amount Pb and Cd are toxic to biological systems. Lead is capable of causing both acute and chronic toxicities. Acute toxicity occurs through accidental exposure to high concentrations of soluble Pb compounds. On the other hand, chronic toxicity can occur through consumption of lead -contaminated foods. Lead is known particularly to reduce intellectual development in children and causes cardiovascular diseases in adults and in extreme cases, death. Chronic exposure to high levels of cadmium can cause

kidney and liver damage in man. Heavy metal pollutions are particularly hazardous contaminants in food and the environment. In general, they are not biodegradable and have long biological half-lives. According to the World Health Organization (World Health Organization 1995) Zinc is an essential micronutrient required in the synthesis and degradation of carbohydrates, lipids, proteins and nuclic acid. It is also an essential element for gene expression and hormone receptor activities in the cell. (Barrene *et al.,* 2008).

Cu plays essential roles as metalloenzymes and as a cofactor of large number of enzymes and is also used for biological electron transport. (Barrento *et al.,* 2007). A range of intake of 1.5-3mg/day for Cu and 12-15mg/day for Zn has been documented to be adequate for the body (Garcia-Rico *et al.,* 2007)

Heavy metals mobility and availability in aquatic environments are primarily controlled by water quality parameters including pH, dissolved oxygen and organic matter content. In Peru, the acid mine drainage from adjacent mining district was flushed downstream from Upamayo Dam, contributing to high concentrations of copper and zinc in sediments (Rodbell *et al.,*2014).

This is the simplest form aquaculture practice, where recently molted crabs are held in confinement for a short period to enhance marketable attributes. This form of culture now forms the basis of a small but vibrant seafood industry in South – East Asian countries. Several aspects of aquaculture of this species have been the basis of intensive research during the last two decades (Keenan 1999; Srinivasagam *et al*.,2000; Kathirvel *et al.,* Shaji *et al*.,2004;Quinitio *et al.,* Shaji *et al.,* 2006).

Mud crabs are preferred due to high quality meat, and rapid growth rate with some species reaching carapace widths of 240 mm from juvenile

sizes of 4-6 mm in just 6-8 months (Joel and Sanjeevaraj 1983, Overton and Macintosh 2002).

Levels of heavy metals in marine ecosystem have been rising at alarming rates due to continuous deposits from industrial activity (David 2003),

Crabs are an excellent bio indicator of metal contamination and can be used to effectively and accurately monitor metal level for several reasons. Anthropogenic pollutants such as industrial, municipal, exposing water flow to a variety of environmental pollutants.

Heavy metal used widely in modern industry. Heavy metals including both essential and non-essential elements have a particular significance in ecotoxicology, since they are highly persistent and all have the potential to be toxic to living organism (Gipson and Barker 1979). The deposition of acids also cause an increased mobility of many metals in the soil, and contributes to increased level of metals in surface waters (Borg, 1987). Decreased pH favours the occurrence of dissolved uncomplexed metal forms, (Campbell and Stokes, 1985). Deve (1992) suggested that release of metals from sediments to the overlaying water and subsequent toxicity is a complex phenomenon. Adverse effects are increased distinctly in soft water (Pistelok and Galas, 1997).

Metals enter into the body of an organism along with nutrients, food and water which result in their accumulation in tissues causing impairment in the physiological functions and may lead to death. Heavy metals are kept under environmental pollutant category due to their toxic effect on plants, animals and human beings (Rajesh Kumar Sharma and Madhoolika Agarwal *et al.,* 2005).)

Toxic metals released into marine environment tend to accumulate in sediments and they are taken up subsequently by filter feeding organisms.

(Saulwood Lin and 1-Jy Hsieh, 1999). Tissues of benthic organism and hence they are often considered as potential indicators of pollution depending on their availability and magnitude of accumulation. The measurement of response to chemical contaminations in sentinel organisms are used as bio-indicators from aquatic environment allowing early detection of biological effects as well as assessment of the extent of contamination of pollutants. Bivalves are filter feeder invertebrates and can accumulate heavy metals from food, water and also from the ingestion of inorganic particulate materials. The use of Donax sp. had been established to monitor the coastal areas (Haynes *et al.,* 1997, Asha *et al.,* 2010).)

It is known that some shrimp, crabs and squids may give a useful means of monitoring such elemental concentration (Pb, Cu, Cd, As, Zn, Ni, Hg, Fe and Cr) levels and their impact on the aquatic environment. Heavy metal pollutions are particularly hazardous contaminants aquatic food and the environment. In general, they are not biodegradable and have long biological half-lives. The heavy metals must be controlled in aquatic food sources to assure public safety. An excessive amount of the attention of meals heavy metals is related to the etiology diseases, specially cardiovascular, renal, neurological, and bone diseases

Squid are themselves important prey items for large fish, sea birds, and marine mammals. Squid and other cephalopod are very efficient accumulators of various trace elements. Toxic metals as cadmium and mercury are bio accumulated and retained in squid and so passed on to predators, therefore potentially increasing the contaminant load in higher trophic levels, including humans

Metals accumulation in living organisms at any time are taken up and stored faster than they are broken down (metabolized or excreted). Bioaccumulation indicates the pollution level in organisms which live in polluted environments. It changes among organisms based on the uptake, detoxification and the outside environment.

The bioaccumulation of heavy metals varied between species, ages, sex and organs. In general, the target tissues of heavy metals are metabolic active ones which accumulate high levels metal in fish such as liver and gills whereas in muscles where the metabolic activity is relatively low accumulates less level of heavy metals.

The measurement of heavy metals concentrations in bio-indicators has been recognized as highly relevant in ecotoxicological terms because of the reflection of bioavailability in the ecosystem that reasonable measurement for public health standards from animals health point.

Functional food should be or look like a traditional food and must be part of our daily diet (The European Scientific community, 2010).

Squid, cuttlefish, and octopus are major seawater catch other then fishes and prawns. These seawater products are grouped under phylum of invertebrate animals known as Mollusca and family of Cephalopoda. Cephalopods are common and important in Malaysia fisheries in terms of domestic consumption and economic values (Rubaie *et al*., 2011)

Humans have used cephalopod ink for many practical and commercial purpose over the millennia, particularly in medicine, cuisine, and art, as well as in even broader applications(Derby, 2014). This by-product can be potential source of good bioactive compounds (Vate and Benjakul, 2013).

Currently, a number of drugs from marine organisms are undergoing clinical trials as antibacterial and anticancer treatments. In addition, scientists have isolated and chemically characterized many unique compounds that have exhibited possible efficacy against microbial infections, tuberculosis, cystic fibrosis, viral infections and other diseases. Many bioactive molecules produced by marine invertebrates have exhibited potent anti-viral and anti-tumour activity. Additionally while bacterial infections can be treated with antibiotics, relatively few compounds are available to treat viruses, parasites and fungi which are responsible for thousands of deaths each year (Zodape, 2016).

Marine crabs are potential sources of new antibiotics. The search for antimicrobial agents has taken a definite direction in developed countries. The first line of defense of arthropods against pathogens and parasites is of physical nature via their hard cuticle. However once this barrier is passed a complex interaction of innate humoral and cellular immune reactions is induced in both tissues and haemocoel, which results in a fast elimination of micro – organisms (Veeruraj *et al*., 2008).

In recent years, a significant number of novel metabolites have been isolated from marine animals especially invertebrates. These metabolites isolated from marine animals showed anti-tumour, antifungal, chemotherapeutic, anticoagulants, inhibition of virus and even for the preparation of the male contraceptive pills (Donia and Hamann, 2003; Zodape, 2016)

REVIEW OF LITERATURE

2.REVIEW OF LITERATURE:

Ink sacs have attracted considerable attention, both casually and scientifically, because of their dramatic black color (derby,2014).

Squid and cuttlefish ink was produced at the end process of maturation in a viscous, colorless medium (Lin et al.,2011).

Natural antioxidants from marine resources, especially from the by – products of seafood processing, can be another alternative antioxidant for food application (Vate and benjakul 2013).

Based on a research by Liu et al. (2011) on broiler chickens, the growth performance, antioxidant functions as well as immunity was affected by squid ink A study of Girija et al.(2014). Majority of fisheries by products are presently utilized to produce fish oil, fish meals, fertilizers, pet food and fish food (Bechtel et al., 2007; Dong and Bech et al., 2010).

Fishes are highly nutritious and consumed as a delicacy food throughout the world. In recent decades, much attention has been paid to the study of both essential and toxic element contents in food stuffs, as a result growing concern about health benefits and risk of food consumption (Guerin et al., 2011)

The effect of dietary fish oil replacement on growth and carcass proximate Composition of juvenile barramundi *(Lates calcarifer)analyzed by Raso and Anderson (2002).*

Deng et al., (2006) carried out an experiment to study the effect of replacing fish meal with soy protein concentrate on feed intake and growth of juvenile Japanese flounder. Field identification guide to the sharks and rays of the Mediterranean and black sea was identified by FAO (2005)

Noda and Narita (1976) was studied about amino acid sequence of eel calcitonin. The fatty acid profile and proximate composition of the thornback ray (Raja clavata, Linnaeus, 1758) from the sinop coast in the black sea was discovered by Turan et al., (2007).

Nair et al., (1973)was determined a new deep sea skate, Rinobatos variegates, with notes on the deep sea sharks Halaelurus hispidus, Eridacnis radcliffei and Eugaleus omanensis from the gulf of mannar.

Thus, determination of harmful and toxic substances in water sediments and biota will give direct information on the significance of pollution in the aquatic environment (Huggest *et al*., 1973).

The natural aquatic systems may extensively be contaminated with heavy metals released from domestic, industrial and other man-made activities Conacher *et al.,* 1993 Velez &Montoro, 1998). Heavy metal contamination may have devastating effects on the ecological balance of the recipient environment and a diversity of aquatic organisms (Ashraj, 2005; Vosyliene&Jankaite, 2006; Farombi *et al* 2007).

The fate of heavy metals introduced by human activities into the aquatic ecosystems has recently become the subject of wide spread concern, since they become toxic beyond the tolerable limits (Rainbow, 1995). Discharge of heavy metals into river or any aquatic environment can change both aquatic species diversity and ecosystems, due to their toxicity and accumulative behaviour (Allen,1995).

The presence of heavy metals in aquatic animals is becoming a threat, thereby making them unfit for human consumption . Heavy metals found in

crustaceans are known to affect the enzyme balance as well as demobilize them (Herkovits *et al.,*1996)

Studies carried out on various fish species have shown that heavy metals may alter the physiological both in the tissues and in the blood (Soegianto *et al.,* 1999).

Several researches revealed heavy metal content in crustaceans and the effects on man who in turn consume the crustacean (Baird and Ulanowics, 1999).

Heavy metals are natural trace components of the aquatic environment, but their levels have increased due to domestic, industrial, mining and agricultural activities (Kalay and Canli 2000).

Discharge of industrial wastes also constitute about 62% of total source of heavy metal such as Lead (Pb), Zinc (Zn), Copper (Cu), Nickel (Ni), Cadmium (Cd), Chromium (Cr) and Manganese (Mn) which are responsible not only for degrading the water quality of a river or sea but for killing a number aquatic organisms (Abubaker and Garba, 2006).

Heavy metals from natural and anthropogenic sources are continually released into aquatic ecosystem and they cause serious threats because of their toxicity, long persistence, as well as bioaccumulation and biomagnifications in the food chain (Kamaruzzaman *et al.,*2007).

A research on heavy metal bioaccumulation in tropical crab from River Aponwa, Ado Ekiti revealed that Cu, Cd, and Zn are evidently bioaccumulated and biomagnified (Falusi and Olanipekun, 2007). Another study on heavy metals in crab and prawn in Ojo River Lagos indicated that the mean concentration of copper

and Zinc have higher concentrations above the range of NAFDAC standard for water and aquatic foods (Olowa *et al* .,2009).

Heavy metals pollution is becoming a significant concern in many countries because of the fact that it is toxic, non-biodegradable, and persistent, and because of the bio-accumulation properties of these contaminants (Idris *et al*., 2007; Abdel-Satar and Geneid 2009; Singh *et al.,* 2005).

Chona Camille *et al.,* 2015 studied the heavy metal (Pb and Cu) in adult mud crabs (Scylla spp.)from the East Bataan Coast. The average concentrations in the samples were 3.37×10^{-3}mg /1 and 1.01 mg/1- both within WHO acceptable limits.

Ramesh *et al* .,(2015) determined the level of Cd, Cu, Cr, Pb and Zn in sea water, sediment, edible crab (*Portunus sanguinolentus*) and edible prawn (*Penaeus merguiensis*) collected from Tuticorin during premonsoon and monsoon 2014. They reported that the level of all metal concentration was high in monsoon season the premonsoon season.

Antimicrobial peptides have been established as key players in animal defense systems. An antimicrobial peptide, which is isolated from a decapods crustacean (*Thalamita crenata*), possess an immense antibacterial activity (Rameshkumar *et al*., 2009a).

In addition to these crustaceans, crabs have an immense antimicrobial property in their hemolymph. One of such antimicrobial protein from the crab hemolymph *Charybdis lucifera* has been extensively studied against *E.coli* and *P. aeuroginosa* type bacteria's (Rameshkumar *et al*., 2009b).

A comparative antimicrobial effect of six brachyuran crabs revealed that the maximum antibacterial effect of crude haemolymph is shown by *Dromiaa brolhensis* against *E. coli* and the minimum is shown by *S. serrata* crab against *Klebsiella oxytoca* (Ravichandran *et al*., 2009).

The influence of crab hemolymph against wide range of clinical pathogens proves that crustaceans are very good source of antimicrobial potency (Anbuchezhian *et al*., 2009)

The presence of antimicrobial compounds in the haemolymph of crustacean species (crabs) has been reported by a number of notable researchers (Chisholm and Smith, 1992; Khoo *et al.*, 1999 and Veeruraj *et al*., 2008).

Antibacterial activity from blue crab *Callinectes sapidus* was studied by (Noga *et al*., 1996) and (Khoo *et al*., 1999). Jayasankar and Subramoniam (1999) evaluated the antibacterial activity of seminal plasma of the mud crab *Scylla serrata*.

Antibacterial activity in four marine crustacean decapods was studied by (Haug *et al*., 2002). Ravichandran *et al*. (2009) analysed antimicrobial lipids from the hemolymph of brachyuran crabs. Anbuchezien *et al.* (2009) studied the influence of crab haemolymph on clinical pathogens. Hoq *et al*. (2003) isolated and characterized the antimicrobial peptides from the mud crab, *Scylla serrata*.

Huang *et al*. (2006) have isolated a 10.8 kDa anionic protein named scygonadin from the male reproductive tract of *S. serrata* that inhibited growth of *Micrococcus luteus* and *Aeromonas hydrophila*. Arul Prakash *et al*. (2011) studied the antimicrobial activity of haemolymph collected from freshwater crab, *Paratelphusa hydrodromous* against clinical pathogens.

Rameshkumar *et al*. (2009) isolated the antimicrobial proteins from the haemolymph of male and female crab *Charybdis lucifera*. Veeruraj *et al*. (2008b) studied the in vitro antifungal activity of crab haemolymph on clinical pathogens

. Identification and characterization of a 11 kDa, antimicrobial protein, named as SSAP from granular hemocytes of the mangrove crab *S. serrata* was reported by (Reddy *et al*., 2009).

Rameshkumar (2007) isolated the antimicrobial peptide from the crab *Thalamita crenata*. Sivasubramanian *et al*. (2010) investigated the antimicrobial activity from the haemolymph of crab *Ocypod macrocera*.

OBJECTIVES

3.OBJECTIVES

The present study has been carried out with the following objectives:

- To analyse the heavy metal concentration in the muscle of squid.
- To analyse the heavy metal concentration in the muscle of fish Sardinella longiceps.
- To probe the bioaccumulation of heavy metals in the muscle tissue of crab *Portunus pelagius* from Thoothukudi coast.
- To discuss the bioaccumulation mechanism and health risk.
- To correlate the metal levels in organisms and the environment.
- To least the antibacterial assay of extracts of whole body tissue of the crab *Portunus pelagius* against human pathogens.
- To investigate the antioxidant activity of squid.
- To study the GC-MS activity of the in the fish Sardinella longiceps.

MATERIALS AND METHODS

4.MATERIALS AND METHODS

5.1.SYSTEMATIC POSITION OF EXPERIMENTAL ANIMAL

5.1.1.Loligo vulgaris

Kingdom : Animalia
Phylum : Mollusca
Class : Cephalopoda
Subclass : Coleoidea
Order : Teuthoidea
Suborder : Myopsida
Family : Loliginidae
Genus : *Loligo*
Species : *Loligo vulgaris*

5.2.1.DESCRIPTION OF STUDY ANIMAL:

Very similar to *Loligo forbesi* , reaching a total length of 750 mm. Distinguished by the tentacle club, on which the median suckers of the middle region are three or four times the diameter of adjacent marginal suckers. Rings of large club suckers are smooth or with irregular small teeth on distal edge. Rings on small suckers on lappets of buccal membrane have distally with 7-10 long sharp teeth (L. vulgaris). The European squid or common squid is a large squid belonging to the family Loliginidae. It occurs abundantly in coastal waters from the North Sea to at least the west coast of Africa. This species lives from sea level to depths of 500 m. Its mantle is up to 40 cm long Mantle long, moderately slender, cylindrical. Fins rhomboid, their length two thirds of mantle length, their posterior border slightly concave. Tentacle club with especially large median suckers, up to four times diameter of marginal suckers. The European squid or common squid (**Loligo**

vulgaris) is a large squid belonging to the family Loliginidae. It occurs abundantly in coastal waters from the North Sea to at least the west coast of Africa. This species lives from sea level to depths of 500 m (1,600 ft). Its mantle is up to 40 cm (16 in) long.

Loligo vulgaris

5.1.2.SYSTEMATIC POSITION OF Sardinella longiceps

Sardinella longiceps

Kingdom :Animalia

Phylum :Chordata

Class :Actinopterygii

Order :Clupeiformes

Family :Clupeidae

Genus :Sardinella

Species :S . longiceps.

5.2.2.DESCRIPITION OF STUDY ANIMAL:

Sardines are most often bought canned, lined up in rows in little tins. The word sardine is actually a general term — it refers to a type of fish, most often a small herring, while a slightly larger one is sometimes called a pilchard. The phrase "packed like sardines," describing people crowded together in a tight spot like an elevator or a subway car, comes from the way sardines look in cans. The word itself comes from the Mediterranean island SardiniaSardines are small, silvery, elongated fishes with a single short dorsal fin, no lateral line, and no scales on the head. They range in length from about 15 to 30 cm (6 to 12 inches) and live in dense schools, migrating along the coast and feeding on plankton, of which they consume vast quantities. The Indian oil sardine (**Sardinella longiceps**) is a species of ray-finned **fish** in the genus **Sardinella**. It is one of the two most important commercial **fishes** in India (with the mackerel). ... These **fish** feed on phytoplankton (diatoms) and zooplankton (copepods). **Sardines** are small, silvery, elongated **fishes** with a single short dorsal fin, no lateral line, and no scales on the head. They range in length from about 15 to 30 cm (6 to 12 inches) and live in dense schools, migrating along the coast and feeding on plankton, of which they consume vast quantities. Schools, or shoals, of sardines swim near the water surface and are primarily marine, although some live in **freshwater**. Most species are migratory; in the Northern Hemisphere, for example, they migrate northward in the summer and southward in the winter. During spring and summer, they spawn. After doing this, the young commonly move closer to the shore to feed. The young sardines eat **plant** plankton (or **phytoplankton**), while adults eat **animal** plankton (**zooplankton**). All sardine species are important **prey** for larger fish.

Sardinella longiceps:

5.1.3.SYSTEMATIC POSITION OF Portunus pelagicus

STUDY AREA

Portunus pelagicus

Kingdom : Animalia

Phylum :Arthropoda

Subphylum :Crustacea

Class :Malacostraca

Order :Decapoda

Infraorder :Brachyura

Family :Portunidae

Genus :Portunus

Species :P. pelagicus

5.2.3.DESCRIPTION OF THE STUDY ANIMAL:

PortunusPelagicus the common name is Blue swimming crab, it is also known as flower crab, blue swimmer crab, blue manna crab or sand crab, rajungan in Indonesian, and alimasag in Tagalog, is a large crab found in the intertidal estuaries of the Indian and West Pacific Oceans, and as a Lessepsian migrant in the eastern Mediterranean Sea. The name "flower crab" is used in East Asian countries while the latter names are used in Australia. The crabs are widely distributed in eastern Africa, Southeast Asia, East Asia, Australia, Persian Gulf, New Zealand and Indonesia. A medium sized marine nocturnal crab (CL males: 7 cm, females: 6.5cm), carapace greenish-brown with irregular pale molting edged dark brown, chelipeds are purplish, mottled and fingers blue. Carapace broad with transverse granulate lines. The front has four acute lobes and the antero-lateral margin bears nine triangular teeth, the last tooth is the largest, projecting laterally. Chelipeds are long, massive, spinous and ridged. A marine nocturnal crab. Active swimmer, but during inactive periods buries in sediment.

Portunus pelagicus:

5.3.HEAVY METAL ANALYSIS

All the glass wares used for the analysis were soaked in 5N nitric acid and thoroughly washed with distilled water. All the reagents used were BDH (Analar grade). Demonized double distilled water was used for the chemical analysis. Nitric acid digestion was employed to analyze heavy metal concentration in samples. (Danielsson et al., 1978).

5.3.1.ANIMAL SAMPLE:

The ice preserved organisms were sorted out, washed in distilled. 1 D.Do water. In mud crab (Scylla serrata) entire organism was used for the tissue analysis of heavy metals excluding the shell.) Infish grouper Epinepheluscoioides only the muscle tissue was removed and used for analysis. The tissues were taken, washed in distilled water and oven dried at 80°C + 1°C for 24 hours. Then dried tissues were powdered using a mortar and pestle and weighed accurately, 500mg in a precision balance in triplicates and transferred to digestion flasks. It is digested with 9ml concentrated nitric acid and 1 ml perchloric acid. The sample was heated by keeping on a hot plate until evaporation of the samples. Care was taken to avoid charring during the digestion process. When the solution became near dryness, added a small quantity of double distilled water along the sides of the flask and rinsed the flask. Filter the solution through Whatmann No: 1 filter paper into 25ml volumetric flask and made up the solution to 25ml using crystal clear double distilled water. Blank solution was also prepared in the same way with the reagents but without sample material. The made up samples were transferred to GENS.

polythene bottles and were analysed for various metals on an Ato Absorption Spectrophotometer (AA SELICO SD 194)

ANTIOXIDANT ASSAY:

5.4.DPPH free radical scavenging assay (Blois, 1958)

The ability of the fractions to annihilate the DPPH radical (1,1-diphenyl-2-picrylhydrazyl) was investigated. Different concentration of the extract of the crab tissue (200 µg/ml, 600 µg/ml and 1000 µg/ml) was added, at an equal volume, to methanolic solution of DPPH (0.1 mM). The reaction mixture was incubated for 30 min at room temperature; the absorbance was recorded at 517 nm. The experiment was repeated for three times. Ascorbic acid was used as standard controls. The annihilation activity of free radicals was calculated in % inhibition according to the following formula:

$$\% \text{ of Inhibition} = \frac{\text{A of control} - \text{A of Test}}{\text{A of control}} \times 100$$

5.5ANTIBACTERIAL ACTIVITY:

5.5.1. Preparation of tissue extracts

The shells were broken and the soft tissues were removed and washed thoroughly with distilled water. And tissue is collected and dried in sunlight after the tissue is prepared in powder form. Approximately 5g of tissue powders were immersed separately into Methanol and Ethanolsolvants and they were cold steeped at -18^{o}c. The extracts from each solvent were filtered twice using WhatmanNo.1 filter paper. Samples were centrifuged at 5000 rpm for 15 min in

rotary evaporator. And the precipitate was collected and it was stored at for further use.

5.5.2. Microbial culture

Five species of human pathogens *Staphylococcus sps, E.coli, Pseudomonas sps,Bacillussps, and Vibrio cholarae*were obtained from Microbiology Lab, St. Mary's college Autonomous Thoothukudi.

5.5.3.Inoculum preparation for bacterial strains

About 6.08g of mullerhinton agar was dissolved in 160ml of distilled water and autoclaved for 15 min at 121^{o}C and left to cool at room temperature. Once the medium was cooled (about 45^{0}C), it was poured into petridish. Each petri dish was left on the flat surface for 30-40 min until completely set. The test microorganisms *(Staphylococcus sps, E.coli, Pseudomonas sps, Bacillus sps, and Vibrio cholarae)* were seeded into respective medium by spread plate method. Further, 20µl was spread onto 20 ml of sterile agar plate by using a sterile cottons swap. The surface of the medium was allowed to dry for about 3 min. the wells (10mm) were punched over the agar plates using sterile gel puncher. Various concentrations (10, 25& 50µl) of extracts were added to the wells. The plates were incubated for 24 hours at 37^{0}C. After incubation the diameter of inhibitory zones formed around each discs were measured in mm are recorded.

5.5.4.Antibacterial activity of tissue extract of *Portunus pelagicus*

The shell extract of the crab *P.pelagicus* were screened for antibacterial activity against five human pathogen were represented in the figures (.)

From the three solvents shell extract of *P.pelagicus* the methanol extract was able to exhibit a broad spectral antibiotic activity against all the human pathogen strain which were concentration dependent.Table (1) &Fig (7)

5.5.5. Effect of methanol extract with DMSO solvent

Different concentration of methanol solvent (10µl, 20µl, 30µl) wereused.The extract was treated with 5ml of DMSO to get pure activity with extract and the zone of inhibition was ranged from 0.5mm to 8mm.

In the present study, the methanol extract of the tissue showed the best activity against all pathogen.The series of concentration in 10mg/µl, 20mg/µl, 30mg/µl of different methanol solvents were tested to determine the inhibitory effect on the growth of pathogens.

The antibacterial activity of methanol extract of tissue of crab showed maximum zone of inhibition against *salmonella typhi and Pseudomonas sps at 20 (10mm),* at 20µl concentration followed by *Pseudomonasaeruginosasps* and Vibrio cholerae(9mm) , *Staphylococcus sps* (5mm) , and *Vibrio cholera sps* (4mm) figures (3,5&6) and Table (1)

The minimum zone of inhibition was observed against V*ibriocholeraesps* (4mm) at 30µl compared to other pathogens.Figure (6),Plate(5) &Table (1)

In ethanol extract of shell of crab the maximum activity was found against *Staphylococcus sps*(6mm) followed by *E.coli*(5mm) and *Pseudomonas sps* (4mm). The least activity was found against *Pseudomonas sps* (0.5mm) at 10µl .Table(2)&Plates (6,7&8)

Among the five pathogen, the maximum zone of inhibition (8mm) was developed against *Bacillus sps* with methanol extract and trace activity was observed against *Pseudomonas sps*(0.5mm) with ethanol extract.Plates (1&6) and Table (1&2)

The result of maximum inhibitory concentration revealed that among the concentrationst,30µl concentration showed maximum activity and 10µl concentration showed and minimum activity. As the concentration increased the activity of shell extract was also increased.

5.6.GC-MS ANALYSIS:

GC-MS analysis of methanol extracts of was carried out by following the method of Hema *et* al., 2010. GC-MS method is a direct and fast analytical approach for identification of chemical compounds. The importance of the study is due to the biological activity of these compounds. Analysis was performed by using a GC, varian CP 3800 and MS, Saturn 2200 (VF 5 ms 30 X0.25 system) equipped with Elite -1, fused silica capillary column composed of 5% phenylArylene-95% Dimethyl poly siloxane. The system comprising a COMBIPAL auto sampler set under the following conditions: helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of1µl EI was employed (split ratio of 1:10) injector temperature 250˚C; the oven temperature was programmed from 100-2700˚C at the rate of 50˚C; total GC running time was 63 minutes interpretation on mass spectrum of GC-MS was done by using the database of national institute standard and technology (NIST) having more than 62000 patterns. The mass spectrum of the unknown components was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

RESULT

6.RESULTS

6.1. HEAVY METAL:

Cadmium:

The Cadmium concentration was observed in the tissue of crab, Squid and Sardine fish. Copper concentration in crab was (2.810µg/g), Accumulation of Cadmium in squid was about (1.016µg/g) and for Fish was about (2.970 µg/g).

At Thoothukudi coast the concentration of Cadmiun was found high in Sardine (2.970µg/g) than in Crab (2.810µg/g) and Squid (1.016µg/g) respectively.

Copper:

The copper concentration was observed in the tissue of crab, Squid and Sardine fish. Copper concentration in crab was (2.421µg/g), Accumulation of copper in squid was about (2.143µg/g) and for Fish was about (2.629µg/g).

At Thoothukudi coast the concentration of copper was found high in Sardine (2.629µg/g) than in Crab (2.421µg/g) and Squid (2.143µg/g) respectively.

Lead:

The Lead concentration was observed in the muscle of crab, Squid and Sardine fish. Lead concentration in crab was (1.011µg/g), Accumulation of Lead in squid

was about (0.761µg/g) and for Fish was about (2.000µg/g). The low level of (0.761µg/g) lead concentration was observed in the tissue of squid.

Zinc:

The zinc concentration was observed in the muscle of crab, Squid and Sardine fish. Zinc concentration in crab was (2.527µg/g), Accumulation of Zinc in squid was about (0.228µg/g) and for Fish was about (3.000µg/g).

At Thoothukudi coast the concentration of Zinc was found high in Sardine (3.000µg/g) than in Crab (2.527 µg/g).

RESULT FOR ANTIOXIDANT ACTIVITY:

6.2.DPPH scavenging activity

From the result obtained,table – showed thattThe free radical scavenging activity of methanolic extract of the crab tissue was assessed by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Ascorbic acid was used as standard.

The DPPH radical scavenging potential of crab tissue extract ranged from 40.31% to 57.42% at varied concentrations of 1µg/ml, 2.5 µg/ml and 5 µg/ml. Scavenging activity was increased with the increasing concentration of the extract and IC 50 value was 46.49.

RESULT FOR ANTIBACTERIAL:

Antibacterial activity of shell extract of the crab *Portunuspelagicus*was tested against five human pathogens (*Bacillussps,Pseudomonassps ,E.colisps, Staphylococcus sps , Vibrio cholerae.)*

6.3. Antibacterial activity of tissue extract of *Portunus pelagicus*

The shell extract of the crab *P.pelagicus* were screened for antibacterial activity against five human pathogen were represented in the figures (.)

From the three solvents shell extract of *P.pelagicus* the methanol extract was able to exhibit a broad spectral antibiotic activity against all the human pathogen strain which were concentration dependent.Table (1) &Fig (7)

6.3.1 Effect of methanol extract with DMSO solvent

Different concentration of methanol solvent (10µl, 20µl, 30µl) were used. The extract was treated with 5ml of DMSO to get pure activity with extract and the zone of inhibition was ranged from 0.5mm to 8mm.

In the present study, the methanol extract of the tissue showed the best activity against all pathogen. The series of concentration in 10mg/µl, 20mg/µl, 30mg/µl of different methanol solvents were tested to determine the inhibitory effect on the growth of pathogens.

The antibacterial activity of methanol extract of tissue of crab showed maximum zone of inhibition against *salmonella typhi and Pseudomonas sps at 20 (10mm),* at 20µl concentration followed by *Pseudomonas aeruginosa sps* and Vibrio cholerae(9mm) , *Staphylococcus sps* (5mm) , and *Vibrio cholera sps* (4mm) figures (3,5&6) and Table (1)

The minimum zone of inhibition was observed against V*ibrio cholera sps* (4mm) at 30µl compared to other pathogens. Figure (6),Plate(5) &Table (1)

In ethanol extract of shell of crab the maximum activity was found against *Staphylococcus sps* (6mm) followed by *E.coli*(5mm) and *Pseudomonas sps* (4mm). The least activity was found against *Pseudomonas sps* (0.5mm) at 10µl .Table(2)&Plates (6,7&8)

Among the five pathogen, the maximum zone of inhibition (8mm) was developed against *Bacillus sps* with methanol extract and trace activity was observed against *Pseudomonas sps*(0.5mm) with ethanol extract.Plates (1&6) and Table (1&2)

The result of maximum inhibitory concentration revealed that among the concentrationst,30µl concentration showed maximum activity and 10µl concentration showed and minimum activity. As the concentration increased the activity of shell extract was also increasednd Squid (0.228µg/g) respectively.

RESULT FOR GC-MS

In this present study three compounds such as were identified in *Sardinella longiceps*. The three compounds are 1,2 Bis(trimethysilyl)Benzene, Silicic acid, diethylbis(trimethysilyl), 2-Ethylacridine with R.T Value 16.223.

TABLE-1

DISTRIPUTION OF HEAVY METAL IN THREE SPECIES

ELEMENTS	CONCENTRATION OF ELEMENTS Mg/g (or) PPm (dry weight)		
	Sardine (Sardinella longiceps)	Squid (Loligo vulgaris)	Carb (Portagius pelagicus)
Copper	2.629	2.143	2.421
Lead	2.000	0.761	1.011
Zinc	3.000	0.288	2.527
Cadmium	2.970	1.016	2.810

TABLE-2

DISTRIPUTION OF ANTIOXIDANT ASSAY

CONCENTRATION	ABSORBANCE	%SCV	IC50
1G	0.422	40.31	
2.5G	0.399	43.56	46.49
5G	0.301	57.42	

TABLE-3

Antibacterial activity of methanol extract of crab tissue against human pathogens

S.NO	NAME OF THE BACTERIA	CONCENTRATION OF SHELL EXTRACT (METHANOL)		
		10µl	20µl	30µl
1.	Bacillus sps	4mm	5mm	7mm
2.	Pseudomonas sps	2mm	4mm	6mm
3.	E.coli	3mm	4mm	5mm
4.	Staphylococcus sps	3mm	4mm	5mm
5.	Vibrio cholera	3mm	4mm	4mm

TABLE-4

Antibacterial activity ethanol extract of crab shell against human pathogens

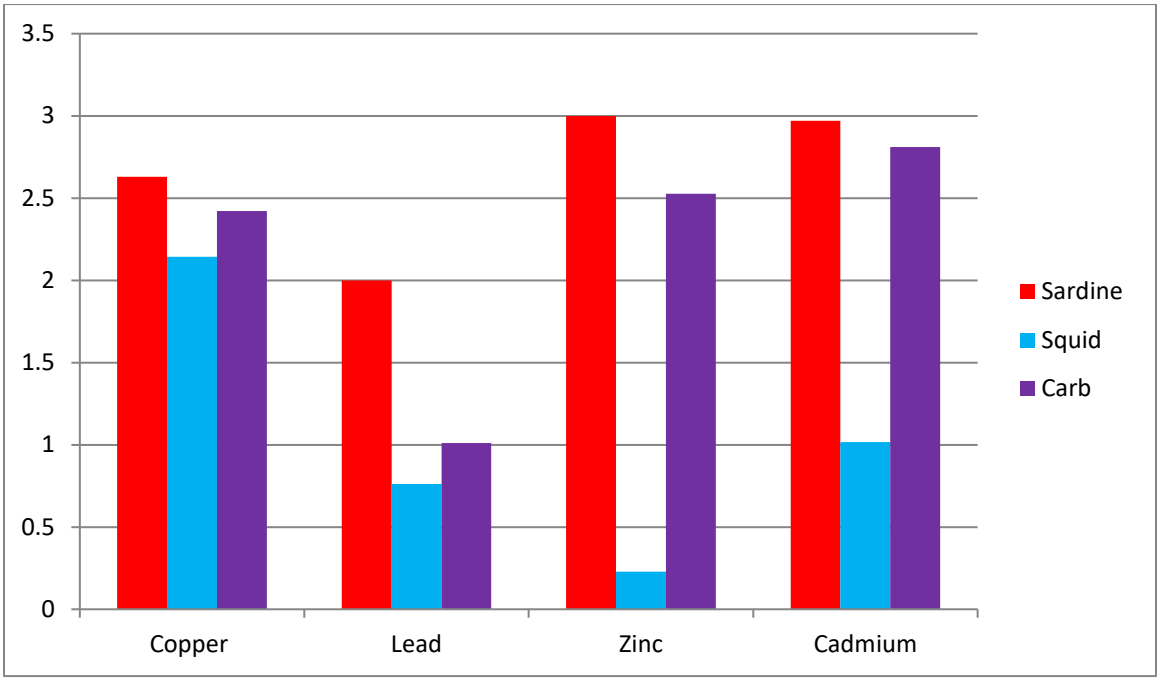
S.NO	NAME OF THE BACTERIA	CONCENTRATION OF SHELL EXTRACT (ETHANOL)		
		10µl	20µl	30µl
1.	Bacillus sps	__	__	__
2.	Pseudomonas sps	0.5mm	2mm	4mm
3.	E .coli	2mm	4mm	5mm
4.	Staphylococcus sps	1mm	3mm	6mm
5.	Vibrio cholera	__	__	__

TABLE-5 ACTIVITY OF COMPOUNENTS IDENTIFIED IN THE MUSCLE SAMPLE (METHANOL) OF *Sardinella longiceps* (GC-MS)

S.NO	RT	COMPOUND NAME	MOLECULAR FORMULA	MOLECULAR WEIGT	PEAKS AREA	ACTIVITY
1.	16.223	1,2-Bis(trimethylsilyl) Benzene	$C_{12}H_{22}Si_2$	222.47g/mol	207.1	Antipyretic, Antibacterial, Antioxidant activity. Antidiabetics
2.	16.223	Silicic acid, diethyl bis (trimethylsilyl)	$C_3H_{12}O_4SIO_2$	296.58g/mol	207.1	Antibacterial activity Antiasthmatics, Antipuritics,
3.	16.223	2-ethylacridine	$C_{15}H_{13}N$	207.27g/mol	207.1	Antimicrobial and antitumor Antiallergic agent, Antiobesity

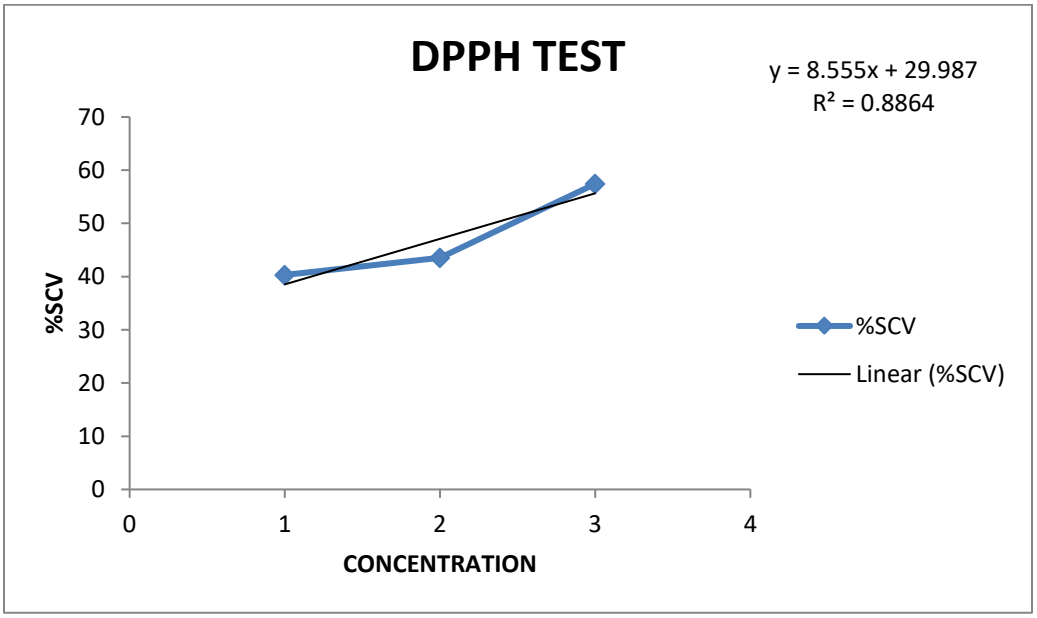
GRAPH-1

DISTRIPUTION OF HEAVY METAL IN THREE SPECIES



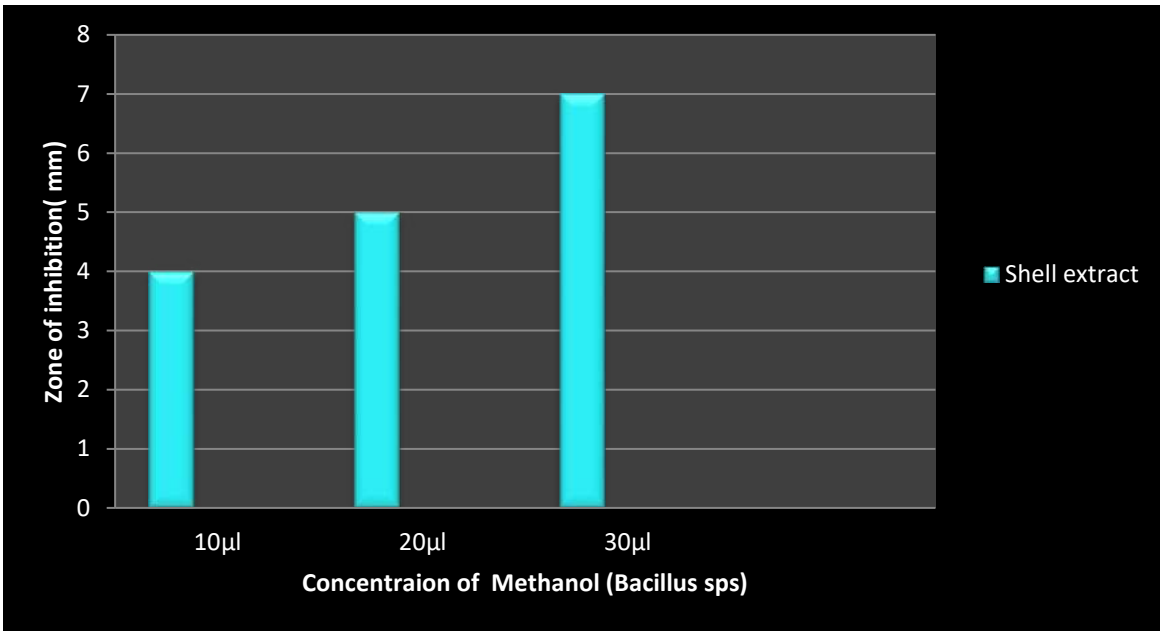
GRAPH-2

DISTRIBUTION OF ANTIOXIDANT ASSAY



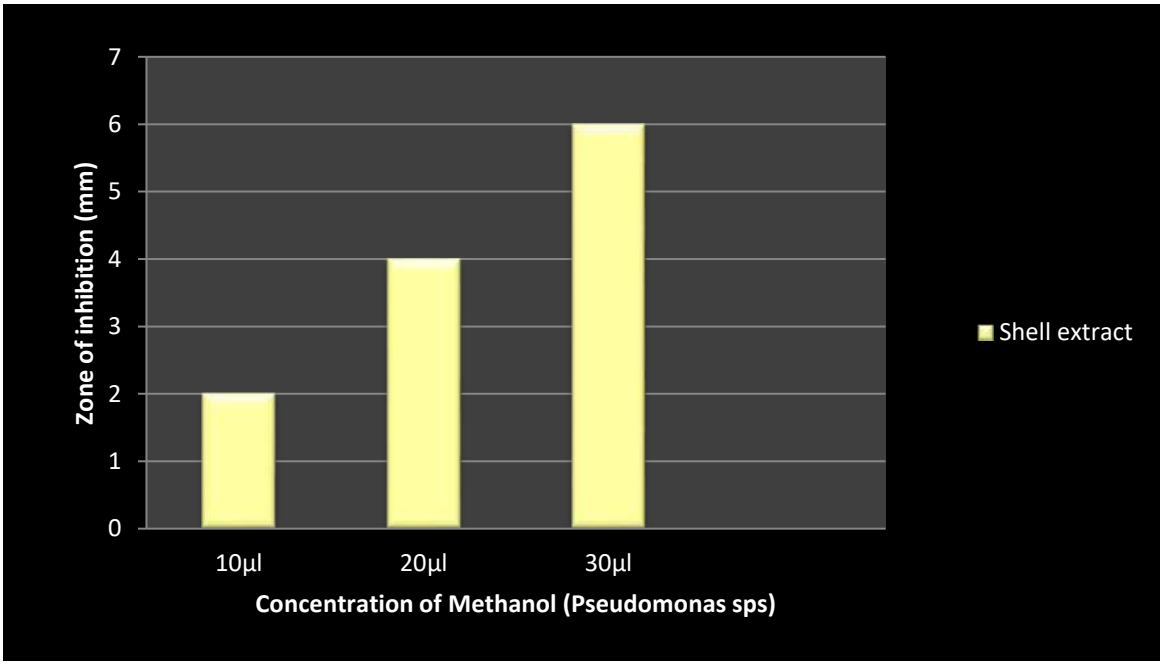
GRAPH-3

Antibacterial activity of crab shell extract against *Bacillus Sps*



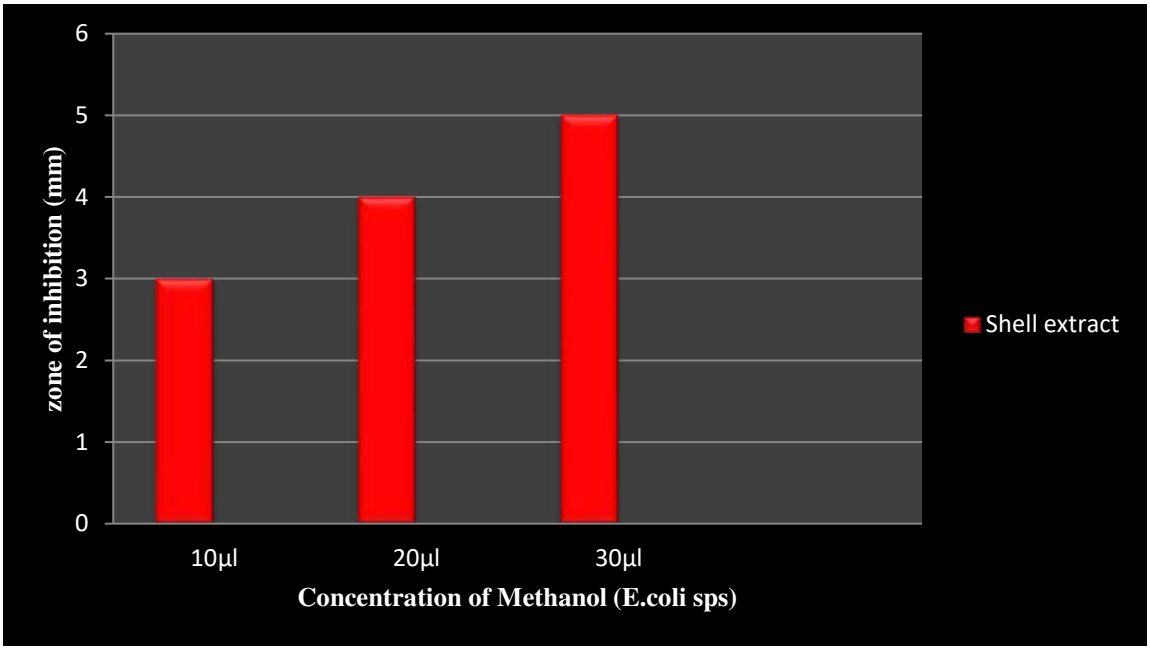
GRAPH-4

Antibacterial activity of crab shell extracts against *Pseudomonas Sps*



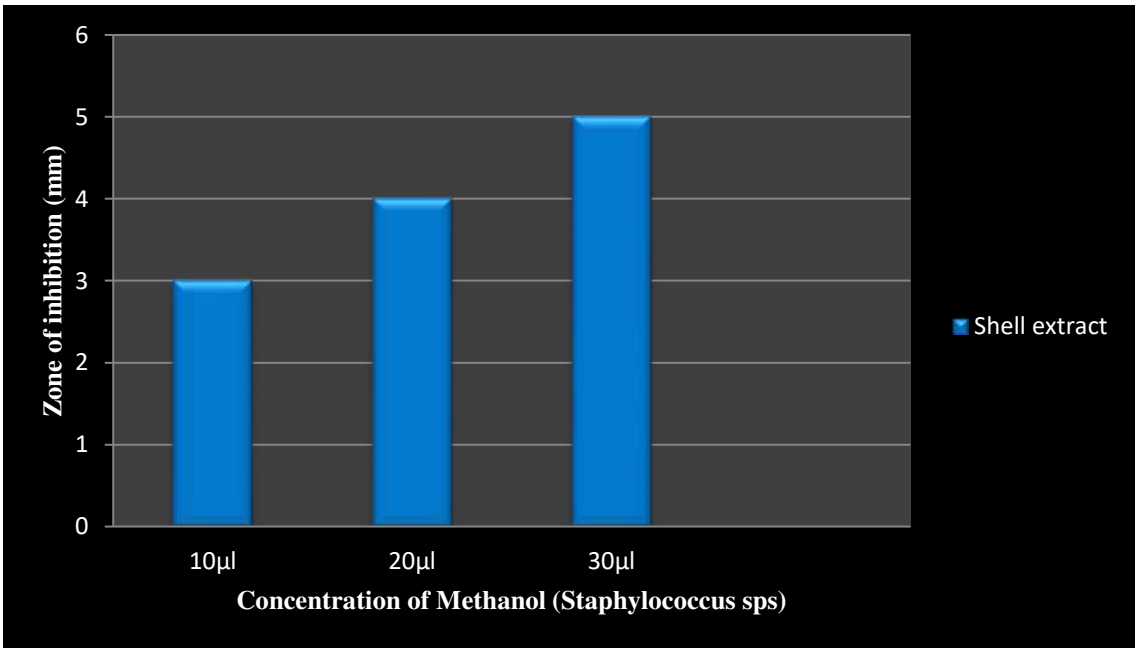
GRAPH-5

Antibacterial activity of crab shell extract against *E.coli Sps*



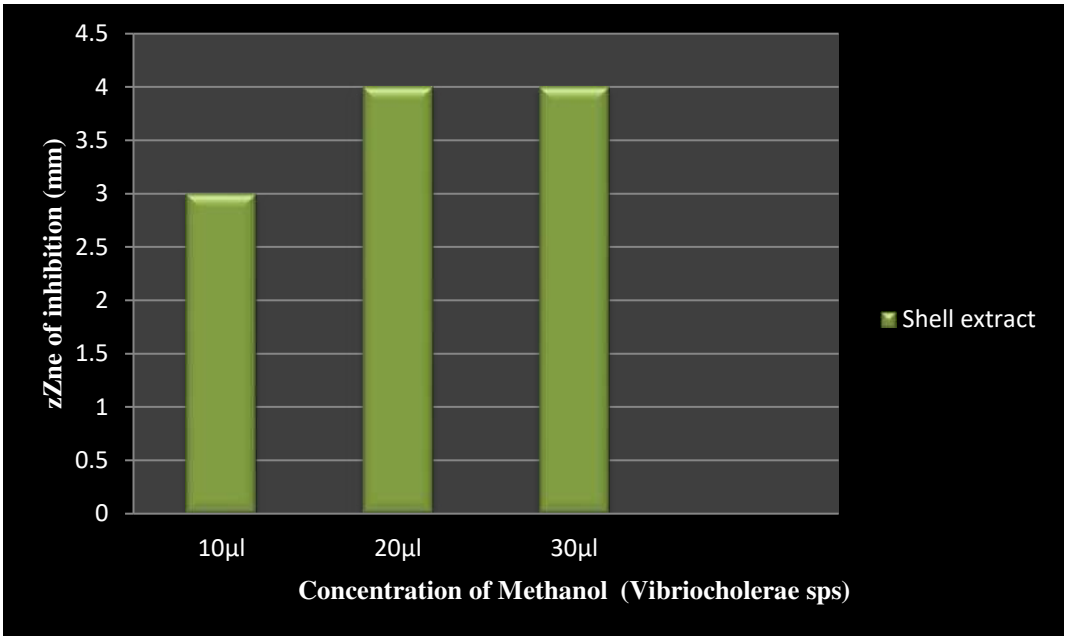
GRAPH-6

Antibacterial activity of crab shell extract against *Staphylococcus Sps*



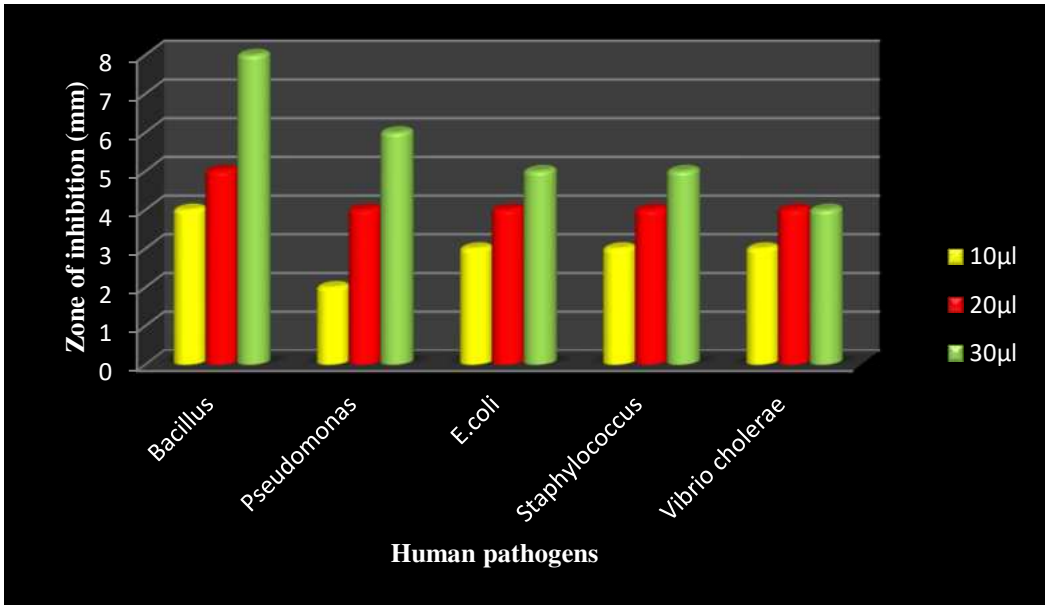
GRAPH-7

Antibacterial activity of crab shell extract against *VibriocholeraeSps*



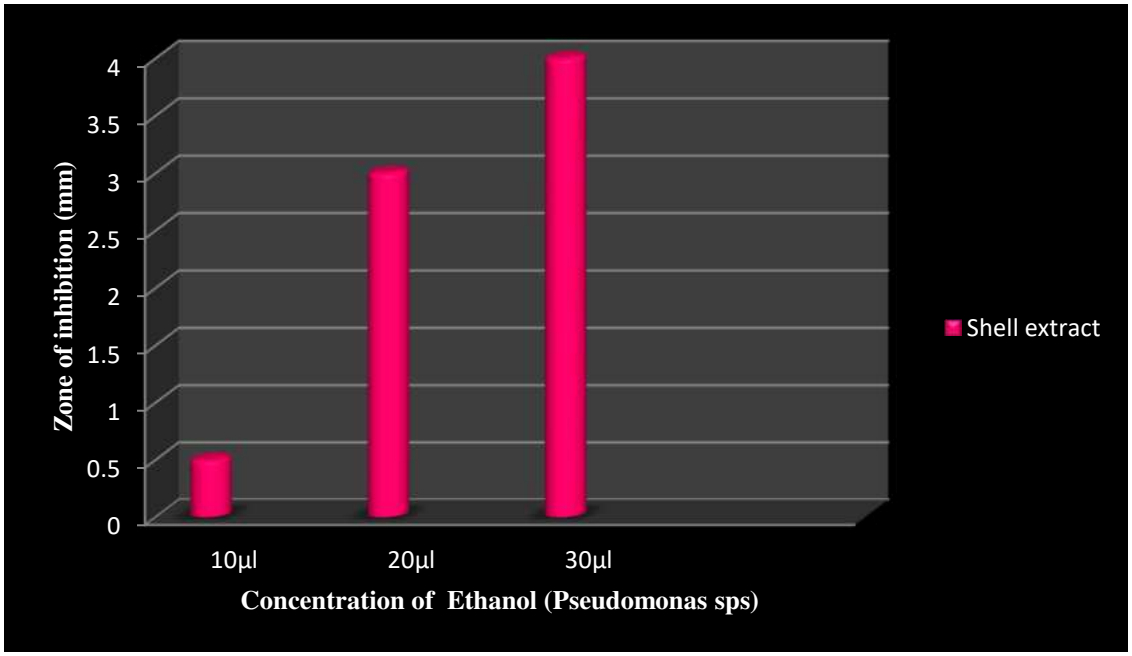
GRAPH-8

Antibacterial activity of crab shell extract of *Portunus pelagicus* against human pathogens



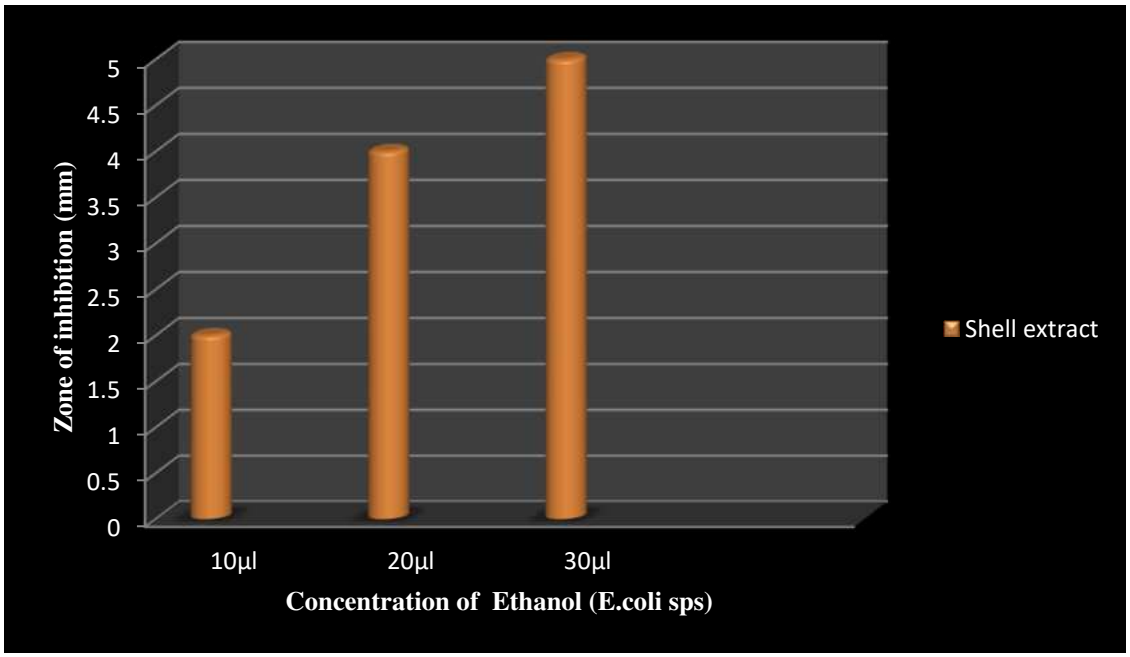
GRAPH-9

Antibacterial activity of crab shell extract against *Pseudomonas Sps*



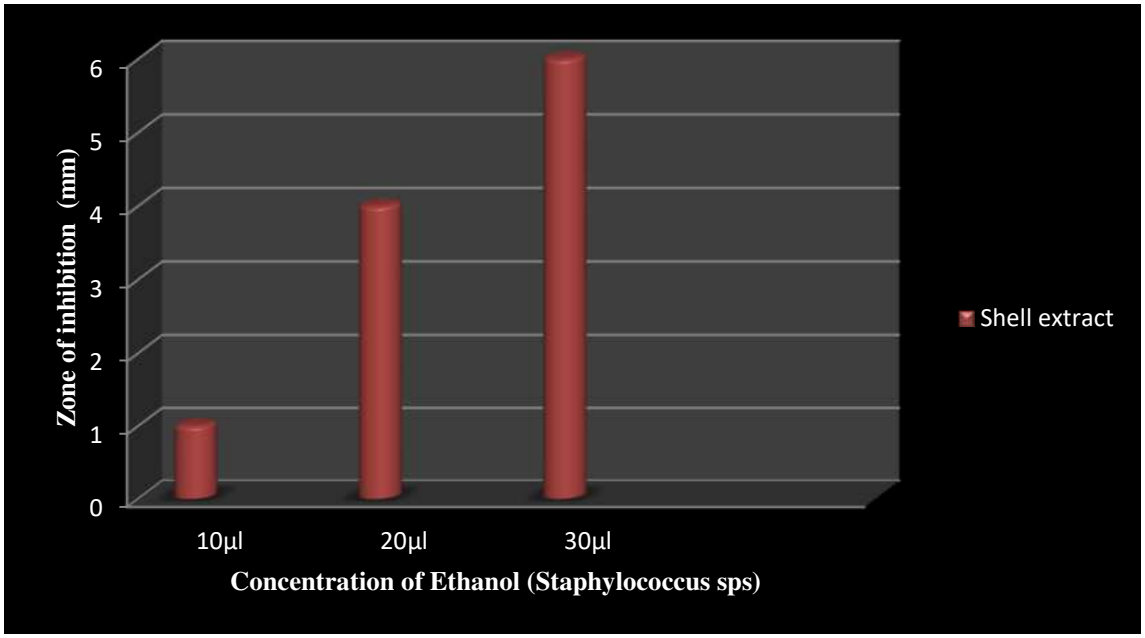
GRAPH-10

Antibacterial activity of crab shell extract against *E.coliSps*



GRAPH-11

Antibacterial activity of crab shell extract against *Staphylococcus Sps*



GRAPH-12

Antibacterial activity Ethanol extract of crab shell against human pathogens

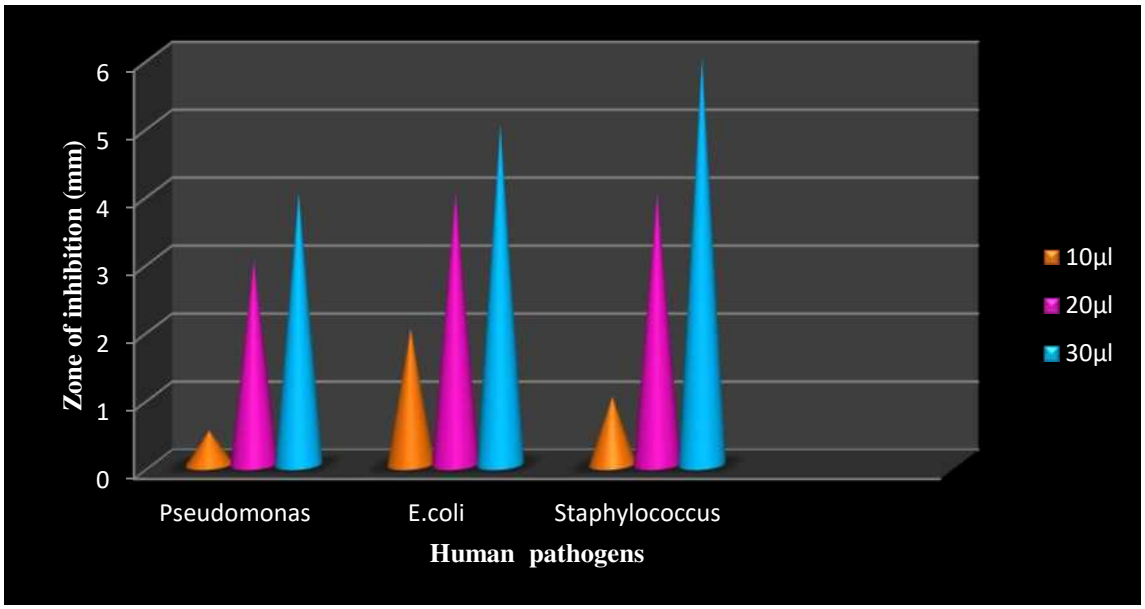


PLATE-1

Antibacterial activity of methanol extract of carb tissue against *Bacillus sps.*

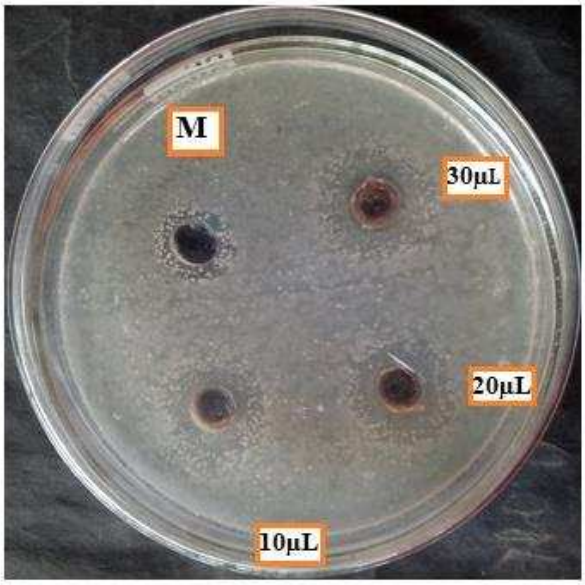


PLATE-2 Antibacterial activity of methanol extract of crab tissue against *Pseudomonas sps*

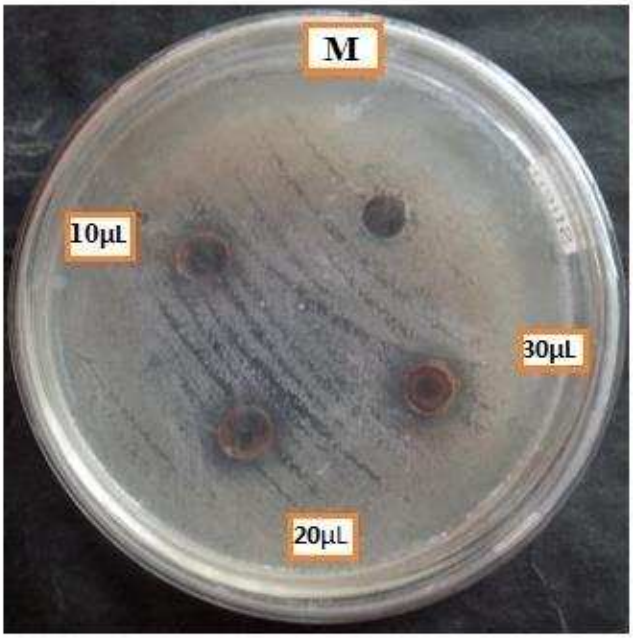


PLATE-3 Antibacterial activity of methanol extract of crab tissue against *E.coli*

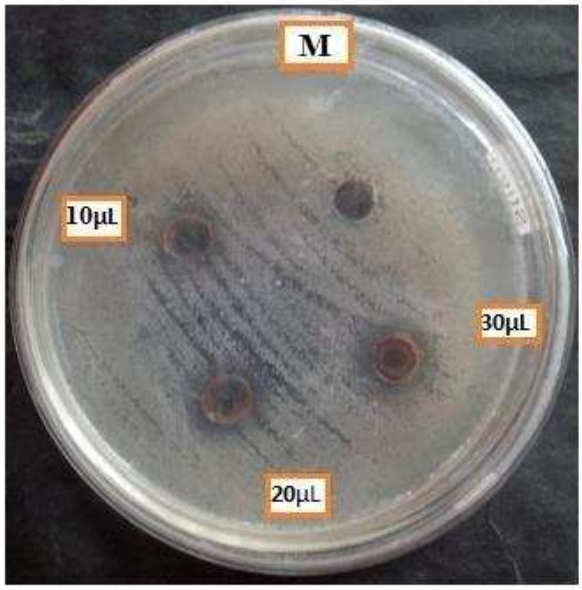


PLATE-4 Antibacterial activity of methanol extract of crabtissue against *Staphylococcus sps*

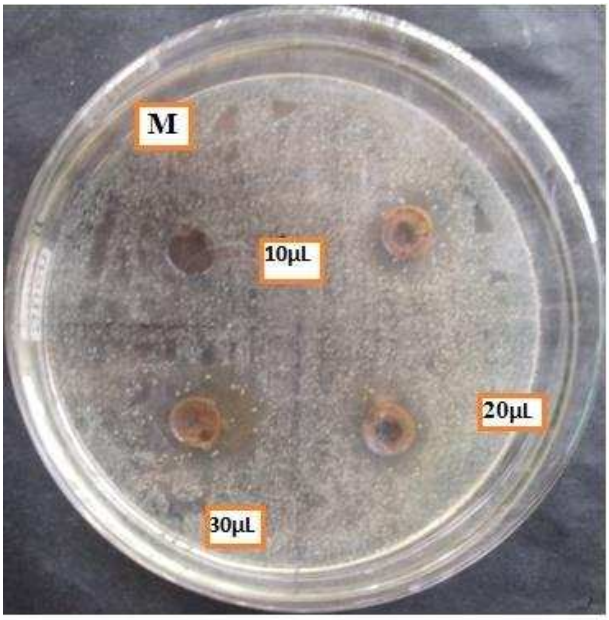


PLATE-5 Antibacterial activity of methanol extract of crab tissue against *Vibriocholerae*

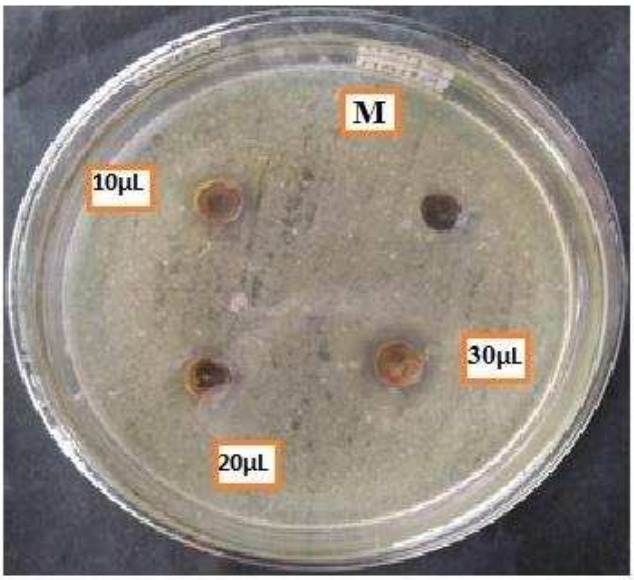


PLATE-6 Antibacterial activity of ethanol extract of crab tissue against *Pseudomonas sps*

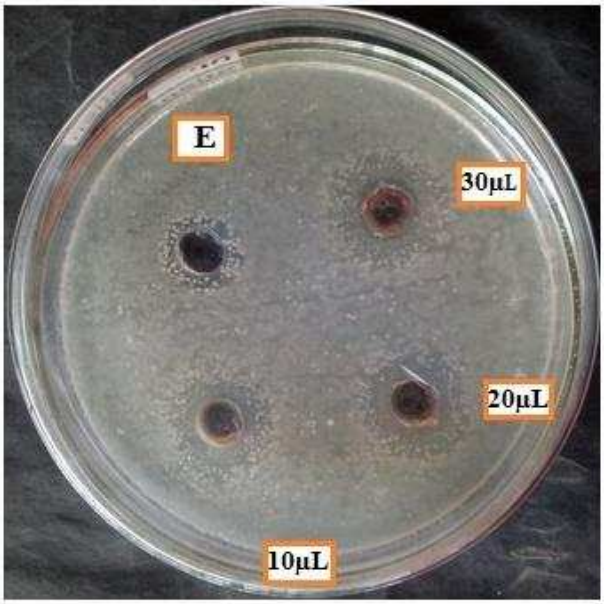


PLATE-7Antibacterial activity of ethanol extract of crab tissueagainst *E.coli*

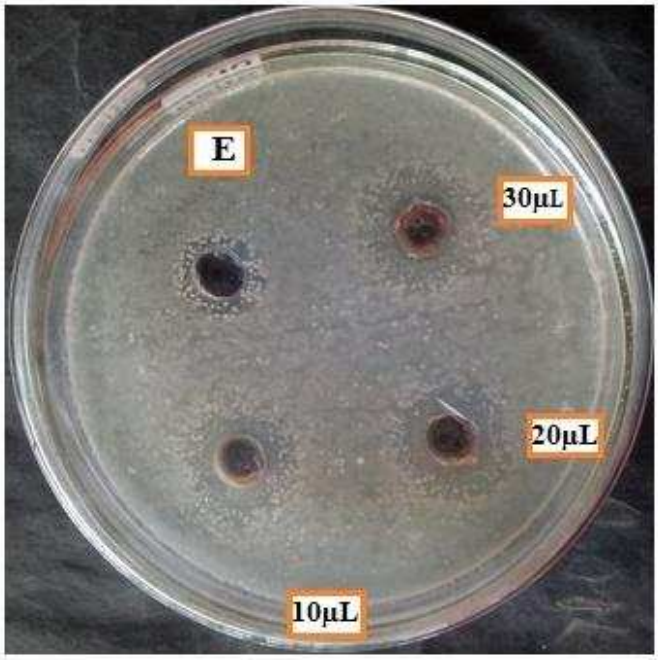
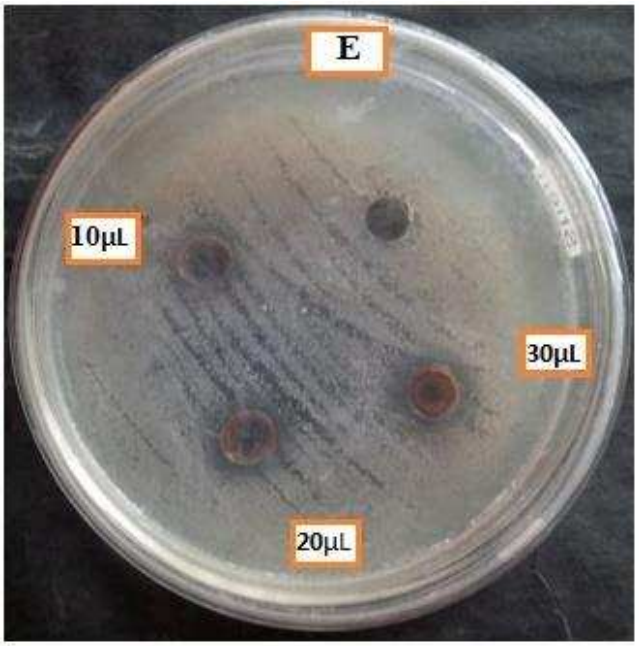


PLATE-8Antibacterial activity of ethanol extract of crab tissue against *Staphylococcus sps*



DISCUSSION

7.DISCUSSION :

Copper is a micronutrient for aquatic life but it becomes toxic at higher level. In the present study the copper concentration was observed higher level in the tissue of sardine fish other two species. The higher concentration of copper at Thoothukudi coast is attributed by industrial effluent, industrial water coolant discharge, domestic sewage and harbour activities. Another possibility for higher concentration of copper is due to the copper industry which situated in the SIPCOT (State Industries Promotion Corporation of Tamilnadu) area.

The results of the present study agreed well with the findings of Selvaraj et al (2003) Jonathan and Ram mohan (2003) and Jeya prakash et al (2008) who studied the metal accumulation in coastal areas.

Lead is known as a snow balling metabolic poison. Its concentration in the coastal region has been altered by human activities. In the present study the maximum level of lead concentration was observed in the tissue of Sardinella longiceps. Likewise the low level of lead concentration was noticed in the tissue of squid than crab.

The high concentration of lead in the coastal transacts is attributed by the sources like automobile exhaust, domestic sewage, agricultural run off, power plant operation, loading and unloading of cargo as well as dredging activities in harbour. This lead transported through the atmosphere later settled in sea water.

The high concentration of lead in Thoothukudi coast is related to the input of industrial effluent from SPIC (Southern Petrochemical Industries Corporation) petrochemical and HWP (heavy water plant) industry which located in Muthiyapuram near Thoothukudi. Leaching from antifouling paints from fisher

man boats is also one of the reason for higher concentration lead at Thoothukudi coast.

The higher level of zinc was observed the tissue of Sardinella longiceps found more when compare to squid and crabs

Cadmium in a non metal for organisms expect some diatoms have its biological role. Cadmium is highly toxic to fresh water and marine organisms. It is bioaccumulative through the food chain. It has been demonstrated as highly toxic metal to wild life and carcinogenic to humans. (Palanichamy and Rajendran 2000).

Cadmium may enter marine environment due to the geology of the catchment soil and run off from phosphate fertilized agricultural soils, disposed of nickel and cadmium based batteries etc.

The low level of copper, lead, zinc and cadmium concentration was found the tissue of squid when compared to Sardinella longiceps, and crab.

Nature has played an instrumental role in providing effective therapeutics entities. Antioxidant activity is fundamental property and much important for life. Many of the biological functions such as anti-mutagenicity, anti-carcinogenicity and anti-aging, among others originate from this property (Cook and Samman, 1996). In the present study antioxidant activity was measured by DPPH scavenging activity.

DPPH is one of the stable free radical used for the assay of scavenging capacity of crab tissue. The DPPH scavenging activity of standard ascorbic acid was found to be 40.31 for (1 µg/ml), 43.56 for 2.5 µg/ml concentration and 57.42% for 5 µg/ml

concentration respectively. Squid posses antioxidant activity. Removal of melanin from squid ink did not affect the antioxidant properties of squid ink where it showed valued when tested with several assays including DPPH assay. The radial scavenging activity of methanol extract of 40.31% to 57.42%.(Vate and Benjakul, 2013). Squid ink polysaccride also ability from antioxidant ability of the sample with DPPH radicals (Luo and Liu, 2013).

Higher content of antioxidant has been found higher in more polar solvents (o'Sullivan *et al.,* 2013). Oroian and Escriche (2015) explained that extraction yield increased with increasing polarity of solvents used with addition combination of water and organic solvent might facilitate the extraction of a compound that soluble in both polar and non-polar solvent.

Many organisms possess antimicrobial properties although most of the antimicrobial agents that have been isolated from marine sources have not been active enough to complete with conventional antimicrobial obtained from microorganisms (Rinehart *et al*., 1981).

The crab haemolymph showed antimicrobial activity against a range of different pathogenic strains of both gram positive and gram negative bacteria. The results suggest that brachyuran crabs were not involved in the economy of finfish resources. It can also produce antibacterial substances instantly to combat bacterial

infection. Similar result was observed in the haemolymph of some mangrove crabs against clinical pathogens (Veeruraj, *et al*., 2008).

Crabs are the wonderful resource of antimicrobial proteins with wide range of antimicrobial properties which is highly supported in the haemolymph study of *C.lucifera*. (Rameshkumar*et al*., 2009 and Latreille, 1829).

Antibacterial activity has been reported earlier in the haemolymph of the blue crab *C.sapidus*. It was highly inhibitory to gram - negative bacteria (Edward, *et al*., 1996). Although there were several reports on antibacterial activity in seminal plasma (Chattopadhyay *et al*., 1993) and (Jayasanker,*et al*., 1999) few antibacterial peptides have been reported in *Syllaserrata*. The antibacterial activity of methanolic extract shell of *P.sanguinolentus* is given in plate (1-8) and figure (2-11). Antibacterial activity was recorded against of five human pathogens at three different concentration. At 10µl concentration the methanol extract showed 4mm inhibitory zone,at 20µl an inhibition zone of 5mm was observed and at 30µl concentration, the inhibition zone of 7mm (maximum antibacterial activity) was observed against *Bacillus sps*.Table (1)

Grasian Immanuel (Immanuel *et al*., 2012) prepared solvent extracts of the shell powder of *C.moneta* solvent extract and investigated the antibacterial effect against three opportunistic human pathogens such as *P.vulgaris*, and micrococcus

sp found that the growth of all the three pathogens was inhibited.In the present study for *Pseudomonas sps* the zone of inhibition at 10µl was observed to be 2mm, for 20µl it was observed to be 4mm and for 30µl it was observed to be 6mm.At 10µl concentration the extract showed 3mm inhibitory zone,a inhibition zone of 4mm was observed at 20µl and 30µl concentration showed 5mm of inhibition against *E.coli*. For *Staphylococcus sps*, the zone of inhibition at 10µl was observed to be 3mm,for 20µl and 30µl , the inhibition zone was observed to be 4mm and 5mm. For *Vibrio cholerae* the zone of inhibition at 10µl was observed to be 3mm, and it was observed to be 4mm in 20µl and 30µlconcentration.Table (1),Plates (1-5) &Figures(2-7)

Crabs are the wonderful resource of antimicrobial proteins with wide range of antimicrobial properties which is highly supported in the haemolymph study of *C.lucifera*. (Rameshkumar, G. *et al*., 2009 and Latreille, 1829).In ethanol extract of shell of crab the maximum activity was found against *Staphylococcus sps*(6mm) followed by *E.coli*(5mm) and P*seudomonas sps* (4mm) at higher concentration The least activity was found against *Pseudomonas sps* (0.5mm) at 10µl .Table(2) ,Plates(6-8) & Figures (8-11)

Among the five pathogen, the maximum zone of inhibition (7mm) was developed against *Bacillus sps* with methanol extract and trace activity was observed against *pseudomonas sps* (0.5mm) with ethanol extract. No activity was

found against Vibrio cholera and Bacillus sps with ethanol extract at three concentrations.Table(2)

The result of maximum inhibitory concentration revealed that among the concentrations,30µl concentration showed maximum activity and 10µl concentration showed and minimum activity.As the concentration increased the activity of shell extract was also increased.

The result suggested that the crab can produce antimicrobial substances instantly to compbat microbial infection. It is an interesting finding that crabs, being marine animal has the ability to dispose the bacteria upon infection. As the bacterium is a human pathogen, it is important that sea water should free from this type of bacteria. Usually it should not be in the water and the peptides can kill more efficiently than the conventional antibiotics.

The higher lipid also produced a high HSI possible because of high lipid content in the liver which is similar to observation in gilthead seabream (Sparus aurata) (Santhinha *et al*.,1999), headlock (melangrammus aegefinus), (Nanton *et al.,* 2000) and Atlantic cod (Gadus morhua L) (morasis *et al.,*2001).

Ravichandrine et al., 1909 revealed the presence of 1-(4-carboxy) phenylnona-2, 5-diene; 3-diydrotryptopan and indolyl carboxylic acid in GC-MS analysis of stingray Dasyatis jenkinsii.

In the present study totally three compounds were identified in the tissue extracts of Sardinella longiceps. These compounds exhibited biological activities such as antiasthmatics, antibacterial agents, antipressants, antiobesity agents, antidabetices, antipyretics, antipuritics, antiallargic agents.

SUMMARY

8.SUMMARY

The increasing industrialization is the major sources of heavy metal pollutants in the natural environment and aq uatic systems. The level of heavy metals above the essential concentration would result in toxicity. Thus monitoring the level of heavy metals in the common edible marine sea food would result in the demand for sea foods. In the present study the concentration of heavy metals

The results of the present study revealed that the heavy metals concentrations in the crabs from both the regions are below the threshold levels associated with the toxicological effects and the regulatory limits. However a long-term study on these heavy metals and their rate of sediments are in need to understand the bioaccumulation in marine food and its biomagnifications to human beings.

This is due to the presence more industries are located in and around Thoothukudi. The waste water and domestic sewage should treated and reused for agricultural and domestic purpose.

The free radical scavenging activity of squid tissue was assessed by the DPPH assay. The DPPH radical scavenging potential of squid tissue ranged from 40.311% to57.42% at varied concentrations of 1g, 2.5g and 5g. Scavenging activity was increased as the concentration of the extract increased.

The present investigation has been undertaken to find out the antibacterial, antidiabetic and biofilm activities of the selected crustacean of *P.pelagicus* Antibacterial activity of the shell extracts of crab were tested against five pathogens (*Bacillussps, Pseudomonas sps , E.coli, Staphylococcus sps , Vibrio cholerae*)by disc method.

- The antibacterial activity of methanol extract of shell of crab showed maximum zone of inhibition against *Bacillus sps*7mm at 30µl concentration followed by *Pseudomonas sps* (6mm) ,*Staphylococcus sps* (5mm) , and *Vibrio choleraesps* (4mm)
- The minimum zone of inhibition was observed against *Vibrio cholerae*sps (4mm) compared to other pathogens.
- In ethanol extract of shell of crab the maximum activity was found against *Staphylococcus sps*(6mm) followed by *E.coli*(5mm) and *Pseudomonas sps (*4mm). The least activity was found against *Pseudomonas sps* (0.5mm) at 10µl .
- Among the five pathogen, the maximum zone of inhibition (7mm) was developed against Bacillus sps with methanol extract and trace activity was observed against *Pseudomonas sps* (0.5mm) with ethanol extract.

The result of maximum inhibitory concentration revealed that among the concentrations, 30µl concentration showed maximum activity and 10µl concentration showed and minimum activity. As the concentration increased the activity of tissue extract was also increased.

Chemical compounds present in the muscle was characterized through GC-MS analysis. The result indicated that there are three compounds in the tissue with Antipyretic, Antibacterial, Antioxidant activity, Antimicrobial, antitumor, Antiasthmatics, Antipressants, Antiobesity, Antidiabetics, Antipuritics, Antiallergic agent.

CONCLUSION AND SUGGESTION

9.CONCLUSION AND SUGGESTION

The concentration of heavy metal in crabs, squid and shrimp showed detectable levels in this study the values were lower in other reported data in Qatar, while this result was in agreement with other studies in Bahrain and in the eastern region coast of the Arabian Gulf, SA. Therefore monitoring programs should continue to maintain the quality of the aquatic environment in eastern region coast and cut the risk of pollution in living fish health and human health.

The investigated species are commonly consumed seafood in many Red sea countries. Therefore, the investigation of heavy metal concentrations in the tissues of these species may provide useful information on the transfer of potentially toxic elements from abiotic compartments (water, sediment) to higher consumers, including man. Therefore, we can conclude that, Cu and Zn concentration of all edible tissues of the species were considerably high but lower than the permissible levels set by FAO and WHO. But the permissible limits for Cd and Pb were exceeded in some of the edible tissues of the analyzed species in present study.

Accumulation of Cd and Pb in edible tissues of the investigated species and other organs may be considered as an important warning signal for fish health and human consumption. The present study shows that precaution measures need to be taken in order to prevent future heavy metal pollution. Therefore, further monitoring programs should be conducted.

Marine natural products isolated from marine organisms have shown wide range of pharmacological properties including antimicrobial, antioxidant, anticancer, and other medicinal So in the present study the pharmaceutically important as well as the stress enzymes related to the environmental conditions were studied. After

clinical trials this could definitely serve as a therapeutically important organism besides its nutritive value.

In the present study it has been recorded that, a wide spectrum at antibacterial activity is found in ethanol and methanol solvents tested and these results indicates that crustaceans were good source for search of new substances for drug development.

From this study, we conclude that the whole body tissue of crabs can be used as an antibacterial agents for many different pathogens and would replace the existing inadequate and cost effective antibiotics.

Commercial antibiotics are highly effective to kill the bacterial and fungal pathogens involved in common infection. Ethanol extracts of tissue and shell of crabs used in the present study showed significant antimicrobial activity compare with other solvent extraction.

The present study indicated that tissue of*P.pelagicus*contains potential antibiotics.The antimicrobial assays done so far and those that will be done will serve as a baseline data for further studies that may confirm the hypothesis that brachyuran crabs muscles are indeed potential source of novel compounds with biological potential.

Today more prevalent pathogens are resistance to the prevailing antibiotics,which are available commercially.

It is worthy to note that the product from natural source is good for health and devoid of side effects.However,further investigation involving application of the extracts as drug for human administration need more research.

GC-MS is a useful tool for chemical analysis. Result obtained clearly indicate that, the muscle extract possess compounds with many biological activities.

However further investigation involving application of the extract as drug for human administration need more research.

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A STUDY ON THE EXPRESSION OF SOME SELECTED HUMAN MORPHOGENETIC TRAITS IN TUTICORIN DISTRICT

A field work submitted to

ST.MARY'S COLLEGE (Autonomous),

THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

In partial fulfillment for the award of the degree of

MASTER OF SCIENCE IN ZOOLOGY

by

1.	**ANANTHASELVI.S**	**20APZO01**
2.	**PANDIMUTHUSELVI.S**	**20APZO02**
3.	**RAJARAJESWARI R**	**20APZO03**

DEPARTMENT OF ZOOLOGY

ST. MARY'S COLLEGE (AUTONOMOUS)

(Re-accredited with A^+ Grade by NAAC)

THOOTHUKUDI

APRIL – 2021

CERTIFICATE

This is to certify that the field work entitled '**A *Study On the Expression Of Some Selected Human Morphogenetic traits in Tuticorin District***' is submitted to St.Mary's College (Autonomous), Thoothukudi affiliated to Manonmaniam Sundaranar University in partial fulfilment for the award of the degree of Master of Science in Zoology and it is a field work done during the year 2020-2021 under my guidance and supervision. It is further certified that this field work report or any part of this has not been submitted elsewhere for any other degree by the following students.

1.	**ANANTHASELVI. S**	**20APZO01**
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EXAMINER

DECLARATION

I do hereby declare that this thesis entitled, "A ***Study On the Expression Of Some Selected Human Morphogenetic traits in Tuticorin District***" submitted by me for the award of the degree of Master of Science in Zoology is the result of my original independent research work carried out under the guidance of **Dr. S.R.T Sherly Cross M.Sc., B.Ed., M.Phil., Ph.D.,** Assistant Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date: 16.04.2021.

R. Rajarajeswari
S. Pandimuthuselvi
S. Angathaselvi

Singnature of the Candidates

INTRODUCTION

1. INTRODUCTION:

Genetic variability is the characteristics of living things, especially in human beings. They show varieties of morphogenetic characters among one population itself. Morphogenetic characters are physical character of an individual and the pattern of inheritance of these traits is autosomal dominant as well as autosomal recessive.

Human population provides an exclusive opportunity to study the morphogenetic variation among the endogamous populations living in different geographical and ecological circumstances. The presence of genetic variation in man is controlled by many factors including assortment, migration and genetic drift.

Human genetics deals with the study of inheritance as it occurs in human beings. The advancement and research in the field of human genetics have made great socio economic contribution for human welfare.

The principle of genetics concern largely with an explanation of the differences existing among individual. It helps in analysing the potentialities of individuals already leaving as well assign predicting the trait of future offspring from a given mating.

In this study, we discuss some human traits which are more prevalent in our community. When one learns about dominant and recessive alleles, there is often a misconception that dominant alleles are the most common and they will tend to crowd out the recessive alleles in course of time. The frequency of a

character in a population is related to whether its phenotypic effect is favorable or unfavorable.

Genetic mechanisms on morphogenetic traits are still not clearly understood. It is seen to occur with variable frequency in different populations. Thus, it is useful in evaluating and analyzing the evolutionary forces and classification *Das and Sengupta (*2003).

The human genetic variations play an important role in bringing about the diversity in human population and contribute to the dynamics of evolution of human. Every population is characterized by a set of gene frequency. Genetic variability is the common feature of human beings. The existence of genetic variation in human is caused by many factors along with selection, migration, temporal variation, gene flow and genetic drift (*Bhasin et al.*, 1992).

The importance of these factors in understanding genetic variation has been described earlier in details *Vogel and Motulsky (1986).* Studies among Indian populations have shown wide genetic diversity. According to a recent estimate, human exhibits 200 traits of Mendelian inheritance (skin colour, hair colour, eye colour). Hence, we want to update the data in order to observe the temporal changes that might have occurred over a long period of time difference.

The main objectives of this study were to assess the frequently expressed morphogenetic traits among the population of selected areas of Thoothukudi district and also to check which trait is more dominantly or recessively expressed in the population and to make a comparison of male and female percentage difference in the expression of the traits.

OBJECTIVES

2. OBJECTIVES:

1. To investigate the distribution pattern and prevalence of some morphogenetic traits among the Tuticorin district.

2. To check which trait is more dominantly or recessively expressed in the population and to make a comparison of male and female percentage difference in the expression of the traits.

3. To find out, on which sex the dominant characters are shown enormously.

REVIEW OF LITERATURE

3. REVIEW OF LITERATURE:

KAWABE, M., 1953.quoted in FUJIKI, K., 1960, on the genetical mechanism of hand- clasping. Japanese Journal of Genetics, 35, 238-246. The manifestation rate of phenocopy is higher in adults than in children.

OA Ebeye, LE Chris – Ozoko, P Ogenevo, A Onoriode, A study of some morphogenetic traits among the Esan ethnic group of Nigeria, East Afr Med J. 91 (11) (2014) 420-422. The study concluded that these traits varies in the population but do not vary with gender.

E Anibor, E Ogbor-Omorie, AE Opiah, The distribution of widow's peak trait among young adults of the Isoko ethnic group in Delta state, Nigeria, Ann Bioanthropol. 3 (2015) 47-49. This study provides normative data for widow's peak distribution among the isoko ethnic group.

AU Usha , S Sidijo, PG Stejin, KS Alisha, CP Anjana, M Anju, A study on the expression of some selected human morphogenetic trait in Thrissur district, Sci. 12 (1) (2016) 94 -100. The survey showed that some typical dominant character was not expressed but the expression of recessive character was prevalent as in the case of widow's peak (absent), smooth chin, crossing of thumb right over left, Hitchhiker's thumb (straight), absence of dimple, etc, all of which are recessive traits.

GO Omotoso, PA Adeniyi, LJ Medubi, Prvalence of facial dimple amongest South-Western Nigerians: A case study of Ilorin, kwara state of Nigeria, Into J Biomed Health sci; 6(4) (2010) 241-244. From this survey, it was observed that 25% of the subjects inherited the two forms of facial dimples from either one or both of their parents who also expressed both phenotypes; a rate higher in females than males.

KS Ordu, BC Didia, N Egbunefu, Inheritance pattern of earlobe attachment Nigerians, Greener J Hum Physiol Anat. 2 (1) (2014) 1-7, doi: 10.15580 GJHPA. 1.0122 14054. The result obtained suggested that observation of pattern of earlobe attachment trait might be suitable for genetic analysis and can help in setting paternal dispate.

E.Anibor, Prevalence of facial dimple among the Niger Deltans in Nigeria, Afr J Cell Path. 6 (2016) 41-43. This study showed a higher prevalence of facial dimples among females than males.

GS Oladipo, DI Amangi-Peters, Incidence of cheek dimples among south – south and south – Eastern Nigerians J Biomed Afr. 3 (2005) 23-25. Results obtained in this study are suitable for medicolegal and anthropological studies.

Anshu S., Navjot K.S., Mahesh K.S., Kanchan K., and Balbir S., 2007. Study of earlobe in Northwest Indian male subjects. Anatomical science international. 82 (2) : 98-104. In present study, the frequency of attached (square) type of lobules was the maximum, followed by free (pendulous) type and tapering lobules.

Sharma, A., Sindhu, N.K., Sharma, M.K., Kapoor, K., and Singh, B. 2007. Morphometry study in Earlobe in Northwest India, Anatomical science International 82, 98-104. The study reported, the frequency of attached type of lobules was the maximum followed by free type and tapering lobules.

Williams G.O., and Hughes A.E., 2008. Frequency of attached and Free Earlobes in Lagos (Nigeria). American Journal of Physical Anthropometry, Wiley – Liss 72 (3): 399-401. In this study, males and females in the population were equally affected by the traits.

Lebow,M.R. and P.B Sawin, 1941.Inheritance of human facial features ; a

pedigree study involving length of face, prominent ears and chin cleft. Journal of Heredity, 32: 127-132.The result concluded that most variations in the teeth and jaws.

Lock-Andersen J, Wulf Hc. 1997. Seasonal variation of skin pigmentation. Acta Derm. Venercol. 77: 219-221. The result showed a considerable seasonal variation for skin pigmentation at the UV- exposed sites.

Das, B and S.Sengupta, 2003. A note on some morphogenetic variables among the Sonowal Kacharis of Assam. Anthropologist.5(3):211-212. They concluded that Sonowal Kachari people stand significantly apart and in no way show resemblance with other Kachari populations of Assam.

Nwaopara, A.O., C.I.P. Anibeze, F.C.Apkuaka and O.F. Agbontaen, 2008. Morphogenetic traits combination pattern amongst the population of Ekpoma, Nigeria: Focus on tongue rolling, earlobe attachment. Blood group and genotypes. African Journal of Biotechnology;7(20):3593-3598. The results suggest that certain morphogenetic traits combinations might be rare.

Bulliyya, G., 2003. Study on anthropogenetic traits in a caste group of Andhra Pradesh. Anthropologist, 5(3):197-199. They concluded that there is no significant association between the combinations of tongue rolling and tongue folding.

Onyije, F.M., 2012. Assessment of morphogenetic trait of AEL and CRT in Relation to Hb genotype. World Applied Sciences Journal, 20(9):1213-1215. The ears and ability to roll tongue were studied in the subject and recorded, as well as genotype as revealed by the subject.

Pandey, B.N., M.D. Jahangir and M.Priyanka, 2013. A morphogenetic study of Badhiya Muslims of Purnia District (Bihar), India. International Journal of Life sciences, 1(3):233-238. It was observed that the frequency distribution of various traits among the studied populations shows the homogeneous distribution. Overall, frequencies of these traits were according to Indian trends.

Odokuma, I.E., O. Eghworo, G. Avwioro and U. Agbedia, 2008. Tongue rolling and tongue folding traits in an African Population. International Journal of Morphology, 26(3):533-535. They concluded that frequency tongue folding was observed to be highest in both males and females.

Razzaq R, Kanwal S, Sajjad N. 2015. Tongue rolling, folding, cheek dimple and chin cleft; study of a morphogenetic traits in Quetta population. World J Zool.10 (3):237-240. They concluded that tongue rolling trait is found in Pathan, tongue folding and facial dimple is found highest in saraiki.

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M.E. Kooffreh, E.V. Ikpeme, E.E. Ekerette, N.O. Eyo 2015. Evaluating the prevalence of five genetic traits of simple inheritance in association with the distribution of ABO and rhesus phenotypes among families in Calabar, Nigeria., Journal Medical Sciences 15(4),185-191. The result showed that the prevalence of ABO phenotypes are associated with the prevalence of genetic traits of simple inheritance (earlobe, hand clasping, facial dimple, mid digital hair and tongue rolling) and gender.

H.K. Odion- Obomhense, E. Emore, O.A. Ebeye, O.P. Otuaga; (2008). A study of genetics using two simple Mendelian inheritance. Clinical Anatomical Journal,3-6. The result showed that the dominant features of both widow's peak and unattached earlobe were approximately at the same ratio in both sexes and was not up to 50% of the population studied, meaning dominant traits are not necessarily seen more in the population.

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Alain F. corcos. Tongue Rolling in the classroom. The American Biology Teacher (1980) 42(5): 311-312. He concluded that there was no evidence for a genetic basis for tongue rolling.

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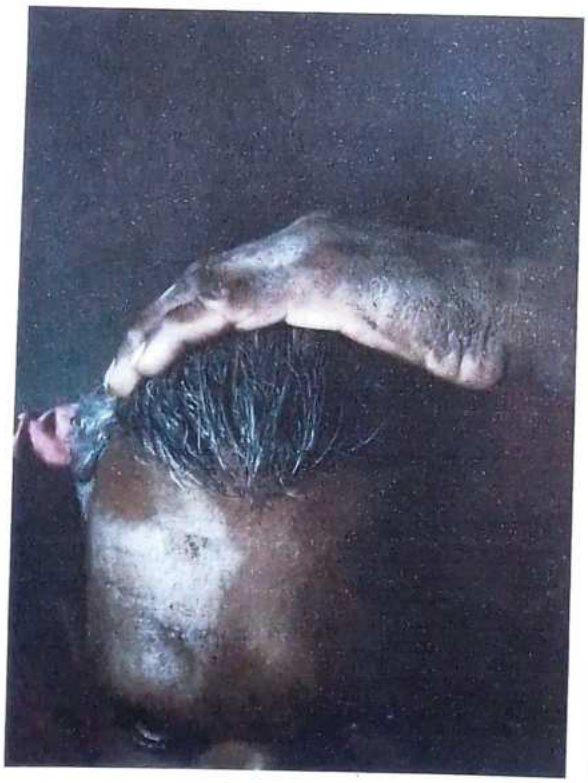
5. Alvergne A, Fauric C, Raymond M, (2007) Differential resemblance of young children to their parents: Who do children look like more Evol Hum Behav 20: 129-135. They found that at all ages, children resemble both their parents more than would be expected by chance, although there is a differential resemblance toward one or the other parent depending on the age and sex of the child.

36. Cruz-Gonzalex l. and Lisker R, (1982) Inheritance of ear wax types, ear lobe attachment and tongue rolling ability. Acta Anthropogenet. 6(4), 247-254. The result clearly showed that the dry ear wax type and the attachment ear lobe type represent the homozygous state for two pairs of autosomal recessive genes.

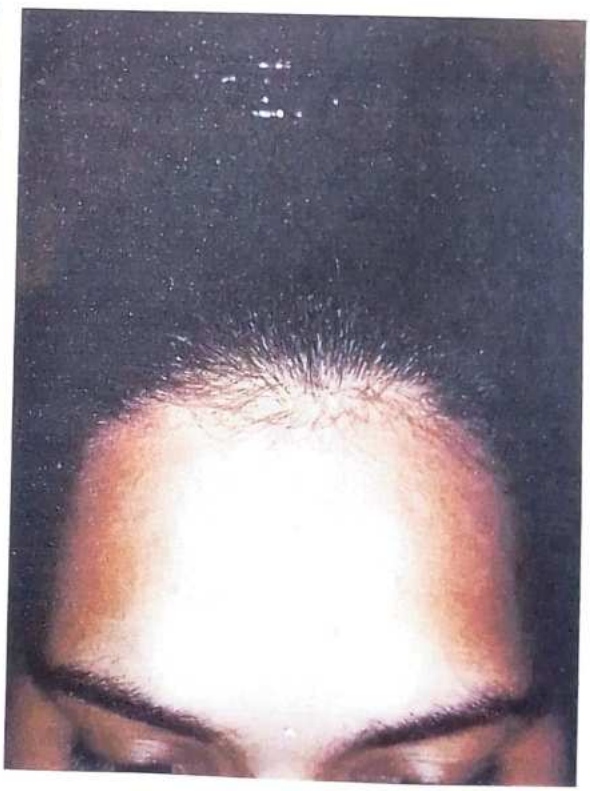
Rabia R., Safoora K., Shandana., Nabeela T., Naheed S., 2015 Tongue rolling, folding, cheek dimple and cleft chin: Case study of a morphogenetic traits in Quett a population. World J Zool. 10(3), 237-240. In conclusion, all the studied morphogenetic traits were found to be present in the different ethnic groups resident in Baluechistan province with variable prevalence.

Curly Hair – Dominant

Straight Hair – Recessive

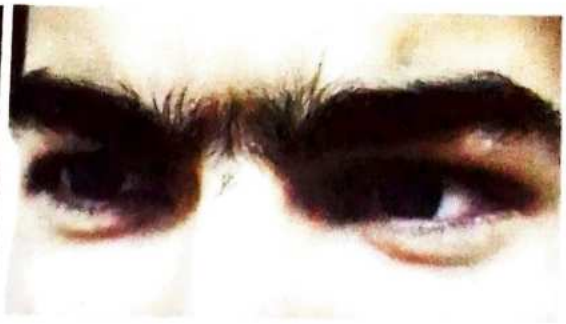


Widows peak – Dominant

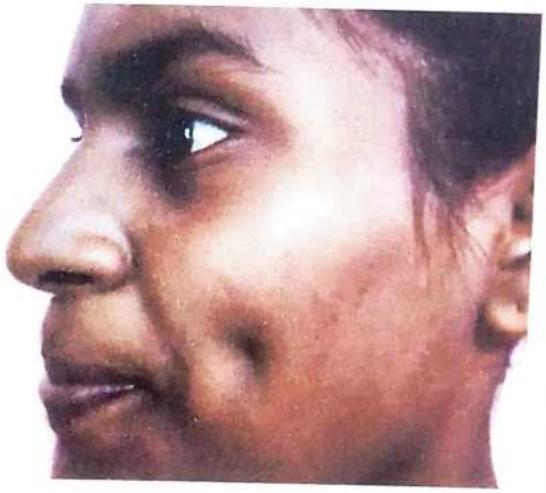


Absence of Widows Peak - Recessive

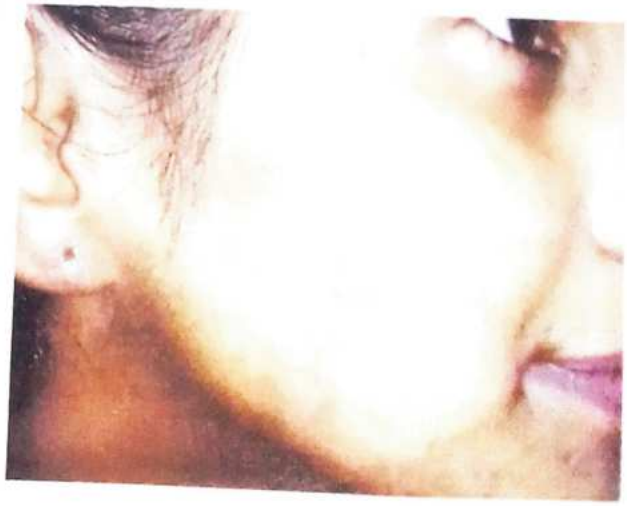
Free Eyebrows – Dominant



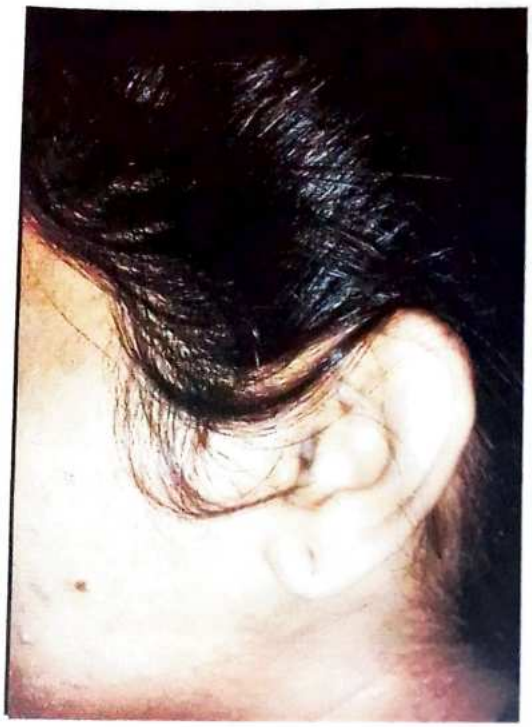
Attached Eyebrows – Recessive



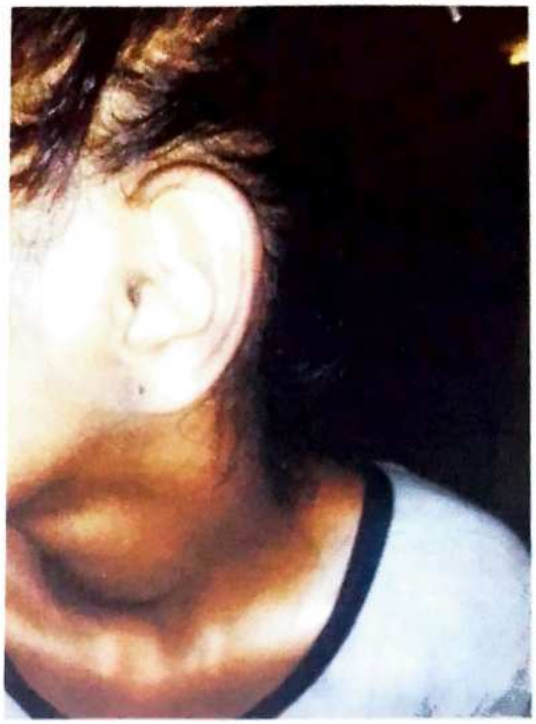
Dimples in cheek - Dominant



Absence of dimples – Recessive

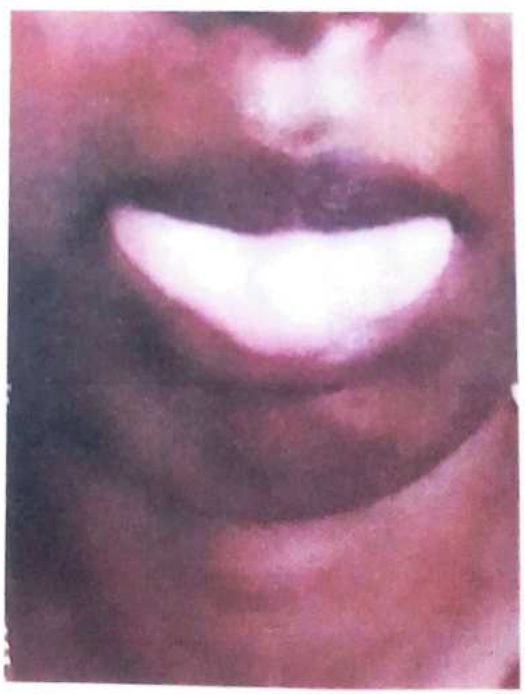


Free earlobes - – Dominant



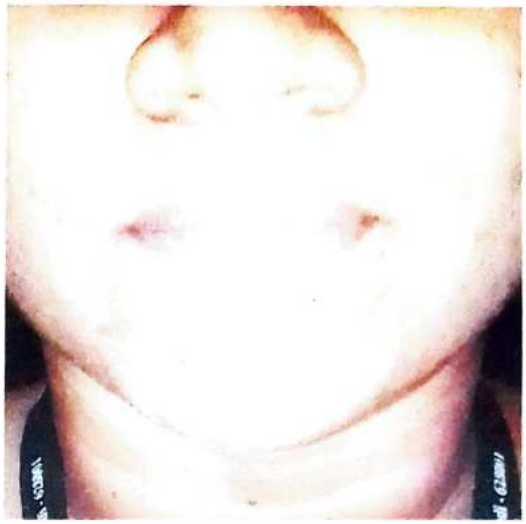
Attachd earlobes -- Recessive

Can roll tongue - – Dominant



Cannot roll tongue — Recessive

Cleft chin - Dominant



Absence of cleft chin - Recessive

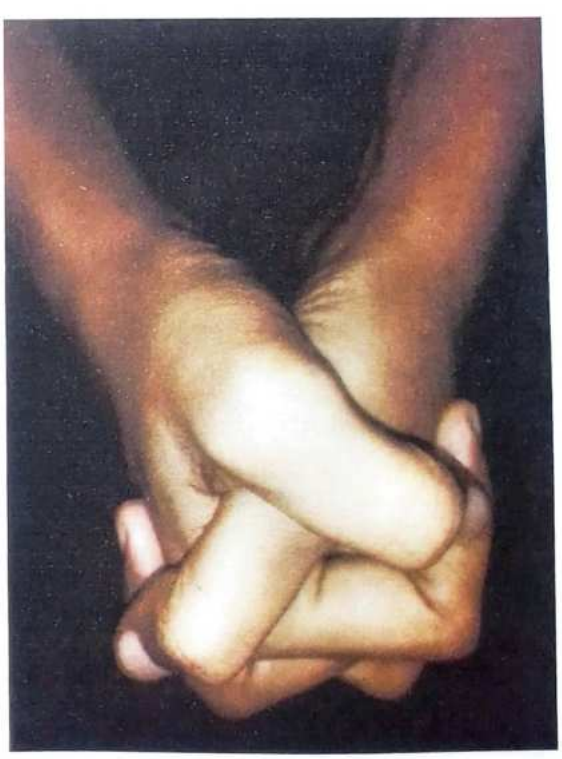
Oval shape in face – Dominant

Other shape in face – Recessive

Black skin colour – Dominant

White skin colour – Recessive

Left Thumb Over Right – Dominant



Right Thumb Over Left – Recessive

St. Mary's College (Autonomous)

Field work

A STUDY ON THE EXPRESSION OF SOME SELECTED HUMAN MORPHOGENETC TRAITS IN TUTICORIN DISTRICT

NAME :

AGE :

AREA :

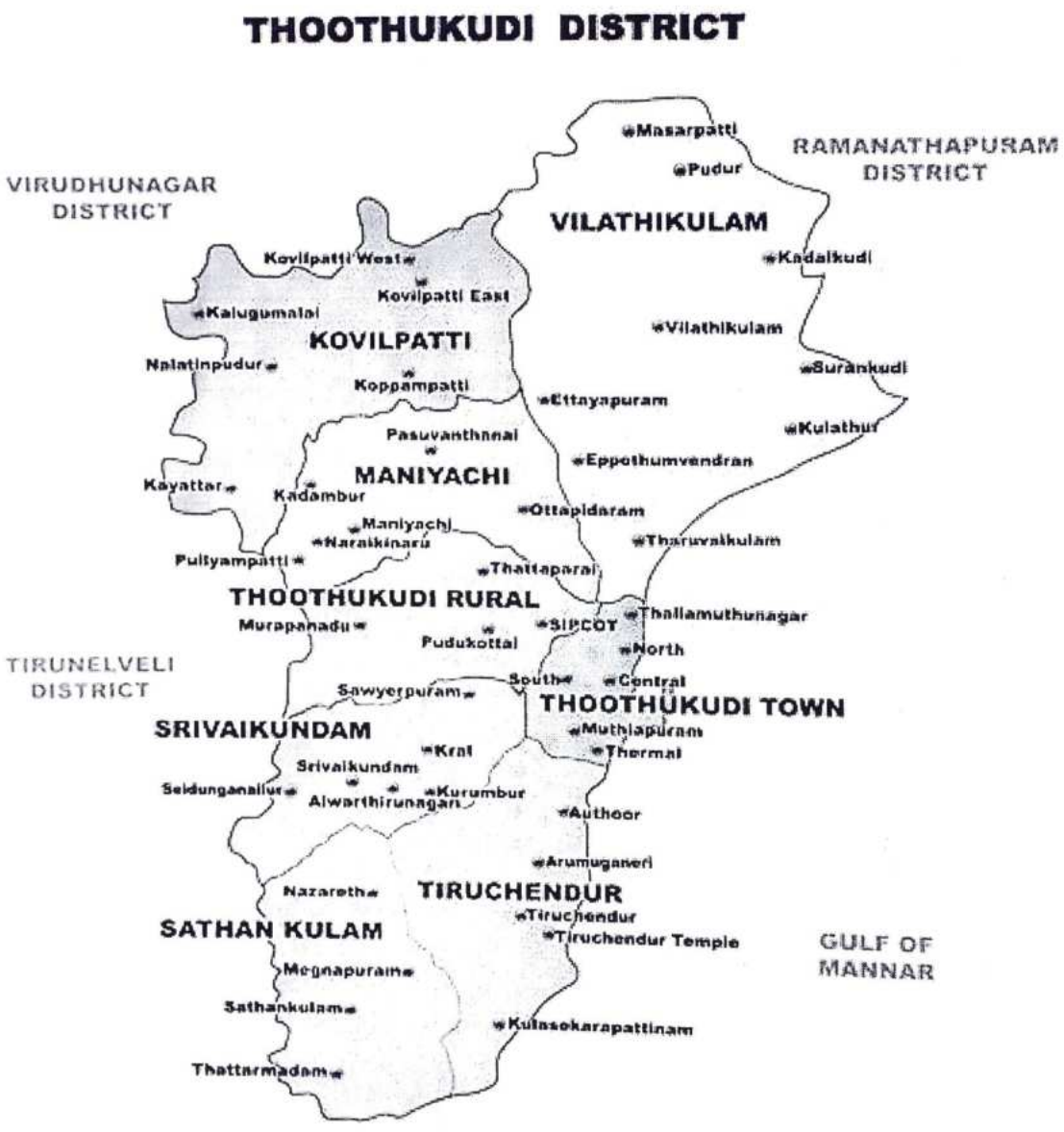
GENDER: DATE:

S.NO	MORPHOGENETIC TRAITS	DOMINANT	RECESSIVE
1.	Hair wave	Curly	Straight
2.	Widows peak	Present	Absent
3.	Position of eyebrows	Free	Attached
4.	Dimples	Present	Absent
5.	Earlobe	Free	Attached
6.	Tongue rolling	Can roll	Cannot roll
7.	Cleft chin	Present	Absent
8.	Face shape	Oval	Others
9.	Skin colour	Black	White
10	Hand clasping	Left thumb	Right thumb

STUDY AREA

4. STUDY AREA:

The survey was conducted in randomly selected areas of Thoothukudi district.



MATERIALS AND METHODS

5. MATERIALS AND METHODS:

The study was conducted in Tuticorin town from December 2020 to March 2021.

A total of 1000 individuals age between 10 to70 years were observed for 10 different morphogenetic traits such as hair , widow's peak, eye brow position, dimples, earlobe, tongue rolling, cleft chin, face shape, skin colour, and hand clasping from which 500 were men and 500 were women. The population was surveyed for observation of different traits.

The collected data was tabulated according to the individual's name, sex, age, trait and whether it was a dominant or recessive trait based on the phenotypic expression in that individual.

Following are the description of selected morphogenetic traits, we have observed for dominance and recessiveness that can be easily observed in people around as.

1. HAIR WAVE:

It is assumed that hair straightness and curliness is controlled by a single pair of alleles showing a partial dominance. An individual with curly hair possesses homozygous dominant allele and straight hair is governed by a homozygous recessive allele.

2. WIDOW'S PEAK:

The trait "widow's peak" is characterized by the presence of a pointed 'V' shaped patterns in the hairline in the center of the forehead. The hairline

drops downward in the center of the forehead. The allele for this trait is dominant over straight or curved hairline Winchester (1979). It is inherited as an autosomal dominant character.

3. DIMPLES IN CHEEK:

In certain individual, fat pads in the cheeks hang downward due to their low placement and thus from a depression called dimple. Dimples are round indentations in the cheek when or dimples in cheek. Dimples (DD or Dd) are dominant over no dimples (dd) (Winchester, 1979). It is inherited as an autosomal dominant trait (Ebeye et al., 2014).

4. EARLOBE:

Earlobes may be attached or free in an individual. If earlobes hang free, they are detached. If they attach directly to the side of the head, they have attached earlobes. The attached earlobe is controlled by a recessive gene while free earlobe is controlled by a dominant gene (Ebeye et al., 2014).

5. TONGUE ROLLING:

Some people, when their tongue extend, are able to roll it into a U-Shaped configuration. The ability to turn up the lateral edges of the tongue is due to the single dominant gene (Liu and Hsu , 1949). The ability to roll the tongue into a U shaped trough is dominant over lack of this rolling ability.

6. CLEFT CHIN:

People can have a cleft chin or smooth chin. Cleft chin or dimple chin refers to a dimple on the chin. It is a Y-shaped fissure on the chin with an underlying bony peculiarity. If there are any malformations in the structure of mandible bone then cleft in is occurred. This is an inherited trait in humans,

where the dominant gene causes the cleft chin, while the recessive genotype presents without a cleft.

7. HAND CLASPING:

In a relaxed interlocking of fingers, left thumb over right results from having one or two copies of the dominant version of the gene. People with two recessive place right thumb over left.

8. SKIN COLOUR:

Skin colour varies continuously in modern population, Each gene has two forms ; Dark skin allele and light skin allele. Dark skin allele is dominant over the light skin allele.

9. FACE SHAPE:

Genes that determine the shape of a person's facial profile. An oval-shaped face is a dominant feature, while a square-shaped face is recessive.

10. POSITION OF EYEBROWS:

The ideal brow position varies between male and female free

Eyebrows are dominant traits while attached eyebrows are recessive.

S.No	Morphogenetic characters	Dominant	Recessive
1	Hair wave	Curly	Straight
2	Widow's peak	Present	Absent
3	Eyebrow	Free	Attached
4	Dimples	Present	Absent
5	Earlobe	Free	Attached
6	Tongue Rolling	Yes	No
7	cleft chin	Yes	No
8	Face shape	Oval	Others
9	skin colour	Black	White
10	Hand clasping	Left Thumb	Right thumb

RESULTS

6. RESULTS:

The survey was carried out in Tuticorin town from December 2020 to March 2021.

A total of 1000 individuals, 500 males and 500 female were observed for morphogenetic traits. Hair wave, widows peak, position of eyebrows, dimples in cheek ,earlobe, tongue rolling, cleft chin, face shape, skin colour, hand clasping characters were visually observed an the data were collected.

The results showed the clear cut view of the expression of traits in each individual in the population. A graph were plotted for dominant and recessive traits classified based on their expression.

Table: 1 shows that out of 1000 individuals, 230 individuals are having curly hair and 770 are having straight hair.

About 204 individuals of the population have widows peak and 796 has normal peak.

About 647 individuals of the population have free eyebrows and 353 has attached eyebrows.

The survey showed that 229 of the population had facial dimples and 771 subjects are without this trait.

About 678 individuals of the population have free earlobes and 322 has attached earlobes.

The survey results showed that 557 individuals were capable to roll their tongue and 443 were not able to rolling their tongue.

Out of 1000 individuals, 149 individuals are having cleft chin and 851 individuals are having smooth chin.

About 521 individuals of the population have oval shape of face and 479 has other shapes

Out of 1000 individuals 601 individuals are black in colour and 399 individuals are white in colour.

The pattern of crossing of thumbs were also surveyed here results showed that 469 individuals are cross their thumb in a pattern of left over right, 531 individuals cross their thumb in a pattern of right over left.

(Fig.1)&(Table 1) shows that traits like earlobe(67.8%) ,tongue rolling(55.7%),face shape(52.1%),skin colour (60.1%),position of eyebrows(64.7%) are expressed as dominant. Whereas traits like hair wave(23%),widows peak(20.4%) ,dimples in cheek(22.9%),cleft chin(14.9%),hand clasping(46.9%) are expressed as recessive .

Table: 1 Distribution of dominance and recessiveness of selected traits in the sampled traits.

S.NO	TRAITS	FEATURES	NO OF INDIVIDUALS	PERCENTAGE
1.	Hair wave	Curly(D)	230	23%
		Straight(R)	770	77%
2.	Widow's peak	Present(D)	204	20.4%
		Absent(R)	796	79.6%
3.	Dimples in cheek	Present(D)	229	22.9%
		Absent(R)	771	77.1%
4.	Earlobe	Free(D)	678	67.8%
		Attached(R)	322	32.2%
5.	Tongue rolling	Can roll(D)	557	55.7%
		Cannot roll(R)	443	44.3%
6.	Cleft chin	Present(D)	149	14.9%
		Absent(R)	851	85.1%
7.	Face shape	Oval(D)	521	52.1%
		Others(R)	479	47.9%
8.	Skin colour	Black(D)	601	60.1%
		White(R)	399	39.9%
9.	Position of eyebrows	Free(D)	647	64.7%
		Attached(R)	353	35.3%
10.	Hand clasping	Left thumb(D)	469	46.9%
		Right thumb(R)	531	53.1%

TABLE 1

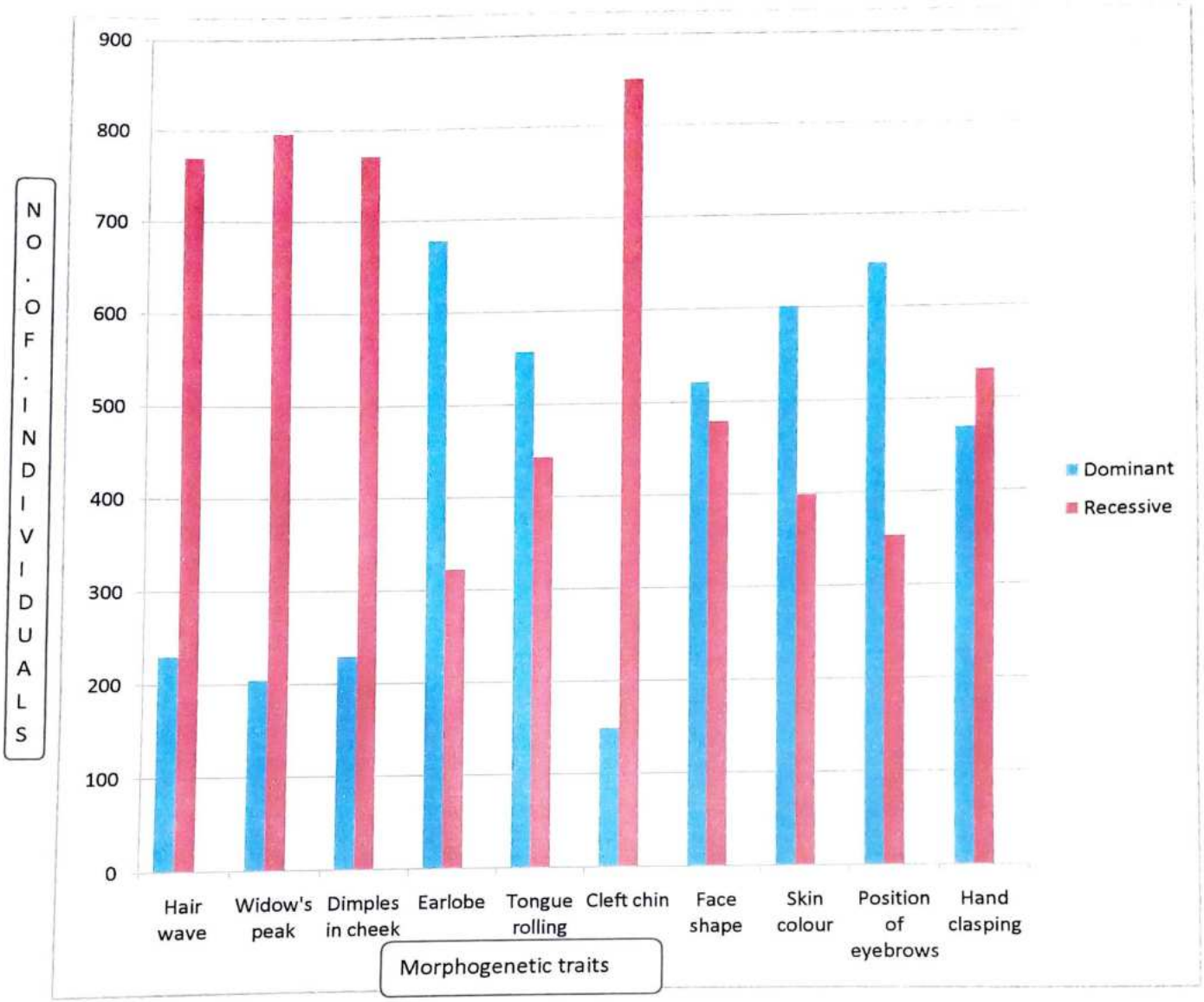


Fig .1 Difference between dominance and recessiveness of the trait among total individuals

Table: 2 shows that percentage wise difference between male and female subjects. About 22% males and 24% females having curly hair. Other 78% of male and 76% females having straight hair. Genderwise, 23.2% of males and 17.6% females had widows peak. About 70.6% males' 58.8%females having free eyebrows. Others are attached eyebrows. The survey showed that 17.8% males and 28%females had facial dimples. Free earlobes were seen in 65.6%males and 70%females.About at 63.6% males and 47.8% females were able to roll their tongue. The survey showed that 14.9% of the population had cleft chin .Only 15% of males and 14.8% of females showed this trait. Oval shape of face were seen in 59.2% males 45% females. About 68.6% males and 51.6% females had black skin colour. Others having white skin.52.2%of males and 41.6%females had cross their thumb left over right. Others are crossed their thumb right over left.

The study showed that there was no significant difference present between male and female in cleft chin (14.9%).

Traits like tongue rolling (63.6%), hand clasping (52.2%) and facial shape (59.2%) are expressed as dominant in males and recessive in females. Males and females both are dominant in traits like eyebrow position (70.6%)(58.8%),earlobe(65.6%)(70%),skin colour(68.6%) (51.6%).

Males and females both are recessive in traits like hair wave (22%)(24%), widow's peak(23.2%)(17.6%),dimples in cheek (17.8%)(28%),cleft chin (15%)(14.8).

Table:2.The percentage wise difference of dominant and recessive traits between male and female.

S.NO	MORPHOGENETIC TRAITS	FEATURES	GENDER	FREQUENCY	NUMBER OF DOMINANT AND RECLESSIVE INDIVIDUALS	PERCENTAGE
1.	Hair wave	1.Curly (D) 2.Straight (R)	Male	500	1. 110 2. 390	1. 22% 2. 78%
		1.Curly (D) 2.Straight (R)	Female	500	1. 120 2. 380	1. 24% 2. 76%
2.	Widow's peak	1.Present (D) 2.Absent(R)	Male	500	1. 116 2. 384	1. 23.2% 2. 76.8%
		1.Present (D) 2.Absent(R)	Female	500	1. 88 2. 412	1. 17.6% 2. 82.4%

3.	Position of eyebrows	1.Free (D) 2.Attached(R)	Male	500	1. 353 2. 147	1. 70.6% 2. 29.4%
		1.Free(D) 2.Attached(R)	Female	500	1. 294 2. 206	1. 58.8% 2. 41.2%
4.	Dimples in cheek	1.Present (D) 2.Absent(R)	Male	500	1. 89 2. 411	1. 17.8% 2. 82.2%
		1.Present (D) 2.Absent(R)	Female	500	1. 140 2. 360	1. 28% 2. 72%

5.	Earlobe	1.Free(D) 2.Attached(R)	Male	500	1. 328 2. 172	1. 65.6% 2. 34.4%
		1.Free(D) 2.Attached(R)	Female	500	1. 350 2. 150	1. 70% 2. 30%
6.	Tongue rolling	1.Can roll(D) 2.Cannot roll(R)	Male	500	1. 318 2. 182	1. 63.6% 2. 36.4%
		1.Can roll(D) 2.Cannot roll(R)	Female	500	1. 239 2. 261	1. 47.8% 2. 52.2%

7.	Cleft chin	1.Present (D) 2.Absent(R)	Male	500	1. 75 2. 425	1. 15% 2. 85%
		1.Present (D) 2.Absent(R)	Female	500	1. 74 2. 426	1. 14.8% 2. 85.2%
8.	Face shape	1.Oval(D) 2.Others(R)	Male	500	1. 296 2. 204	1. 59.2% 2. 40.8%
		1.Oval(D) 2.Others(R)	Female	500	1. 225 2. 275	1. 45% 2. 55%
9.	Skin colour	1.Black(D) 2.White(R)	Male	500	1. 343 2. 157	1. 68.6% 2. 31.4%
		1.Black(D) 2.White(R)	Female	500	1. 258 2. 242	1. 51.6% 2. 48.4%

10.	Hand clasping	1.Left thumb(D) 2.Right thumb(R)	Male	500	1. 261 2. 239	1. 52.2% 2. 47.8%
		1.Left thumb(D) 2.Right thumb(R)	Female	500	1. 208 2. 292	1. 41.6% 2. 58.4%

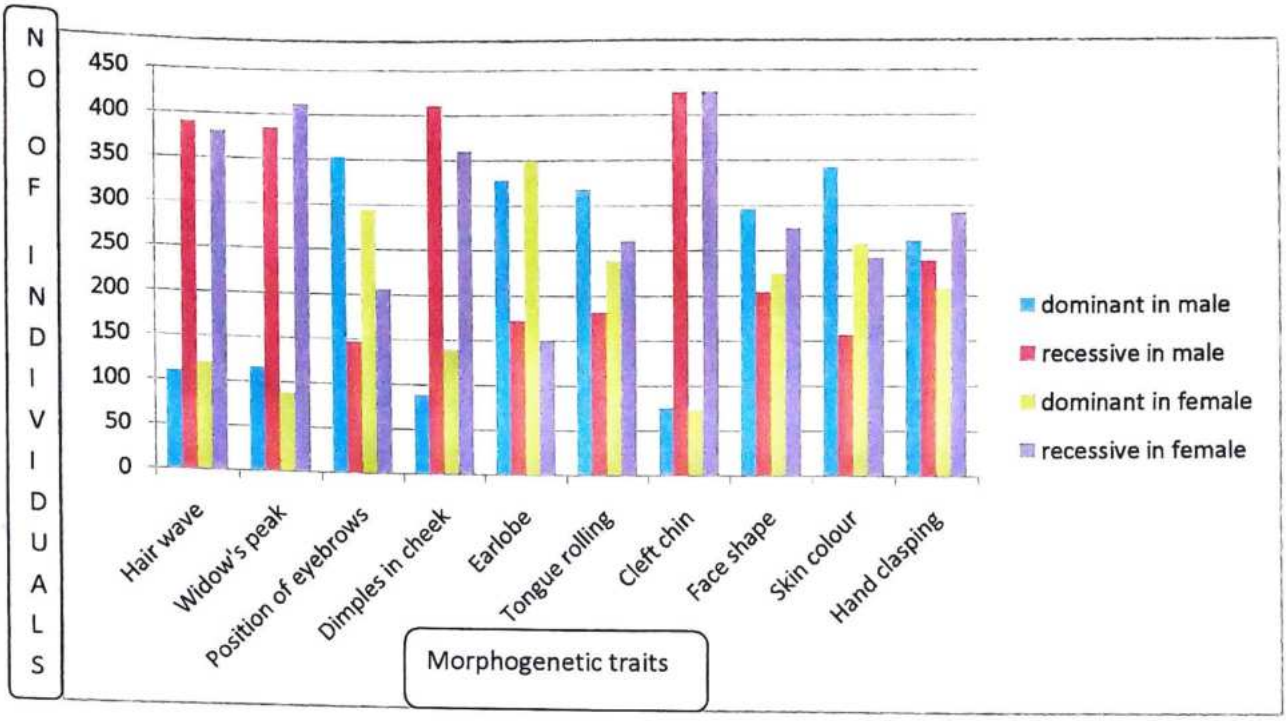


Fig 2: Difference between dominance and recessiveness in males and females

Plate 1

Plate 2

Plate 3

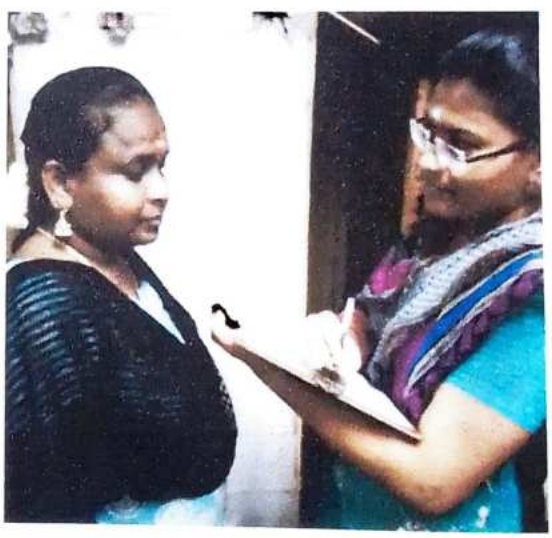


Plate 4

Field Work - Survey of Data Collection

DISSCUSSION

7. DISCUSSION:

A phenotypic trait is an obvious, observable, and measurable trait; it is the expression of genes in an observable way. An example of a phenotypic trait is a specific hair color. Underlying genes, which make up the genotype, determine the hair color, but the hair color observed is the phenotype.

The phenotype is dependent on the genetic make-up of the organism, and also influenced by the environmental conditions to which the organism is subjected across its ontogenetic development, including various epigenetic, processes. Regardless of the degree of influence of genotype versus environment, the phenotype encompasses all of the characteristics of an organism, including traits at multiple levels of biological organization, ranging from behavior and evolutionary history of life traits (e.g., litter size), through morphology (e.g., body height and composition), physiology (e.g., blood pressure), cellular characteristics (e.g., membrane lipid composition, mitochondrial densities), components of biochemical pathways, and even messenger RNA.

Heredity is **important** to all living organisms as it determines which **traits** are passed from parent to child. Successful **traits** are more frequently passed along and over time can change a species. Changes in **traits** can allow organisms to adapt to specific environments for better rates of survival.

Traits are **important** and interesting because they describe stable patterns of behavior that persist for long periods of time (Caspi, Roberts, & Shiner, 2005). Importantly, these stable patterns can have broad-ranging consequences for many areas of our life (Roberts, Kuncel, Shiner, Caspi, & Goldberg, 2007). The expression of morphogenetic traits varied among human populations in which they have been reported, and are known to be inherited as autosomal dominant or autosomal recessive manner (D. L. Hartl, 2011) (K. Molly. Houghton, J.Dawei, 2010.)

Genes are the tiny pieces of molecular information that determine who we are. They are responsible for everything, from our curly or straight hair to whether or not will develop certain health conditions later in life.

There are two types of genetic traits: dominant and recessive. When combined together in an offspring, the dominant trait will always be expressed over the recessive trait. For example, traits such as immunity to poison ivy, normal eyesight and hearing, and normal blood clotting abilities are all expressions of dominant genes. Traits such as albinism, hemophilia, deafness, and poor eyesight are all less desirable recessive traits. .

For example, the gene for having an extra finger is actually dominant, while the gene for having a tall stature is a recessive trait. If it happens to be a hairy person, it can guarantee that the children will also inherit this particularly fuzzy trait because it's caused by a dominant gene.

The ability to sing well is recessive so you may be able to pass it on to your children even if you can't hold a note yourself. Other more obscure dominant traits are the ability to roll our tongue and a tendency to cross our left thumb over our right when folding our hands.

The expression of dominant and recessive characters may vary and may depend on various factors. A study showed dominant trait such asposition of eyebrows(64.7%),earlobe(67.8%),skin colour(60.1%) in both males and female then tongue rolling(63.6%),face shape(59.2%),hand clasping(52.2%) in males.

In the result of curly hair of male and female is 22% and 24% and straight hair of male and female is 78%and 76%.It was reported that straight hair is specifically present in an Asian population frequently(Fujimoto et al., 2007,2009).The investigation showed that there are three types of cells in the cortical region of hair namely orthocortex , mesocortex and paracortex. As the curly nature of hair increases, the mesocortical cells decrease while in straight hair mesocortical macrofibirils have more in concentration (Thibaut et al., 2007).

Result from widows peak showed 23.2%in males and 17.6% in females had widows peak while individuals who had straight hair line were 76.8%in males and 82.4% in females. Inheritance of cheek dimples, widows peak and earlobe attachment follow the simple Mendelian law of inheritance as no difference was observed in both with gender (Ordu et al., 2014).

Result from eyebrow position showed 70.6% in males and 58.8% in females had free eyebrow while individuals who had attached eyebrows were 29.4% in males and 41.2% in females. Measurements of eyebrows location and rate of change vary between genders and within ethnic groups (Lora Rabin Dagi Glass et al., 2014).

The prevalence of facial dimple of present study was 22.9% total in which 17.8% were male and 28% were female. Facial dimple were observed in Greek children showed low frequency in male(12.7%) and high frequency in female(13.08%) reveals same results when compared with current study

(Pentozos et al.,2004).Another study conduct in south west Nigeria showed high frequency in female 13.2% has dimple while male has low frequency rate 9.0%(Omotoso et al.,2010).

The prevalence of earlobe of present study was 67.8% total in which 65.6% were male and 70% were female .The attached earlobe present in 32.2%which is close to the result of Indian and Ekpoma Nigeria population containing 35.1%(Sharma, A .et al.,2007)and 31.61%(Ordu .K.S. et al .,2014).

The percentage of tongue rollers 55.7% was higher compared to non rollers 44.3% with male dominance 63.6%compared to female 47.8%. This is in tandem with the results of the studies conducted among the Bini (Anibor E. et al., 2014) and Southern Nigerians (Onyije F.M, 2012). The finding of the present study is also in tandem with a previous report of a study among European ancestry that the percentage of tongue rollers was higher than those of non rollers (Sturtevant A., 1940). Matlock (1952) studying 33 pairs of identical twins observed seven pairs who were discordant. From these observations he had to conclude that the tongue rolling is not entirely heredity.

In the current study the frequency of the cleft chin was 14.9% out of which 14.8%were females showing low frequency and 15%male with high frequency .Study conducted in south west Nigeria chin dimple more frequent in female 2.0%as compared to male 1.6% show dissimilarity with current study(Omotoso G.O et al.,2010)

In the current study the frequency of the facial shape was 52.1% out of which 59.2% were males showing higher frequency and 45%females with lower frequency. According to Mortaza Bonakdarchian et al., who studied in

2009 that results showed higher bite force in men and those with square face form.

The prevalence of black skin of present study was 60.1% total in which 68.6% were male and 51.6% were female. Most studies of age trends report more consistent trends in females than in males (Kahlon, 1976).

In the current study the frequency of the left thumb cross over right thumb was 46.9% out of which 52.2%were males showing higher frequency and 41.6% females with lower frequency. Wiener (1932) and Lai and Walsh (1965)agreed that hand clasping had no relation to genetic factor.

CONCLUSION AND SUGGESTION

8. CONCLUSION:

This study helps us

Better understand ourselves and improve our health.

Know the frequency of distribution of morphological, genetical and behavioral traits among people

Recognize the presence of diversity in a population

Appreciate the uniqueness in each of us.

SUGGESTIONS:

Data from this study will be useful in genetic analysis and determination of percentage.

Study of the inheritance pattern of human traits is one of the significant methods for analyzing the genetic history of the population.

This study will help to trace their ancestral inheritance pattern and reaches at accurate understandings about the family history.

This type of study always helps for the treatment of genetic diseases in the family through the pedigree analysis.

Our data with some more characters to be studied in future can throw light on the origin and evolution of the population under study.

We believe that these studies may greatly enrich our knowledge of human evolution history and elucidate the genetic basis of complex traits in human.

SUMMARY

9. SUMMARY:

The survey was carried out in Tuticorin town from December 2020 to March 2021.

A total of 1000 individuals, 500 males and 500 female were observed for morphogenetic traits. Hair wave, widows peak, position of eyebrows, dimples in cheek ,earlobe, tongue rolling, cleft chin, face shape, skin colour, hand clasping characters were visually observed an the data were collected.

Through this type of study we easily understand the normal skin with melanin / albino.

Hormonal factors are also depended on dominant and recessive characters.

The shape and type of hair are determined not just during embryogenesis but also repeatedly in each hair growth cycle. It may be depend on particular type of cell present in the hair.

The recessive character of widow's peak may be a reason of age factor. We concluded the position of eyebrow is a inherited trait.

Cleft chin and cheek dimple are the defects of mandible and musculature of human.

Tongue rolling ability is not inherited as a simple mendelian dominant recessive trait.

Free or attached earlobes may be depend on age factor or it based on facial shape.

Facial shape and hand clasping may be transmitted in the mendelian pattern or non-mendelian pattern.

Environmental factors play a major role in determining skin colour.

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STUDIES ON DISTRIBUTION OF MOLLUSC SHELLS FROM DIFFERENT STATIONS OF THOOTHUKUDI DISTRICT

A field work submitted to

ST.MARY'S COLLEGE(Autonomous),

THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

In partial fulfillment for the award of the degree of

MASTER OF SCIENCE IN ZOOLOGY

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ARRIL- 2021

CERTIFICATE

This is to certify that the field work entitled '**Studies on distribution of Mollusc shells from different Stations of Thoothukudi District'** is submitted to St. Mary's College (Autonomous), Thoothukudi affiliated to Manonmaniam Sundaranar University in partial fulfilment for the award of the degree of Master of Science in Zoology and it is a field work done during the year 2020-2021 under my guidance and supervision. It is further certified that this field work report or any part of this has not been submitted elsewhere for any other degree by the following students.

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EXAMINER

DECLARATION

I do hereby declare that this thesis entitled, "***Studies On Distribution Of Mollusc Shells From Different Stations Of Thoothukudi District***" submitted by me for the award of the degree of Master of Science in Zoology is the result of my original independent research work carried out under the guidance of **Dr. M. Paripooranaselvi M.Sc., M.Phil., B.Ed., Ph.D., SET**., Assistant Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date: 16.04.21

Signature of the Candidates

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CONTENTS

INTRODUCTION

In the geological time scale, molluscs evolved about 600 million years ago and this phyla is a very old monophyletic lineage, dating from before cambriam, occurring in many habitat types (G.M Barker, 2001). Mollusca (Latin : Mollis = soft) comprise the second largest phylum of invertebrates in the number of species, over 80,000 living species have been described, and in addition about 35,000 fossil species are known. Possession of hard shells increases the chance of preservation, this has resulted in a rich fossil record of molluscs which came into existence in the early Cambrian. Mollusca appear to be a heterogenous group with great diversity of form, but all of them are built on the same fundamental plan. The phylum molluscan, which includes soft – bodied invertebrates. These are the commonest organisms of Indian sea beaches and distributed all over the world in almost all types of habitats .

Molluscan are primitively bilaterally symmetrical animals with soft short bodies which show no segmentation, bilaterally symmetry may be lost in some. The body has an anterior head, a dorsal visceral hump, a ventral muscular foot modified for crawling, burrowing or swimming. Around the body is a fleshy mantle which secretes a calcareous shell, the shell is usually external, though it may be internal, reduced or absent. The shell may be of one piece and called univalve, or of two parts are known as bivalve. Between the mantle and the body is a mantle cavity into which the anus and kidneys open, and in which lie a pair f ciliated gills or ctenidia having an axis bearing

leaf – like branches on both sides, but the number of gills may be much larger in some molluscs. The schizocoelic coelum is reduced to cavities of pericardium, gonads and kidney, the main body cavity is a haemocoel. The large amount of blood in the haemocoel can be manipulated by body wall muscles, this brings about change of shapes, dilution of foot, and extension of proboscis and head. There is a dorsal heart with one or two auricles and a single ventricle, the respiratory pigment is haemocyanin. Respiration occurs by the mantle, epidermis, one to several ctenidia or by a lung in the mantle cavity. Sexes are usually separate.. Fertilization is external or internal. development is either direct or there is modified trochosphere called a veligar larva.

Molluscan are mostly marine, though some are found in freshwater and a few are terrestrial. The body is clothed with one layered often ciliated epidermis. Body is commonly protected by an exoskeletal calcareous shell of one or more pieces, secreted by the mantle. Head is distinct, bearing the mouth and provided with eyes, tentacles and other sense organs except in pelecypoda and scaphopoda. Ventral body wall is modified into a muscular flat or plough like surface, the foot which is variously modified for creeping, burrowing, and swimming. Digestive tract is simple with an anterior mouth and posterior anus but in gastropods, scaphopods and cephalopods the intestine becomes U–shaped bringing the anus to an anterior position. pharynx contains a rasping organ, the radula except in pelecypoda.

Circulatory system is open except in cephalopods which shows some tendency towards a closed system. The members of this phylum show a great diversity of form, they include such familiar animals such as chitons, snails, slugs, clams, oysters, squids and octopus.

Gulf of Mannar is the richest source of marine molluscs which is situated in the south east coast of India. The mollusc constitutes a significant part of the world fauna today. Mollusca are second only to arthropoda in numerical abundance. The number of species identified under phylum molluscan varies between 80,000 to 1,00,000 around the world ocean. They are more abundant in the littoral zones of tropical seas.

Among the molluscs, the gastropoda constitutes an important group. Gaster = belly + podos = foot. They are characterized by the presence of shell, a fold of body wall, thee mantle and a muscular organ of locomotion, the foot. Gastropods are marin, freshwater, terrestrial and few parasitic on echinoderms. Body is unsegmented, asymmetrical typically with a univalve, spirally coiled shell. Head is distinct bearing tentacles, eyes, and mouth. Foot is ventral, broad, flat and muscular forming the creeping sole and often bearing dorsally a hard piece, the operculum on its posterior end. Visceral mass spirally coiled exhibiting torsion. Mantle is a collar like fold of body wall, lining the body whorl leaving a space, the mantle cavity between itself and body. Buccal cavity contains an odontophore with a radula bearing rows of chitinious teeth. Digestive system comprises a muscular pharynx, long

oesophagus, stomach and long coiled intestine and anteriorly placed anus. Respiration occurs by gills in most form, through the wall of the mantle cavity in some forms and in many by lungs. Circulation system is open and the heart is enclosed in a pericardium. Excretory organs comprise metanephridia which are paired in primitive forms and reduced to a single nephridia in most forms. Nervous system comprises distinct cerebral and pleural besides buccal, pedal, parietal and visceral ganglia. Sexes are separate in most forms, while in some forms united. Development includes trochosphore and veliger larval stages. The magnificent shells of gastropods are used for decoration, ornamentation and in jewellery and lime making (Somneuk, 1991) and in ancient times as utensils. The shells are cleaned polished and sold as curious. Further the opercula of gastropods have also received considerable attention since they are used in the preparation of dyes, perfumes, cosmetics and medicines (Vovells, 1967).

Some molluscs are indirectly harmful to man but most of them are beneficial. The harmful molluscs are slugs and shipworms. Slugs are injurious in gardens and cultivations, they not only eat leaves but also destroys plants by cutting up their roots and stems. Teredo, the shipworm burrows into wooden structures immersed in the sea, it causes serious damage to wharves, piers and ships. But molluscs are a great source of human food in various parts of world, millions of maunds and clams, oysters, scallops and mussels are eaten in China, Japan, Malaya, Europe and America, oysters

being regarded as a delicacy. Bivalves, octopuses and cuttlefishes furnish large quantities of food in Europe. Shells of freshwater mussels are used in a pearl button industry in all parts of the world, they are made from the nacreous layer of shells, no other material stands laundering as these buttons. Shells of oysters are mixed with tar for making roads in America and lime from these shells is used in feeding for poultry for forming of their egg shells. Lime is also used in building in many parts of the world, molluscan shells are used for making ornaments and jewellery, in some parts shells of cryprae are used as money and as ornaments. Many freshwater clams and marine oysters produce pearls, but the most valuable pearls are produced by pearl oysters *pinctada margaritifera* and *pinctada mertenci* which inhibit the warmer parts of Indian and pacific oceans along the coasts of China, India, Srilanka and Japan. A pearl is made when a small foreign object, such as particle of sand or a parasite, lodges between the shells and the mantle. The foreign object becomes a nucleus around which concentric layers of nacreous are laid by mantle, in this manner a pearl is formed, but pearls are also produced by most pelecypods including freshwater clams. In Japan, pearl culture is practiced by artificially introducing a small solid or liquid irritant below the mantle of the oyster, the resultant one year old pearl is then transplanted to another oyster, a pearl good size is obtained in three years after transplanting.

OBJECTIVES

In the present study a systematic investigation on the occurrence of molluscs in four different stations of Thoothukudi district - Station 1 – Vellapatti, Station 2 – Tharavaikulam, Station 3 – Keelavaipar and Station 4 – Threspuram to find out

1. Species composition of different stations
2. Distribution of species

REVIEW OF LITERATURE

Bouchet and Rocroi (2005) described that according to the current classification of Gastropoda, B. spirata belongs to order Neogastropod. The order Neogastropoda is a highly diversified group of predatory marine shelled gastropods with more than 16,000 living species of many well known ecologically significant super families including Buccinoidea, Muricoidea, Olivoidea, Pseudolivoidea, Conoidea and Cancellarioidea. Heulsken (2008) observed the genus Babylonia belongs to the family Buccinidae of super family Buccinoidea. Buccinidae is one of the most diverse families of Neogastropoda. Therefore the phylogenetic status of B. spirata mains quite ambiguous. Hellberg and Morrisey (2011) reported that the DNA is more informative than protein and can be easily extracted from small traces of organic material. Ramussen and Morrissey (2008) described that the PCR based methods are extremely sensitive, often more rapid than other techniques and are widely used in the fishery industry. Ravindra kumar *et al*., (2009) reported that the genetic characterization of fish and shellfish species that are particularly threatened or economically important species is usefull for planning their conservation strategies for safeguarding biodiversity. Arularasan *et al*., (2014) studied the species diversity of *B. spirata* in Tamil Nadu coast, less genetic diversity even though distinct population structure. He also described that highly informative for characterization of diversity in this gastropod species. Skujiene and Soroka (2005) observed the 18S rRNA is

currently one of the most widely used gene for phylogeny, systematic and the identification of species for gastropod tissue; however, there is only little information available on the amplification success of the 18S rRNA gene fragment as a function of preservation storage media, storage duration or DNA extraction. Saitou and Nei (1987) introduced the Neighbor- Joining method and it has become the most widely used methods for building phylogenetic tree distances. The Neighbor-Joining method is a greedy algorithm, which attempts to minimize the sum of all branch-length on the constructed phylognetic tree. Conceptually, it starts out with a star formed tree where each leaf corresponds to a species and it relatively picks two nodes adjacent to the root and joins them by inserting a new node between the root and the two selected nodes (Thomas Mailund *et al*., 2006). Studies of a using the 18S gene sequence of the ribosomal gene to evaluate the relationships among the various bivalve subclasses have been conducted on a small number of Heterodonta species Adamkewicz *et al*., (1997) and Canapa *et al*., (1999).

Jayalakshm (2016) have reported that the edible body tissue of *Babylonia* sp an excellent source of high protein, low lipid content but enriched with essential vitamins and minerals. It is can be taken regularly as animal protein supplement or nutritive seafood which supplies all vital nutrients for the growing children, pregnant women and people suffering from malnutrition. Ansari *et al*., (1981) has observed that the gastropod meats are considered to be on par with other animal foods in terms of nutritional

values. However, nutritionally *B. spirata* remains under utilized in the country while the shell utilized for ornamental purposes. Chandrasekaran (1985) has also observed the operculum is having great economic importance, yielding good income to the poor fisher- folk. In general animals in the aquatic environment carry bacterial flora, which is a reflection of the flora in the environment. Works on *B. spirata* in India are very limited and are of preliminary natural particularly on fishery status only. Hahn (1989 and 1989) has described that the artificial diets allow mechanized production to increase survival rates and generally to produce better growth in weight and length than natural food. The slow growth and poor survival of spiral babylon's resulting from inappropriate or inadequate food supply during the growing out period will prolong holding time and increase hatchery expenditure. Periyasamy *et al*., (2014) has reported that the shellfish proteins are rich in essential amino acids, which are required for the growth, reproduction and synthesis of vitamins. Aquatic animal fats are good sources of essential fatty acids that are not synthesized in the human body. The fatty acids have a very distinctive character compared to fatty acids from other sources. Patterson Edward *et al*., (2006) also reported the status of the feasibility of culturing spiral babylon, *B. spirata* in Tuticorin coast. The male and female ration was observed to be 1:1.5 Male had an average length of 45.4 mm and weight 26.36 g and Female (47.4 mm) in length and 28.62 g in weight during the three years culture period. Sogbesan *et al*., (2006) has reported that the

protein from fish meal is most suitable for snail feed. Due to its high cost, adequate substitution of this ingredient will significantly reduce operating cost in aquaculture farms. Nowadays, the lack of research and development on appropriate feeds to culturing Spiral Babylon is a major constraint for it malacological studies. Zhou *et al*., (2007) reported that the weight gain of juvenile *B. aerolata* increased by increasing dietary lipid level from (1.83% to 5.91%) and slightly decreased weight gain and shell length increment hereafter with further increasing in dietary lipid level above 7.80% and the optimal dietary lipid requirement for maximum protein gain of juvenile of about 6.54% of dry diet. Sirusa Kritsanapuntu *et al*., (2013) reported the partial replacement of tuna oil by corn oil in formulated diets of *B. areolata* under hatchery conditions have no effects on growth performance but fat content of the whole body reduced to half than those contained in formulated diets. Similarly, Chelladurai *et al*., (2014) have reported that the *B. spirata* showed a good prospect for commercial aquaculture due to its resistance to the environment. Its growth seems to be rapid and its meat was delicious for soups and salads. Its market demand for this gastropod was noted to be rapidly increasing. It has been reported that the overfishing the notable cause the sharp decline in their natural stocks.

MATERIALS AND METHODS

Current study involves identifications and quantitative analysis of mollusc of Thoothukudi coast. Samples of mollusc were collected from four locations covering one coastal district. The investigations were carried out in the following 4 stations of Thoothukudi district.

- Station 1 – Vellapatti
- Station 2 – Tharavaikulam
- Station 3 – Keelavaipar
- Station 4 - Threspuram

STATION 1 - VELLAPATTI:

This station is situated near Ayyanpuram. Samples were collected from the seashore and littoral zone, which offers excellent habitat for the settlement of mollusc during low tides.

STATION 2 - THARAVAIKULAM:

Observation were made and this station is situated near samathuvapuram, samples were only collected from the seashore.

STATION 3 - KEELAVAIPAR:

Keelavaipar station is situated near Vaippar. Shells were collected from the fishingnets and the seashore.

STATION 4 - THRESPURAM:

Threspuram is situated near Poobalrayerpuram, samples were collected from fishingnets.

RESULT

In the current study 44 species of molluscs were identified in 4 stations of Thoothukudi district. Figure 1 and 2 shows the total of 14 species including 10 gastropods and 4 bivalve were recorded from station 1. From station 2, 09 species (7-Gastropods and 2-Bivalves), from station 3, 09 species (8-Gastropods and 1-Bivalve) and from station 4, 12 species (7-Gastropods and 5-Bivalves) were recorded.

In station – 1 (Plate:1 & 2), the available species were *lambis lambis, Cerastoderma edule, Semicassis granulate, Spondylus gaederopus, Chicoreus ramosus, Paratapes textali, Lophiotoma indica, Megastraea turbanica, Conus arenatus, Crypraea onyx, Americoliva sayana, Turritellinae acropora, Babylonia spirata and Chione cancellata.* From station-2 (Plate: 3), the observed species were *Lambis lambis*, *Chicooreus ramosus*, *Anadara rhombea*, *Cerastoderma*

edule, *Turritellnae acropora*, *Murex trapa*, *Lophitoma indica*, *Conus arenatus*, *Hemifusus pugilinus.* In station – 3 (Plate: 4), the collected species were *Chicoreus ramosus*, *Acanthocardia aculeate, Melongena melongena, Laevistrombus turturella , Strombus Campbellium, Fasiolaria filamentosa , Xancus pyrum , Strombus canarium, Crypraea tigris.* In station – 4 (Plate:5), the collected species were *Chicoreus ramosus*, *Conus arenatus, Cerastoderma edule, Donax faba, Hemifusus pugilinus, Anadara rhombea,*

cypraea moneta, Cymatium perryl, Mactra stultorum, Pecten albicans, Murex ternispina, Semicassis granulate.

STATION 1 - VELLAPETTI :

DESCRIPTION OF THE SPECIES:

Lambis lambis

Phylum : Mollusca
Class : Gastropoda
Family : Strombidae
Genus : *Lambis*
Species : *Lambis lambis*

Key characters:

1. The maximum shell length for this species is up to 29 cm, and average length stands for 18 cm.
2. *Lambis lambis* has a very large, robust and heavy shell. One of the most striking characteristics is its flared outer lip, ornamated by six hallow marginal digitations.
3. The colour of the shell is highly variable, being white or cream externally and often presenting brown, purplish or bluish black patches. The interior is glazed and may be pink, orange or purple.

Cerastoderma edule

Phylum : Mollusca
Class : Bivalva
Family : Cardiidae
Order : Cardiida

Genus : *Cerastoderma*

Species : *C.edule*

Key characters :

1. It typically reaches from 3.5 centimeters to 5 centimeters in length, but sometimes it reaches 6 centimeters.
2. The shells are pale or whitish yellow, grubby white or brown.
3. The shells are oval, are covered by ribs, which are flattened in the middle part of the shells.

Semicassis granulate

Phylum : Mollusca

Class : Gastropoda

Family : Cassidae

Genus : *Semicassis*

Species : *S.granulata.*

Key characters:

1. *Semicassis granulate* is a medium sized sea snail, a marine gastropod mollusc is a sub family of cassine, the helmet shells and bonnet shells.
2. In the spring, the adult females of this species lay eggs in the tower shaped structures.
3. It alludes to the general outline and coloured pattern of the shell.
4. The shell is egg-shaped and fairly large 2 to 4 inches in maximum dimention with the regular pattern of yellow, orange or brown.
5. The surface sculpture of the shell is highly variable, the surface can be smooth and polished.

Spondylus gaederopus

Phylum : Mollusc

Class : Bivalve

Order : Pectinida

Family : Spondylidae

Genus : *Spondylus*

Species : *S.gaedropus*

Key characters:

1. *Spondylus gaedropus* attaches itself to the substrates with its lower valve, which is usually white.
2. The upper valve is usually purple. Specimens that are all white or all purple do, however exist.
3. The mollusc is edible and is consumed in Sardinia.

Chicoreus ramosus:

Phylum : Mollusc

Class : Gastropoda

Family : Muricidae

Genus : *Chicoreus*

Spcies : *C . ramosus*

Key characters:

1. *Chicoreus ramosus* has a very large, solid, very rugged and heavy shell of upto to 330 mm in length.
2. It has a relatively globose outline, possessing a short spire, a slightly inflated body whorl, and a moderately long siphonal canal.
3. One of its most striking ornamentations are the conspicuous, leaf-like, recurved hollow digitations.

4. It also presents three spinose axial varices per whorl, with two elongated nodes between them.
5. The shell is coloured white to light brown externally with a white aperture, generally pink towards the inner edge, the outer lip and the columnella.

Paratapes textilis

Phylum : Molluscan
Class : Bivalve
Order : Venerida
Family : Veneridae
Genus : *Paratapes*
Species : *P. textalis*

Key characters:

1. Shell of *paratapes textalis* can reach a length of 3-4 centimeters with a maximum length 8 centimeters.

2. These shells are elongate, elliptical-ovate and moderately inflated , with rounded margins

3. The outer shell surface is smooth, glossy, pale yellowish – white, with pale purplish grey inverted v shaped markings. hinge is narrow, with three radiating cardinal teeth.

Lophitoma indica

Phylum : Mollusca
Class : Gastropoda
Family : Turridae
Genus : *Lophitoma*

Species :*L. indica*

Key characters:

1. The size of an adult shell varies between 35 mm and 90 mm.

2. The fusiform shell is somewhat less ridged and striated and has a long siphonal canal. The shoulder angle is very slight, the central ridge forming a carina.

3. The shell is covered with sharply carinaated whorls**,** the carina consisting of a pair of narrow ribs the length of fusiforrm shell is 65 mm, the diameter 20 mm.

4. The whorl surface is covered with close, raised revolving lines, of which two or three below the carina are more prominent. The colour of the shell is whitish with minutely numerously brown spots and with usually a row of larger spots and with usually a row of larger spots below the suture.

Megastraea turbanica

Phylum : Mollusca

Class : Gastropoda

Order : Trochida

Family : Turbinidae

Genus : Megastraea

Species *: Megastraea turbanica*

Key characters:

1. Turbinidae have a strong, thick calcareous operculum radially distinguishing them from the somewhat similar trochidae or top snails, which have a corneous operculum.

2. This strong operculum serves as a passive defensive structure against predators that try to enter by way of the aperture or what would break the shell at the outer lip.

Conus arenatus

Phylum : Molluscan

Class : Gastropoda

Family : Conidae

Order : Neogastropoda

Genus : *Conus*

Species : *C. arenatus*

Key characters:

1. The size of the shell varied from 25 mm and 90 mm

2. The shell is stoutly turbinated, coronated on the spire. The colour of the shell is white, sprinkled in a waved longitudinal manner with very small, close brown dots, sometimes forming indistinct bands. The aperture has usually a light flesh – colour.

3. This marine species is occurs in the red sea and in the indo pacific and off Australia.

4. The species now also occurs in the Mediterranean off Israel, having invded as a lessepsian migrant through the Suez canal.

Crypraea onyx

Phylum : Molluscan

Class : Gastropoda

Order : Sorbeoconcha

Family : Crypraeidae

Genus : *Crypraea*

Species : *Crypraea onyx*

Key characters;

1. The shells of these quite common cowries reach an average 32-38 mm of length, with a minimum size of 24mm and maximum size of 57mm.
2. The dorsal surface of *Crypraea onyx* is smooth and shiny and generally golden brown , with alternating longitudinal fuzzy bands of translucent blush and reddish colors.
3. The base and the margins are dark brown or black, sometimes the teeth are orange .
4. In the living coweries mantle is dark brown, quite thin, with bluish papillae.

Americoliva sayana

Phylum : Molluscan

Class : Gastropoda

Family : Olividae

Genus : *Americoliva*

Species : *A.sayana*

Key characters:

1. The lettered olive, *americoliva sayana* is a species of large predatory sea snail, a marine gastropod mollusk in the family olividae.
2. The shell of the species can be found 6cm long.

3. It is smooth, shiny, cylindrical – shaped shell with a short spire.
4. The aperture is narrow and extending almost the length of the shell, continuing around bottom and ending in the notch on the other side.
5. The suture is v- cut and deep.the lower part of the whorl is just above the where the suture extends outward and then at a sharp shoulder drops into the suture.

Turritella acropora:

Phylum : Molluscan
Class : Gastropoda
Family : Turritellidae
Genus : *Turritella*
Species : *T.acropora*

Key characters:

1. Shell whitish, violet or pale rose colour with longitudinal flammules of dark reddish brown, and light coloured primary spiral threads articulated with elongated distant brown spots .
2. Whorls about 15, those after the nucleus with the distinct median keel and obscure suture, the later ones with less prominent median or perhaps several indistinct keels. the last two whorls less closely coiled in the adult , showing the posterior edge overhung by the preceding whorl at the suture
3. The striation appears very fine to the naked eye.

Babylonia spirata:

Phylum : Molluscan

Class : Gastropoda

Family : Buccinidae

Genus : *Babylonia*

Species : *B.spirata*

Key characters:

1. They are thick , heavy shell, having an almost flush sided body whorl
2. The spire seems pushed down into the body , separated by a deep channel wrapping around the body
3. The shells has rings of brown and alternating cream colour spiraling around the body.
4. The spirata is common across the Indian ocean and has been seen thru the indo – pacific region.

***Chione cancellata**:*

Phylum : Molluscan

Class : Bivalvia

Order : Venerida

Family : Veneridae

Genus : *Chione*

Species : *Chione cancellata*

Key characters:

1. This species grows to be 1 ¾ inches across, and has a rounded, triangular shell with both strong concentric ridges and strong radial ribbing, which is together form a raised crisscross pattern of ridges, hence the specific name, *cancellata* or *cancellate*.

2. The interior of the shell possesses crenulations on its bottom edge, and like most veneridae it has well developed lateral and cardinal teeth on the hinge line.
3. The shell of *c.cancellata* is quite brightly coloured and patterned having colonized every medium available except the air.

STATION 2 -THARAVAIKULAM:

DESCRIPTION OF THE SPECIES;

Lambis lambis

Phylum : Mollusca

Class : Gastropoda

Family : Strombidae

Genus : *Lambis*

Species : *Lambis lambis*

Key characters:

1. The maximum shell length for this species is up to 29 cm, and average length stands for 18 cm.
2. *Lambis lambis* has a very large, robust and heavy shell. One of the most striking characteristics is its flared outer lip, ornamated by six hallow marginal digitations.

3. The colour of the shell is highly variable, being white or cream externally and often presenting brown, purplish or bluish black patches. The interior is glazed and may be pink, orange or purple.

Chicoreus ramosus :

Phylum : Molluscan

Class : Gastropoda

Family : Muricidae

Genus : *Chicoreus*

Spcies : *C. ramosus*

Key characters :

1. *C.ramosus* has a very large, solid , very rugged and heavy shell of upto to 330mm in length.
2. It has a relatively globose outline, possessing a short spire, a slightly inflated body whorl, and a moderately long siphonal canal.
3. One of its most striking ornamentations are the conspicuous, leaf-like, recurved hollow digitations.
4. It also presents three spinose axial varices per whorl, with two elongated nodes between them.
5. The shell is coloured white to light brown externally with a white aperture, generally pink towards the inner edge, the outer lip and the columnella.

Anadara rhombae

Phylum : Molluscan

Class : Bivalvia

Order : Arcida

Family : Arcidae

Genus : Anadara

Species : A. rhombae

Key characters:

1. Anadar is a genus of saltwater bivalves, ark clams, in the family Arcidae. It is also called Scapharca

2. This genus is known in the fossil record from the Cretaceous period to the Quaternary period.

3. These fossils have been found all over the world.

Cerastoderma edule

Phylum : Mollusca

Class : Bivalva

Family : Cardiidae

Genus : *Cerastoderma*

Species : *C.edule*

Key characters :

1. It typically reaches from 3.5 centimeters to 5 centimeters in length, but sometimes it reaches 6 centimeters.
2. The shells are pal or whitish yellow, grubby white or brown.

3. The shells are oval, are covered by ribs, which are flattened in the middle part of the shells.
4. The digestive glands are light brown to dark green.

Turritella acropora:

Phylum : Mollusca

Class : Gastropoda

Family : Turritellidae

Genus : *Turritella*

Species : *T.acropora*

Key characters:

1. Shell whitish, violet or pale rose colour with longitudinal flammules of dark reddish brown, and light coloured primary spiral threads articulated with elongated distant brown spots.
2. Whorls about 15, those after the nucleus with the distinct median keel and obscure suture, the later ones with less prominent median or perhaps several indistinct keels. The last two whorls less closely coiled in the adult, showing the posterior edge overhung by the preceding whorl at the suture.
3. The striation appears very fine to the naked eye.

Lophitoma indica

Phylum : Mollusca

Class : Gastropoda

Family : Turridae

Genus : *Lophitoma*

Species : L. *indica*

Key characters:

1. The size of an adult shell varies between 35 mm and 90 mm.
2. The fusiform shell is somewhat less ridged and striated and has a long siphonal canal. The shoulder angle is very slight, the central ridge forming a carina.
3. The shell is covered with sharply carinaated whorls**,** the carina consisting of a pair of narrow ribs the length of fusiforrm shell is 65 mm, the diameter 20 mm.
4. The whorl surface is covered with close, raised revolving lines, of which two or three below the carina are more prominent. The colour of the shell is whitish with minutely numerously brown spots and with usually a row of larger spots and with usually a row of larger spots below the suture.

Conus arenatus

Phylum : Molluscan

Class : Gastropoda

Family : Conidae

Genus : *Conus*

Species : *C. arenatus*

Key characters :

1. The size of the shell varied from 25 mm and 90 mm

2. The shell is toutly turbinated, coronated on the spire. The colour of the shell is white, sprinkled in a waved longitudinal manner with very small , close brown dots, sometimes forming indistinct bands. the aperture has usually a light flesh – colour.
3. This marine species is occurs in the red sea and in the indo pacific and off Australia.
4. The species now also occurs in the Mediterranean off Israel, having invded as a lessepsian migrant through the Suez canal.

Hemifusus pugilinus:

Phylum : Molluscan
Class : Gastropoda
Order : Neogastropoda
Family : Melongenidae
Genus : *Hemifusus*
Species : *H.pugilinus*

Key characters:

1. *Hemifusus* is a genus of sea snails, marine gastropod molluscs in the family melonginidae, the crown conches and their allies.

2. Unequally fusiform, the spire being shorter than aperture; shell ponderous, coronated with compressed spines; and internal and ascending canal at the top of aperture.

STATION 3 - KEELAVAIPPAR

DESCRIPTION OF THE SPECIES:

Melongena melongena

Phylum **:** Mollusca

Class : Gastropod

Family **:** Melongenidae

Genus : *Melongena*

Species : *melongena*

Key characters:

1. *Melongena* is a common name the Caribbean crown conch, is a species of large maraine gastropods molluscs in the family melongenidae.
2. *Melongena* are carnivorous, primarily preying on small bivalves.
3. They shell have large and thick. They are a tough operculum made of a horn like material
4. They lay egg capsules in orderly rows on rocks and other hard surface.

Laevistrombus turturella

Phylum : Mollusca

Class : Gastropod

Order : Littorinimorpha

Family . : Strombidae

Genus : *Laevistrombus*

Species **:** *turturella*

Key characters:

1. *Laevistrombus turturella* is a species of sea snail a marine gastropod molluscs in the family strombidae.

2. The shell margin has an indentation near the anterior end which accommodate one of the eye stalks.

3. The large shells, attaining a wide variety of lengths depending on the species (20-40) mm.
4. They also develop wider and thicker shells with fewer but spines in deeper water.

Strombus Campbellium

Phylum : Mollusca
Class : Gastropo
Order : Littorinimorph
Family : Strombidae
Genus : *Strombus*
Species **:** *Campbellium*

Key characters:

1. Strombidae family and sea snail then species name was *Strombus campbellium*.
2. Strombid gastropods have a characteristics means of locomotion, using their pointed, sickle – shaped, horny in a so called leaping motion.
3. Borrowing behaviour, sometimes complex behaviour characters.
4. They have usually large strombid gastropods and useful of food, some strombid snails species have been used in human culture for centuries.

*Fasiolaria filameatosa***:**

Phylum : Mollusca
Class **:** Gastropod
Order : Neogastropoda
Family **:** Fasiolariadea
Genus : *Fasiolaria*

Species : *Fasiolaria filameatosa*

Key characters:

1. Filifuscus filamentosus is a species of sea snail, a marine gastropod Mollusca in the family Fasiolariadea, the spindle snails, tulip snails and their allies.
2. The species occurs in the Red sea and in the Indian ocean off Tanzania, Madagascar and the Mascarene Basin.

Xancuspyrum

Phylum : Mollusca

Class : Gastropod

Order : Neogastropoda

Family : Turbenellidae

Genus : *xancus*

Species : *Xancus pyrum*

Key characters:

1. The shell is thick and heavy, biconical to fusiform, often noduloseto spinose on shoulder.
2. Periostracus conspicuous.
3. Siphonal canal present.
4. Inner lip with strong folds.
5. Operculum is corneous in nature.

Strombus canarium

Phylum : Mollusca

Class : Gastropod

Order : sorbeoconcha

Family : Strombidae

Genus **:** S*trombus*

Species : S*trombus canarium*

Key characters:

1. *Strombu scanarium* is a species of edible sea snail, a maraine gastropods molluscs family in the strombidae the true conchs.
2. The shell of adult individuals is coloured light yellowish brown to golden to gray.
3. It has a inflated a body whole, a flared and thick outer lip and a shallow stromboid notch. Although is considered to have value as an ornament, because the shell is heavy and compact it's often used as sinker for fishing nets.
4. The external a natomy of the soft parts of the species is similar to that of other stromboid snails the animal has a elongated with snout, thin eyestalks is well developed eyes and tentacles and a narrow, strong food with a sickle shaped operculum attached.

Crypraea tigiris

Phylum : Mollusca

Class : Gastropod

Order : Sorbeoconcha

Family **:** Crypraeidae

Genus : *Crypraea*

Species *: crypraea tigiris*

Key characters:

1. Roughly egg shaped and dextral the glossy shell is large and heavy for a cowry.
2. It measures up to 15 cm (16) in length and the upper dorsal side is white, pale bluish white or buff, densely covered with dark brown or blackish sparely circular spots.
3. There is sometimes a blurred red along the length of the at the midline on the dorsal surface. The lower margins are rounded (that is there is no Sharpe between the upper and lower of the shell as in the found in some other cowries).
4. The ventral side is white or whitish, and the shell opening is line with tooth like serrations.
5. The mantel can also withdraw in to the shell opening when threatened. In this species, the exterior surface of the mantle has numerous pin like projections that are white tipped.

Acanthocardia aculeata

Phylum : Mollusca
Class : Gastropod
Order : Cardiida
Family : Cardiidae
Genus **:** *Acanthocardia*
Species : *Aculeata*

Key characters :

1. *Acanthocardia aculeata* , the cockle, is a species of salt water calms, maraine bivalve Molluscusin the family Cardiidae
2. The shell of *Acanthocardia aculeatacan* reach a size of 50- 115 mm
3. The shell is robust , broadly oval with a heart shaped profile
4. The surface shows 20 – 22 prominent radial ribs, with rows of sharp spines, especially at sides.

Chicoreus ramosus

Phylum **:** Mollusca
Class **:** Gastropod
Order **:** Sorbeoconcha
Family : Muricidae
Genus : Chicoreus ramosus
Species : ramosus

Key characters :

1. It has a large, solid, very rugged and heavy shell of up to 327 – 330 mm in length .
2. It has a relatively globose outline, possessing a short spire a slightly inflated body whole and a moderately siphonal canal.
3. One of it's most striking ornamentation is the conspicuous leaf like, recurved hollow digitations.
4. It also presents three spinose axial varices per whole, with two elongated nodes.

STATION 04 - THERESPURAM:

DESCRIPTION OF THE SPECIES:

Conus arenatus

Phylum : Molluscan

Class : Gastropoda

Family : Conidae

Order : Neogastropoda

Genus : *Conus*

Species : *C. arenatus*

Key characters:

1. The size of the shell varied from 25mm and 90mm
2. The shell is stoutly turbinated, coronated on the spire. The colour of the shell is white , sprinkled in a waved longitudinal manner with very small , close brown dots, sometimes forming indistinct bands. The aperture has usually a light flesh – colour.
3. This marine species is occurs in the red sea and in the indo pacific and off Australia.
4. The species now also occurs in the Mediterranean off Israel, having invded as a lessepsian migrant through the Suez canal.

Cerastoderma edule

Phylum : Mollusca

Class : Bivalvia

Family : Cardiidae

Order : Cardiida

Genus : *Cerastoderma*

Species : *C.edule*

Key characters :

1. It typically reaches from 3.5 centimeters to 5 centimeters in length,but sometimes it reaches 6 centimeters.
2. The shells are pal or whitish yellow, grubby white or brown.
3. The shells are oval, are covered by ribs, which are flattened in the middle part of the shells.
4. The digestive glands are light brown to dark green.

Donax faba

Phylum : Molluca

Class : Bivalvia

Order : Cardiida

Family : donacidae

Genus : *Donax*

Species : *Donax faba*

key characters:

1. It is a genus of small, edible salt water clams, marine bivalve molluscs. The genus is sometimes known as bean calms or wedge shells. Donax species have numerous different common names in different parts of the world .
2. Species of donax live, sometimes in high concentrations, vertically aligned in the sand on exposed beaches, on tropical and temperate coasts worldwide.

3. When the waves wash these small clams out of sand, they can dig back in again quite rapidly. They are filter feeders.

Hemifusus pugilinus:

Phylum : Mollusca

Class : Gastropoda

Order : Neogastropoda

Family : Melongenidae

Genus : *Hemifusus*

Species : *H.pugilinus*

Key characters:

1. It is a genus of sea snails, marine gastropod molluscs in the family melonginidae, the crown conches and their allies.
2. Unequally fusiform, the spire being shorter than aperture; shell ponderous, coronated with compressed spines; and internal and ascending canal at the top of aperture.

Anadara rhombae

Phylum : Molluscan

Class : Bivalvia

Order : Arcida

Family : Arcidae

Genus : Anadara

Species : A. rhombae

Key characters:

1. Anadar is a genus of saltwater bivalves, ark clams, in the family Arcidae. It is also called Scapharca

2. This genus is known in the fossil record from the Cretaceous period to the Quaternary period.
3. These fossils have been found all over the world.

Crypraea moneta

Phylum : mollusca

Class : gastropoda

Order : sorbeoconcha

Family : crypraeidae

Genus : C*rypraea*

Species : *Cryprea moneta*

Key characters:

1. *Cypraea* is a genus of medium – sized to large sea snails.
2. The size ranges from 10 to 44 mm, typically about 20-25 mm.
3. The most common species of porcelain, thee is huge area: the population is estimated at hundreds of billions of individuals.
4. Both lips with teeth. absence of operculum.

Cymatium perryl

Phylum : molluscan

Class : gastropoda

Order : littorinimorpha

Family : cymatiidae

Genus : *Cymatium*

Species : *perryl*

Key characters:

1. *Cymatium perryl* is a species of predatory sea snail, a marine gastropod mollusk in the family cymatiidae.
2. This species of marine snal lives in the indo – pacific oceans.

Mactra stultorum :

Phylum : Molluscan

Class : Bivalvia

Order : venerida

Family : mactridae

Geus : *Mactra*

Species : *stultorum*

Key characters :

1. Mactra is a genus of medium – sized marine bivalve mollusks or clams , commonly known as trough shells or duck clams .
2. Mactra is the type genus within the family Mactridae.
3. The word 'trough' is the common name refers to the fact that all Mactra shells have a large ligamental pit at the hinge line, which in life contains a large intestinal ligament.
4. Most of the bivalves in other familie have an external ligament instead.

Pectin albicans

Phylum : molluscs

Class: bivalvia

Order : pectinida

Family : pectinidae

Genus : *Pecten*

Species : *P. albicans*

Key Characters:

1.Pecten albicans, common name Japanese baking scallop, is a species of marine bivalve molluscs in the family Pectinidae,

2. *Pectenalbicans* has a shell reaching a size of 95 mm, with about 12 radiating ribs.

3. The color of t value for fishing in Japan.

4. The color of the surface usually ranges from light brown to dark brown, but it may be also orange or purple.

5. This species is of commercial value for fishing in Japan.

Chicoreus ramosus :

Phylum : Molluscan
Class : Gastropoda
Family : Muricidae
Genus : *Chicoreus*
Spcies : *C. ramosus*

Key characters:

1. *Chicoreus ramosus* has a very large, solid, very rugged and heavy shell of upto to 330 mm in length.
2. It has a relatively globose outline, possessing a short spire, a slightly inflated body whorl, and a moderately long siphonal canal.
3. One of its most striking ornamentations are the conspicuous, leaf-like, recurved hollow digitations.

4. It also presents three spinose axial varices per whorl, with two elongated nodes between them.
5. The shell is coloured white to light brown externally with a white aperture, generally pink towards the inner edge, the outer lip and the columnella.

***Murex ternispina**:*

Phylum :mollusk

Class : gastropoda

Family : muricidae

Genus : murex

Species : M. ternispina

Key characters:

1. The common name murex.

2. It is still used for many species in the family Muricidae which were originally given the Latin generic name *Murex* in the past, but have more recently been regrouped into different newer genera.

4. The word *murex* was used by Aristotle in reference to these kinds of snails, thus making it one of the oldest classical seashell names still in use by the scientific community.

Semicassis granulate

Phylum : Mollusca

Class : Gastropoda

Family : Cassidae

Genus : *Semicassis*

Species : *S.granulata.*

Key characters:

1. The scotch bonnet is a medium sized sea snail, a marine gastropod mollusk is a sub family of cassine, the helmet shells and bonnet shells.
2. In the spring, the adult females of this species lay eggs in the tower shaped structures.
3. It alludes to the general outline and coloured pattern of the shell.
4. The shell is egg-shaped and fairly large 2 to 4 inches in maximum dimention with the regular pattern of yellow, orange or brown.
5. The surface sculpture of the shell is highly variable, the surface can be smooth and polished.

STATION 1 : VELLAPETTI **CLASS : GASTROPODA**

lambis lambis

Lambis lambis

Lophitoma indica

Americoliva sayana

Turritellinae acropora

Babylonia spirata

Megastrada turbanica

Conus arenatus

Scotch bonnet

Cryprae onyx

Chicoreus ramosus

CLASS : BIVALVE

Chione cancellata

Cerastoderma edule

Paratapes textalis

Spondylus gaederopus

STATION 2: THARUVAIKULAM **CLASS : GASTROPODA**

Lambis lambis

Chiroreus ramosus

Turritellnae acropora

Murex trapa

FAMILY: Turridae

Laphitoma indica

FAMILY:Conidae

Conus arenatus

FAMILY*:Volamidae*

Hemifusus pugilinus

CLASS : BIVALVIA

Anadara rhombea

Cerastoderma edule

STATION 3: KEELAVAIPPAR **CLASS : GASTROPODA**

Chicoreus ramosus.

Melongena melongena

Laevistrombus turturella

Strombus Campbellium

Fasiolaria filamentosa

Xancus pyrum

Strombus canarium

Crypraea tigris

CLASS : BIVALVE

Acanthocardia aculeate

STATION 4: THRESPURAM **CLASS : GASTROPODA**

Conus arenatus

Murex ternispina

Chicoreus ramosus

Hemifusus pugilinus

Senicassis granulata

Cypraea moneta

Cymatium perryl

CLASS : BIVALVIA

Mactra eximia

Pecten albicans

Cerastoderma edule

Donax faba

Anadara rhombea

Figure 1: Number of Species at different stations of Thoothukudi District

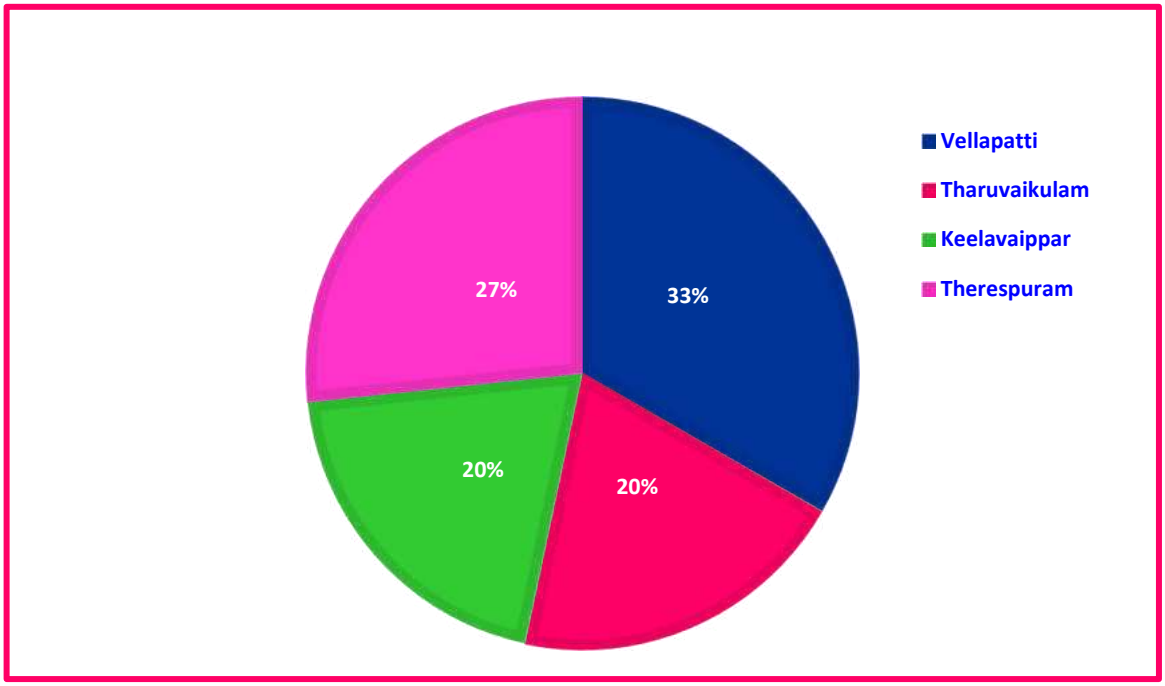
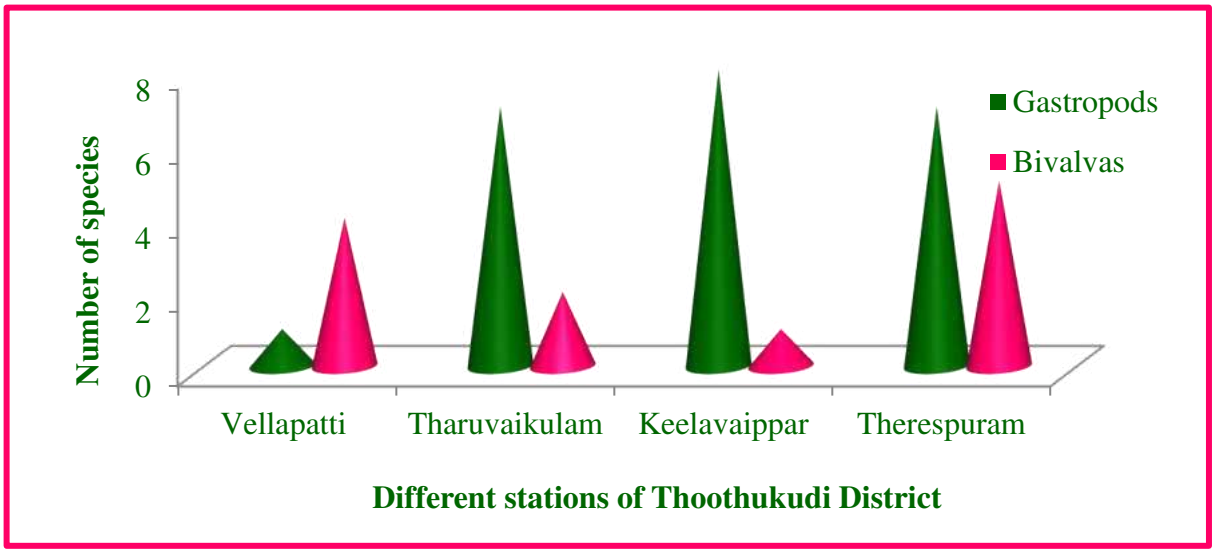


Figure 2: Species Composition of different stations of Thoothukudi District



DISCUSSIION

According to K.S. Mohammed (2006), in the phylum- mollusc , about 3270 have been reported from India belonging to 220 families and 591 genera. Among these the bivalves are the most diverse followed by gastropods. The biodiversity study of marine mollusc of Thoothukudi district in Tamilnadu observed about 21 species in the class gastropoda and 9 species of class Bivalvia were reported.

A total of 44 species of molluscs were associated with corals in Gulf of Manner and Gastropods represented the numerically dominant group with species. In the current study 21 species of gastropods, 9 species of bivalves were recorded.

CONCLUSION

In the current study 44 species of molluscs were identified in 4 stations of Thoothukudi district. A total of 14 species including 10 gastropods and 4 bivalve were recorded from station 1. From station 2, 09 species (7-Gastropods and 2-Bivalves), from station 3, 09 species (8-Gastropods and 1-Bivalve) and from station 4, 12 species (7-Gastropods and 5-Bivalves) were recorded.

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MANAGEMENT OF FACE MASK DISPOSAL POST COVID - A SURVEY

A field work submitted to

ST.MARY'S COLLEGE (Autonomous),

THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

In partial fulfillment for the award of the degree of

MASTER OF SCIENCE IN ZOOLOGY

by

1.	**SESILIPRIYA .A**	**20APZO04**
2.	**SHANTHI .J**	**20APZO05**
3.	**SHERINE .S**	**20APZO06**

DEPARTMENT OF ZOOLOGY

ST. MARY'S COLLEGE (AUTONOMOUS)

(Re-accredited with A^+ Grade by NAAC)

THOOTHUKUDI

APRIL – 2021

CERTIFICATE

This is to certify that the field work entitled ' **MANAGEMENT OF FACE MASK DISPOSAL POST COVID - A SURVEY'** is submitted to **St.Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Master of Science in Zoology** and it is a field work done during the year 2020-2021 by the following students

1.	**SESILIPRIYA .A**	**20APZO04**
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INTRODUCTION

INTRODUCTION

2020 came with a new source of pollution. A certain type of waste has been accumulating in huge quantities, all over the globe. We see it soiling the streets, parks and forests. It is found floating in seas and oceans, proving that most of it end up in nature, foremost, for people. These items carry a higher risk of contamination with the coronavirus that other kinds of waste. It is the face mask which we wear daily due to the Covid19 pandemic.

The origin of the novel human coronavirus (SARS-CoV-2) and its potential for harm increased face mask and medical waste in the environment, thereby necessitating the urgent prevention and control of the pandemic. The current work estimates the face mask and medical waste generation in Thoothukudi during the pandemic to convince the waste management and scientific communities to find ways to address the negative impact that the waste disposal has on the environment. Standardisation, procedures, guidelines and strict implementation of medical waste management related to COVID-19, community habitats and public areas should be carefully considered to reduce pandemic risks in hospitals, as proper medical waste disposal effectively controls infection sources.

World Health Organization(WHO) and the US centers for disease control and prevention, the National Centers for Disease Control and local governments have announced various guidelines, including frequent handwashing, social distancing and quarantine (home, local and state quarantine), to reduce the spread and health risks associated with COVID-19. These institutions have also recommended medical masks, non-medical face masks (including various forms of self-made or commercial masks of cloth, cotton or other textiles), face shields, aprons and gloves. More and more countries have recommended wearing masks when going out in public places. The press conference study of the joint prevention and control mechanism of the state council of china found that approximately 468.9 tons of medical waste are generated every day in association with COVID-19. On the other hand, it was found in Indonesia(Jakarta) that the medical waste scale had reached 12,740 tons approximately 60 days after people were first infected by coronavirus in the area.

One of the social measures applied during the COVID-19 pandemic has been the use of personal protective equipment (PPE)—face masks and gloves. As a result, this waste category has expanded enormously. This study investigates waste management issues from multiple perspectives, including local governments, waste collection companies, and individual citizens in Poland using a telephone survey for institutions and an online questionnaire for individuals. The results of this study

show that approximately 80% of local governments in the Silesian region have applied special measures for handling and collection of waste PPE. Only 13% of waste collection companies have applied special collection schedules for the waste generated at quarantine collection points due to the high costs of changing collection schedules, providing additional vehicles, and paying for more labor. The information campaigns focusing on new methods of PPE waste collection have been difficult to introduce on a large scale, and citizens need better information regarding how to handle and dispose of waste PPE. Results indicated the most helpful method in supporting waste PPE collection would be automatic PPE dispensers with waste PPE collection options and waste bags of a designated color. The respondents identified waste PPE pollution of the environment as an issue and the necessity for proper recovery of this waste stream

The new PPE products are considered disposable after brief use. As a consequence, a new waste category has appeared. The mandatory use of PPE has been extended to many locations such as public transport, shops, supermarkets, or medical centers. In many countries, this requirement has also been applied to open spaces. This use has resulted in a significantly growing volume of disposed PPE items. In the European Union (EU), waste PPE can be classified as medical, separated, or mixed waste depending on the source of its generation [EU Directive]. Due to concerns of possible virus transmission from the surfaces of face

masks or gloves and the increasingly common use of PPE in society, management of PPE waste has emerged as a challenging task that includes legal, business, and social aspects [Maria et al., 2020, Kargar et al., 2020]. Management should combine methods of storage, collection, transport, and treatment of PPE. Also, all resources containing various raw materials are subject to further recovery, including for energy generation or recycling. The natural consequence of the introduction of the common and broad use of PPE is the need to implement a system and methods of collecting this waste, which requires cooperation between local governments, waste collection companies, local institutions, and each individual in society. Proper disposal also requires additional tasks, including preparation of waste collection schedules and adequate handling of waste from households and quarantined locations, including from persons with positive SARS-CoV-2 results and medical centers.

Consequently, one of the many problems that will inevitably occur is contagious waste, which, if not managed properly, may be the root cause of severe diseases and environmental problems. Hence, the purpose of this work is to estimate face mask use and medical waste during the COVID-19 pandemic, thereby calling on the waste management and scientific communities to express their concerns and take the requisite actions for the formulation of appropriate solid waste management policies and strategies.

OBJECTIVES

OBJECTIVES

The objectives of the current field work are

1. To take survey of people in Thoothukudi who use disposable and reusable masks.

2. Survey the number of disposable masks used per day by an individual.

3. To survey the methodology of disposal of the face masks.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

On March 11, 2020, the World Health Organization (WHO) has declared the novel corona virus (COVID-19) outbreak a global pandemic at a news briefing held at its headquarter (Cucinotta et al., 2020). COVID-19 is a new public health crisis that originated in bats and transmitted to humans through unknown intermediary animals in Wuhan, China in December 2019 (Singhal, 2019). The disease has spread over several countries of the world with millions of confirmed cases and hundreds of thousands of deaths.

The world is not new for pandemic diseases, several pandemics have occurred in the past far and near times. All of these pandemics had left unforgettable memories in the world. COVID-19 is a global pandemic with a complicated viral character to contain. It kills healthy adults in addition to people with existing health problems and its transmission is quite efficient with an exponential rate of increase (Gates, 2020, Rothe et al., 2020). Moreover, the human corona viruses can persist on inanimate surfaces like metal, glass, and plastic for up to 9 days (Kampf et al., 2020, Chin et al., 2020). The study also shows the stability of the corona virus in water and wastewater (Gundy et al., 2009). The corona virus is stable in a pH range of 3–10 and on plastic surfaces up to 72 hours (Van Doremalen, 2020). However, inactivation of the virus is possible with surface disinfection

procedures involving 71% ethanol, 0.5% hydrogen peroxide or 0.1% sodium hypochlorite in 1 minute, and at a higher temperature.

It is estimated that more than 1,720,000 cases have occurred worldwide since January 2020 (Gardner, 2020). On 11 March, the World Health Organization (WHO) characterized COVID-19 as a pandemic (COVID-19, 2020). One month later, the worldwide death rate has reached around 6.2% (Gardner, 2020). SARS-CoV and MERS-CoV also belong to the large coronavirus family, all of which are believed to have originated in bats (COVID-19, 2020). From November 2002 to July 2003, more than 8000 probable severe acute respiratory syndrome (SARS) cases were reported from 29 countries with an estimated 10% death rate (>50% in people older than 60) (CDC, 2004), but since 2004, no cases of SARS have been reported worldwide (CDC, 2020). Middle East Respiratory Syndrome (MERS), first reported in Saudi Arabia in 2012, has reached 27 countries globally, with a death rate of 34.3% and 2519 confirmed laboratory cases to the end of January 2020 (WHO, 2020a).

COVID-19 indiscriminately attacks people of all ages and all nations and transmitted by inhalation and contact with infected droplets. The incubation period of the disease varies from 2 to 14 days. Symptomatic patients and asymptomatic people can transmit the corona virus through droplets during coughing and

sneezing (Zou *et al*., 2020). Many people infected by the COVID-19 virus might not develop symptoms of illness. Those developed the symptom of the disease usually experience high-grade fever, cough, sore throat, breathlessness, fatigue, and malaise. The disease is severe among chronic disease patients and old age people due to their decreasing immune system. Most of these patients often die of their original comorbidities. The disease could pose severe consequences in developing countries, where healthcare facilities and services are poor.

Most countries of the world have applied case detection, quarantine, and social distancing measures to reduce the transmission of the virus. These measures were effective to contain the virus and reduce new infections in many developed countries (Anderson *et al.,* 2020). Countries that applied strict social distancing have reduced new cases by more than 90% in a short time (Remuzzi *et al.,* 2020). However, the infectivity of the virus during the incubation period and its transmission from asymptomatic people enforce many countries to declare a complete lockdown. Countries in the developing world also considered social distancing measures to contain the virus.

Environmental risks of face mask

The use of personal protective equipment (PPE), social distance, travel restrictions and lockdown were currently employed to reduce this spreading level of coronavirus (Rubio-Romero *et al.*, 2020). This ongoing pandemic situation

created that wearing mask is must for every human life. There are various types of masks such as surgical , N95 , and commercial fabric/cloth masks used to tackle the ongoing pandemic situation. According to the World Health Organization (WHO) study, in USA about 89 million medical masks are anticipated to be required to respond the COVID-19 as this crisis is likely to persist for some time (Xiang *et al.,* 2020). Further, the plastic innovation hub has identified. that the domestic demand for the mask in UK is around 24.37 billion per year (Liebsch, 2020). As of February 2020, China has raised its daily production of medical masks to 14.8 million. The Japanese ministry of finance, trade, and industry recorded that more than 600 million face masks required per month of April 2020 (Fadare and Okoffo, 2020). The increasing use of mask significantly increases the production of mask and it consumes higher amount of energy. A study by Klemeš et al., 2020a,b shows that a mask production consumes about 10-30 Wh energy and releases 59 g CO2-eq greenhouse gas to the environment. Further, ever increasing uses of face mask also increase the landfill and medical waste. Most of these face mask wastes contains either polypropylene and/or polyethylene, polyurethane, polystyrene, polycarbonate, polyacrylonitrile, which add plastic or microplastic pollution to the environment (Akber *et al.,* 2020). This indicates that current ongoing pandemic, increases the environmental pollution and negative impact to human and animal health.

Initially, discarded masks may risk spreading coronavirus to waste collectors, litter pickers or members of the public who first come across the litter. We know that in certain conditions, the virus can survive on a plastic surgical mask for seven days. Plastics break down into smaller pieces over time, and the longer litter is in the environment, the more it will decompose. Plastics first break down into microplastics and eventually into even smaller nanoplastics. These tiny particles and fibres are often long-lived polymers that can accumulate in food chains. Just one mask can produce millions of particles, each with the potential to also carry chemicals and bacteria up the food chain and potentially even into humans.Littered areas also tend to encourage further littering, making the problem worse.

In March, the World Health Organization estimated that 89 million additional disposable masks were needed globally per month in medical settings to combat COVID-19. In addition, a recent working paper by the Plastic Waste Innovation Hub at University College London has put the current domestic demand for the UK at 24.7 billion masks a year. However, the demand for domestic face masks in the UK drops dramatically – to around 136 million a year – if only reusable masks are used (Roberts et al., 2020).

But even with reusable masks, their specific design and how you choose to clean them makes a difference. The University College London team examined the

manufacture, use and disposal of masks that were disposable, reusable, and reusable with disposable filters, to calculate their overall environmental impact. They found machine washing reusable masks with no filters had the lowest impact over a year.

Hand washing masks increased the environmental impact as – while machine washing uses electricity – manual washing uses more water and detergent for each mask. Disposable filters also increase the environmental impact because the small filters are often made from plastic similar to the disposable masks, with a filter discarded after every use.

Perhaps surprisingly, it is estimated that hand washing reusable masks with disposable filters had the highest environmental impact overall – higher even than using fully disposable masks. Therefore, sustainable solutions need to reduce the environmental impacts, while meeting the mask demand. Despite millions of people being told to use face masks, little guidance has been given on how to dispose of or recycle them safely. And as countries begin to lift lockdown restrictions, billions of masks will be needed each month globally. Without better disposal practices, an environmental disaster is looming.

The majority of masks are manufactured from long-lasting plastic materials, and if discarded can persist in the environment for decades to hundreds of years. This means they can have a number of impacts on the environment and people.

Hazards of face mask to the human health

Patients infected by human coronavirus being treated at home are generating infected waste possibly discarded as domestic waste, which can pose risks to workers and the environment, depending on the conditions of transport and disposal. In particular, the spread of the coronavirus may be increased by inadequate waste management, highlighting poor handling conditions associated with inappropriate use of personal protective equipment and other unfavourable conditions presented mainly in developing countries.

MATERIALS AND METHODS

MATERIALS AND METHODS

Study area:

Data was collected from residences of Beach Road, Caldwell colony, Shanmugapuram, Government Hospital. Mattakadai and Krishnarajapuram areas of Thoothukudi City.

Data Collection:

In order to collect data a questionnaire was prepared Fig. 1. The questionnaire is attached below.

ST.MARY'S COLLEGE (Autonomous)

THOOTHUKUDI-628001

I M.SC ZOOLOGY FIELDWORK PROJECT

SURVEY ON WASTE MANAGEMENT

1. NAME :

2. AGE :

3. ADDRESS :

4. OCCUPATION :

5. TYPE OF MASK USED : REUSABLE ☐ DISPOSABLE ☐

6. METHOD OF DISPOSNAL :

7. NUMBER OF MASK USED PER DAY : OFFICE ☐ HOME ☐

8. FREQUENCY OF GARBAGE DISPOSAL AT OFFICE : DAILY ☐ WEEKLY ☐ MONTHLY ☐

9. FREQUENCY OF GARBAGE DISPOSAL AT HOME : DAILY ☐ WEEKLY ☐ MONTHLY ☐

RESULTS

RESULTS

Data collection:

Data on usage of facial mask among the people of Thoothukudi, was collected by circulating a questionnaire. Data was collected from 150 individauals. The informations on the type and number of masks used by each person was also collected. The data is tabulated in Table 1. Among 150 people surveyed 76 people use disposable mask that comes to 50% of total samples surveyed. The data on frequency of disposing the masks per day was also collected. The data showed that 60 people used 1 mask per day, 30 people 2 masks per day and 10 individuals use more than 4 masks per day (Table 2, Figure 1).

Method of Disposal of the masks:

From the data collected it as observed that disposable masks were disposed in the garbage. The garbage disposal was done in a regular basis either daily or on alternative days. But the data on the method of disposal was not obtained in the survey as there were a lot of ambiguity in the method of disposal.

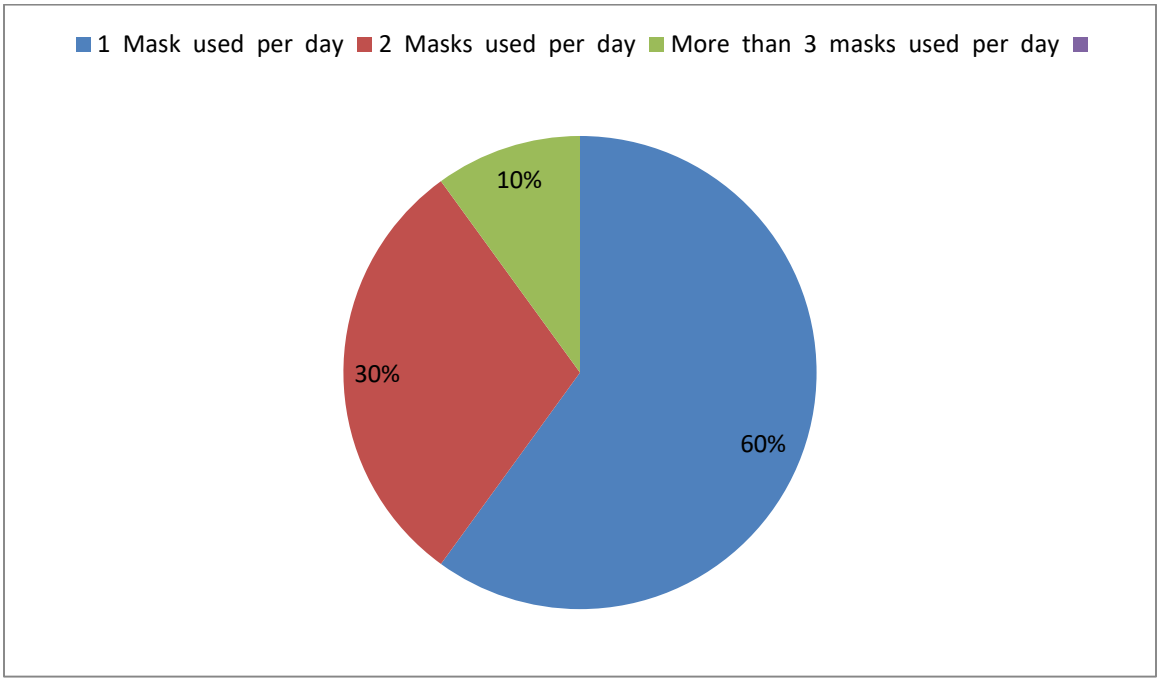
TABLE :1 Type of mask used by people of Thoothukudi

S. NO	TYPES OF MASK USED	NO.OF.INDIVIDUALS
1	**DISPOSABLE MASKS**	76
2	**REUSABLE MASKS**	74
	TOTAL	150

TABLE :2 Frequency of disposal of masks per day

	MASK USED PRE DAY		
	1	**2-3**	**>3**
Number of individuals	60	30	10

Figure 1: Graphical representations of the frequency of mask disposal by people of Thoothukudi



DISCUSSION

DISCUSSION

. Single use surgical masks have become in some way the standard of protection during the COVID-19 pandemic. The main reason is that they are very practical: they are lightweight, flexible, and comfortable to wear, offering a right balance between filtration efficiency, cost, and ease of breathing. Disposable face masks (single use) are used to slow down the transmission rate of Covid-19 from person to person. However, the staggering increase in the number of disposable masks being used globally on a daily basis exacerbates the environmental risk connected to improper disposal. The surgical mask has the potential to release in the marine environment thousands of microscopic fibers. Unprecedented rise in the global production of face masks present a new environmental challenge due to Covid-19 pandemic. In the present survey it is observed that 50% of the samples surveyed, use disposable masks. It is also observed that 40% of the people surveyed dispose more than one mask per day. This shows that a very high number of masks are disposed per day.

In most of the places in Thoothukudi city there is no proper method of waste disposal. Most of the time the garbage is discarded in open spaces. There is no proper landfill facility for waste disposal. This may result in indiscriminate dumping of a large quantity of masks. The masks may pose a number of problems

to the environment and the health of humans. There is a great risk of the masks contaminating the sea and hence polluting the marine ecosystem.

Action is therefore urgently needed to limit the amount of discarded surgical masks reaching the marine ecosystem, *i.e.,* campaigns to promote the correct disposal and improvements in managing these new waste streams. Furthermore, research efforts may provide more eco-friendly alternatives based, for example, on biodegradable components or higher quality solutions that can be used over extended periods.

CONCLUSION AND SUGGESTIONS

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Pieces of equipment for personal protection such as masks, gloves, antibacterial wipes have been used, this year, by people all over the world, to stay safe from the coronavirus. If not disposed of for recycling, like other plastic wastes, disposable masks can end up in the environment, freshwater systems, and oceans, where weathering can generate a large number of micro sized particles (smaller than 5 mm) during a relatively short period (weeks) and further fragment into nano plastics (smaller than 1 micrometer.

Suggestions

1. Use reusable masks without disposable filters. Machine wash them regularly following the instructions for the fabric.
2. Try to carry a spare so if something goes wrong with the one you're wearing you don't need to use or buy a disposable mask.
3. If you do need to use a disposable mask, take it home (maybe in a bag if you have to take it off) and then put it straight into a bin with a lid. If this isn't possible, place it in a proper public bin.

4. Don’t put disposable masks in the recycling. They can get caught in specialist recycling equipment and be a potential biohazard to waste workers.

5. Whatever you do, don’t litter them!

SUMMARY

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A survey on the usage of facial mask and its method of disposal was done. It was observed that

- 50% of the total people surveyed use disposal mask
- 40% of the people surveyed discard more than 1 mask per day
- The disposal of mask is not done in a proper manner as it is suggested by the WHO.
- This results in the dumping of used face masks in the marine environment.

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