COMPARATIVE PROFILING OF SENSORY, PHYSICO-CHEMICAL AND MICROBIAL CHARACTERISTICS OF RAW COW AND GOAT MILK SAMPLES

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in partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

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CERTIFICATE

This is to certify that the project entitled "**Comparative profiling of sensory physico-chemical and microbial characteristics of raw cow and goat milk samples**" is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a record of the work done during the year 2022-2023 by the following students.

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INTRODUCTION

1.0 INTRODUCTION

Milk is known as ideal food with unique quality for nourishment of human being long before recorded history. It is recognized as complete meal because of its wholesome nutrients for all mammals, including human being. In native conformation, milk shows the apex food value. It meets the nutritional requirement of the body more perfectly than any other single food as it carries each of the absolutely necessary nutritional components to perform every physiological activities of the body system (Hossain *et al.*, 2010).

Mammals produce milk to feed their offspring. In many areas of the world humans consider milk as an important treasure of their life because they are used to it all over this era. In addition to milk, several dairy products such as cream, butter, yogurt, kefir, and cheese have been produced and consumed worldwide. Milk is the best source of calcium that we can supply to our body. It also functions as a healthy aid in losing unwanted fats and reducing weight (Thomsen *et al.*, 2003). In India, the presence of cows and goats in excavation sites suggests that dairy may have been in use since at least the Harappan Civilization (3300-1300 BCE).

Milk is an important source of all basic nutrients for mammals. Milk from cows and goats are being used for producing different dairy products including cream, butter, yogurt, ghee, sour milk etc., (Nicolaou *et al.*, 2010). It supplies

nutrients like high quality proteins, fats, carbohydrates, vitamins and minerals in significant amount than any other single food (Neumann *et al.*, 2002). Goat milk has more calcium, potassium and vitamin A and cow milk has more vitamin B_{12}, selenium and folic acid. Cow's milk is a rich and cheap source of protein and calcium, and a valuable food for bone health. Goat's milk contains vitamins, minerals, trace elements, electrolytes, enzymes, proteins, and fatty acids that are easily assimilated by the body. Goat's milk has a similarity to human milk that is unmatched in cow milk and also has several medicinal values (Kumar *et al.*, 2012).

The average production of milk in India, as per 2015-16 statistics, was 155.5 million tones which make India the largest producer in the World (Nalwaya *et al.*, 2018). According to World Health Organization (WHO) standards the quality milk should contain 2.6% fat, 3.5% protein, 0.17% TA, 7.71% SNF and SG 1.030. The pH 6.6 ensured the milk freshness at boiling point 100°C -117°C (Hossain *et al.*, 2013).

The quality of raw milk encompasses such milk characteristics as chemical composition, physical properties, microbiological and cytological quality, sensory properties, technological suitability and nutritive value (Shahida *et al.*, 2015).

Organoleptic analysis is important for every application of milk. It is necessary to understand the sensory qualities of milk in part because of the

widespread familiarity of raw milk and its typical organoleptic profile. Organoleptic analysis of the flavor or at least the aroma of raw milk can identify handling or production problems before milk is processed. The first standardized method for the sensory analysis of dairy products was dairy product judging (Clark and Costello, 2008). The organoleptic perception of raw milk is heavily influenced by the balance of its macronutrient components. Milk fat plays a critical role in the sensory analysis of fluid milk (Richardson-Harman *et al.,* 2000; McCarthy *et al.,* 2017). Visual, texture, and flavor attributes of raw milk are all influenced by milk fat (Phillips *et al.,* 1995). Descriptive organoleptic analysis of raw milks of varying fat percentages demonstrated that opacity, thickness, mouthcoating, viscosity, milk fat flavor, and yellow color increased with fat content (Francis *et al.,* 2005).

The physico-chemical analysis is an important tool to scrutinize the quality of milk that encompasses with chemical composition, physical properties, microbiological and nutritive value (Czerniewicz *et al.,* 2006). The physico-chemical properties of milk show some natural variations depending upon factors like method of manufacture, age and condition of the sample, species, breed, individuality of animal, stage of lactation, number of lactation (age of animal), season of the year, region of the country, feed of the animal etc., (De, 2005; Aneja *et al.,* 2002).

Milk in the udder of a healthy cow is almost free of bacteria. The milk can get contaminated, during the milking process, by the skin of udder, unclean milking equipment, brittle gum parts of the milking system or dust and dirt in the air of the cow-shed. The milk is also contaminated when it is passed through the teat-channel. The microbial population can vary from 100 to 10000 cfu/ml with significantly higher count in mastitis cows (Bytyqi *et al.,* 2009).

Milk is an ideal medium for microbial growth because of its high-water content, neutral pH and biochemical composition. Therefore, raw milk may contain various kinds of microorganisms with variable characteristics in respect to classification, morphology and physiology. Very important for the quality of raw milk and dairy products are bacteria that predominate among all kinds of milk microorganisms.

Bacteria in raw milk can be spoilage or pathogenic with mesophilic, psychrophilic or thermophilic behavior. In brief, bacterial growth is divided into four phases: i.e., lag, exponential or log, stationary and dying-off phases (Walstra *et al.*, 2006). Multiplication of bacteria shows a geometric progression and the bacterial growth during the log phase is described by the generation time (g) that is the time needed for a full cell division. Generation time in raw milk depends mainly on species or strains of bacteria as well as temperature, pH, level of oxygen, inhibitors and nutrients. Thus, the profile of initial microflora and the handling of raw milk

regarding hygienic and temperature conditions are the determinative factors for raw milk quality before processing.

Raw milk microflora is of critical importance for consumers safety and quality and shelf-life of dairy products. Raw milk microflora could be grouped as indigenous or contaminants and also as spoilage or pathogenic microorganisms. Microbes can have profound effects on milk flavor whether the contamination occurs before or after pasteurization. Raw milk quality can affect the rate of development of off-flavors (Ma *et al.*, 2000; Barbano *et al.*, 2006). Microorganisms allowed to grow in raw milk can affect the sensory quality of the milk before pasteurization. Malty flavors are produced by growth of *Streptococcus lactis* var. maltigenes, and musty potato aromas can be attributed to pyrazine compounds produced by *Pseudomonas perolens*. Bacterial growth is a common cause of premature milk spoilage.

Early studies on bacterial spoilage lacked both the sensory and analytical methods needed to draw conclusions about the off-flavors caused by specific species of bacteria. The genus *Pseudomonas* consists of psychrotrophic, gram-negative rod bacteria that are responsible for the majority of post pasteurization contamination of raw milk. Milk contaminated with *Pseudomonas* is characterized by a fruity (pineapple- or strawberry like) off-flavor as well as lower levels of sour, rancid, and soapy flavors (Whitfield *et al.*, 2000).

Cow's milk had contained most compounds that meet the human bodies requirements such as; protein content and which has an important role in growth and human nutrition. Its rich sources of minerals, vitamins, and protein, such as vitamins D, niacin, vitamin A, vitamin B12, riboflavin and potassium, calcium, phosphorus (Ajai et al., 2012). Cow's milk is lower in fat, but it is rich in lactose compared to goat milk and similar to other animals in minerals content (Guetouache *et al.,* 2014). Goat's milk contains a higher amount of magnesium, phosphors, and calcium than cow and human milk. Goat milk is very important for stimulating immunity and disease prevention (Ibrahim *et al.,* 2020). It contains various nutrients and can be utilized in various dairy products. Goat milk is superior to cow milk in terms of nutritional value (Kumar *et al.,* 2012). Goat milk has high nutritive value and beneficial properties as a purposeful diet for humanoid healthiness (Abbas *et al.,* 2014). Be used pasteurization and fermentation with some food additives with good manufacturing practices for ensuring protection and safety of fermented milk products (Leroy *et al.,* 2014). Goat milk can be consumed as an alternative to cow milk in the treatment of persons with cow milk allergies and gastrointestinal disorders, on the other hand, stimulating immunity (Chen *et al.,* 2016). In the ancient time Indians have used different varieties of milk from different dairy breeds but at the most they have consumed the milk of cow and goat. In this study the attempt has been made to distinguish between raw milk samples of cow and goat.

AIMS AND OBJECTIVES

2.0 AIMS AND OBJECTIVES

Food is a basic material that a body needs for its survival and well-being. Eating food that is healthy and rich in nutrients is vital for proper body functioning. Healthy foods give us a healthy life and longevity. Vitamins are important elements that helps the body to fight against disease. That's why milk plays a vital role in the human life. Milk keeps the body hydrated for hours. Milk boosts up the immunity.

In Tamil Nadu most of the people are using cow milk in their day-to-day life and in all over the world and many areas of India the mostly used milk variety is the milk of goat, so to test the basic difference between these two milks we started this project by doing the sensory, physico-chemical and microbial quality analysis of the samples. The aim of this research was to determine the quality of fresh milk physically and chemically and microbiologically obtained from cow and goat raw milk samples.

The main objectives are:

- To enrich our knowledge in dairy field.
- To fulfill our interest in having a study on milk varieties especially goat milk.
- To analyse the difference between cow and goat milk.
- To inculcate scientific thoughts.
- To isolate and identify lactic acid bacteria.

- To analyse the sensory quality of the collected milk samples.
- To estimate the physico-chemical analysis.
- To compare and elucidate the microbiological colonies and counts of the sample.
- To find which one is the best among these two according to their quality.
- To identify the foodborne pathogen.

REVIEW OF LITERATURE

3.0 REVIEW OF LITERATURE

Due to the nutritive value of milk, its testing and quality control is an essential component. In terms of milk production, India is considered the country with the largest number of milk producers, accounting for 22% of the world total. The negative effect of non-protected fat sources on milk fat content of cows is modulated by their inclusion rate and the main source of forage (Dhiman *et al.*, 2000). Haenlein (2004) gives an anecdotal evidence for the lower allergenicity of goat milk.

Raiha *et al.*, (2002) reported that feeding undiluted cow or goat milk to infants is not recommended as the high protein and mineral content of both milks can result in excess amino acid intake. Detection of coliforms bacteria and pathogens in milk indicates a possible contamination of bacteria either from the udder, milk utensils or water supply used (Bonfoh *et. al.*, 2003). Preservation techniques for milk is mainly performed to destroy or inactive all the harmful or pathogenic microorganisms by using heat treatment (De *et al.*, 2005).

The physico-chemical analysis is an important tool to scrutinize the quality of milk that encompasses with chemical composition, physical properties, microbiological and nutritive value. Total lipids in goat milk found to have higher physical characteristics compared to cow's milk, but may vary among different reports (Czerniewicz *et al.*, 2006).

According to Wendorff *et al.*, (2006) the studies in some seasonal areas, milk yield is high in summer while the fat and protein contents are low and during winter, the milk yield may low, but the fat and protein concentrations are higher. Vitamin B1, B2, and B6 were analysed using High-Performance Liquid Chromatographic methods (HPLC) after alkaline saponification by Agostini-Costa *et al.*, (2007). Perez-Chabela *et al.*, (2008) reported that four strains of lactic acid bacteria including *Lactobacillus plantarum*, *Lactobacillus curvatus*, *Pediococcus pentosaceus* and *Pediococcus acidilacti* are able to survive under thermal treatment at 70 °C for 60 minutes. Besides the proliferation of pathogens in milk, an important consideration has to be given on the antibiotic resistance, which is a major clinical obstacle in medicating disease especially in the developing countries (Jilani *et al.*, 2008).

The microbial population can vary from 100 to 10000 cfu/ml with significantly higher count in milk samples (Bytyqi *et al.*, 2009). The comparative macronutrient features of goat and cow milk have been reviewed previously (Ceballos *et al.*, 2009). Goat milk has lower allergic potential compared to cow milk (Silanikove *et al.*, 2010). The general total bacterial count regardless of milk types used as the main quality and safety assessment may not adequate to be a proper guideline research (Wasiksiri *et al.*, 2010). Samples of raw goat milk were made into 3 aliquots (25 ml each) in sterilized 50 ml Scotch. Each platform test, including

organoleptic test, COB test, and alcohol test was being repeated 3 times and the results were found. Hygienic role of raw milk is characterized by contamination levels and distribution of microorganisms (Zucari *et al.,* 2011). Common pathogenic microbes in milk include *Salmonella* sp., *Listeria monocytogenes, Yersinia enterocolitica, Campylobacter jejuni, Staphylococcus aureus, Escherichia coli,* etc., and these are responsible for many of food-borne diseases (Anderson *et al.,* 2011). Psychrotrophic microorganisms constitute a major cause of milk spoilage due to their ability to produce heat-resistant enzymes such as proteases, lipases, and phospholipases under refrigeration, this was reported by Samaržija *et al.,* (2012) and Xin *et al.,* (2017).

Yamazi *et al.,* (2013), the total loads of mesophilic bacteria, coliforms bacteria, Escherichia coli and psychrotrophic bacteria of milk stored for 48 hours or longer were relatively higher than the storage for 24 hours or less. Milk stored for 48 hours or longer were relatively higher than the storage for 24 hours or less.

Milk also provides an excellent growth of environment for microorganisms and their propagation in milk leads to spoilage and physicochemical properties of milk (Claeys *et al.,* 2013). Although raw milk contained lower bacteria, it has the possibility in having various bacterial populations (Quigley *et al.,* 2013).

Dairy production has been considered as a potential means of alleviating large-scale unemployment, especially in rural areas (Marichamy *et al.,* 2014).

Pseudomonas, Arenobacter, Aeromonas, Bacillus, Lactococcus, Mycobacterium, Staphylococcus, Clostridium are reported as the main spoilage bacterial genera associated with raw milk (Addis *et al.*, 2016).

Ahmed *et al.*, (2016) reported that milk processing sectors and suppliers add different adulterants and preservative for increasing the shelf life to get rid from the problem of quick deterioration of milk. Mandal *et al.*, (2017) has employed that High-Pressure Processing (HPP) normally employs pressure of 300–600 MPa at room temperature for 2–30 min to eliminate pathogenic microorganisms and extends the shelf life of milk with minimal alteration of nutritional and sensorial attributes. Presence of multi nutrients in goat milk is however may be the factor which encourages the growth and accumulation of microorganisms (Liam *et al.*, 2018).

Ndahetuye *et al.*, (2019) identified that high SCC and TBC in milk may lead to the production of enzymes that degrade fat and protein, reducing the quality of the milk and its products. Zhang *et al.*, (2019) were examined the microbiota of raw milk using 16s rRNA technology to characterize the major microorganisms present in raw milk and to analyze the patterns of variation throughout the year and correlations with milk quality parameters (TA, TBC, SCC, milk fat and protein).

Berhanu *et al.*, (2021) have done an analysis on microbial quality of raw cow milk and its predictors along the dairy value chain and as the result they found that common foodborne pathogens present in the contaminated milk can lead to the

development of various foodborne diseases, posing a potential risk to human health. Zhang *et al.*, (2022) have revealed in their study that, consumption of improperly preserved or expired raw and pasteurized milk may pose health risks due to contamination by various pathogenic microorganisms, which can lead to a series of symptoms such as abdominal pain and diarrhea, and even death in severe cases.

The research on microbiological, physico-chemical and nutritional properties of fresh cow milk treated with industrial high-pressure processing (HPP) during storage by Shu Huey Lim et al., (2023) has shown the potential of HPP treatment in preserving milk quality for the dairy industry. HPP-treated milk had a storage shelf life beyond 60 days, with all microbial testing meeting permitted safety levels. It had high stability for physicochemical properties with consistent pH and acidity during the entire storage. HPP treatment has successfully retained calcium, phosphorus, magnesium, and zinc contents by 99.3, 99.4, 99.1, and 100%, respectively.

MATERIALS AND METHODS

4.0 MATERIALS AND METHODS

4.1 Study area (Plate A)

Narippaiyur village is located Kadaladi taluk of Ramanathapuram district in Tamil Nadu, India. The total geographical area of village is 1798.06 hectares. Narippayur has the total population of 9,861 people. Sayalkudi is the nearest town to Narippaiyur village for all major economic activities. Main occupation is cow and goat shepherding, goat and cow rearing, palm jaggery manufacturing.

4.2 Selection of breeds (Plate A)

Following two varieties of cow and goat are selected.

4.2.1 *Bos indicus*

The *Bos indicus* is called in tamil as "Kangeyam". It is an Indian breed of drought cattle belongs to Tamil Nadu in South India. The milk of *Bos indicus* has a high nutritive value, though it is a poor milker. The annual production range of *Bos indicus* is 342 - 1455 Kg.

4.2.2 *Capra hircus*

The *Capra hircus* is called in tamil as "Vellaadu". It is the domestic goat. Vellaadu's milk has small, well-emulsified fat globules, it helps the milk to stay in

DESIGN OF THE EXPERIMENT

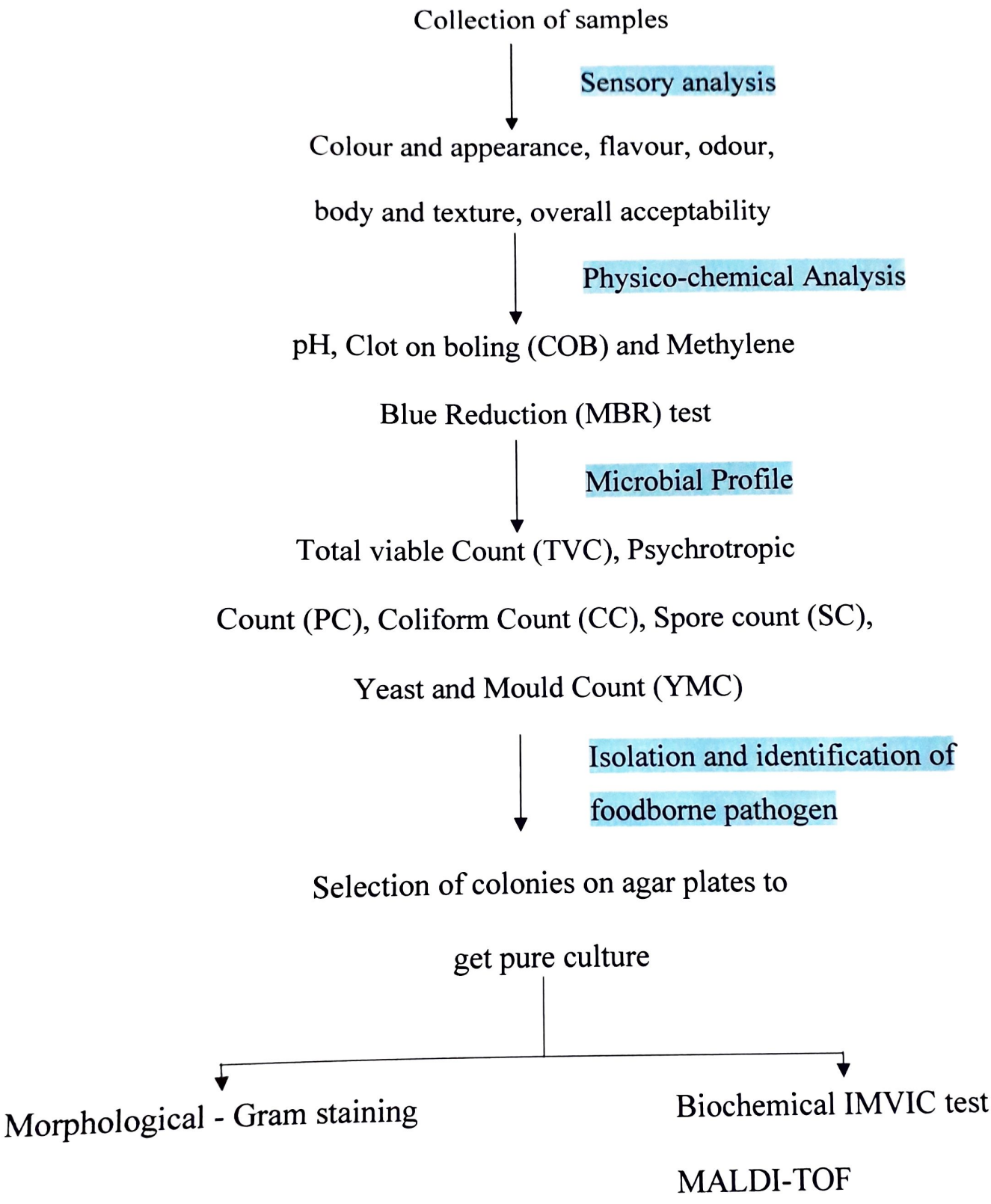
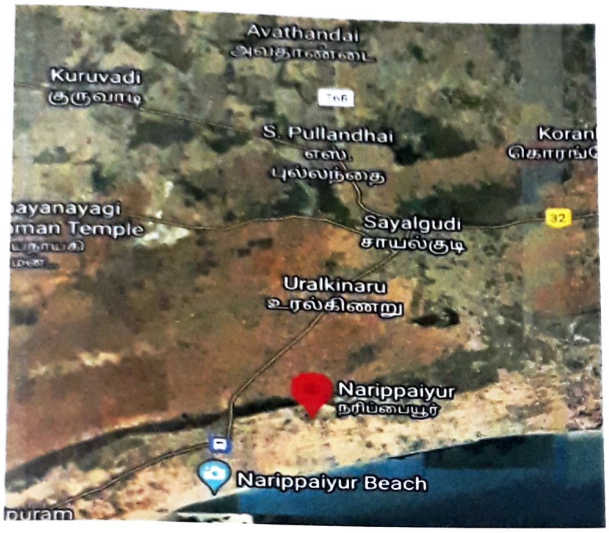


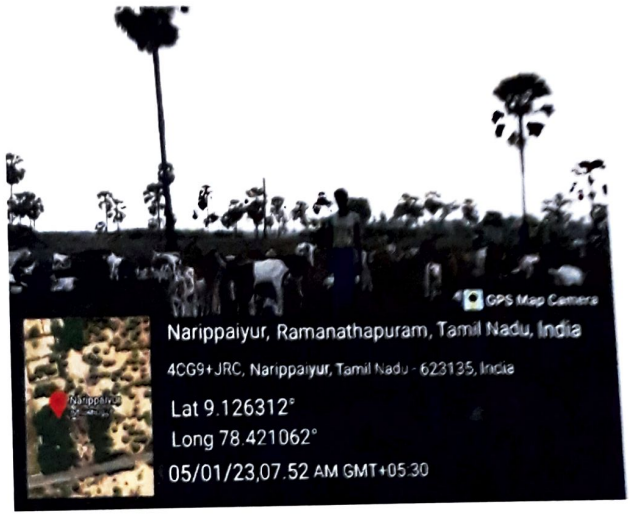
PLATE A

Study Area and Dairy Breeds

Study area – Narippaiyur, Ramanathapuram



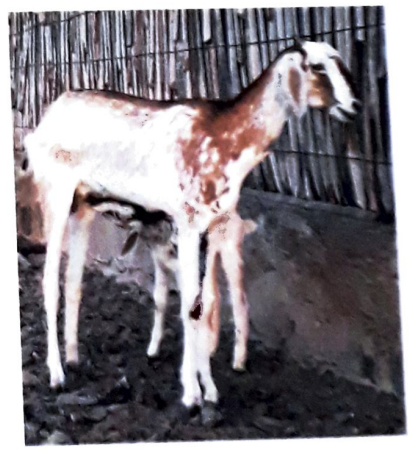
Map



Area

Dairy Breeds

Kangayam (*Bos indicus*)



Vellaadu (*Capra hircus*)

suspension for a long period. An important component present in *Capra hircus*'s milk is bioorganic sodium.

4.2.3 Collection of milk sample

The samples of *Bos indicus* and *Capra hircus*'s raw milk were procured locally from a farmer at Narippaiyur, Ramanathapuram. The samples were collected and brought to the laboratory.

4.3 Analysis of milk sample

4.3.1 Sensory quality (BIS 1975)

A panel of four members were arranged to test the sensory quality of milk. They evaluated the colour and appearance (10), flavor (10), odour (10), body and texture (10) and overall acceptability (10). The scores were given to the sample for 100 and average was calculated.

4.3.2 Physico-chemical quality

I. Estimation of pH (Scott *et al.*, 2000)

The pH of the milk sample was measured with the help of the pH meter.

II. Clot on boiling test (COBT)

Clot on boiling test was performed following the procedure described by BIS (1981).

III. Methylene blue reduction test (MBRT)

10 ml of samples was transferred into labelled sterile screw cap test tubes and 1 ml of methylene blue solution was added. The contents were mixed by inverting the tubes gently for 4 – 5 times. The tubes were incubated in a water bath at 37°C for 6-8 hours. After every half an hour the tubes were observed for the reduction of methylene blue and reduction time was noted and accordingly and the quality of sample was noted.

Proximate analysis of raw cow and goat milk samples

Methods described by AOAC, (2005) was used to analyze moisture content, ash, crude protein, fat, carbohydrates, titratable acidity and total solids.

4.3.3 Microbial Quality

Methods for bacteriological analysis

The method enumerated by Speck (1992) was adopted in this study. Following bacterial count was made.

Total Viable Count (TVC)

The bacteriological test routinely executed was TVC. A suspension of the milk was made by blending 10 ml of sample with 90 ml of sterile maximum recovery diluents namely saline peptone diluent (0.85% W/V saline and 0.1% peptone). From

initial suspension, further tenfold dilution series were used to prepare agar plates using nutrient agar. The medium was autoclaved at 121°C for 15 minutes.

Pour plate technique was followed in this study. Plates were incubated at 30°C for 2-3 days to give total viable count or aerobic colony count. Triplicate was prepared for each dilution.

At the end of incubation, the plate was removed from incubator and inspected for the presence of visible colonies. Plates containing 30-300 colonies were counted and expressed in "colony forming units" log cfu/ml. The plates used for counts were also used for counting acid producing colonies, proteolytic colonies and chromogenic colonies.

4.3.3 Coliform count (CC)

To obtain coliform count plates were made with Eosin Methylene Blue (EMB) agar and inoculated with selected milk samples.

Eosin – Methylene blue (EMB) agar (Levine)

The ingredients were dissolved and autoclaved at 121°C for 24 hours. All the metallic sheen colonies that were greater than 0.5 mm in diameter and surrounded by halo were counted for coliform and expressed as log cfu/ml.

4.3.4 Psychotropic count (PC)

The plates were inoculated with selected milk samples and incubated at 7°C for 10 days for enumeration for psychrotropic count and expressed as log cfu/ml.

Spore count (SC)

The number of spore bearers is important in dairy industry. 10 ml of sample bromocresol purple milk in triplicates was inoculated with 10, 1.0, and 0.1 ml of milk. The samples were heated at 80°C for 10 minutes and overlaid with 3ml of 2 % agar. The tubes were incubated for 7 days. Gas formation was noted and Jacobs and Gerstein's (1960) MPN table was consulted to estimate the count.

Yeast and Mould count (YMC)

Plates of potato glucose agar (pH adjusted to 3.0 with tartaric acid) were inoculated and incubated at 23°C ± 2°C for 5 days. Observation were made for the yeast and moulds and expressed as log cfu/ml.

4.4 Identification of coliforms - E.coli

Coliforms were identified by following standard method as described in sneath *et al.*, (1986)

Gram staining (Hucker's version)

Bacterial smear was stained with crystal violet solution for 1 minute and washed with tap water. The smear was stained with Gram's iodine for 1 minute. The

smear was decolourized with 95% ethanol until no more stains comes away and washed with tap water. The smear was counter stained with the safranin solution for 2 minutes. Gram's reaction together with the shape of the cells, size and arrangement was observed.

4.4.1 IMVIC Test

It is combination of following 4 tests.

Indole production test

I. Kovac's method

Peptone broth was inoculated with the test culture and incubated for 24-48 hours. After incubation 0.5 ml of Kovac's reagent was added and agitated gently. Development of a rose pink colour indicated indole.

II. Methyl red (MR) test

Tubes of MR-VP broth were inoculated with the test culture and incubated at 37°C for 48 hours. After incubation few drops of methyl red solution was added and observed for ring of colour formed. A bright red colour indicated positive reaction and yellow and orange colour indicated negative reaction.

III. Voges-Proskauer (VP) test

The test colour were inoculated into MR-VP broth and incubated at 37°C for 48 hours. After incubation 1ml of 40% potassium hydroxide and 3ml of 5% alcoholic solution of alpha-napthal were added. A positive reaction was indicated by the development of bright pink or eosin colour in 5 minutes.

IV. Citrate utilization test

Simmon's citrate agar was incubated with the test culture by means of stab and streak the inoculation and incubated 37°C for 24-48 hours. The slants were observed for the growth of the organism and color change from green to Prussian blue.

4.5 MALDI TOF method of bacterial identification

Matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) is replacing traditional methods for identifying microorganisms in the clinical laboratory. This relatively simple technique overcomes many of the challenges of identifying bacteria and fungi. MALDI-TOF MS is an analytical technique in which particles are ionized, separated according to their mass-to-charge ratio, and measured by determining the time it takes for the ions to travel to a detector at the end of a time-of-flight tube. The resulting spectrum, with mass-to-charge values along the x-axis and intensity along the y-axis, is compared to a database of spectra from known organisms.

4.6 Statistical analysis

The statistical analysis of the data was performed as per the method described by Snedecor and Cochran (1967).

1. Mean

The average ($\bar{x}$)

$$\bar{x} = \frac{\sum x}{n}$$

Where,

$\bar{x}$ = data obtained

$\sum X$ = sum of value of samples

N = total number of samples

2. Standard Deviation (SD)

$$SD = \sqrt{\frac{\Sigma d^2}{n-1}}$$

d = deviation of each score from mean

n = total no of samples

3. Standard error of mean ($SE_{\bar{x}}$)

$$SE_{\bar{x}} = \frac{SD}{\sqrt{n-1}}$$

SD = standard deviation

n = total number of samples

4. Finding out 95% confidence limit

Student's t-test was used to compute 95% confidence limits. Lower and upper limits were estimated using the following methods.

$$\bar{x} \pm t_{0.05} \frac{s}{\sqrt{n}}$$

Lower limit $= \bar{x} - t_{0.05} \frac{s}{\sqrt{n}}$

Upper limit $= \bar{x} + t_{0.05} \frac{s}{\sqrt{n}}$

Critical value of $t_{0.05}$ was referred from 't' distribution table.

5. Correlation

Correlation coefficient r was calculated by the following formula.

$$r = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{n}}{\sqrt{\left[\Sigma x^2 - \frac{(\Sigma x)^2}{n}\right]\left[\Sigma y^2 - \frac{(\Sigma y)^2}{n}\right]}}$$

To test the significance of 'r', a quantity was calculated which has the same distribution of student 't'.

$$t = \frac{r}{\sqrt{\frac{(1 - r^2)}{N - 2}}}$$

r = absolute value of the correlation coefficient

N = number of paired observations that were made

Critical value of 't' is compared with the calculated values and decision about the acceptance or rejection of H_o or H_a was made.

6.Analysis of variance (ANOVA)

Steps in computation

1. Grand total(N) = $n_1 + n_2$
2. Sum of squared observations = $\sum x_1^2 + \sum x_2^2$
3. Sum of the squared observation is divided by n = $\frac{(\sum x_1)^2}{n_1} + \frac{(\sum x_2)^2}{n_2}$
4. Grand total squared and divided by total sample size (CF) = $\frac{(\sum x)^2}{N}$
5. $SS_{total} = [\sum x_1^2 + \sum x_2^2] - \frac{(\sum x)^2}{N}$
6. $SS_{between} = \left[\frac{(\sum x_1)^2}{n_1} + \frac{(\sum x_2)^2}{n_2}\right] - \frac{(\sum x)^2}{N}$
7. $SS_{within\ groups} = SS_{total} - SS_{between}$

8. The ANOVA table was constructed as follows.

Source of variation	DF	SS	MS	F value
Between groups	K-1	$SS_{between\ groups}$	$\frac{SS_{between\ groups}}{K-1}$	$\frac{MS_{between}}{MS_{within}}$
Within groups	N-K	$SS_{within\ groups}$	$\frac{SS_{within\ groups}}{N-K}$	
Total	N-1	SS_{total}	-	-

The calculated F_s was compared with critical value of $F_{0.05}$ for V_1, V_2 degrees of freedom to draw conclusion about the variance component.

RESULTS

5.0 RESULTS

The current project was developed to explain the organoleptic, physico-chemical and microbial quality of raw milk samples of two different species. With this analysis we found a great variation between the two samples, not because of the species difference but also their microbiological quality shows a lot of variation too.

5.1 Organoleptic Analysis:

The Table I and II portray the scores of sensory quality of the tested raw milk samples. The total score was found to be the highest for goat milk. It has scored A grade. Milk of cow has scored B grade. The scores are illustrated in the Figure 1 and 2.

5.2 Physico-Chemical Analysis:

The result of this investigation demonstrates the physico-chemical analysis of the raw milk samples and they are shown in the Table III. The pH of the raw milk sample of goat is 8.8 and cow is 7(Neutral).

Clot on Boiling test was found to be negative for both the samples. Methylene Blue Reduction test reflected the quality of both the milk samples. The quality of goat milk is good and cow milk is also good.

5.3 Microbial Quality Analysis:

5.3.1 Microbial quality of cow milk:

The microbial profile of raw cow milk sample is illustrated in Table IV and Figure 3. The microbial colony count in TVC, acid producers, proteolytic colonies, chromogenic colonies, psychrotrophic colonies, coliform count and yeast mould count are found to be $\log_{10}$ 1.7859 cfu/ml, $\log_{10}$ 0.5689 cfu/ml, $\log_{10}$ 0.3112 cfu/ml, $\log_{10}$ 0.1798 cfu/ml, $\log_{10}$ 0.5187 cfu/ml, $\log_{10}$ 1.9314 cfu/ml and $\log_{10}$ 2.0195 cfu/ml. Spore counts were not observed. Plate B clearly depicts the TVC, PC, YMC and CC sample from the cow milk.

5.3.2 Microbial quality of goat milk:

The microbial colony count in TVC, acid producers, proteolytic colonies, chromogenic colonies, coliform count and yeast mould count are found to be $\log_{10}$ 2.2507 cfu/ml, $\log_{10}$ 0.3597 cfu/ml, $\log_{10}$ 0.4407 cfu/ml, $\log_{10}$ 0.3010 cfu/ml, $\log_{10}$ 2.6673 cfu/ml and $\log_{10}$ 2.7569 cfu/ml. Psychrotrophic count and spore count were not observed. Plate C provides the reference to TVC, PC, YMC and CC of the sample from goat milk. The microbial profile of raw goat milk sample is illustrated in Table V and Figure 4.

Cultural and morphological characteristics of the bacterial colonies on agar plate are shown in Table VI. The variation revealed by the statistical analysis of the samples reflected the distribution of microbes during the analysis.

With the 95% confidence limit the upper and lower confidence limits were specified with 95% probability for all counts of both the samples.

5.4 Correlation Analysis:

The correlation analysis which was performed to estimate the degree and direction of association between the microbial counts of the two varieties of milk revealed some relationship of statistical significance, mentioned in table VII. Null and alternative hypothesis were tested. A very high degree of positive correlation was observed and there is a statistically significant correlation between the two samples, was also observed ($p<0.05$).

5.5 ANOVA:

The results of ANOVA are given in Table VII. F value in ANOVA was found to be statistically non-significant ($p>0.05$) for microbial counts of raw milk samples from the cow and goat.

5.6 Chemical constituents:

The proximate composition analysis (Table IX) revealed the presence of moisture (80.82%), carbohydrates (4.34%), proteins (3.71%) and fats (5.21%) in *Bos indicus*. The milk of *Capra hircus* contains moisture (82.21%), carbohydrates (4.58%), proteins (4.39%) and fats (6.31%). So, as per the result, goat milk contains more nutrients than cow and that's good for health, especially for the heart by lowering the blood cholesterol level.

5.7 Identification of *Bacillus cereus*:

Bacillus cereus was identified by MALDI TOF Mass Spectrometry with the result of 2.10 score value (>2.0: Highly Probable Species identification).

Table I

Organoleptic analysis of raw milk of cow

Panel No.	Colour and Appearance	Flavour	Odour	Body and Texture	Overall Acceptability	Total		Grade
						50	100	
1.	10	10	9	9	9	47	94	A
2.	10	10	10	9	10	49	98	A
3.	9	10	9	9	9	46	92	A
4.	7	7	7	8	7	36	72	C
Average	9±0.61	9.25±0.65	8.75±0.55	8.75±0.22	8.75±0.55	44.5	89	B
Colour: Yellowish White								
Consistency (Porcelain tile test): Thick (Didn't run off)								

Figure 1: Organoleptic Analysis of Raw Milk of Cow

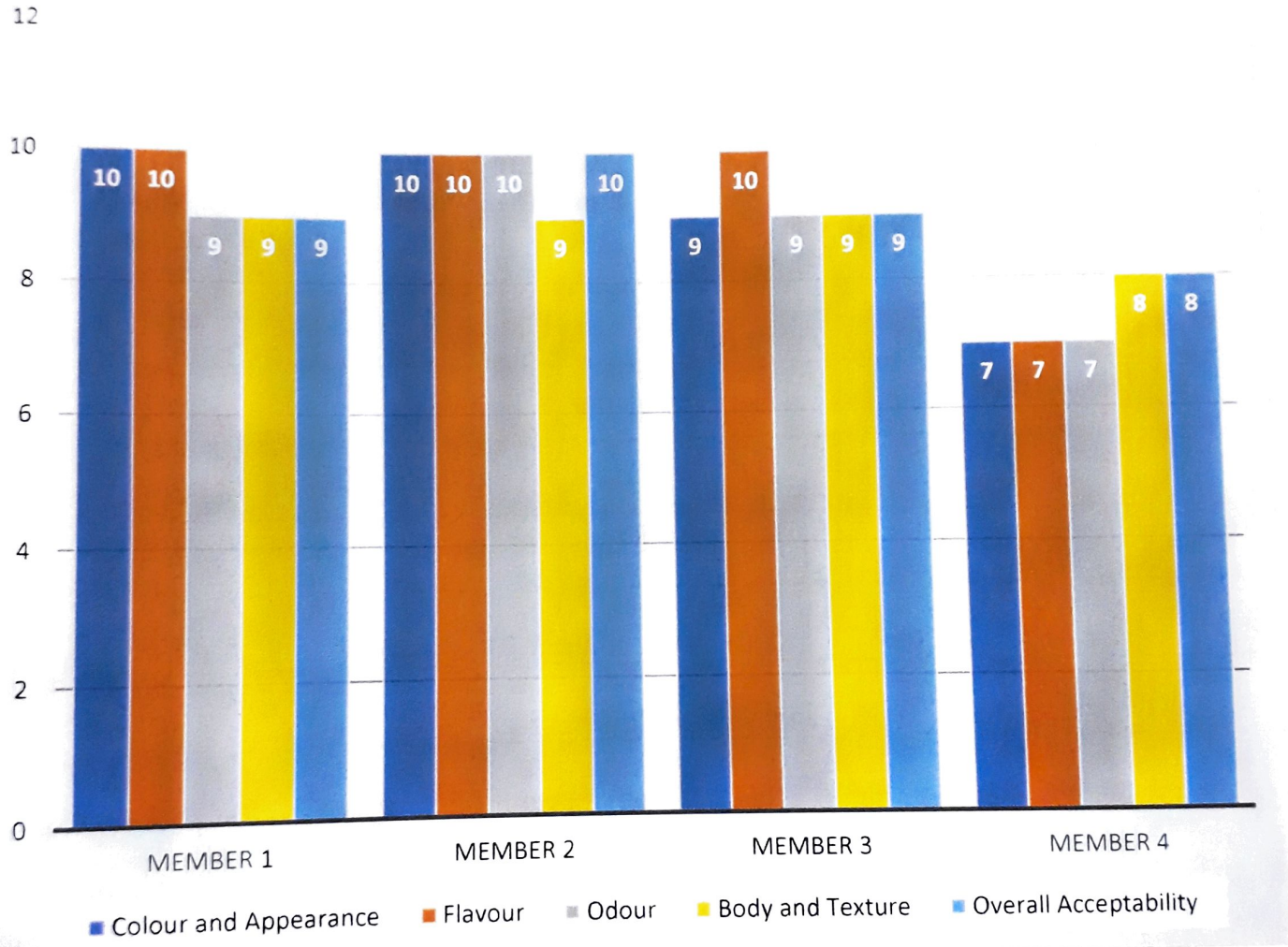


Table II

Organoleptic analysis of raw milk of goat

Panel No.	Colour and Appearance	Flavour	Odour	Body and Texture	Overall Acceptability	Total		Grade
						50	100	
1.	10	10	10	10	10	50	100	A
2.	10	8	9	9	9	45	90	A
3.	10	7	8	10	8	43	86	B
4.	9	8	8	10	9	44	88	B
Average	9.75±0.23	8.25±0.55	9.75±0.65	9.75±0.22	9±0.35	46.5	93	A
Colour: Yellowish White								
Consistency (Porcelain tile test): Thick (Didn't run off)								

Figure 2: Organoleptic Analysis of Raw Milk of Goat

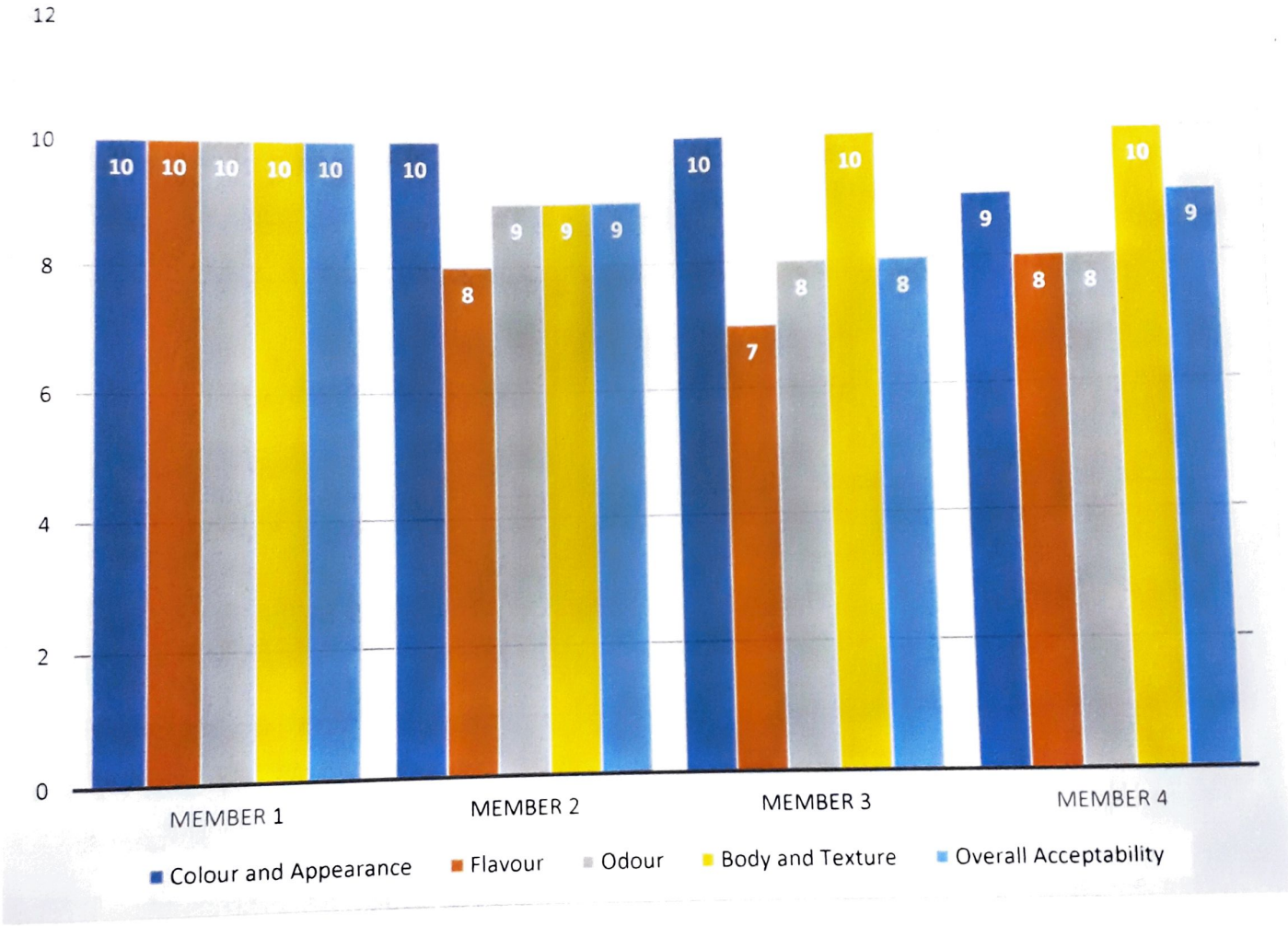


Table III

Physico-chemical analysis of raw milk samples

S.No	Samples	pH	COB Test	MBR Test	
				Hours	Quality
1.	Cow Milk	7	Negative	4 hours	Good
2.	Goat Milk	8.8	Negative	4 hours	Good

COB – Clot on Boiling

MBR – Methylene Blue Reduction

Table IV

Microbial profile of raw milk of cow

Microbial Count log10 cfu/ml	Mean	Standard Deviation	Standard Error	Confidence Limit	
				Upper	Lower
Total Viable Count (TVC)	1.7859	0.69817	0.3122	2.5885	0.9833
Acid Producers	0.5689	0.2561	0.1145	0.8632	0.2746
Proteolytic Colonies	0.3112	0.3896	0.1948	0.8519	0.2295
Chromogenic Colonies	0.1798	0.3178	0.1421	0.5451	0.1855
Psychrotropic Colonies	0.5187	0	0	0	0
Coliform Count	1.9314	2.0518	1.4508	8.1733	4.3105
Yeast and Mould Count	2.0195	1.3748	0.9721	6.2024	2.1634
Spore Count	-	-	-	-	-

Figure 3: Microbiological Quality of Raw Milk of Cow

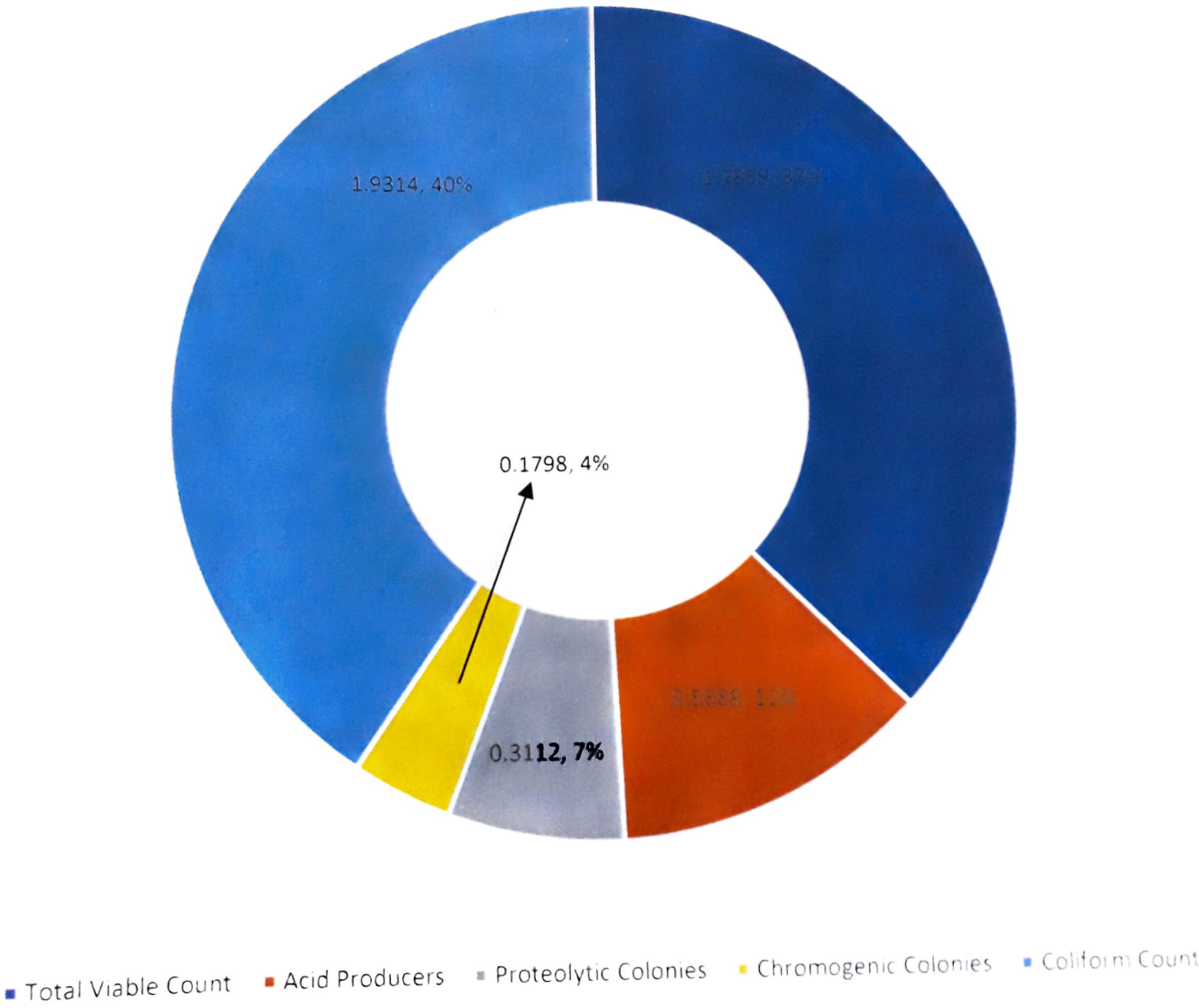
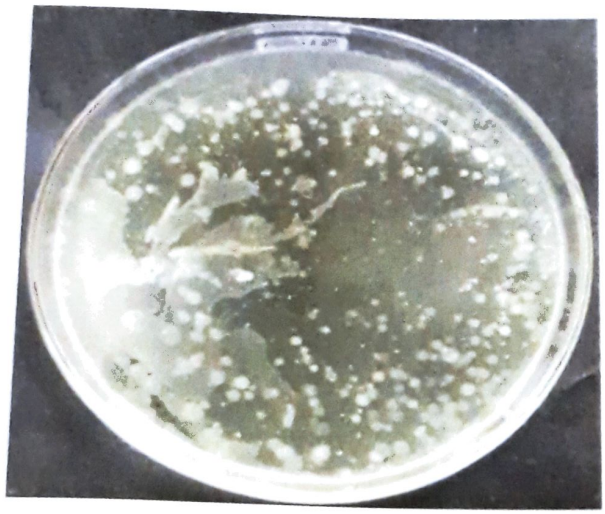
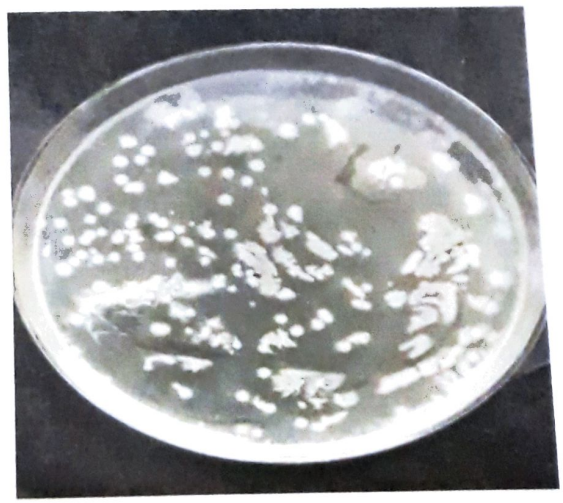
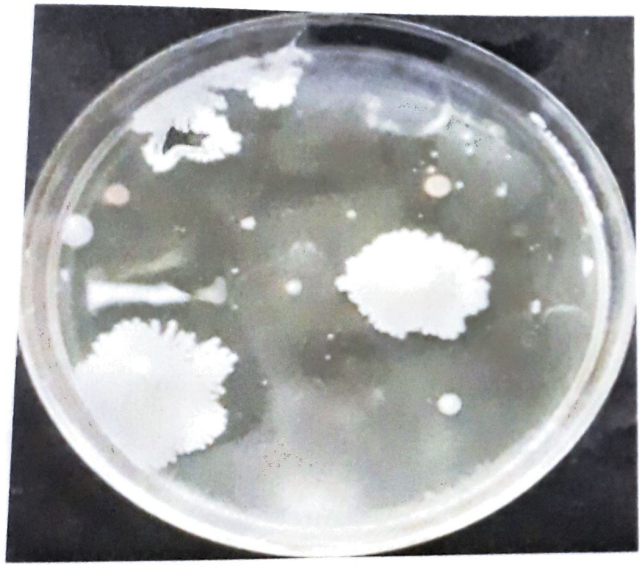


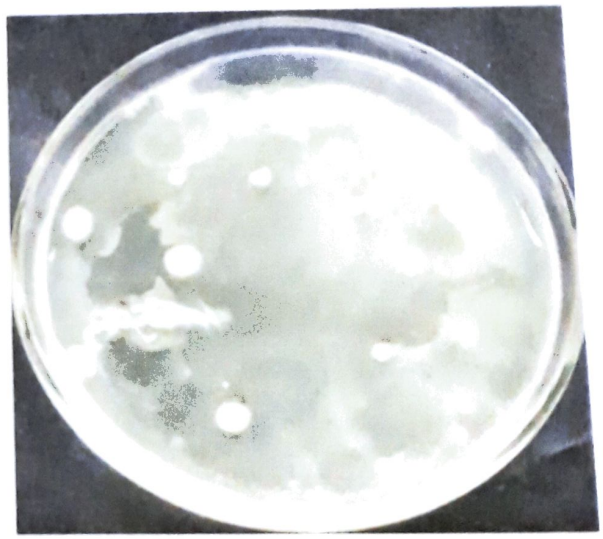
PLATE B

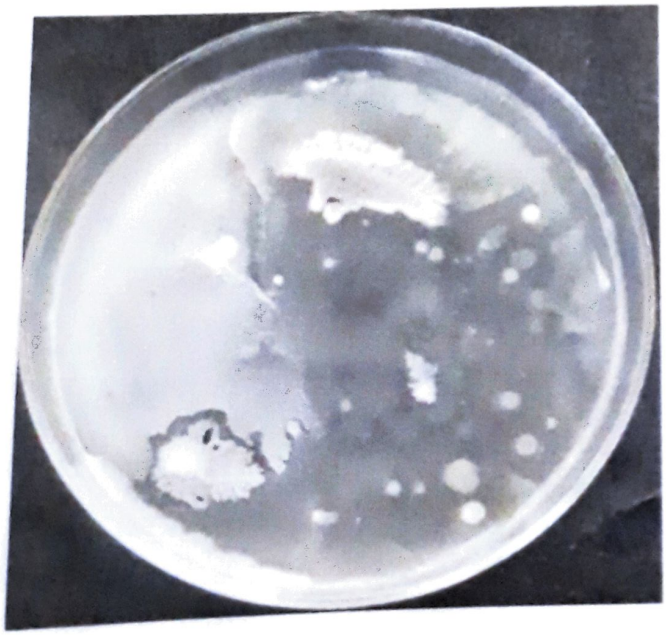
Total Viable Count of raw milk of cow











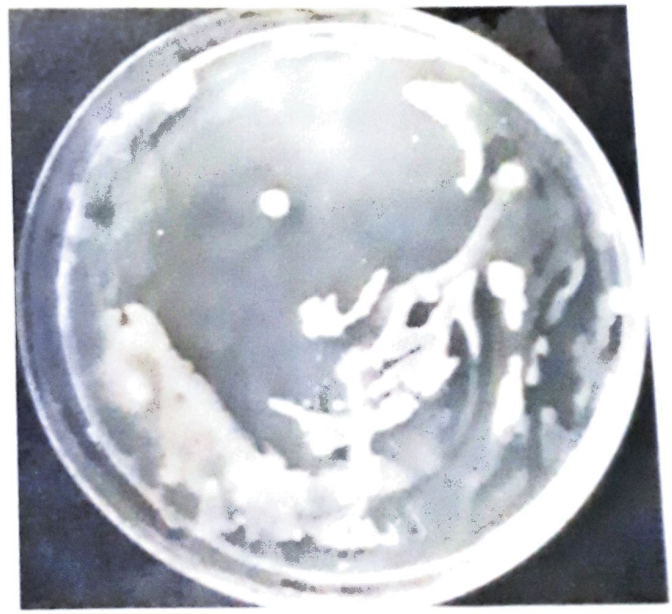
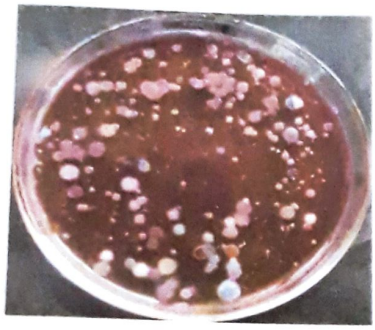
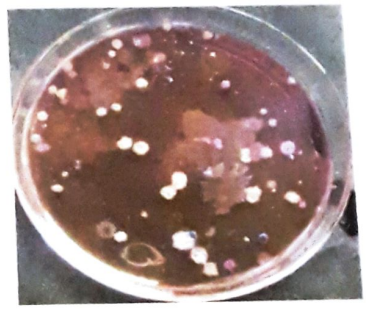
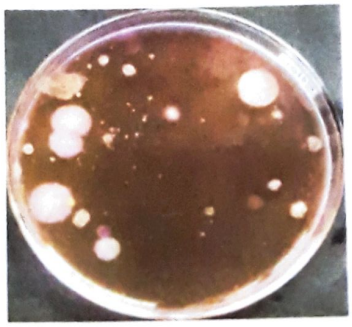


PLATE C

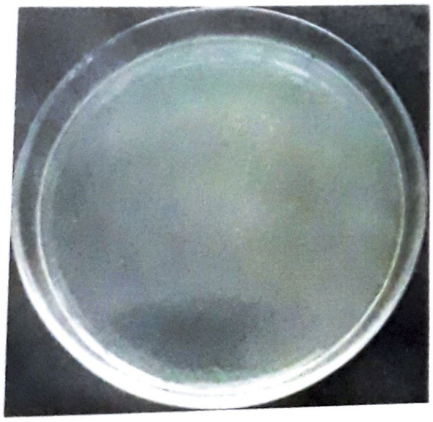
Coliform count

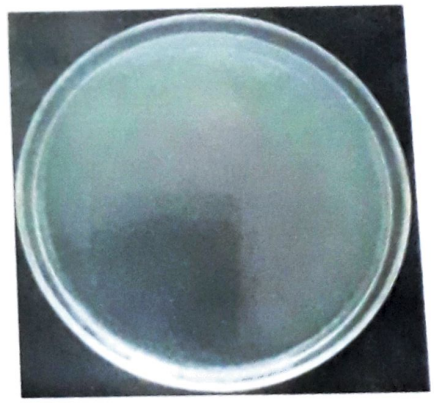






Psychrotrophic count





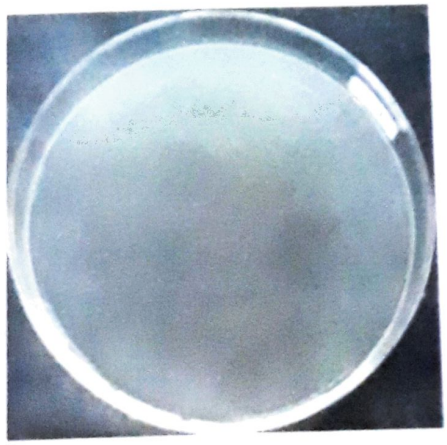
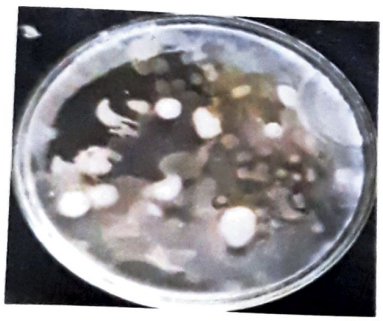
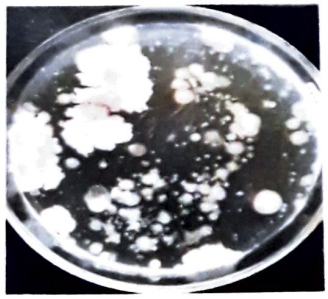
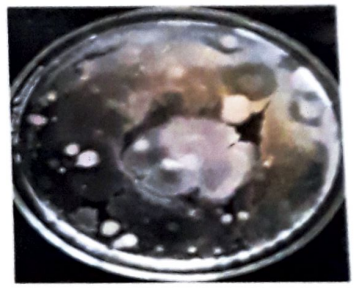
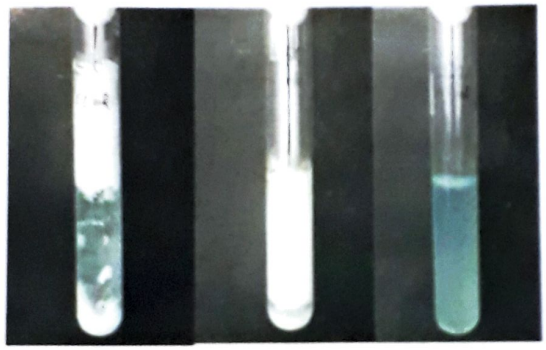


PLATE D

Yeast and Mould Count

Spore Count



Methylene Blue Reduction Test

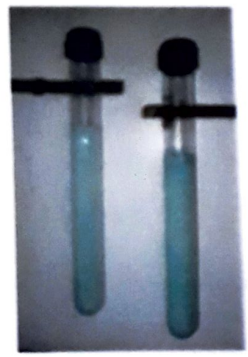




Table V

Microbial profile of raw milk of goat

Microbial Count log10 cfu/ml	Mean	Standard Deviation	Standard Error	Confidence Limit	
				Upper	Lower
Total Viable Count (TVC)	2.2507	0.2561	0.0968	2.6672	1.8342
Acid Producers	0.3597	0.40995	0.2899	1.6071	0.8877
Proteolytic Colonies	0.4407	0.4237	0.2996	1.7298	0.8484
Chromogenic Colonies	0.3010	0	0	0	0
Psychrotrophic Colonies	-	-	-	-	-
Coliform Count	2.6673	0.2826	0.1998	3.5270	1.8076
Yeast and mould count	2.7569	0.1714	0.1714	4.9347	0.5791
Spore count	-	-	-	-	-

Figure 4: Microbiological Quality of Raw Milk of Goat

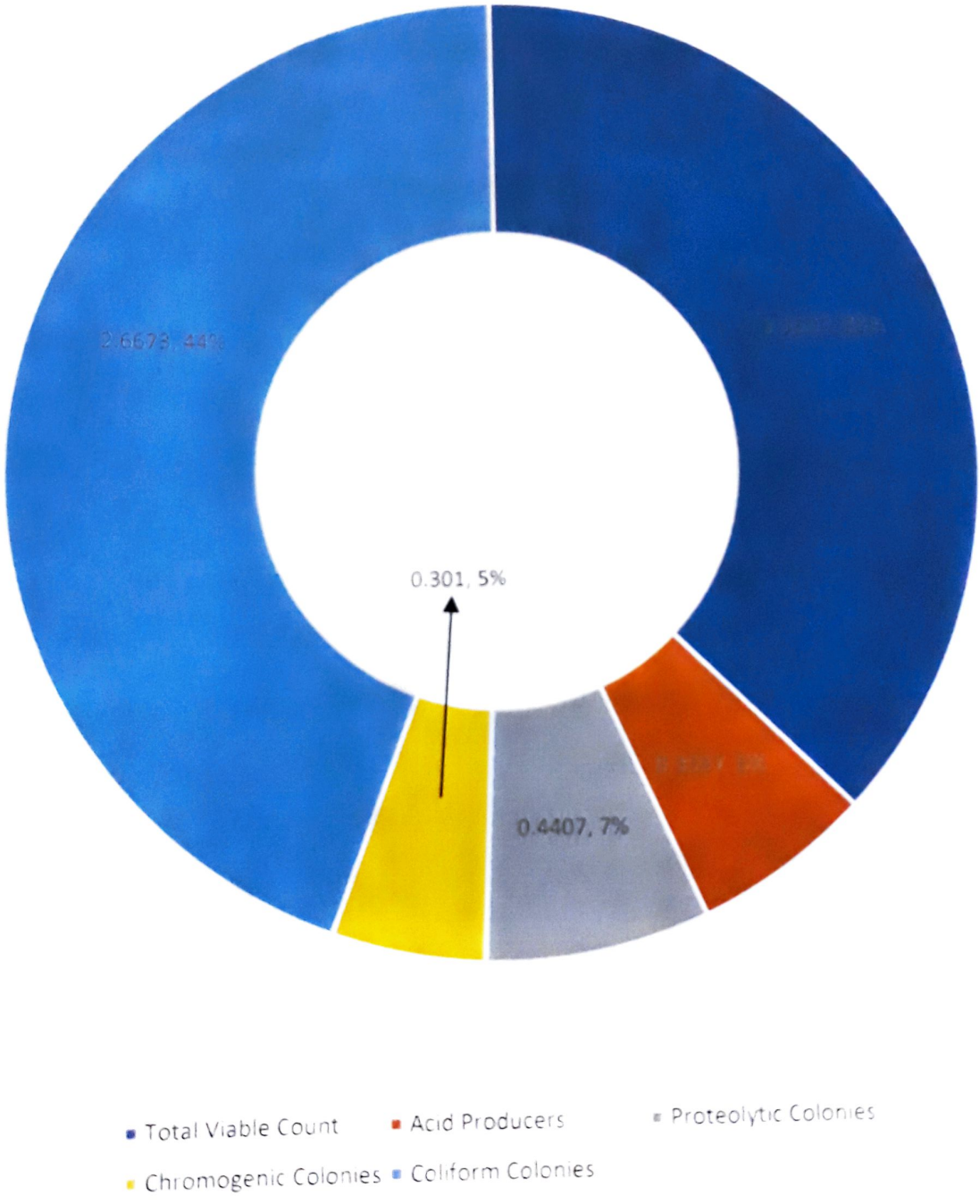
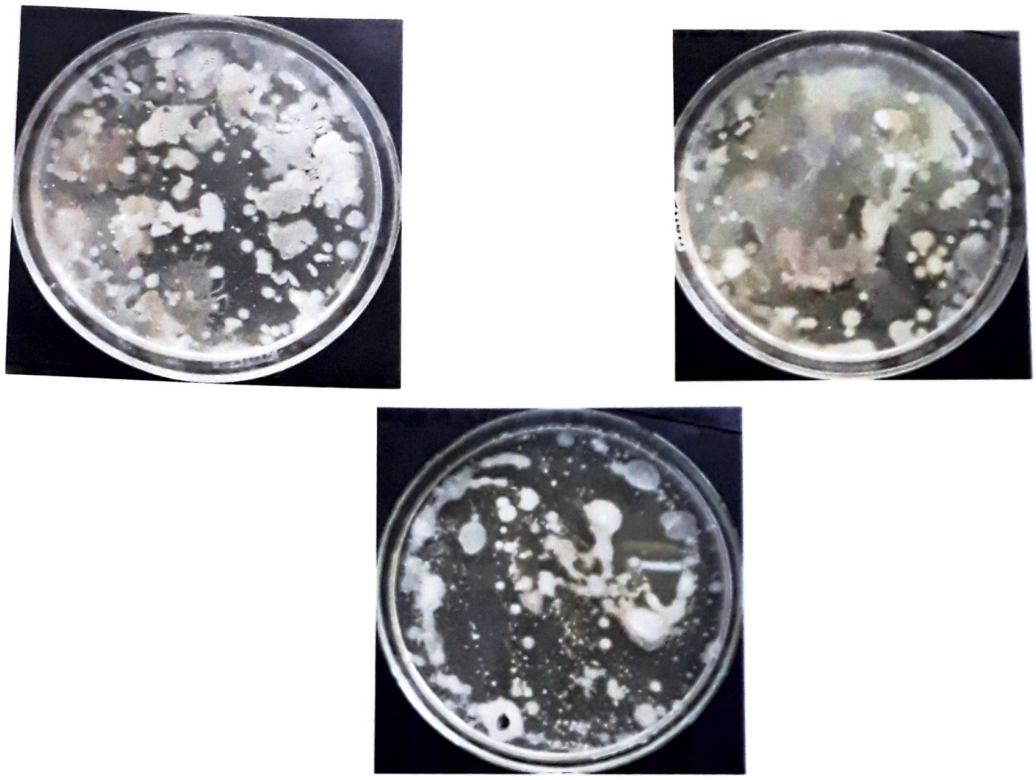


TABLE E

Total Viable Count of raw milk of goat



Coliform Count

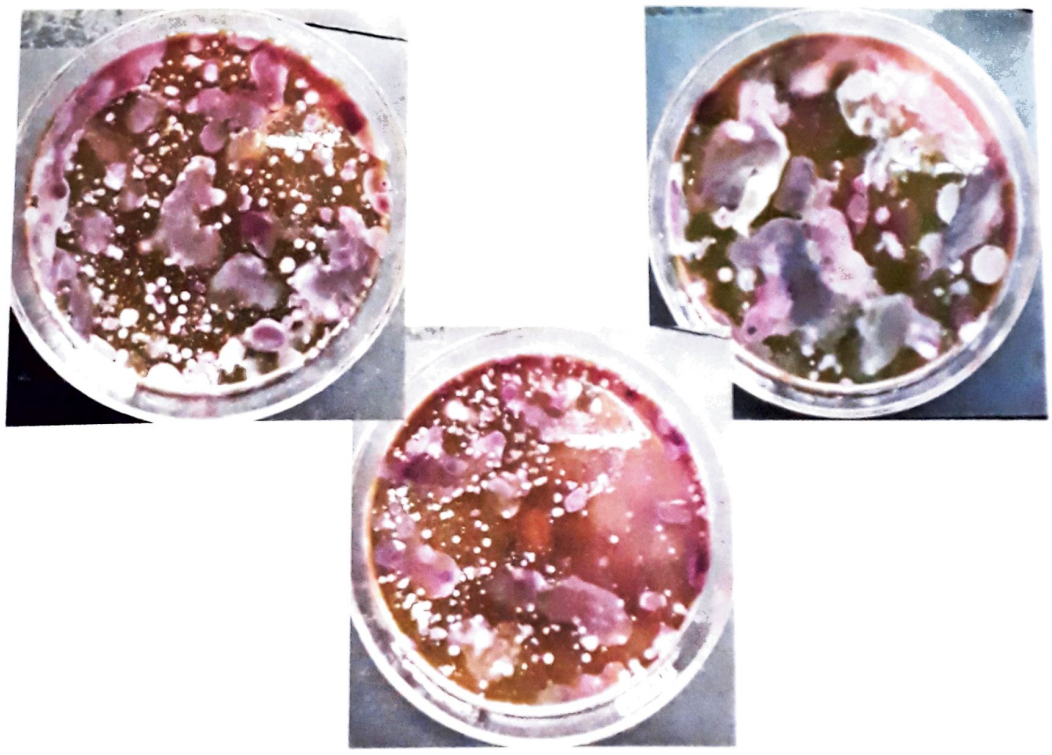
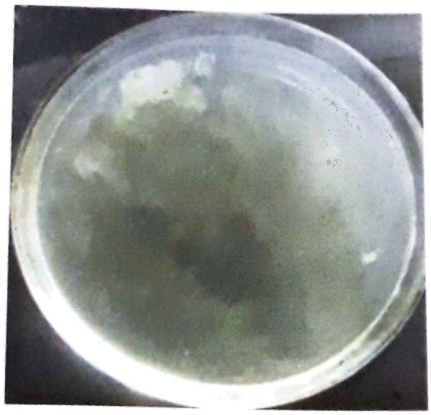
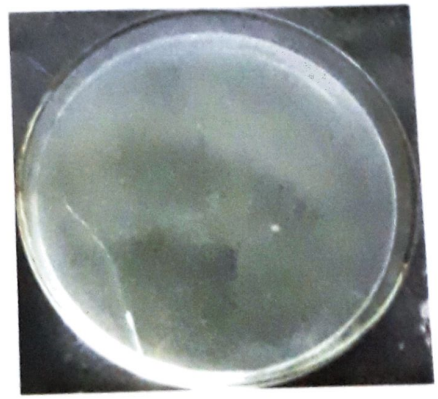
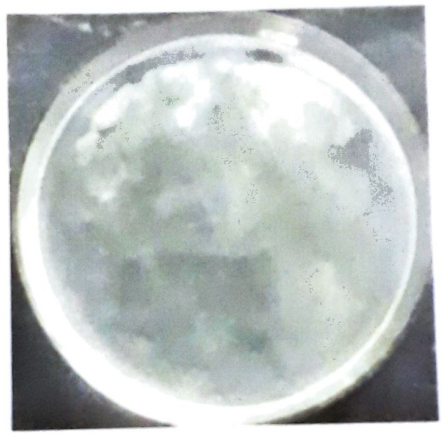


PLATE F

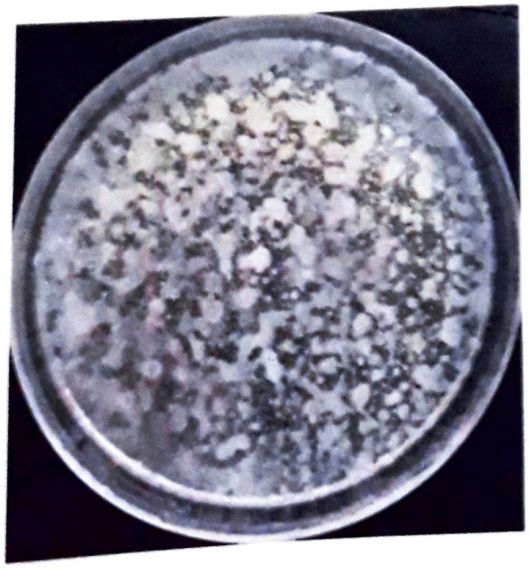
Psychrotropic Count







Yeast and Mould Count



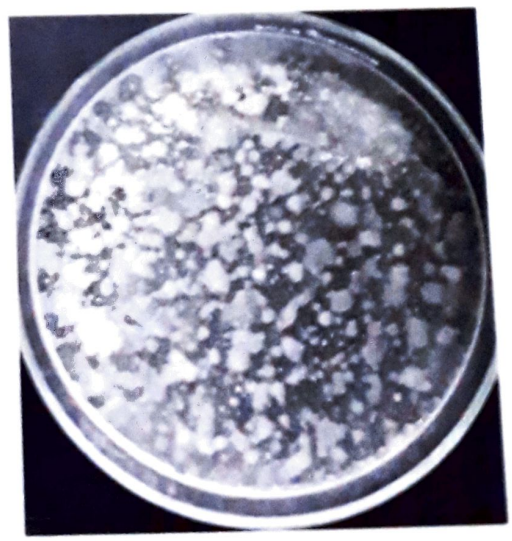
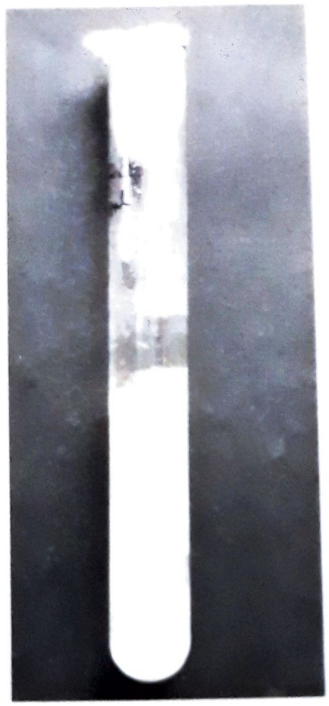
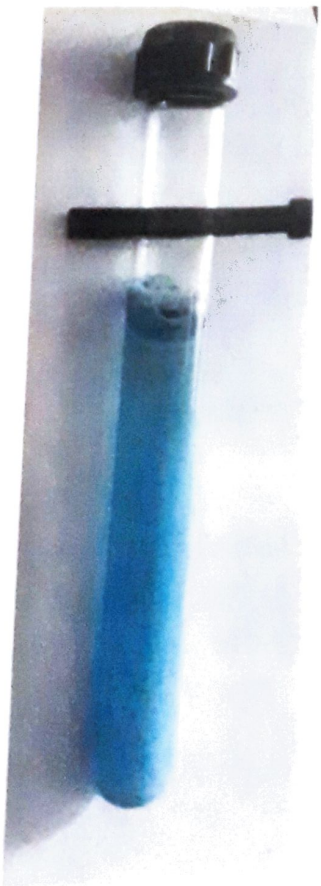


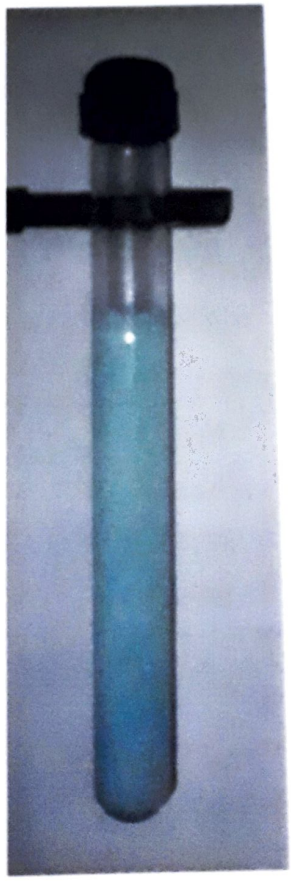
PLATE G

Spore Count



Methylene blue reduction test





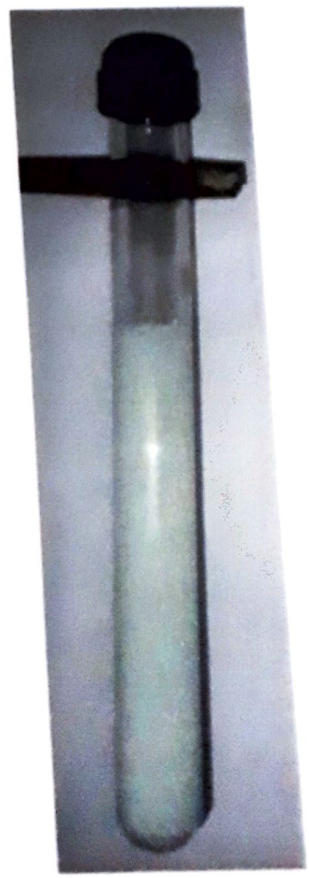


Table VI

Cultural and morphological characteristics of colonies on agar plate - Raw milk of cow and goat

Breed	Cow	Goat
Size	Pinpoint Small Moderate Large	Pinpoint Small Moderate Large
Pigmentation	White Red Blue Pink Orange Violet Yellow	White Red Blue Pink Orange Violet Yellow
Form	Circular Irregular Rhizoid	Circular Irregular Rhizoid
Margin	Entire Lobate Undulate Serrate Filamentous	Entire Lobate Undulate Filamentous
Elevation	Flat Raised Convex Umbonate	Flat Raised Convex Umbonate

Table VII

Correlation analysis between bacterial counts of raw milk samples of Kangayam and Vellaadu

Variable	'r' Value	't' Value	H_O/H_A accepted
Bacterial counts	0.99	17.2174	H_A accepted

Critical value of $t_{0.05} = 2.447$

$p < 0.05$ statistically significant

H_O: There is a no significant correlation between bacterial counts of breeds

H_A: There is a significant correlation between bacterial counts of breeds

H_A is accepted.

There is a statistically significant very high degree of positive correlation between bacterial counts of breeds.

Table VIII

ANOVA for microbial counts of raw milk of cow and goat milk

Source of variation	DF	SS	MS	F value	F Critical value
Between groups	1	0.1338	0.1338	0.1158	2.44
Within groups	14	16.1799	1.1557		
Total	15	15.7305	-	-	

$p > 0.05$ statistically non-significant

Table IX

Chemical constituents of raw milk samples of cow and goat

Parameters	Cow milk	Goat milk
Moisture %	80.82±0.05	82.82±0.89
Protein %	3.71±0.32	4.39±0.22
Fat %	5.21±0.23	6.41±0.36
Carbohydrate %	4.34±0.26	4.58±0.07
Total solids %	13.26±0.35	15.64±0.48

Table X

Biochemical characterisation and MALDI-TOF MS scores

Characteristics	Isolate 1	Isolate 2	Isolate 3
Gram Staining	Gram positive rod	Gram positive rod	Gram positive rod
Indole Production test	-	-	+
Methyl Red test	-	+	+
Voges-Proskauer test	+	-	+
Citrate Utilization test	+	+	-
MALDI-TOF MS Score	2.10	1.84	1.80
Bacterial Identification	*Bacillus cereus* 994000168 LBK	*Bacillus cereus* 994000168 LBK	*Bacillus* spp.

>2.0: Highly probable species identification

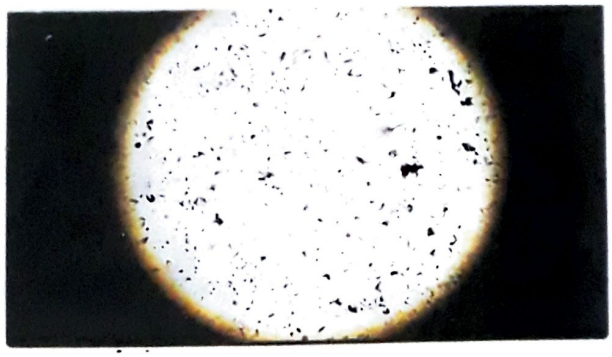
1.75-1.9: Secure genus identification, probable spp identification

1.0-1.75: Probable genus identification

<1.0: Not reliable identification by MALDI-TOF

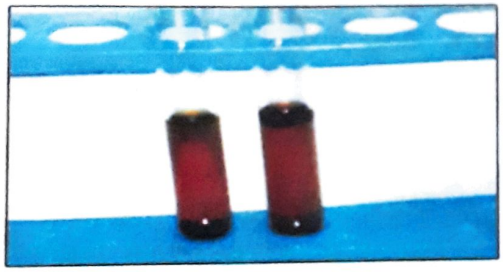
PLATE H

Gram's Staining

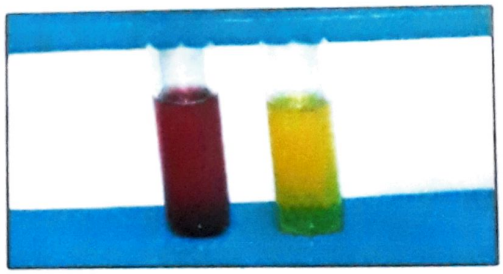


Biochemical tests - IMVIC

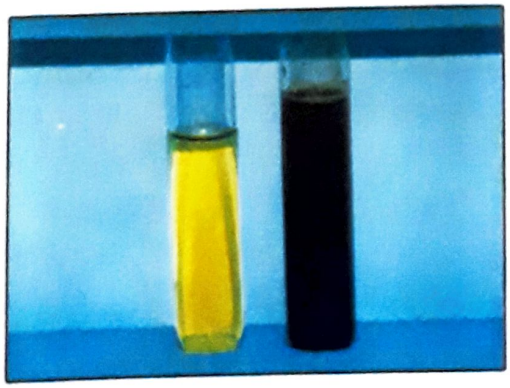
Indole Production test



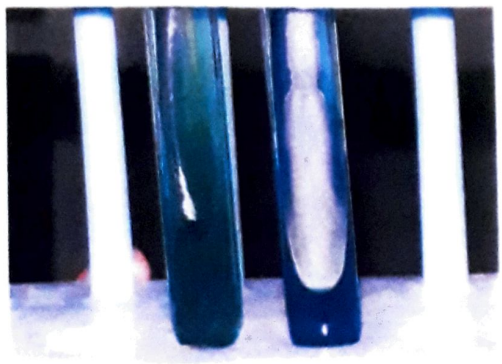
Methyl Red test



Voges Proskauer test



Citrate Utilization test



DISCUSSION

The major constituents of milk are water, fat, proteins, lactose, ash or minerals matter. The minor constituent are phospholipids, sterols, vitamins, enzymes, pigments etc., Milk is almost an ideal food, with high value. Milk proteins contain all essential amino acids in fairly large quantities. Milk is an excellent source of calcium and phosphorus, together with vitamin D for bone formation. Milk is also a good source of thiamine, riboflavin, etc., Milk fat imparts a soft body, smooth, texture and rich taste to dairy products. It increases the incentive of eating good taste.

The quality of raw milk encompasses such milk characteristics as chemical composition, physical properties, microbiological and cytological quality, sensory properties, technological suitability and nutritive value (Shahida *et al.,* 2015). The present inquiry emphasizes the organoleptic, physico-chemical and microbial quality of raw milk samples from *Bos indicus* and *Capra hircus.*

As reported by the panel members the raw milk of cow and goat has specific taste and consistency. This can depend on proper processing and handling of the milk as well as the diet of the animal. Goat milk is an excellent alternative to cow milk. The physico-chemical analysis is an important tool to scrutinize the quality of milk that encompasses with chemical composition, physical properties, microbiological and nutritive value (Czerniewicz *et al.,* 2006). Measurement of

6.0 DISCUSSION

some of the physico-chemical properties is used to assess milk quality (Hassan, 2005). Nutritionally enriched milk and its products with enhanced biological potential and without health risks are generally demanded (Khan and Zeb, 2007; Baloch *et al.*, 2006). The changes in the pH may be related with the interaction between lactose and milk proteins with the hydrolytic dephosphorylation of casein and with changes in calcium, phosphorous equilibrium (Heisch and Ren *et al.*, 2001). A positive clot on boiling test is dependent upon the initial microbial load.

The data of microbial count obtained from this study revealed the information about the microorganisms, potential shelf-life and possible public health hazards of milk products. The microbial composition of milk can also have health – related implications in that the consumption of raw milk contaminated with pathogens can lead to illness (Oliver *et al.*, 2009).

Incidences of coliforms belonging to the genera Enterobacter and E. coli in raw milk have received considerable attention and the majority of the coliforms are the *Aerobacter* spp. (Thomas and Druce, 1972). Coliforms can rapidly build up in moist, milky residues (biofilms) on milking equipment and then become a major source for contamination of the milk being collected. The presence of Coliform means that there is contamination during milking or the dairy animal might have mastitis. Coliform is one of the fastest-growing bacteria. The mastitis infection process

involves a pathogen carrier source, means of transfer, the opportunity to invade, and a susceptible host. This is due to poor plant hygiene.

Psychrotrophic microflora are those microorganisms that can thrive under refrigerated temperatures (3-7°C). An excellent review about the psychrotrophs found in milk and dairy products is given by Cousin (1982). Some of these bacteria are of considerable importance in manufactured milk products and are most commonly associated with post-pasteurization. The incidence of psychrotrophic strains of the *Bacillus* spp. containing spores in individual producer milk is low, seldom exceeding $10ml^{-1}$ Those species found in raw milk include *B. coagulans*, *B. circulars*, *B. cereus*, *B. pumilus*, and *B. subtilis*.

Milk cans are known to be a primary source of *B. cereus* spores in milk. During our microbiological analysis on raw milk samples we also have identified a pathogenic bacterial species, *Bacillus cereus*. It is a foodborne pathogen that can produce toxins, causing two types of gastrointestinal illness: the emetic (vomiting) syndrome and the diarrhoeal syndrome. When the emetic toxin (cereulide) is produced in the food, vomiting occurs after ingestion of the contaminated food (Bottone, 2010). Generally, food matrices rich in carbohydrates, such as pasta and rice, as well as milk and dairy products are associated with a highest risk of causing cereulide intoxications (Delbrassinne *et al.*, 2012, Messelhausser *et al.*, 2014).

Bacillus cereus is a common and ubiquitous foodborne pathogen with an increasing prevalence rate in dairy products in China. High and unmet demands for such products, particularly milk, raise the risk of *B. cereus* associated contamination. The presence of *B. cereus* and its virulence factors in dairy products may cause food poisoning and other illnesses.

Bacillus cereus is a Gram-positive aerobic or facultatively anaerobic, motile, spore-forming, rod-shaped bacterium that is widely distributed environmentally. While *B.cereus* is associated mainly with food poisoning, it is being increasingly reported to be a cause of serious and potentially fatal non-gastrointestinal tract infections. The pathogenicity of *B.cereus*, whether intestinal or non-intestinal, is intimately associated with the production of tissue destructive exo-enzymes. Among these secreted toxins are four hemolysins, three distinct phospholipases, an emesis-inducing toxin, and proteases.

The major hurdle in evaluating *B.cereus* when isolated from a clinical specimen is overcoming its stigma as an insignificant contaminant. Outside its notoriety in association with food poisoning and severe eye infections, this bacterium has been incriminated in a multitude of other clinical conditions such as anthrax-like progressive pneumonia, fulminant sepsis, and devastating central nervous system infections, particularly in immunosuppressed individuals, intravenous drug abusers, and neonates.

Matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) is replacing traditional methods for identifying microorganisms in the clinical laboratory. The identification of a microorganism in MALDI TOF Mass Spectrometry can be established down to the genus, and in many cases to the species and strain level (Fagerquist *et al.*, 2010). This relatively simple technique overcomes many of the challenges of identifying bacteria and fungi. As the technology has evolved, the expansion of the databases containing spectra of known organisms has allowed for the identification of species with similar phenotypic, genotypic, and biochemical properties that was not previously possible.

Identification of bacteria and fungi by traditional methods can be a time consuming and complex task. Workup of bacteria and yeasts may include assessing colony and Gram stain morphology followed by phenotypic and biochemical testing. MALDI-TOF MS was first approved for clinical use in China in 2012 when bio Mérieux VITEK MS system was approved by China State FDA for *in vitro* diagnostic (IVD) purposes (Luo *et al.*, 2015).

MALDI-TOF MS is an analytical technique in which particles are ionized, separated according to their mass-to-charge ratio, and measured by determining the time it takes for the ions to travel to a detector at the end of a time-of-flight tube. Recently, a very novel application of MALDI-TOF MS was shown by Guembe *et al.*, (2014). The resulting spectrum, with mass-to-charge values along the x-axis and

intensity along the y-axis, is compared to a database of spectra from known organisms. This technology can identify Gram-positive, Gram-negative, aerobic and anaerobic bacteria as well as mycobacteria, yeasts and moulds, typically at the species level, with accuracy as good and often better than traditional methods when compared to sequencing. It has also been used for rapid identification of atypical, Gram-negative environmental organisms and respiratory tract pathogens which chronically infect patients with cystic fibrosis (Alby *et al.*, 2013). The method is also more reliable than traditional and molecular methods for microorganism identification. Exceptions to this are species not included in the database and species that are inherently similar to one another. This contributes to a reduction in the time to identification by at least one day for most bacteria.

Among the organisms that are particularly difficult to identify to the species level using traditional methods, but readily identified by MALDI-TOF MS, are the coagulase-negative staphylococci and bacteria with complex nutritional requirements such as the nutritionally variant streptococci and organisms. MALDI-TOF MS has also been applied successfully in food microbiology for various purposes like, identification and classification of lactic acid bacteria in fermented food (Nguyen *et al.*, 2013), detection of bacteria involved in spoilage of milk (Nicolaou *et al.*, 2011), identification of bacteria isolated from milk of dairy cows (Barreiro *et al.*, 2010). The speed with which MALDI-TOF MS can identify

microorganisms helps to quickly guide treatment decisions, which is especially critical when the infecting pathogen is unexpected.

SUMMARY

7.0 SUMMARY

Milk plays an important role in this rotating globe. That's why we all are celebrating World Milk Day on June 1st every year. Milk provides humans with strong bones, healthy teeth, muscle growth, brain development, fatigue reduction and supports normal immune functioning. In our developing nation milk becomes a source of life. To the citizens, obtaining safe and uncontaminated milk is a potential problem which brings out serious health consequences and has a public health significance.

In the current investigation an evaluation on the sensory, physico-chemical and microbial quality and safety of raw milk samples from cow and goat was made. The following are the major findings of the current study.

- Milk of *Capra hircus* scored high in organoleptic analysis – A grade.
- pH was higher for *Capra hircus* and the pH was 8.8.
- Clot on boiling was negative for both the milk samples.
- Methylene Blue Reduction test revealed that the milk of both *Bos indicus* and *Capra hircus* was in a good quality.
- Amount of carbohydrates, proteins, and fats was higher in goat milk.
- TVC of *Bos indicus* was $\log_{10}1.7859$ cfu/ml. Acid producers, chromogenic colonies, coliform counts and psychrotrophic counts were recorded.

- TVC of *Capra hircus* was $\log_{10}2.2507$ cfu/ml. Acid producers, chromogenic colonies and coliform counts were recorded.
- Spore counts were not observed.
- Pathogenic species *Bacillus cereus* was indicated with the help of MALDI TOF – MS.

SUGGESTIONS AND CONCLUSIONS

8.0 SUGGESTIONS AND CONCLUSION

"We're organisms; we're conceived, we're born, we live, we die, and we decay. But as we decay we feed the world of the living: plants and bugs and microbes." We all are living a coexisted life with the microbes. India has the world's largest dairy herd with over 300 million bovines, producing over 187 million tonnes of milk. India is first among all countries in both production and consumption of milk. In India there is a lack of nutritional and health security.

The suggestions are:

- Hygienic practices have to be followed from cow and goat to consumers.
- Care during production of food is required to prevent contamination and growth of microorganisms that will extend the storage life.
- For the milk we have to keep the microbial load as low as possible and hopefully, there is a less chance of food borne illness when the food is ingested.
- Water is commonly used for clearing the udder of dairy breeds and for adulteration contribute bacteria to milk. Checking microbial quality of water is very important as it affects the quality of milk.

- Rather than *Homo sapiens*, who will safe guard the milk of dairy breeds? Due to the increased knowledge in microbes and sanitation, as well as increased regulations, we are the only one to took the responsibility.
- People should be aware and beware of safety, keeping quality and microbial criteria for evaluation.
- Stringent guidelines have to be followed regarding Hazard Analysis Critical Control Point (HACCP).
- Education, dissemination of information and creating awareness in people are the pressing demands of the hour.
- Milk available should be safer today than in the good old days, however due to large-scale high-speed processing, alteration of traditional preservative method and proliferation of heat and eat convenience, there is a new door to overcome these problems to benefit the population in future.
- The programmes to conserve and enhance the productivity of India's dairy cattle breeds in mission mode

Conclusion:

The results from the study concluded that goat milk had the best physico-chemical properties and sensory characteristics as compared to cow milk.

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9.0 BIBLIOGRAPHY

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APPENDIX

10.0 APPENDIX

i. **Indicators Methyl red**

Methyl red	-	0.1 g
Ethanol	-	300 ml
Distilled water	-	200 ml

ii. **Gram staining – Hucker's version**

1. **Crystal violet**

Solution

Crystal violet (90%) dye content	-	2 g
Ethyl alcohol (95%)	-	20 ml

Solution B

Ammonium oxalate	-	0.8 g
Distilled water	-	80 ml

2. **Gram's iodine**

Iodine	-	1 g
Potassium iodide	-	2 g
Distilled water	-	300 ml

3. **Ethyl alcohol (95%)**

Ethyl alcohol (100%)	-95 ml
Distilled water	-5 ml

4. **Safranin**

Safranin	-	0.25 ml
Ethyl alcohol (95%)	-	10 ml
Distilled water	-	100 ml

iii. **Reagent Kovac's Reagent**

P.dimethyl amino benzaldehyde	-	5 g
Amyl alcohol	-	75 ml
Conc.Hydrochloric acid	-	25 ml

The aldehyde was dissolved in the alcohol at 50-55°C, cooled and acid was added.

iv. **Microbiological Media**

1. **Eosin- Methylene blue (EMB) agar (Levine)**

Peptone	-	10 g
Lactose	-	5 g
Dipotassium hydrogen phosphate	-	2 g
Agar	-	13.5 g
Eosin	-	0.4 g
Methylene blue	-	0.065 g
Distilled water	-	1000 ml
pH	-	7.2

The ingredients were dissolved and autoclaved at 121°C for 10 minutes.

2. **IMVIC: MR-VP broth**

Peptone	-	5 g
Dipotassium hydrogen phosphate	-	5 g
Glucose 10% solution	-	5 ml
Distilled water	-	1000 ml

Peptone and phosphate were dissolved. pH was adjusted to 7.6, dispensed in 5ml amounts in test tubes and sterilized at 121°C for 15 minutes.

3. **Nutrient Agar**

Peptone	-	5 g
Beef extract	-	3 g
Agar	-	15 g
pH	-	7.0

4. **PDA- Potato dextrose agar**

Potato	-	200 g
Glucose	-	20 g
Agar	-	15 g
Distilled water	-	1000 ml
pH	-	5.6

5. **Simmon's citrate medium**

Sodium chloride	-	5 g
Magnesium sulphate	-	0.2 g
Ammonium dihydrogen phosphate	-	1 g

Dipotassium hydrogen phosphate	-	1 g
Sodium citrate	-	5 g
Agar	-	20 g
Bromothymol blue	-	40 ml
pH	-	6.8
Distilled water	-	1000 ml

The medium was autoclaved at 121°C for 15 minutes.

6. <u>Brilliant Green Lactose Bile (BGLB) broth:</u>

Brilliant Green	-	0.0133 g
Lactose	-	10 g
Oxyall	-	20 g
Peptone	-	10 g
Distilled water	-	1000 ml
Ph	-	7.2

BIOADSORPTION KINETICS OF CUTTLEFISH AND STARFISH SHELLS

A project submitted to

ST. MARY'S COLLEGE (Autonomous), THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

By

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DEPARTMENT OF ZOOLOGY

ST. MARY'S COLLEGE (Autonomous),

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

THOOTHUKUDI – 628 001

April - 2023

CERTIFICATE

This is to certify that the project entitled 'Bioadsorption kinetics of cuttlefish and Starfish shells' is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a record of the work done during the year 2022-2023 by the following students.

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INTRODUCTION

INTRODUCTION

Cuttlefish (*Sepia officinalis*) or cuttles are marine molluscs of the order Sepiida. The belong to the class Cephalopoda which also includes squid, octopuses and nautiluses. Cuttlefish have a unique internal shell, the cuttlebone, which is used for control of buoyancy.

Cuttlefish have large, W-shaped pupils, eight arms, and two tentacles

furnished with denticulated suckers, with which they secure their prey. They are generally range in size from 15 to 25 cm (6 to 10 in), with the largest species, thegiant cuttlefish (*Sepia apama*), reaching 50cm (20 in) in mantle length and over10.5 kg (23 Ib) in mass.

Small molluscs, crabs, shrimp, fish, octopi, worms, and other cuttlefish areall consumed by cuttlefish. Dolphins, sharks, fish, seals, seagulls, and othercuttlefish are some of their predators. A cuttlefish typically lives between one and two years.

Cuttlefish are reportedly among the most intellectual invertebrates, according to studies. Cuttlefish have a single spawning season towards the end of their life cycle and often have short lifespans (1-2 years). Additionally, because most cuttlefish have low fecundity, they produce large eggs and benthic hatchlings with limited potential for dispersal.

As a result, it is essential that each generation lay sufficient numbers of eggs across suitable habitats to ensure adequate annual recruitment.Cuttlebone, also known as cuttlefish bone, is a hard, brittle internal structure(an internal shell) found in all members of the family Sepiidae, commonly known as cuttlefish, within the cephalopods. In other cephalopod families it is called a gladius.

In the past, cuttlebones were ground up to make polishing powder, which was used by goldsmiths. The powder was also added to toothpaste, and was usedas an antacid for medicinal purposes or as an absorbent. They were also used as anartistic carving medium. Today, cuttlebones are commonly used as calcium-richdietary supplements for caged birds, chinchillas, hermit crabs, reptiles, shrimp, andsnails. These are not intended for human consumption. Due to their availabilityrecent studies are exploring the usage of cuttle bone as bioadsorbants.

The star fish (*Asterias rubens*) belong to the phylum Echinodermata. Thebody wall is composed of three layers: an outer, ectodermal epidermis, whoseapical surface is in contact with the environment; a middle, mesodermal layerknown as the dermis and an inner, coelomic epithelium, also mesoderm ally derived, whose apical surface is in contact with the perivisceral coelomic fluid.

The existence of spines, papulae and pedicellariae that protrude from the body walls dorsal and lateral areas, and tube-feet in the

ambulacral (ventral) region allcomplicate this basic organisation. The body wall; s endoskeleton is found in thedermis and is made up of ossicles, which are joined at mobile articulations byinterossicular ligaments and muscles.

These ossicles are made of a three-dimensional, mostly calcific network (stereo). The ossicles in the lateral anddorsal parts of the arm body wall connect to a single longitudinal row of carinal ossicles at the dorsal midline to form a regular mesh.

A longitudinal series of paired, rafter-like ambulacral ossicles with smaller adambulacral ossicles attached to their outer edge makes up the ambulacral body wall; s endoskeleton. The star fish bones are uses as show pieces and for artistic works.

The current study is focused on using the cuttle fish bone and star fish bone as absorbents.

Industrial pollution due to dyes

The usage of organic dyes in a variety of industries, including textile,pharmaceutical, leather, and paper manufacturing, has led to an annual the

generation of wastewaters that contain dye. These wastewaters have the potentialto poison water sources and harm the entire ecosystem if improperly managed.

Malachite rise in green (MG), a dye of the triphenylmethane family that is frequently usedin the textile and dyeing industries, is genotoxic and carcinogenic and can harm theimmunological and reproductive systems of live beings even at very low doses(Choudhary et al.,2020: You et al., 2022).

In order to protect ecosystems and to reduce potential problems that couldjeopardise human health, dye removal is essential. Dye-containing wastewater has

previously been treated using a variety of technologies, such as ultrafiltration,membrane filtration, oxidation, flocculation, ion exchange, and adsorption.

Adsorption is regarded as one of them because of its benefits of ease of use,high removal effectiveness, and design flexibility. Although several reports of both natural and syntheticadsorbents have been made, the creation of highly effective, affordable, andsustainable adsorbents continues to be the key challenge to maintaining thesecurity of the world' s water environment (Wu et al., 2022a: Xiao et al., 2020).

Adsorbents made of activated carbon perform better than those made ofother materials. The majority of activated carbons are manufactured from non-renewable fossil fuels and are therefore expensive, making it challenging toimplement them widely. Researchershave recently focused a lot of emphasis on the utilisation of low-cost wastebiomass resources, like agricultural wastes,

livestock manure, or municipal sludge,to create adsorbent biochar's for wastewater purification.

OBJECTIVES

AIMS AND OBJECTIVES

To analyse the efficacy of biosorption of biochar of shells of cuttle fish and to study the dynamics of biosorption.

The objectives of the study are:

1. To investigate the biosorption capacity of Cuttlefish bone and Starfish in adsorption of malachite green dye solution.

2. To investigate the effects of initial dye concentrations, amount of adsorbent, temperature of pyrolysis and pH on efficacy of biosorption.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Bioremediation

The term of bioremediation has been made of two parts: "bios" means life and refers to living organisms and "to remediate" that means to solve a problem. "Bioremediate" means to use biological organisms to solve an environmental problem such as contaminated soil or groundwater. Bioremediation is the use of living microorganisms to degrade environmental pollutants or to prevent pollution. In other words, it is a technology for removing pollutants from the environment thus restoring the original natural surroundings and preventing further pollution (Sasikumar and Papinazath 2003). Bioremediation could simply be defined as a biological process of the decontamination of contaminated environment. The environment may be either terrestrial, aqueous, or both. However, a more comprehensive definition is presented below: Bioremediation is a means of cleaning up contaminated environments by exploiting the diverse metabolic abilities of microorganisms to convert contaminants to harmless products by mineralization, generation of carbon (IV) oxide and water, or by conversion into microbial biomass. A point to emphasize here is that bioremediation and biodegradation should not be confused with each other. Bioremediation as a technique may include biodegra-dation as only one of the mechanisms involved or applied in the process of bioremediation. (Walsh 1999). Bioremediation can prove less expensive than other technologies

used for clean-up of hazardous waste (Vitali 2001). Nutrient imbalance can hinder biodegradation. Inadequate provision of nitrogen, phosphorus, potassium, and Sulphur could limit the rate of hydrocarbon degradation in the terrestrial environment (McGill and Nyborg 1975).

Types of Bioremediation

The bioremediation includes plant-microbe-based remediation, which is different in the process/mechanism by which plants/microbes can immobilize, remove or degrade pollutants (Khalid et al., 2017). It is including phytoremediation and microorganism remediation or physical methods like bio adsorption.

Phytoextraction

It is also known as phytoaccumulation, Phyto adsorption or phytosequestration. It is the removal of pollution from the soil or water by the plant's roots and translocation, accumulation on the biomass,(MuthuSaravanan et al., 2022). Pollutant translocation suitable for crucial biochemical process and it is effective for phytoextraction because the harvestry root biomass is not feasible. The continuous phytoextraction used for plants that is accumulate the high level of pollution over the entire life time.

Phyto filtration

The term Phyto filtration also known as rhizofiltration. It involves adsorption or precipitation of pollution (Khan et al.,2019). In this mechanism related synthesis of chemical, with the roots.

The rhizofiltration straight forwardly. It is connected to effluents and contaminated water ways, or ground water frame works. The ideal plant for rhizofiltration should have rapidly growth roots with ability to remove the contamination from that solution for over long period of time. It is used for the extensive root architecture and fibrous root. It is helps to draw out the contamination from the ground water and rhizopheric zone (Pilon Smits et al., 2005: Ali et al., 2013: Khan et al., 2019).

Phyto stabilization

The Phyto stabilization also known as Phyto immobilization. In this process of using plant's ability to decrease the mobility. The Phyto stabilization can be divided into 2 characters is i)The restoration of a pollutant media aggregated by the roots, absorption into roots. ii) The development of plants and plant roots avoid continent movement from the wind and water, draining and dispersion of soil (USEPA, 1999). The Phyto stabilization is not permanent solution to contamination. Because the Phyto stabilization doesn't reduce the pollutants but reduces the contamination nearby media.

Phytovolatilization

Phytovolatilization is plant mediated uptake of a contaminants, and transforms volatile compounds and release the compound from the atmosphere. The plant species are extensive root system. It is uptake of contaminants and produce some of specific enzyme or genes.

Phytodegradation

The phytodegradation otherwise called as Phyto transformation. It is referred to the contaminants and nutrients from the water, sediment, or soil and chemicals. Some plants are can be degraded contaminants into the less toxic compounds by the plants and metabolic process or enzymes (Muthusaravanan et al.,2018).

Bioadsorption

A metabolically inactive process known as biosorption is primarily produced by nonliving microbes or biological materials (such as agricultural waste), but bioaccumulation necessities the presence of live organisms and is accomplished through subsequent biosorption phases. Hence, biosorption is the initial stage in bioaccumulation; following this stage, the pollutant is carried inside cells mostly via energetic active transportation systems (Chojnacka 2009).

Biosorption or bioremediations consists with wide range of applications which involve the detoxification of hazardous substances instead of transferring

them from one medium to another by methods of microbes and plants. This process is considered as less disruptive and can be often carried out on site, eliminating the need to transport the toxic, materials to treatment sites (Gavrilescu, 2004). Bio sorbents are prepared from naturally abundant waste biomass. Due to the high uptake capacity and very cost-effective source of the raw material, biosorption is a progression towards a perspective method not only utilization of naturally available materials as emergent need of present and future to reduce the waste generation not only in industrial processes but also from pollution remediation.

The use of naturally available materials as biosorbents has many advantagesover the aforementioned methods (Wang and Chen, 2006) as they are considered as an effective remediation strategy due to its low cost and relatively simple design. To date, many such absorbents have been studied, including chitosan, zeolites, microorganisms, clay, and waste products from industrial operations such as flyash, coal and oxides (Babel and Kurniawan, 2003).

Adsorption is the ability of the adsorbate to adhere or attach to the adsorbent. It is a well-established separation technique to remove dilute pollutants as well as to recover valuable products from aqueous solutions. In the conventional adsorption process, the particle size of the adsorbent is restricted because ofhydrodynamicphenomena such as pressure drop (Chia-Chang andHawaii-Shen, 2000). Adsorption is divided in two categoriesaccording to the

forces that are made in between adsorbate and adsorbent. First one is generated due to forces of physical nature called van der Waals force which is relatively weak, since they are not sufficiently strong to influence appreciably the reactivity of the molecule adsorbed. The second type is considerably stronger. In there the adsorbed molecules are held to the surface by valence force of the same type as those occurring between bound atoms in molecules. This is known as chemisorption and the heat evolved is of the order 10 to 100 kcal/mole, compared to physisorption which has less than 5 kcal/mole (Moto Yuki, 1990).

Organic Pollutants: Dyes and Xenobiotics

The vast manufacturing and use of synthetic organic compounds in modern society's essential goods and processes results in a significant amount of xenobiotic chemicals lost in the industry's effluents, which, in many cases, are released into the environment. They are hazardous compounds because they can affect ecosystems and species, including people. The fundamental characteristics, including solubility, volatility, and there are as many different types of organic xenobiotic chemicals as there are potential sources and applications for them (Donner et al.,2010).

If natural substances are ingested by another organism, such as the chemical defences, they can turn into xenobiotics. Some species create this to ward off predators. When referring to compounds that are foreign to a whole biological system and did not exist in nature.

The term "xenobiotic" refers to compounds that were synthesized by humans and are foreign to a complete biological system (Csaba and Csaba 2011). The features include the toxicity in nature, stability and insolubility to water, large molecular size which determines difficulty to be input into the microbial cell, non-recognizability as a substrate by microbes to act upon and degrade it, and the absence of permease essential for its recalcitrance of xenobiotic compounds as a result of their recalcitrant properties(Hlihor *et al., 2001*)

Depending on their chemical makeup, the resistant xenobiotic substances can be categorized into many classes. Halogenated aliphatic and aromatic hydrocarbons, xenobiotics include huge. (Eke rue 2014).

These substances have a significant toxicity and can have an impact on life of all living things on the basis of persistence (their ability to persist in an environment over time, resulting in bioaccumulation and/or biomagnifications) and their ability to find their way into the food chain. These attributes contribute to high substances found in organisms that do not immediately come into contact with xenobiotics. Due to their toxic, mutagenic, and carcinogenic properties as well as their chronic toxic effects, 16 specific PAH chemicals have been identified as priority pollutants and were suggested as potential persistent organic pollutants (POPs). Sediments from benthic and aquatic organisms have been shown to contain high quantities of PAH (Perala 2010).

The manufacturing of coke produces PAHs, a common pollution byproduct. Coal tar, too. The incomplete combustion of organic compounds results in the formation of a wide range of distinct PAH chemicals. Some are related to industry, such PAHs utilized in the creation of colors', polymers, and insecticides as well as in medicine. As a result, due to their low water solubility and hydrophobicity, they frequently adsorb to and accumulate in sediments, where high molecular weight PAHs are degraded especially sluggish (Zaki and Hammam 2014). Complex and varied coal tars.

The primary component of food dyes is combinations of phenols, PAHs, and heterocyclic chemicals. Complex and varied coal tars the primary component of food dyes is combinations of phenols, PAHs, and heterocyclic chemicals. For instance, the food pigment Erythrosine B's tetra-raid counterpart of fluorescein is created by the interaction of iodine or iodide with as resorcinol and phthalic anhydride condense, potassium iodate in an ethanolic solution transforms to the sodium salt) of fluorescein.

Environmental problems have resulted from xenobiotic chemicals produced by industry. Research aimed at developing strategies that can be successfully applied to various environmental elements in order to reduce their hazardous effects.

For you to the utilization of biological remediation procedures employing by-products or crops should be the subject of in-depth research because it can contribute to sustainable development.

Due to their bioconcentrating and metabolic properties, microbes (or other forms of biomaterials of natural origin) can be used in bioremediation to breakdown, sequester, or remove pollutants from the environment or eliminate environmental toxins. As a subfield of environmental biotechnology, detoxifying dangerous pollutants is what bioremediation entails (Fingerman and Nagabhushanam 2005: Gavrilescu 2004).

In nature, biomass has a significant impact on the detoxification of various waste streams. In the process known as biosorption, soluble chemicals interact with substances of biological origin and are bonded to cellular surfaces; in the process known as bioaccumulation, these substances build up inside the cells (Kadu ova and Versova2005: Chojnacka2009).

The biosorption and bioaccumulation processes can involve numerous pathways, making it challenging to identify or quantify these processesunderstood (Volesky 1990: Gadd 2009: Gavrilescu 2010). Surface complexation, ion exchange, and other non-rectified physical-chemical mechanisms are all involved in biosorption. (Volesky 1990: Gadd 2009: Gavrilescu 2010).

Over the past ten years, a wide variety of microbial biomass has been examined for biosorption and bioaccumulation research. Heavy metals and biomaterials interact well, opening up new opportunities for recycling, recovery, and pollution prevention (Gadd 2009). Heavy metal exposure can have a variety of biological impacts on microorganisms, depending on the metal species and the tested model organism (Polisar et al., 2009). From the perspective of organic pollutants, these compounds' hydrophobicity, hydrophilicity, or polarity or no polarity had a role in the adsorption process's success.

Removal Efficiency by Biosorption and Bioaccumulation: Role of Environmental Factors and Process Parameters

pH

The pH of the solution is one of the most significant influencing elements for biosorption and bioaccumulation of inorganic and organic chemicals. Not only does it affect the rivalry between metallic ions for active sites as well as the chemical speciation of metal ions or the charges on the biosorption sites of biological mass. Itis critical that think about the metal solution chemistry at various pH levels as well as the ionic states of the functional groups in the bio sorbent.

As pH affects microbial metabolism, food availability, metal bioavailability, and solubility, microbial growth is significantly reduced by pH values in the system at pH values 3.0 and >9.0 or 10 (Vijayaraghavan and Yun 2008; Mudhoo and Moher 2012; Hlihor et al.,2014). For instance, Trichoderma viroid's biosorption and bioaccumulation for Cd (II) bio removal from aqueous solutions were studied by Hlihor et al., (2015).

The pH of the solution had a significant impact on the process. The scientists discovered that dead biomass's ability to absorb cadmium increases with pH, reaching a maximum of 7.31 mg/g at pH 6.0 for 100 mg/L of solution,

while the effectiveness of living biomass decreases with pH60% of the process was completed.

Gül and Dönmez (2014) investigated the decolorization potential of Aspergillus in the context of bioremediation experiments using living organisms.

Biosorbent Dosage

The specific absorption of inorganic or organic pollutants is influenced by bio sorbent dosage or biomass concentration in solution; for lower values of biomass dosages, there is a rise in the specific metal uptake. Reduced bio sorbent dosages are frequently necessary yield greater uptakes but lower removal percentages. The amount of solute adsorbed typically increases with an increase in biomass concentration due to the increased thebio sorbent's surface area, which in turn boosts the number of binding sites (Samone et al., 2011: Hlihor et al., 2014).

The phase where the concentrations of the bio sorbent are determined Xenobiotic substances in solution and on the surface of the bio sorbent reach equilibrium with one another. As an illustration, in research on pumpkin by-products as a bio sorbent, the concentration at equilibrium for erythrosine B was 20 mg/L (Apostol et al., 2015a) and for RR120, it was 0.5 g/L. The AspergillusNiger and Trichoderma sp. fungal biomass concentration

for Bishop- Environmental Bioremediation via Biosorption and Bioaccumulation...Sivasamy and Sunderaraj (2011) established 304 adsorptions of Orange G at 90 g/L.

A single parameter variation was considered in the investigationsbyand Sivasamy and Sunderaraj (2011), taking the proportion of pollutant removed, whereas Apostol et al. (2015a) included both the quantity and the percentage of pollutant removed in their investigation.

Temperature

According to a comprehensive survey of the literature, the study of the thermodynamics of the biosorption process demonstrates that temperature is a crucial factor because it affects the effectiveness with which various types of pollutants are removed from the environment. It is intimately connected to the metal ions' kinetic energy. As a result, it can explain how diffusion occurs. The amount of metal removed should alter when the temperature rises or falls or by the biomass being adsorbed. In the processes of biosorption and bioremediation, temperature is crucial. The positive value of enthalpy change (H°) in demonstrated the endothermic nature of the biosorption process, with increased equilibrium Erythrosine B uptake by pumpkin seed hulls as temperature rises (Apostol et al., 2015). Elaichiet al., (2014) have reported that the biosorption mechanism for the elimination of RR120 by pumpkin husk is endothermic. Before applying the adsorption process, the temperature of the industrial

effluents must be adjusted in the event of an exothermic operation, where the dye uptake reduces as the temperature rises. The critical parameter for microorganism growth, reproduction, and enzyme activity in bioremediation is the process temperature. The data obtained thus far point to the existence of two distinct biosorption mechanisms, either energy dependent or energy independent, for various metal-biomaterial systems (Fengetal.,2012). The temperature is a limiting factor in the case of living things. Only in the case of thermotolerant culture treatment of xenobiotics or heavy metal effluents is temperature advantageous.

The current study is done to analyses the efficacy of biosorption of two different substrates, Cuttlefish shell and Star fish shell which are often considered as wastes.

MATERIALS AND METHODS

MATERIALS AND METHODS

Collection of Sample

Molluscan and Crab shell samples were collected from the shore line of Siluvai Patti beach in Thoothukudi and from the Vella Patti beach in Thoothukudi. Two samples, Cuttlefish bone and Starfish were collected and used for this experiment. (Fig.1 (a and b))

Sample processing

The samples were cleaned off the dust and it was rinsed with fresh water and shade dried. (Fig. 2 (a and b))

Pyrolysis of sample

The collected samples were shade dried and crushed into a fine powder. The powder was taken in a China Dish and sealed with a silver foil to maintain an oxygen free environment. The sample was then pyrolyzed in a muffle furnace for 30 minutes. The pyrolyzed sample was taken for further analysis.

Bioadsorption

50mg of biochar was taken in 100ml of malachite green 50 mg /l concentration. The set up was placed in a shaker at room temperature. Samples of dye were taken at regular intervals (5, 10. 15, 20, 25 min). Absorbance at 617 nm was taken in the Spectrophotometer. A standard graph for concentration of malachite green was plotted by measuring the OD of known concentrations of

dye. The concentration of the dye in the experimental sample was obtained from the standard graph.

Fig.1 Area of study

a.

b.

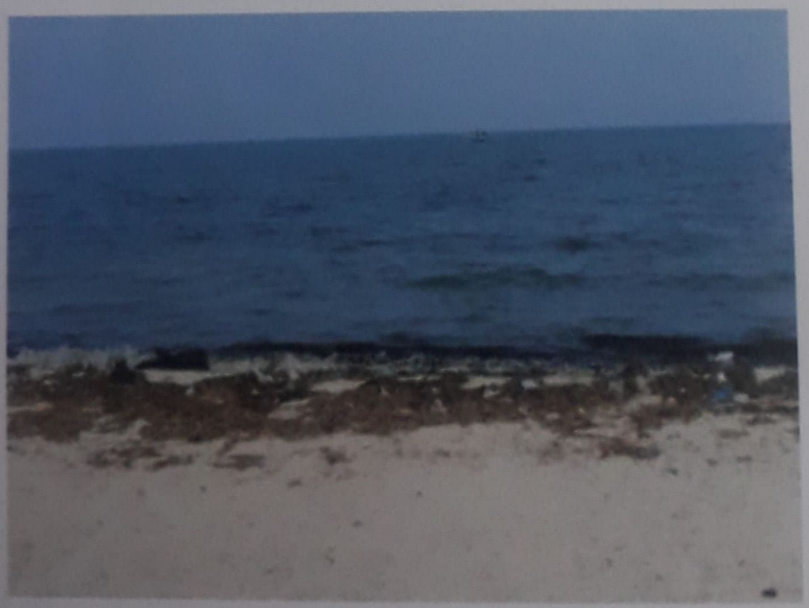


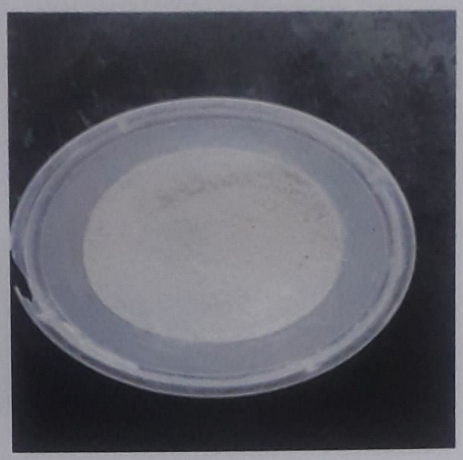
Fig.2 Samples used for the study

b. Cuttlefish

b. Star fish

Figure.3 Bioadsorbants

a. Cuttlefish bone powder



b. Starfish powder

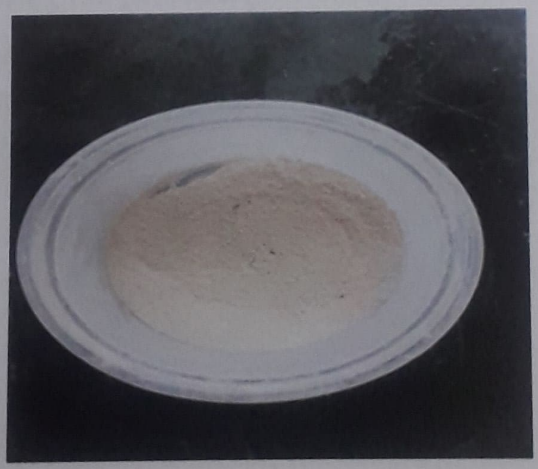


Fig.4 Graph representing the percentage of biosorption between Cuttlefish sample Vs Starfish sample

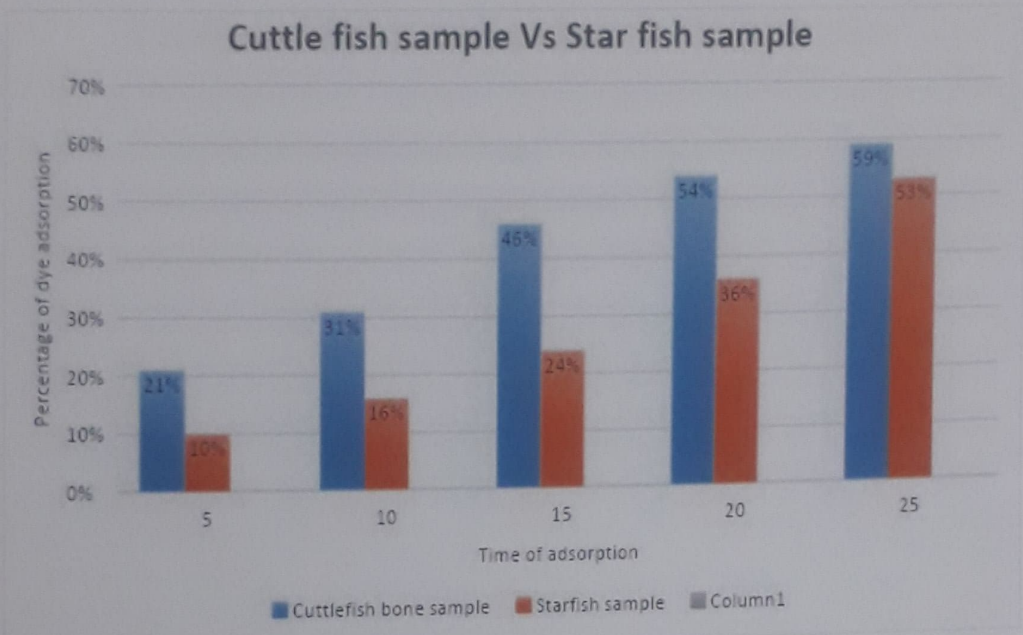


Fig.5 Graph representing the percentage of biosorption between samples pyrolyzed at 300^0C and 600^0C

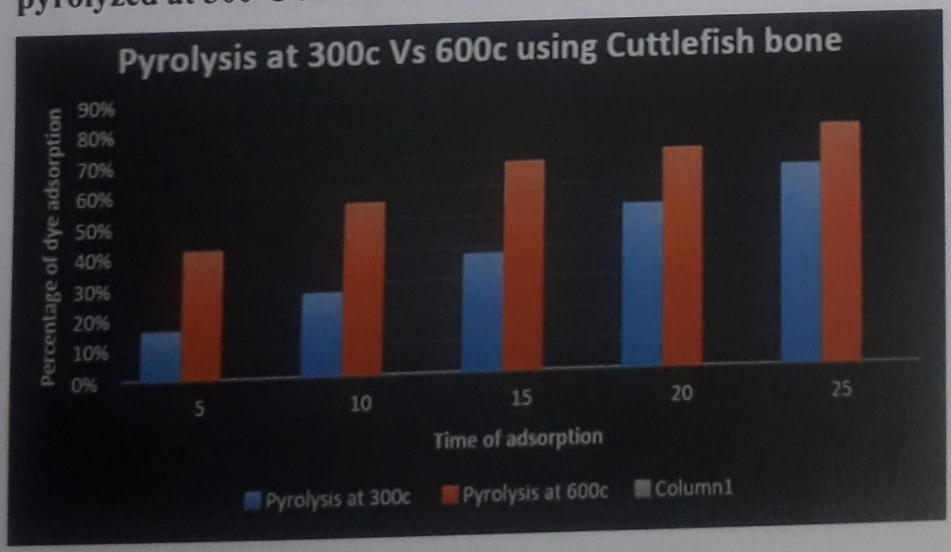


Fig.6 Graph representing the percentage of biosorption and Cuttlefish shell sample and its biochar

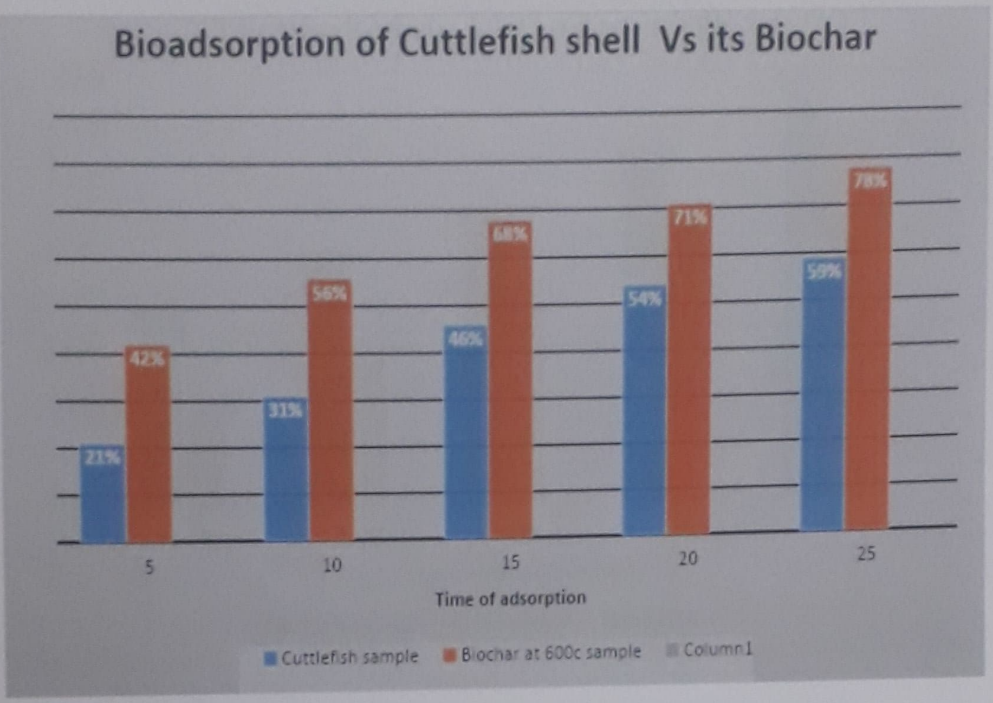
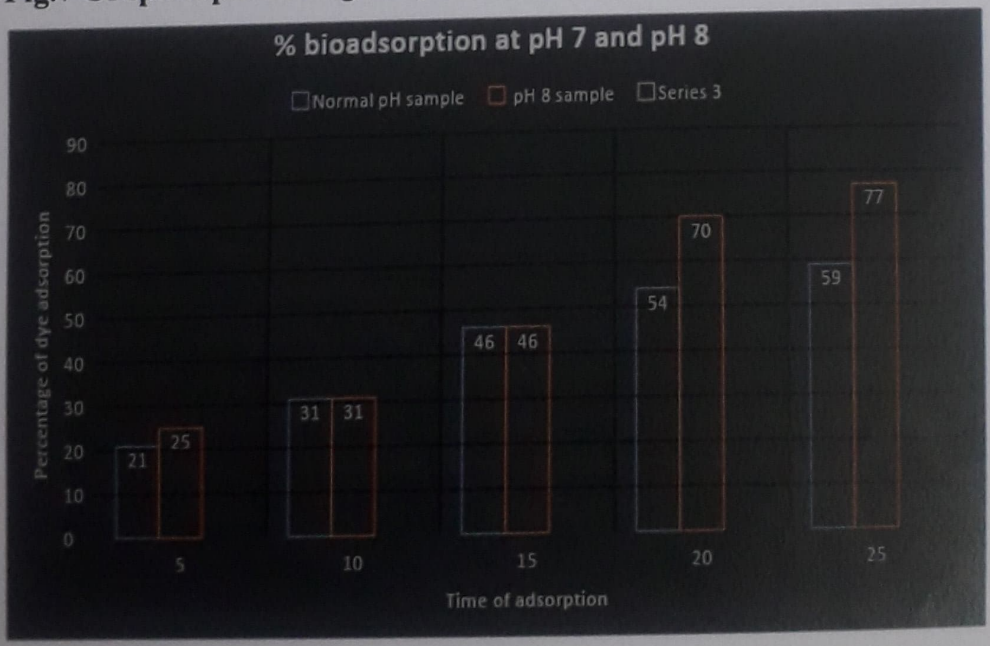


Fig.7 Graph representing the percentage of biosorption at pH7 and pH8



RESULTS

RESULT

Bioadsorption using Cuttle fish and Star fish shell powders

Bioadsorption of malachite green dye using Cuttlefish shell and Star fish shell powders of particle size 0.75 mm were used for bioadsorption of malachite green dye to find the efficiency of their bioadsorption potential. 50 mg of the adsorbent was used for the adsorption of malachite green. Percentage of dye adsorbed by the adsorbent was calculated at time interval of 5 mins, 10 mins, 15 mins, 20 mins and 25 mins and are tabulated in Table 1a & 1b. The comparison of bioadsorption between the cuttle fish bone adsorbent and the star fish bone adsorbent was done and plotted in a graph (Fig. 4). The cuttle fish bone sample was found to be more efficient in adsorption when compared to the starfish bone sample. The functional groups present in the different tissues may be attributed to the difference in bioadsorption potential.

Bioadsorption using Biochar prepared at different pyrolysis temperature

Biochar of the cuttle fish shell powder was done in a muffle furnace at different temperatures. Pyrolysis was performed at two different temperatures 300^0C and 600 ^{0}C for 30 minutes. 50 mg of the biochar was used for adsorption of malachite green dye. Percentage of dye adsorbed by the adsorbent was calculated at time interval of 5 mins, 10 mins, 15 mins, 20 mins and 25 mins and are tabulated in Table 2a & 2b. The comparison of bioadsorption between the biochar prepared at 300^0C and 600^0C was done and the percentage of

bioadsorption was plotted in a graph (Fig. 5). Bioadsorption by the samples pyrolyzed at 600^0C was more efficient than the adsorbent pyrolyzed at 600^0C.

Comparison of Bioadsorption of Cuttle fish shell powder and its biochar

A comparison was done between the efficiency of bioadsorption of Cuttle fish shell powder and its biochar. 50 mg of the fish shell powder and biochar was used

for adsorption of malachite green dye. Percentage of dye adsorbed by the adsorbent was calculated at time interval of 5 mins, 10 mins, 15 mins, 20 mins and 25 mins and are tabulated in Table 3a & 3b. The comparison of bioadsorption between the bioadsorption of Cuttle fish shell powder and its biochar was done and the percentage of bioadsorption was plotted in a graph (Fig. 6). Bioadsorption was found to be more efficient by the biochar than the shell powder.

Effect of pH on bioadsorption efficiency

Bioadsorption of malachite green using the biochar of cuttle fish shell was done at two different pH. The adsorption of malachite green was done at pH 7 and also by adjusting the pH of the reaction to pH 8 with 2N NaOH. Percentage of dye adsorbed by the adsorbent was calculated at time interval of 5 mins, 10 mins, 15 mins, 20 mins and 25 mins and are tabulated in Table 4a & 4b. The comparison of bioadsorption at pH 7 and pH 8 was done and the percentage of bioadsorption was plotted in a graph (Fig. 7). There was not much difference in

efficiency of bioadsorption in pH 7 and pH 8 initially but at 25 minutes the bioadsorption was found to be more efficient at pH 8.

Effect of concentration of adsorbate on bioadsorption efficiency

The efficiency of the adsorbate concentration was studies using cuttle fish shell biochar. 100 mg and 25 mg of the adsorbate was used for adsorption of malachite green. Q value for the two samples were calculated at different time points (Table 5a and 5b) and the results were compared (Fig. 8). 100 mg of the adsorbate was found to be more efficient in bioadsorption than at lower concentrations.

Table.1 Comparison of percentage adsorption of Cuttlefish shell VS Starfish shell

a. Percentage adsorption of Cuttlefish sample

S.No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617nm	Final concentration of dye mg/l	Amount of dye adsorbed	% of dye adsorption
1.	5	10	0.889	7.9	0.21	21%
2.	10	10	0.773	6.9	0.31	31%
3.	15	10	0.613	5.4	0.46	46%
4.	20	10	0.524	4.6	0.54	54%
5.	25	10	0.465	4.1	0.59	59%

b. Percentage adsorption of Starfish sample

S.No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617nm	Final concentration of dye mg/l	Amount of dye adsorbed	% of dye adsorption
1.	5	10	0.879	9	0.1	10%
2.	10	10	0.636	8.4	0.16	16%
3.	15	10	0.577	7.6	0.24	24%
4.	20	10	0.480	6.4	0.36	36%
5.	25	10	0.362	4.7	0.53	53%

Table.2 Comparison of percentage adsorption of cuttle fish shell pyrolysed at 300^0C vs 600^0C

a. Bioadsorption of Cuttlefish shell pyrolysed at 300^0C

S.No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617nm	Final concentration of dye mg/l	Amount of dye adsorbed	% of dye adsorption
1.	5	10	0.635	8.4	0.16	16%
2.	10	10	0.552	7.3	0.27	27%
3.	15	10	0.467	6.2	0.38	38%
4.	20	10	0.351	4.7	0.53	53%
5.	25	10	0.264	3.5	0.65	65%

b. Bioadsorption of Cuttlefish shell pyrolysed at 600^0C

S.No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617nm	Final concentration of dye mg\l	Amount of dye absorbed	% of dye adsorption
1.	5	10	0.578	5.8	0.42	42%
2.	10	10	0.436	4.4	0.56	56%
3.	15	10	0.305	3.2	0.68	68%
4.	20	10	0.280	2.9	0.71	71%
5.	25	10	0.212	2.2	0.78	78%

Table.3 Comparison of percentage adsorption of Cuttlefish shell sample VS its Biochar

3. Bioadsorption of Cuttlefish shell biochar

S.No.	Time of adsorption	Initial concentration of dye mg/ml	OD Value at 617nm	Final concentration of dye mg/ml	Amount of dye adsorbed	% of dye adsorption
1.	5	10	0.889	7.9	0.21	21%
2.	10	10	0.773	6.9	0.31	31%
3.	15	10	0.613	5.4	0.46	46%
4.	20	10	0.524	4.6	0.54	54%
5.	25	10	0.465	4.1	0.59	59%

b. Bioadsorption of Cuttlefish shell biochar

S.No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617nm	Final concentration of dye mg/l	Amount of dye adsorbed	% of dye adsorption
1.	5	10	0.578	5.8	0.42	42%
2.	10	10	0.436	4.4	0.56	56%
3.	15	10	0.305	3.2	0.68	68%
4.	20	10	0.280	2.9	0.71	71%
5.	25	10	0.212	2.2	0.78	78%

Table.4 Comparison of percentage adsorption at pH 7 and pH 8

a. Percentage adsorption at pH 7

S.No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617nm	Final concentration of dye mg/l	Amount of dye adsorbed	% of dye adsorption
1.	5	10	0.889	7.9	0.21	21%
2.	10	10	0.773	6.9	0.31	31%
3.	15	10	0.613	5.4	0.46	46%
4.	20	10	0.524	4.6	0.54	54%
5.	25	10	0.465	4.1	0.59	59%

b. Percentage adsorption at pH 8

S.No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617nm	Final concentration of dye mg/l	Amount of dye adsorbed	% of dye adsorption
1.	5	10	0.740	7.5	0.25	25%
2.	10	10	0.686	6.9	0.31	31%
3.	15	10	0.532	5.4	0.46	46%
4.	20	10	0.305	3	0.7	70%
5.	25	10	0.235	2.3	0.77	77%

Table.5 Calculation of Q values for different concentrations of adsorbate

a. Calculation of Q value for 100 mg of adsorbate

S.No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617 nm	Final concentration of dye mg/l	Amount of dye adsorbed	Q= (Amount of dye adsorbed/Weight of adsorbent) g/g
1.	10	10	0.501	6.6	0.34	0.0034
2.	20	10	0.426	5.6	0.44	0.0044
3.	30	10	0.374	4.9	0.51	0.0051
4.	40	10	0.282	3.7	0.63	0.0063
5.	50	10	0.189	2.4	0.76	0.0076

b. Calculation of Q value for 25 mg of adsorbate

S.No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617nm	Final concentration of dye mg/l	Amount of dye adsorbed	Q= (Amount of dye adsorbed/Weight of adsorbent) g/g
1.	10	10	0.635	8.4	0.16	0.0016
2.	20	10	0.552	7.3	0.27	0.0027
3.	30	10	0.467	6.2	0.38	0.0038
4.	40	10	0.351	4.7	0.53	0.0053
5.	50	10	0.264	3.5	0.65	0.0065

DISCUSSION

DISCUSSION

The release of organic and inorganic pollutants into the environment can cause severe changes (contamination in the water, death of trees and grassland, more problems in wildlife, contamination in the food chain, negative health effects on the fora and fauna, severe human health problems etc.) in the atmosphere and generates various types of pollutions. This is because these pollutants are toxic, mutagenic and carcinogenic, and their presence in ecosystems determines the decrease of the ecosystem quality. Over the recent past, biological treatment has been one of the cost-effective technologies compared to other processes.Compared to other conventional methods, biosorption is considered as one of the most effective treatment techniques, both techno centric and eco centric. Biobased sorbents (or biosorbents) such as fungi, bacteria, polysaccharide sorbents, algae, and agricultural wastes are cheap, available in large quantities, and their performances depend; the type of dye and the experimental conditions (including initial dye concentration, pH, temperature, contact time, etc.). Therefore, biosorbents with a higher potential to bind/take dyes molecules are desirable.

The experimental results presented in many investigations suggested that the solution's pH has a significant impact on the absorption due to its impact on

the surface characteristics (the activity of the functional groups on the surface) and speciation processes including ionisation (protonation/deprotonation) and dissociation of organic xenobiotic chemical molecules (such as dyes) that make up the biomass. The maximal removal efficiency was determined to be 95.75% was attained at pH 6.0. The solution pH has a direct influence, not only on the adsorption capacity but also on the surface chemistry of the biochar as well as the accessibility of dye molecules of the binding sites (Vijayaraghavan and Ashokkumar 2019). Form most of the previous studies it is inferred that the optimum pH for dyes removal is often neutral or slightly alkaline (Sadhasivam et al., 2007). The percentage of dyes removal is maximum only at optimum conditions, and tends to decrease rapidly in acid or alkaline conditions. The solution pH is directly associated with the competition ability of hydrogen ions with adsorbate. These results concur with Mishra et al. (2013). The researchers found that whereas metal absorption capability significantly decreased, removal efficiency from aqueous solutions increased as biomass dose increased at a constant Zn (II) ion concentration. Although organic pollutants can have enormous molecule sizes when exposed to heavy metal compounds, the biosorbent features (such as concentration and particle size) have a significant impact on the biosorption of pollutants. In our studies pH 7 was found to be optimal for high efficiency of adsorption.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

- The biosorption capacity of Cuttlefish bone and Starfish in adsorption of malachite green dye solution was studied.
- The effects of initial dye concentrations, amount of adsorbent, temperature of pyrolysis and pH on efficacy of biosorption.
- It was found that the cuttle fish was more efficient than the starfish samples for bisorption.
- More experiements have to be standardized the efficacy of cuttle fish bones as good biosorbents

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A STUDY ON MACROBENTHOS WITH REFERENCE TO QUALITY OF WATER TO ASSESS THE INTENSITY OF POLLUTION ALONG THE TUTICORIN COAST.

A project submitted to

ST. MARY'S COLLEGE (Autonomous), THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

by

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DEPARTMENT OF ZOOLOGY

ST. MARY'S COLLEGE (Autonomous),

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

THOOTHUKUDI – 628 001

April – 2023

CERTIFICATE

This is to certify that the project entitled **A study on macrobenthos with reference to quality of water to assess the intensity of pollution along the Tuticorin coast** is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a record of the work done during the year 2022-2023 by the following students.

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CONTENTS

INTRODUCTION

INTRODUCTION

Benthos refers to those which live on or in the bottom of any body of water (Bostwick, 1983). Benthic organisms are sometimes found on hard substrate such as rock, wood or in soft sediment. Benthic organisms are divided in to three categories according to their size (1) macrobenthos (>500 μ) (2) meiobenthos (500 μ to 63 μ) and (3) macrobenthos (63 μ) (Mare, 1942). The benthos retained on 0.5 sieve are treated as microbenthos.

The term 'pollution indicator species' is often (Reish, 1979) refer to certain macrofaunal benthic species occurring in organically enriched coastal waters. Even though the term has been employed in somewhat different way by different investigators, the most common use appears to be in reference to species that numerically dominate the macrobenthos in organically enriched areas (Young and Young, 1982). 'Indicators species' will simply mean species that have been reported as numerical dominants in organically enriched water. Two major explainations have been put forth to account for this dominance (1) superior tolerance to presumably stressful conditions (Reish, 1979) (2) Superior invasion abilities (i.e.opportunism) following sporadic pollution including disturbances (Pearson, 1980 and Rosenberg, 1981).

Many of them are treated as sentinel organisms and biomarker in the assessment of health of the marine environment because of their direct relationship with the type of bottom and the physical nature of substratum. Thus benthos may be

treated as sensitive indicators of the accumulation of organic matter and its nature in the sediments (Butkasetal, 2011). Apart from the above some of the macrobenthic organisms like gastropods, crabs, prawns etc contribute well to the economy of the region.

Numerous publication have critically reviewed the use of macrobenthic communities as bioindicators as well as the appropriateness and shortcoming of certain indices (Yap *et al.*,2003). Survival distribution and abundance of macrobenthos depend on the characteristic of their environment such as salinity, organic matter content, soil texture, sediment particles and the ability to construct permanent burrows in the substratum (Dahanayakar and Wijeyaratne,2006).

Macrobenthos such as polychaetes, decapods and molluscs are important sea-bed fauna. The macrobenthos are mostly non migrant inhabitants and can be used as indices of ecological changes in the sea water environment. Human activities associated with industries and farming have adversely affected aquatic ecosystem.

The benthic organisms are commonly considered as bio-indicators to assess the pollution impacts of the aquatic environment. Numerous authors have depicted the beneficial usages of macrobenthic organisms for biological assessment in the natural environment (Hellawell, 2012). Alternations in the chemical compositions of water and sediment along with a change in primary productivity

can have a greater effect on the sedentary community along with mass mortality and physiological interchanges (Uwadie, 2016).

Precise difference in physiological activities like feeding, locomotion and life cycle depict the sensitivity of the benthic individuals to tolerate certain types of (Rosenberg and Resh, 1993). Through the changes that occurred in the natural system due to contamination can predict the degree of pollution by studying these organisms. The spatial and temporal distribution of the coastal biological community may be directly or potentially affected by the changes in water and sedimentary environments (Shi, 2014).

Macrobenthos diversity is closely related to both environmental factors and anthropogenic alternations (Nouri *et al.*, 2008). Macrobenthic assemblages have been used to indicate stress as they are sensitive to pollution and are also different due to sensitivity degree. The reason is that benthic organisms lives in sediment and are able to accumulate contaminants over long periods, also these organisms cannot move and migrate due to environmental pressure and disturbances (Paul *et al.*,2001).

When the water body is subjected to the influence of sewage and industrial pollution, a considerable stress on their faunal communities results as evidenced by the population elasticity of the macrobenthic community (Ramkumar, 2010). Oil spills result in mass pollution and lead to death of benthic communities.

(Elmrgen *et al.*, 1983). Therefore aquatic organisms have an important role in bio assessment. (Mooraki *et al.*, 2009).

Macrobenthos have been used for decades to measure and describe ecological status and variations of marine and estuarine environments (Angradi *et al.*, 2001; Manoliadis, 2002; Aalm *et al.*, 2007; Sivadas *et al.*, 2008). The discharge of heated effluents in the coastal water by thermal plant and industries not only produce adverse effect on coastal water but also can affect planktonic community and bottom fauna. The water of anthropogenic and industrial origin are of complex characters and have an considerable percentage of heavy metals. Quantitative description of soft bottom macrobenthos are often and important part of the studies of the effect of pollutions are marine communities.

Benthic communities are important to marine ecosystem and form important food source for most of the marine organisms especially fishes. The estimation of benthic production would serve as a useful index for assessing the fishery potentials, interaction, pollution and intertidal ecology.

One of the aims of benthic ecologists is to understand the ecological process, which is achieved by examining the inter-relationship between environmental parameters and benthic community structure, anthropogenic impacts and modeling of the ecosystem. (Frouin, 2000). The macrobenthos is widely used as an indicator of ecological health in marine monitoring and assessment due to the

relatively weak ability of macrobenthos species to migrate, their long life cycles and their different tolerances to stressors (Xu and Li, 2021).

The discharge of heated effluent in the coastal water by Tuticorin Thermal Power Station is a regular problem. Thermal effluent not only can produce adverse effects on the coastal water but also affect the aquatic organisms such as planktonic community and bottom fauna (Easterson *et al.*,2000).

With regard to different stressor factor in the Gulf of Mannar, the goal of this study is to assess the ecological status of the Tuticorin coast which is necessary for the prevention of future portable risks.

OBJECTIVES

PURPOSE OF STUDY

The objectives of the present study is to

- Analyze the physico-chemical parameters of water in our study area.
- Assess the THB load in the water of the selected area.
- Study the distribution and numerical abundance of macrobenthos in our study area.
- Analyze the impact of all the above factors on the distribution of macrobenthos.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

A survey of literature adds knowledge regarding the enormous amount of research works that have been carried out on macrobenthic fauna in aquatic environment.

The pioneering work on quantitative study on benthos was done by Peterson in Danish water in 1999 Petersen, 1913). In addition, the bottom fauna was studied by Annandale and Kemp (1915) in Chilka lake.

Kurian (1967) gave a detailed account of the benthos of southwest coast of India. Desai and Krishnankutty (1976) conduced investigation on the bottom fauna of the Cochin brakish waters. They also made a comparative study of marine and esturine benthic fauna of the near shore regions of the Arabian sea.

Pilli (1978) investigated the macro-benthos of Vembanadu lake. Pearson & Rosenberg (1978) has studied the macro-benthic succession in relation to organic enrichment and pollution of the marine environment. Reish (1979) studied the use of benthic animals in monitoring the marine environment.

Jegaseesan and Ayakannu (1992) investigated the seasonal variation of benthic fauna on marine zone of coleroon estuary and inshore waters of Southeast coasts of India.

Asha (1999) studied the impact of effluent discharge from Thermal Power Station on the hydrological conditions of Tuticorin bay.

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Shi (2014) reported the effect of spatial and temporal distribution of the coastal biological community on the quality of water and sediment.

O'Brien *et al.,*(2016) have investigated the sensitivity of macrobenthos to the change in physico-chemistry of the environment. Uwadie (2016) has investigated the effect of alterations in the chemical composition of water and sediment on the sedimental community.

Gu *et al.,* (2017) have analysed the role of macrobenthos in regulating the physico-chemical and biological state of an aquatic ecosystem.

Zhao *et al.,* (2019) and Hajilizadeh *et al.,* (2019) have worked on the role of macrobenthic community on the energy flow, material circulation and information transfer and as an indicator of ecosystem health.

Xu and Li (2021) have analysed the benefit of longlife cycle and sedentary life of macrobenthos as an indicator of ecological health in marine monitoring. Wrang *et al.,*(2021) have studied the impact of distribution and abundance of macrobenthos in utilizing them as ecological indicators to assess the health of benthic habitat. Eriksen *et al.,*(2021) have reported the high sensitivity of macrobenthos to the physicochemical changes in the environment and their role as indicator organism in aquatic ecosystem.

EXPERIMENTAL

DESIGN

EXPERIMENTAL DESIGN

STATION I

STATION II

STATION III

SAMPLES COLLECTED

WATER

MACROBENTHOS

PHYSICAL PARAMETERS

- PH
- SALINITY
- TEMPERATURE
- DISSOLVED OXYGEN

BACTERIOLOGICAL ANALYSIS

- THB

DISTRIBUTION OF MACROBENTHOS

- GASTROPODS
- BIVALVES
- ECHINODERMS
- CRUSTACEANS

MATERIALS AND METHODS

MATERIALS AND METHODS

For our present investigation on 'A study on macrobenthos with reference to quality of water to assess the intensity of pollution along the Tuticorin coast', three sampling sites were selected.

STUDY AREA – TUTICORIN COAST

Thoothukudi also known as Tuticorin, is a port city. Thoothukudi is known as 'Pearl City' due to pearl fishing carried out in the town. It is a commercial seaport which serves the inland cities of Southern India and is one of the Sea Gateways of Tamil Nadu.

Industries like Southern Petro Chemical Industrial Corporation (SPIC), Tuticorin Alkali Chemicals (TAC), Heavy water Plant, Sterlite and KSPS Salt pans have their manufacturing units in Thoothukudi. Thoothukudi is often threatened by pollutants from the industries and sewage effluents. Among the industrial pollution sources Tuticorin Thermal Power Station (TTPS) which has commissioned with three units in 1980 plays a major role. The effluent discharge mainly the fly-ash from TTPS has a already resulted in filling up of an extensive portion of the bay that has caused irreversible damages to the ecosystem. Presently each of the five units produce about 210 MW of electricity consumes 2800 tonnes of coal per day. In addition to ash containing slurry it also discharges heated water effluents used for cooling the boilers there by affecting the aquatic

PLATE – 1 STATION – I HARBOUR BEACH

PLATE – 2 STATION – II HARE ISLAND

PLATE – 3 STATION – III THERESPURAM

PLATE – 4 STUDENT COLLECTING SAMPLE

life of the bay. Majority of the people of the city are employed in salt pan, seaborne trading and fishing. The 21 islands between Thoothukudi and Rameshwaram are in the Gulf of Mannar which is notified as the first Marine Biosphere Reserve of India having around 36,000 species of flora and fauna.

SAMPLING SITES:

STATION – I: HARBOUR BEACH

The Harbour beach is located closed to the port guest house. It is situated near the Tuticorin Thermal Power Station (TTPS) is a clean beach and a recreation site for the people of Tuticorin. There is also a park near it where people can stroll in the evenings amidsts the refreshing sea breeze.

STATION – II: HARE ISLAND

Hare island is an island which lies adjoining the V.O.C Port Trust in Tuticorin. It forms a part of Gulf of Mannar Marine National Park with an area of 1.29 sq.kms. It is the largest island in the Gulf of Mannar. It is calm and clean beach. The island has two light houses and one can spot numerous shells on the shore. The weather is usually hot. It attracts numerous visitors over the weekends all the time.

STATION – III: THERESPURAM

It is located 8 km away from Tuticorin Thermal Power Station and 6 km distance from Tuticorin Fishing Harbour. Loads of untreated sewage, human and animal

feacal matters are mixed with the coastal water in this station. Salt pans and small fish processing industries located around this station release fish wastes and salt pan effluent in this area. It is a heavily polluted area with organic enrichment of sewage waste and effluents directly discharging into sea.

SAMPLING METHODS

WATER:

Water samples were collected from surface using sterile acid wash and plastic bottles from all the stations for the measurement of temperature, salinity, pH and dissolved oxygen. Temperature of water was measured in the field itself. For estimation of dissolved oxygen, water was taken in 125ml stoppered glass bottles taking care that no air bubbles is trapped in the sample and fixed with Winkler's solution. For pH and salinity water samples were collected in plastic bottles taken to the laboratory and stored in an insulted box till they were analyzed.

BENTHOS:

Benthic sampling was done in all the three stations. Sampling was carried out using along armed Van-Veen Grap. The larger organisms were hand picked immediately and then sieved through 0.5mm mesh screen. The organisms retained in the sieve were placed in a labled container and fixed in 5% formalin.

The organisms were stained with Rose Bengal (0.1gm in 100ml of distilled water) for enhanced visibility during identification. All the specimens were sorted

enumerated and identified to the group level (Mc Intyre, 1984; Aswandy *et al.*, 1991).

ANALYSIS PHYSICAL PARAMETERS

TEMPERATURE:

The temperature of the surface water was recorded using digital stem Thermometer (50-200°C).

DISSOLVED OXYGEN:

It was estimated by the classic Winkler's titration (1883) method. The values are expressed as mg/l.

SALINITY:

It was determined by hand held refractometer.

HYDROGEN ION CONCENTRATION (pH):

It was measured using a digital pH meter.

BACTERIAOLOGICAL ANALYSIS

TOTAL HETEROITROPHIC BACTERIA (THB):

One ml of water sample was added in 99ml of 50% of filtered sea water bottle separately and then serially diluted using the same diluents 0.1ml of the serially

diluted sample are inoculated into the Zobell marine agar to enumerate the THB and also to isolate the specific pathogens. After inoculation the petridishes were inoculated in an inverted positions at a temperature of 28±2°Cfor 24 to 48 hours after incubation the colony was enumerated. THB was expressed as THB × 10^{10} in water.

STATISTICAL ANALYSIS

Separate two way ANOVA was performed to find out the significance in the parameters like physical parameters and numerical abundance of macro benthos.

RESULT

RESULTS

PHYSICO-CHEMICAL PROPERTIES:

1. TEMPERATURE:

The water temperature in different stations during the study period ranged from 24^0C to 27^0C. The temperature of coastal water was 25^0C in Station- I (Harbour beach). It was 27^0C in Station – II (Hare Island) and 24^0C in Station-III (Therespuram beach). (Table – 1).

2. pH:

The water collected from different stations showed a tendancy toward slight alkalinity. The pH was found to be 7.39, 7.42 and 7.54 respectively in Station- I, Station- II and Station- III. (Table- 1).

3. SALINITY:

As presented in Table-2. The salinity of coastal water collected from different stations during the study period. Ranged from 30-34. The salinity was observed to be 32ppt in Station-I, 34 ppt in Station-II and 30 ppt in Station-III.

4. DISSOLVED OXYGEN:

As presented in Table-2. The dissolved oxygen content in the sea water from the study area showed much variation. There was no O_2 in the water

TABLE 1: TEMPERATURE, pH AND SALINITY OF SEA WATER FROM SELECTED ZONES

(Values are expressed in ^{0}C & PPT)

STATIONS	TEMPERATURE(^{0}C)	pH	SALINITY(ppt)
STATION I	25^{0}C	7.39	32
STATION II	27^{0}C	7.42	34
STATION III	24^{0}C	7.54	30

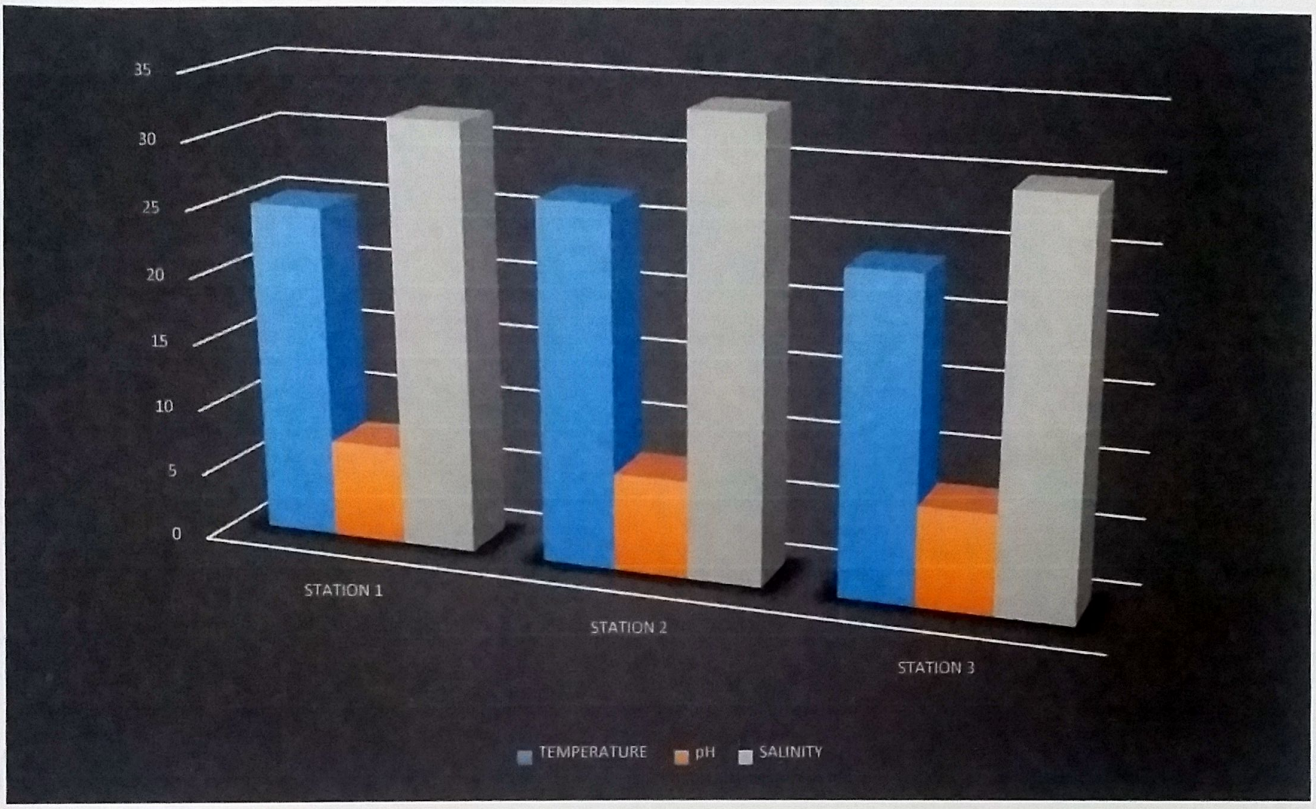


FIG:1 TEMPERATURE, pH AND SALINITY OF SEAWATER FROM SELECTED ZONES

TABLE 2: DISSOLVED OXGEN CONTENT OF WATER FROM SELECTED COAST

(Values are expressed in ml/l)

STATIONS	DISSOLVED OXYGEN (ml/l)
STATION-I	5.132
STATION-II	2.763
STATION-III	NIL

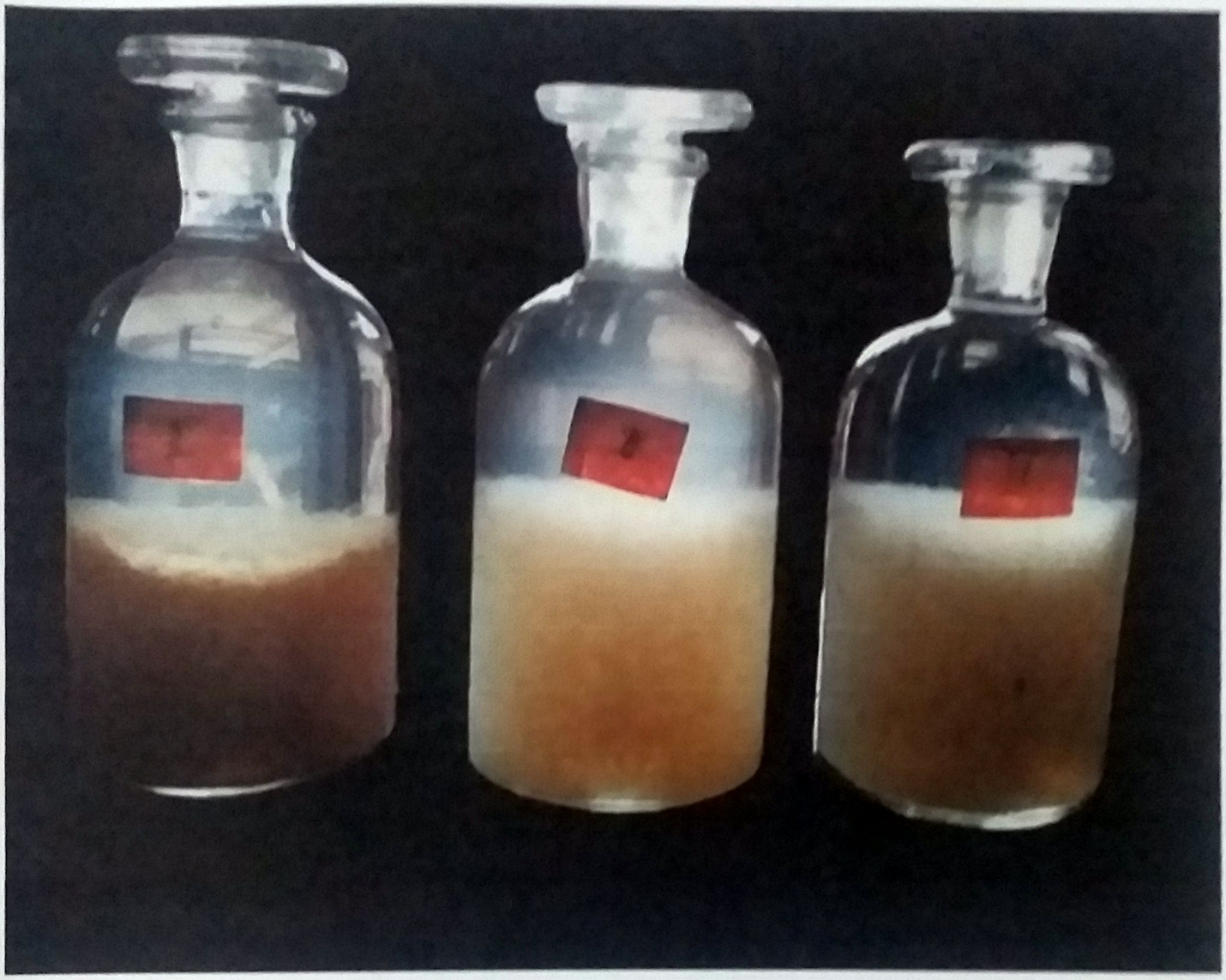


PLATE: 5 LOW CONTENT OF WATER FROM THERESPURAM

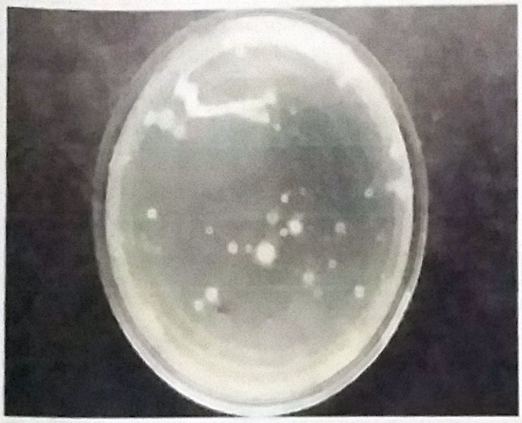
INDICATED DURING WRINKLE'S METHOD (ACHROMIC WITH NO

COLOUR)

TABLE 3: TOTAL HETEROTROPHIC BACTERIA LOAD IN THE SELECTED COASTAL WATER

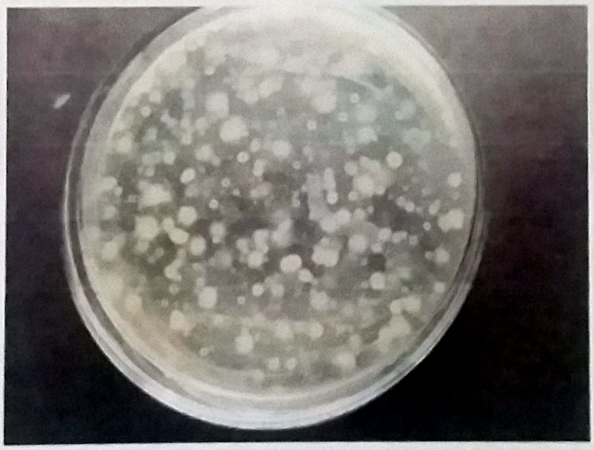
(Values are expressed in THB/10^{10})

STATIONS	WATER
STATION – I	231
STATION – II	136
STATION – III	497



STATION – I

STATION – II



STATION - III

PLATE-6: THB OF WATER FROM THE SELECTED COAST

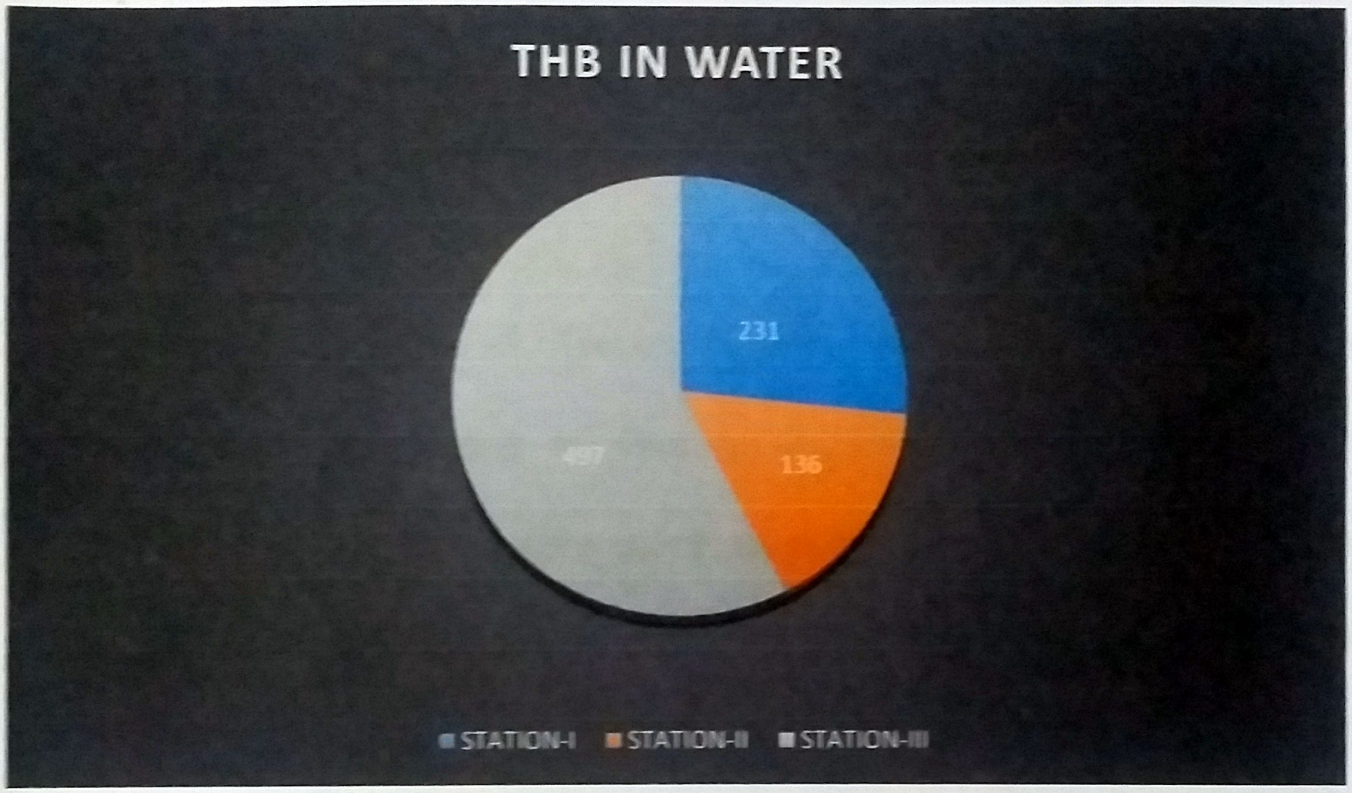


FIG:2 TOTAL HETEROTROPHIC BACTERIA LOAD IN THE SELECTED COAST

from Station-III. The dissolved oxygen was 5.13ml/l in Station-I and it was 2.76ml/l in Station-II.

5. TOTAL HETEROTROPHIC BACTERIAL LOAD IN WATER:

As presented in Table-3. The maximum THB load (497 $\times 10^{10}$) was observed in the water sample collected from Therespuram (Station- III). The THB load was minimum (136×10^{10}) in the coastal water from Hare Island (Station-II). The THB was (231×10^{10}) in Harbour beach (Station-I).

6. MACROBENTHOS:

As given in Table -4. The macrobenthic population collected from the sampling sites belong to Gastropoda, Bivalvia, Echinodermata and Crustacea. Bivalves were more abundant in Station-I and Station-II. Station-III has very few bivalves than other stations. The gastropod population ranked second among the macrobenthos collected from different study area, but for Station-II where gastropods were abundant (123). Very few crustaceans were collected from Station-I and Station-II and it was completely absent in Station-III. Echinoderms were collected few in numbers from Station-I where as no echinoderms were sited in other stations. The total macrobenthic population was high in Therespuram (Station-III) where the gastropods (micromolluscs) were the only macrobenthos along with few bivalves. Station-II was recorded with the least number of macrobenthos.

TABLE: NUMERICAL ABUNDANCE OF MACROBENTHOS FROM THE THREE COASTAL STATIONS UNDER STUDY

MACROBENTHOS	STATION - I	STATION - II	STATION - III
GASTROPODS	33	28	123
BIVALVES	40	51	5
ECHINODERMS	7	–	–
CRUSTACEANS	3	3	—

PLATE - 7 MACROBENTHOS COMMUNITY FROM THE SELECTED COAST

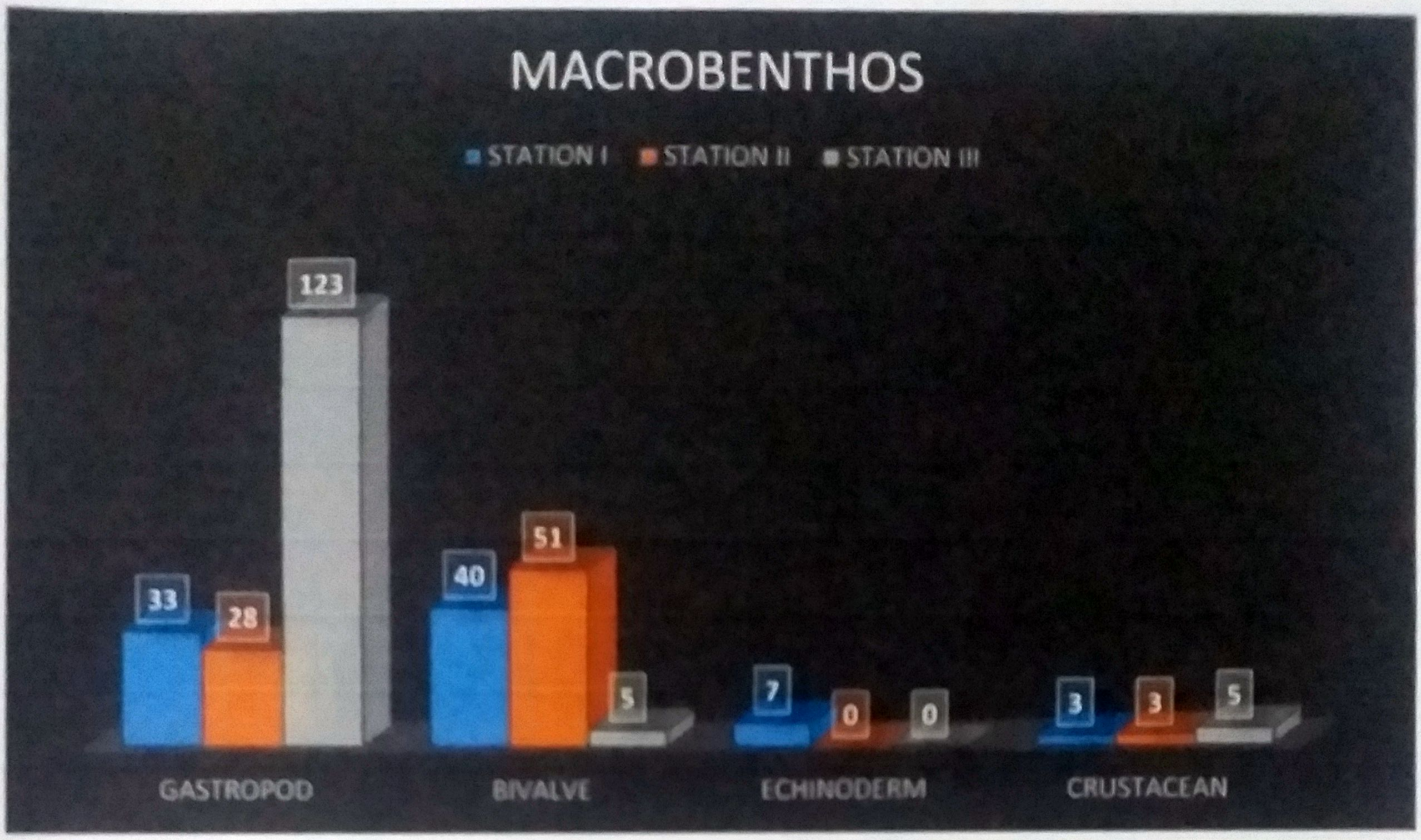


FIG:3 POPULATION OF MACROBENTHOS COLLECTED FROM THE SELECTED COASTAL STATIONS

DISCUSSION

DISCUSSION

WATER QUALITY

1. TEMPERATURE:

Temperature variation is one of the most important factors in the coastal eco-system which influence the physico-chemical characteristics. It also influence the distribution and abundance of flora and fauna. The temperature of coastal water during the study period was the highest in Station-II (Hare Island) which may be due to the discharge of thermal effluent from TTPS. Our result supported by (Ashok Prabhu *et.al.*,2008). Minimum water temperature was reported from Therespuram which may be attributed to the facts that inflow of sewage from buccal canal may reduce the temperature.

2. pH:

pH is recorded from different study areas ranged from 7.39 to 7.54. Extensively buffering capacity of the sea water allows only little pH change to be pronounced normally in closed portion whereas biological activity can cause variations. Among the stations studied low pH was recorded in Station-I (Harbour beach) which may be due to the increase in carbon dioxide resulting from the raise in temperature due to discharge from TTPS. Though no much variation was noted in pH among stations, high pH was recorded in Station-III (Therespuram) and this may be

correlated to the intense photosynthetic activity of the algae. Our result is supported by (Usha Nateson *et al.*, 2017).

3. **SALINITY:**

Slight variation was noted among the three stations under study. High salinity (34 ppt) was recorded in Hare Island. This might be due to the tidal influence. Tidal action may increase the salinity due to large quantity of salt water that lashes the coast (Bhakhtiari and Zeinodalini. 2011). Our result is supported by the results of (Julia *et al.,* 2017) in the study of wave current interaction in Southern North Sea. More over the temperature recorded in Station –III was also high compared to the other stations, which may also the reason for high salinity due to the evaporation. Low salinity (30 ppt) was recorded in the Station-III (Therespuram). This may be correlated to the heavy freshwater inflow through domestic sewage. Similar result were recorded by (Usha Natesan *et al.,* 2017).

4. **DISSOLVED OXYGEN:**

Dissolved oxygen was reported to be nil in Therespuram coastal water. This might be due to the uncontrolled discharge of domestic sewage which might have increased the bacterial load, turbidity, heavy metals and suspended solids in water column. Heavy metals bind with the dissolved oxygen in water. Bacteria discharged through sewage and effluents also deplete O_2 from the water. The depletion of oxygen is also enhanced by the presence of degradable organic matter. Result of the present study area

are similar to the reports of (Fatoki *et al.*, 2003) and (Jagi *et al.*, 2007). Among the stations under dissolved oxygen was high in coastal water of Harbour beach. The well oxygenated water is Harbour beach might be due to the wind and wave action is horizontal and vertical dirction which promotes the distribution of oxygen. This might also be attributed to the fact that there is no flow of domestic sewage and less anthropogenic activity.

5. TOTAL HETEROTROHIC BACTERIA:

Microbial indicators have been utilized worldwide to show if there is contamination in water body. Microbial impairment of recreational seawaters is generally monitored which is useful in defining the quality of seawater body. Various researchers have documented an elevated risk of contracting gastro intestinal diseases, skin infections as well as acute respiratory infections after exposure with recreational seawater body with increased concentration of bacteria. In our study the maximum THB load was observed in the water sample collected from Station-III (Therespuram). It is understood that the increase in THB is proportionate to the degree of sewage and human pollution. So the THB load in Therespuram could be attributed to the surplus dumping of domestic sewage and waste into the coast which increases the nutrient deposits for microbes. Similar trail was also reported by (Sugumar *et al.*,2008) from Tuticorin coast. Our result also coincide with the report of (Arasamuthu *et*

al.,2017) from seagrass meadows of Tuticorin coast of Gulf of Mannar. The THB load was minimum in the coastal water from Hare Island (Station-II). This might be due to the absence of domestic sewage and human waste in this water column which might be the reason for less nutrient load to the microbes. Human intrusion is also low in this area which might be the reason for low bacterial load. This report is also supported by the report of (Jagi *etal.*,2007) from Mannakudy estuary. Moderate levels of THB load was reported from Harbour beach (Station-I). This might be attributed to the fact that Harbour beach is recreational site for Tuticorin people and so anthropogenic activities might have added a moderate level of bacteria in this area. It might also be due to the effluent from SPIC and TAC which increase the the nutrient load and enhance the bacterial growth. But pollution due to domestic sewage run off were low in Harbour beach than Therespuram. Earlier study reports from Bhavnagar coast (Vaidia *et al.*,2001) and Nagore coast (Mohandas and Bharathi.,2003) supports our result. Nutrients stimulate the bacterial growth in water system. High bacterial load was also reported from Andhra coast where high mount of sulphate, nitrate and ammonium ions were present (Swami *etal.*,2006).

6. DISTRIBUTION OF MACROBENTHOS:

Variation in the composition and abundance of macro benthos has been mainly related to the changing environmental conditions (Gao.,2011) and

substrate concentration (Uwadie.,2016). The present study gives an insight into the impact of pollution on the macrobenthic fauna. (Carvalho *et al.*,2006) explained the study of macro fauna is an useful guide to assess the ecological condition.

The present study indicates that Gastropods were numerically abundant species in Station-III (Therespuram). But in Station-II and Station-III, bivalves were the abundant species, followed by Echinoderms in Station-I (Harbour beach) and few Crustaceans in Station-I (Harbour beach) and Station-II (Hare Island). This is in agreement with the finding of (Kathiresan *et al.*,2000) in Vellar estuary on the Southeast coast of India. It is reported that high tolerance to different environmental situation and coastal pollution from industries was the reason for the numerical abundance of Gastropods in Therespuram.

Environmental factor such as temperature, pH, salinity, dissolved oxygen and nutrient load are the main factors influencing the distribution of faunal communities in tropical coasts.

SUMMARY

SUMMARY

A present study on 'Macrobenthos with reference to quality of water to assess the intensity of pollution along Turticorin coast' revealed that the water temperature of Harbour beach (Station-I) is having moderate temperature, low pH, moderate salinity, well oxygenated and moderate levels of THB and supports more bivalves than the gastropods and few echinoderm and crustaceans (wide variety of macrobenthic population).

In Hare island (Station-II) the coastal water was having very high temperature, moderate pH and high salinity. The water was moderately oxygenated and minimum total heterotrophic bacterial load when compared to the other stations. A moderate macrobenthic community is reported including more bivalves than the gastrropodss and very few crustaceans. Echinoderms were reported nil. This seems to be a less polluted coast among the stations studied.

In Therespuram (Station-III) water sample is reported to have very less temperature comparatively with the maximum pH and minimum salinity.

The dissolved oxygen is observed to be nil with a heavy total bacterial load. The nutrient load from sewage has enhanced the bacterial load (Uwadie.,2016.) This has dramatically altered the macrobenthic population in this station. There use to be a positive correlation between the DO and the benthic population but in the present study though there was no dissolved oxygen in Station-III there are more gastropods and few bivalves. The maximum gastropod population with few

bivalves were observed in Station-III though the dissolved oxygen level is nil (Ramkumar.,2010). Ability of molluscs specially the gastropods to withstand anaerobic condition reported by (Gao.,2011) which support our results.

In our present study the highly oxygenated Station-I (Harbour beach) water has harboured a variety of macrobenthic population of gastropods, bivalaves, echinoderms and few crustaceans.

The study revealed the presence of pH range from 7.39-7.54 which is the preferable range for aquatic life. Though the pH was considered insignificant in distribution of macrobenthic population.

Though Therespuram coast is reported to have low temperature, high pH, very low salinity with high bacterial load and no dissolved oxygen, it seems to support maximum macrobenthic population gastropods among the stations studied.

This may be attributed to the high nutrient load in this station and the adaptation and tolerance of gastropods and bivalves to the polluted environment.

CONCLUSION AND SUGGESTIONS

CONCLUSION AND SUGGESTIONS

The present investigation has given an insight into the impact of water quality and bacterial load in the distribution of macrobenthic community in the coastal zones of Thoothukudi. It is understood that the abundant distribution of the macrobenthic fauna are affected and influenced by the degree of pollution in the environment. It is also evident that through domestic sewage discharge drastically pollute the environment, the organic enrichment in this coast support benthic community which indicates the adaptation and tolerance exhibited by the benthic community.

The present study reveals the fact that Therespuram coastal water is highly polluted to the level where it leads to anoxic condition. Though harbour beach seems to be less polluted and well oxygenated, thermal pollution, fishing activities and human instrusion has induced bacterial load. Hare island was found to be less polluted zone but supported less macrofauna.

Accordingly sewage disposal, thermal pollution, industrial pollution and fishing activities have ruthlessly affected the biota of Thoothukudi bay signaling the disturbed nature of the system. Therefore, it warrants an extensive survey covering entire area of the bay to have a holistic view of the system for better conservation and management of biodiversity.

Further researches could be done on:

- ❖ Long term changes in the structure of benthic community of Gulf of Mannar.
- ❖ Physical factors influencing macro fauna.
- ❖ Seasonality of Meio-benthos of Tuticorin coast.
- ❖ Benthic communities in tropical mangrove and coral reef.
- ❖ Effect of anthropogenic disturbance on tropical benthic communities.

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ISOLATION OF BIOACTIVE COMPOUNDS AND PHARMACOLOGICAL PROPERTIES OF MARINE GASTROPOD *LAMBIS TRUNCATA* (Lightfoot, 1786)

A project submitted to

ST. MARY'S COLLEGE (Autonomous), THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

by

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DEPARTMENT OF ZOOLOGY

ST.MARY'S COLLEGE (Autonomous)

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

THOOTHUKUDI - 628001

April - 2023

CERTIFICATE

This is to certify that the project entitled **Isolation of Bioactive Compounds and Pharmacological Properties of Marine Gastropod *Lambis truncata* (Lightfoot, 1786)** is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a record of the work done during the year 2022-2023 by the following students.

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CONTENTS

1. INTRODUCTION

Marine organisms make up approximately half of the total global biodiversity, with the Mollusca containing the second largest number of species, including snails and bivalves. The marine environment is highly competitive, hostile and aggressive, which has led to the production of specific and potent bioactive compounds by the mollusca and their associated microorganisms, in abid to protect themselves and ensure their survival. A diverse array of bioactive compounds can be isolated from the extracts of marine molluscs of which linear, cyclic, and conjugated peptides and depsipeptides form some of the most important bioactive compounds that have been well characterized and some of have already reached clinical trials or been approved for use as therapeutic agents and supplements (Queensley *et al*., 2019).

Molluscs are said to be pharmacologically significant outlet. There are more than thousand of bioactive compounds discovered in molluscs. They are peptide, depsipeptide, sterols, sesquiterpene, terpenes, polypropionate, nitrogenous compounds, macrolides, prostaglandins and fatty acid derivatives, sterols, miscellaneous compounds and alkaloids (Blunt *et al*., 2006). The availability of molluscs is too high and their utilization is extremely low compared to other marine organisms.

In the recent past, molluscs have been screened for antitumour, antileukemic, antibacterial and antiviral properties world over (Mayer and Hamann, 2005; Diane *et al*., 2009). Extraction of antibacterial and antifungal compounds from marine organisms has been in vogue since many years, perusal of literature revealed that a large number of works have been carried out in other groups organisms but only a few studies were made in molluscs (Chandren *et al*., 2009; Annamalai *et al*., 2007 and Diane *et al*., 2009). Studies of antimicrobial mechanisms and compounds in mollusc may provide valuable information for new antibiotic discoveries and give new insights into bioactive compounds.

The phylum Mollusca stand second to Arthropoda in numerical abundance. The number of species identified under phylum mollusca vary between 80,000 to 1,00,000. They are more abundant in the littoral zones of tropical seas. Gastropods and bivalves constitute 98% of the total population of mollusca. The gastropods and bivalve fisheries are of sustenance nature and used for edible purpose, source of lime, as decorative shells or for industrial purposes. The class Gastropoda has an extraordinary diversification of habitats. They have the greatest numbers of named molluscs species.

Natural products have served as an important source of drugs since ancient times and about half of the useful drugs today are derived from the natural sources. Natural products isolated from molluscs have been tested for

an extensive range of natural activities. Molluscan metabolites have been most frequently tested for neuromuscular blocking action, anti-predator, antimicrobial, anti-neoplastic and cytotoxic activity. The use of crude or more sophasticated products from nature in order to acquire health benefits is an ancient human habit; nevertheless, the expenditure of many of these products has been scientifically proven to offer chemoprevention for several human diseases. Among the invertebrates the molluscs are very good source of biomedically important products and have developed very effective mechanisms that are a part of their innate immunity (Tincu and Taylor, 2004).

The Marine environment represents an excellent source for bioactive compounds (Benkendorff, 2010; Jirge *et al*., 2010 and Datta *et al*., 2015) due to the magnitude of the oceans and the high biodiversity of the organisms therein. Several marine organisms are generally soft-bodied and completely immersed in their environment. Some organisms are sedentary. Hence, they have developed biological defense systems, including the secretion of mucus containing bioactive compounds in order to protect themselves from the harsh nature of their environment. These defense systems can be exploited and used in rational drug design (Jha and Xuzi-Rong, 2004). Several molecules isolated from a vast number of marine species have clinical application as supplements and drugs while other molecules are currently in clinical trials or are under study (Jirge *et al*., 2010). Many of them have novel chemical

structures which may lead to the development of entirely new drugs and therapeutic agents.

Bioactive compounds isolated from marine sources include secondary metabolites which have been produced by sponges, algae, scale-less fishes, seaweed, marine molluscs and even associated microorganisms. These secondary metabolites include nitrogen heterocyclics, and sulphur containing nitrogen heterocyclics as well asterpenoids, quinones, steroids and isoprenoids) (Datta *et al*., 2015). Bioactive low molecular weight peptides and depsipeptides, as well as lectins, have also been obtained from marine sources (Suarez-Jimenez *et al*., 2012). Exploitable biological activity that marine organisms have been shown to possess include: antimicrobial activity (Datta *et al*., 2015 and Salehi *et al*., 2014), anti-cancer and anti-proliferation activity (Benkendorff, 2010; Suarez-Jimenez *et al*., 2012 and Chakraborty *et al*., 2009), free radical scavenging and antioxidant activity (Apriandi Azwin, 2011 and Purwaningsih, 2012) and analgesic and anti-inflammatory effects (Cheung *et al*., 2006).

In many culture molluscs, especially shelled gastropods and bivalves, are regarded as food delicacies (Jimmy and Okonkwo, 2016 and Ogamba *et al*., 2016). Furthermore, they provide a wide range of human resources including: the use of the shells for improvising mixed aggregates for building construction (Ogamba *et al*., 2016) and the production of dyes especially

from molluscs such as the Muricid whelk, *Trunculariopsis trunculus* (Benkendorff, 2010). Molluscs have also been used in a variety of traditional natural remedies (Benkendorff *et al*., 2015) although the active ingredients are typically unknown, this is due to the fact that, very few scientific studies had been undertaken to evaluate and verify the health benefits of the molluscs (Prabhakar and Roy, 2009).

A vast number of bioactive compounds have been isolated from the mollusca, including metabolites like complex alkaloids, macrolides and terpenes. However, among the most active of them are the cyclic peptides and depsipeptides or linear peptides. Currently, peptides isolated from molluscs as well as their synthetic structural analogues are in clinical trials as anticancer compounds (Benkendorff, 2010 and Simmons *et al*., 2015) and have been approved for use in pain management (Pati *et al*., 2015).

The earliest records of employing herbal medicines in managing health issues and microbial infections refer back to the Sumerian, Egyptian, and Chinese civilisations (Suntar, 2020 and Luo *et al*., 2021). Thus, the search for new anti-infectives, especially antimicrobial drugs, is still in demand. With the rapid development of screening technology for marine antimicrobial substances, the screening process has been continuously promoted with the expansion of the scope of application (Fenical *et al*., 2020).

Bioactive peptides isolated from marine molluscs specifically those isolated from bivalves and abalone, have displayed potent antimicrobial efficacy. A saccharothrixmicine peptide with antimicrobial activity against *Candida albicans* and *Xanthomonas sp* was isolated from the marine mollusc *Anadara broughtoni* in association with *Saccharothrix espanaensis* AN113. A cysteine-rich peptide, myticin, hasbeen isolated from the Mediterranean mussel (*Mytilus galloprovencialis*) Myticin has potent antibacterial activity against both gram- negative and positive bacteria (Shukla, 2016). Peptides with antifungal properties have also been isolated from the blue mussel (*Mytilus edulis*) and from *Mytilus coruscus* (Sun *et al*., 2014).

In the most of the publications concerning antimicrobial activity in Mollusca, either single body compartment alone, like haemolymph and egg masses, or extracts of whole bodies have been tested for activity (Haug *et al*.,2003). Further the cuttlefish also reported antibacterial and antifungal activities against some of the human pathogenic microorganism. A screening of antibacterial activity in cuttle fishes extracts of *Sepia* sp. and *Loligo* sp. And marine snail of *Tibia insulaechorabcurta* were conducted. Antibacterial activity has previously been described in wide range of mollusc species (Shanmugam, 2008; Benkendorff *et al*., 2001 and Rajaganapathi, 2001).

Oxidation is essential to many living organisms for the production of energy to fuel biological process. However, oxygen-centre free radicals and

other reactive oxygen species (ROS) which are continuously produced *in vivo*, results in cell death and tissue damage. Scientific evidence has suggested that under oxidative stress conditions, oxygen radicals such as superoxide anions (O_2^-), hydroxyl radical (OH) and peroxyl radicals (H_2O_2) are produced in biological system. These reactive oxygen species can damage DNA which causes mutation and chromosomal damage (Sumitra Chanda *et al.,* 2015).

Antioxidant based drug formulations are used for the prevention and treatment of complex diseases like atheroscelerosis, stroke, diabetes, Alzheimer's disease and cancer. Therefore, antioxidants are vital substances which possess the ability to protect the body from damage caused by free radicals induced oxidative stress (Ozsoy *et al.,* 2008). Natural antioxidants are responsible for inhibiting or preventing the deleterious consequences of oxidative stress.

In general, only about 1% of the population of molluscan species has been investigated for bioactive secondary metabolites. This could be as a result of the vast number of species in the phylum, the diversity in the habitats and ecological niches of species in the phylum and our relatively poor knowledge of the biology of the different taxa. There is little ethnomedical history of marine molluscs (Benkendorff, 2010). This means that most studies on these organisms have to undergo screening from scratch and experience

quite a bit of trial and errors. Where ethnomedical accounts do exist, the active ingredients contributed by the mollusca are typically unknown, as very few scientific researches have been undertaken to authenticate the health benefits of molluscs, as attention is usually given to the bioaction of plant species (Prabhakar and Roy, 2009).

Diabetes mellitus is a chronic metabolic disorder that exhibited great expansion all over the world. It is becoming an epidemic disease adding a major burden to the health care system, particularly in developing countries.Diabetes triggers the production of reactive oxygen species (ROS) (Savu *et al*., 2012 and Son, 2012). Consequently, agents exhibiting radical-scavenging activity can abolish ROS-induced oxidative damage (Pietta, 2000 and Kucharska *et al*., 2004).

Type 2 diabetes is a complex metabolic disorder associated with developing insulin resistance, impaired increased oxidative stress, inflammation, insulin signaling, abnormal glucose metabolism, and so on. The disorder leads to a consequent decrease in quality of life and an increase in the rate of mortality (Brown *et al*., 2004).

Chronic anti-inflammatory diseases including rheumatoid arthritis are still one of the main health problems of the world's population. At present, although synthetic drugs are dominating the market but element of toxicity

that these drugs entail, cannot be ruled out. In addition, inflammation, which is a major defense reaction of the immune system to harmful stimuli, including infection and injury, is a serious threat to health and exists in many diseases, such as bronchitis, pneumonia, gastritis, nephritis, and rheumatism. At present, steroidal and nonsteroidal anti-inflammatory drugs are commonly used in clinics; nevertheless, their side effects, such as gastrointestinal tract damage and allergic reactions cannot be ignored (Blumenthal *et al*., 2017).

Hence, an attempt has been made to analyze the bioactive components through FT-IR, GC-MS and their pharmacological activities viz., *in vitro* antibacterial, antifungal, antioxidant, antidiabetic and anti-inflammatory properties of marine molluscan shell extract *Lambis truncata* were evaluated. Consequently, there is a need to develop new drugs with minimum side effects. Search for safe and effective natural agents have been given priority in scientific research. So, the marine gastropod *L. truncata* can be regarded as a new, healthy and natural drug or additive that is associated with antibacterial, antifungal, antioxidant, antidiabetic and anti-inflammatory activities for the pharmaceutical and food industries.

2. REVIEW OF LITERATURE

Oceans possesses nearly three lakh described species of plants and animals from the marine ecosystem (Jimeno, 2004; Kijjoa and Swangwong, 2004).The number of potential compounds isolated from marine realm has virtually soared and this number now exceeds to 10,000 with hundreds of new compounds still being discovered every year (Proksch *et al*., 2002).

With the combined efforts of marine natural product chemists and pharmocologists, a number of promising identified molecules are already in market, clinical trails or in pre-clinical trials. Interestingly, these precious natural products have been obtained from marine microorganism as well as invertebrates such as sponges, molluscs, bryozoans, ascidians etc (Thakur and Muller, 2004). Natural product is a source for bioactive compounds and has potential for developing some novel therapeutic agent. Marine molluscs are heterogenous group of soft-bodied animals with great diversity. Considerable research have been carried out on the various aspects of this group. So in the present study, a critical review of literature is carried out on the bioactive compounds and biological activities.

Vennila *et al*., (2011) investigated antimicrobial and plasma coagulation property of some molluscan ink extracts: Gastropods and Cephalopods. Screening on antimicrobial activity of marine gastropods

Babylonia zeylanica (Brugiere, 1789) and *Harpa conoidalis* (Lamarck, 1822) from Mudasalodai,South coast of India was reported by Suresh *et al*., 2012. Suresh *et al.* (2012) identified various forms of the compounds present in the molluscan extracts.

Mohanraj *et al*. (2014) screened the biomedical properties of whole body and ink fluid extracts of marine sea slug from South Coast of India. The antimicrobial, cytotoxic, thin layer chromatography and functional characterization of whole body and ink fluid crude extracts from *Kalinga ornata* and *Bursatella leachii* was investigated.

Skingsley *et al.* (2000) described the analysis of pulmonate mucus by infra red spectroscopy. Furuhashi *et al*. (2009) evaluated pyrolysis GC-MS and FTIR spectroscopy in chitin analysis of molluscan shells. Dhivya T. Dharan (2018) observed FTIR and GC-MS studies on the ethanol extract of *Aplidium multiplicatum* from Vizhinjam, Kerala. GC-MS analysis of the milk from *Plicopurpura pansa* revealed four compounds that could be identified as tyrindoleninole 3,6-bromoisatin,6-bromo-2 methyl sulfinyl-3H,indole-3-one (Felipe Javier, 2009).

Nuzhat Afsar *et al.* (2012) reported GC-MS analysis of fatty acid (FAs) of prosobranch gastropod species *Thais carinifera* from Pakistan Coast (North Arabian Sea). Periyasamy *et al.*(2013) analyzed agarose gel

electrophoresis, FT-IR and anticoagulant activity of marine gastropods *Babylonia spirata* (Lin. 1758) and *Phalium glaucum* (Lin. 1758) collected from Cuddalore, Southeast Coast of India. Gayathri and BaskaraSanjeevi (2014) described FT-IR spectral analysis and antipathogenic activity of freshwater gastropod *Pila virens* (Lamarck, 1822) from Lower Grand Anaicut Reservoir, Tamil Nadu.

Tamil Muthu and Selvaraj (2015) analyzed the bioactive constituents from the flesh of *Turbo brunneus* (Roeding, 1798) by GC-MS. Jemma Hermelin Jesy Diaz and Thilaga (2015) investigated GC-MS analysis of methanolic extract from the internal shell of Cephalopods. Hermina Giftson and Jamila Patterson (2016) evaluated the biochemical composition, FT-IR spectral analysis and antibacterial activity of crude extracts of gastropod *Harpa davidis* (Roeding, 1798) from Kanyakumari coast against isolated human and fish pathogens.

Edwards (2012) reported the effects of bioactive compounds from the marine mollusc *Dicathais orbita* on human reproductive cancer cells. Benkendorff *et al*. (2011) studied the bioactivity of the murex homeopathic remedy and of extracts from an Australian muricid mollusc against human cancer cells. Elumalai *et al.* (2011) showed the antibacterial activity of variuos leaf extracts of *Merremiae margianta*. Sri Kumaran *et al.* (2011)screened the antimicrobial activities of marine molluscs *Thais tissoti*

and *Babylonia spirata* against human, fish and biofilm pathogenic microorganisms.

Immanuel *et al*. (2012) studied the antipyretic, wound healing and antimicrobial activity of processed shell of the marine mollusc *Cypraea moneta*. Suresh *et al*. (2012) reported the screening on antimicrobial activity of marine gastropods *Babylonia zeylanica* and *Harpa conoidalis* from Mudasalodai, Southeast coast of India. Periyasamy *et al*. (2012) elucidated the antimicrobial activities of the tissue extracts of *Babylonia spirata* from Thazhanguda, Southeast coast of India.

Suarez-Jimenex *et al*. (2012) analyzed the bioactive peptides and depsipeptides with anticancer potential: sources from marine animals. Salehi *et al*. (2012) studied the isolation and characterization of some kind bioactive protiens sponge as antibacterial agent. Hermina Giftson and Jamila patterson (2014) evaluated the antibacterial activity of the shell extracts of marine mollusc *Donax faba* against pathogens. Suganya *et al.* (2014) investigated the *in vitro* anti-diabetic, antioxidant and anti-inflammatory activity of *Clitoria ternatea*.

Benkendorff *et al*. (2015) investigated the traditional medical uses of Muricidae molluscs substantiated by their pharmacological properties and bioactive compounds. Pati *et al*. (2015) reported marine molluscs as ac

potential drug cabinet :An Overview. Queensley Eghianruwa *et al.* (2019) investigated the bioactive peptides from marine molluscs.

Harekrishna Jana *et al*. (2017) analyzed the comparative study on the antimicrobial activity, anti-oxidant, anti-diabetic and anti-inflammatory property of freshwater molluscs (*Bellamya bengalensis*) and marine molluscs (*Saccostrea cucullata*). Al-Thubiani *et al*. (2018) studied the identification and characterization of a novel antimicrobial peptide compound produced by *Bacillus megaterium* strain isolated from oral microflora. Yang *et al*. (2019) studied the purification and characterization of antioxidant peptides derived from protein hydrolysate of the marine bivalve mollusc *Tergillarca granosa*.

Ji *et al*. (2019) analyzed the antibacterial effect and components of crude extract from *Bacillus megaterium* L2. Zhao *et al.* (2019) evaluated the antibacterial mechanism of fermentation product from *Bacilllus megaterium* L2 against Erwinia carotovor. Ding *et al.* (2020) reported the isolation and identification of antifungal components synthesized by *Bacillus megaterium* LB01 from special environment and its action mechanism.

Liu *et al*. (2013) explained the isolation and structural characterization of novel antioxidant mannoglucan from a marine bubble snail, *Bullacta exarata*(Philippi).Fahmy and Soliman (2013) reported *invitro* antioxidant, analgesic and cytotoxic activities of *Sepia officinalis* ink and *Coelatura*

aegyptiaca extracts. Sreejamole and Radhakrishnan (2013) studied antioxidant and cytotoxic activities of ethyl acetate extract of the Indian Green Mussel *Perna viridis*. Subhapradha *et al*. (2013) investigated antioxidant potential of crude methanolic extract from whole body tissue of *Bursa spinosa*(Schumacher, 1817).

Sivaperumal *et al*. (2013) showed antimicrobial peptide from crab haemolymph of *Ocypoda macrocera*(Milne Edwards, 1852) with reference to antioxidant: A case study. Sadeesh Kumar *et al*. (2014) carried out the potential activity of *invitro* antioxidant on methanolic extract of *Babylonia zeylanica*(Bruguiere, 1789) from Mudasalodai, South Coast of India. Ramesh *et al*. (2014) described the effects of drugs against antioxidant and cytotoxic (Hep 2 cell line) activity compounds from marine animals *Conus amadis* venom (Gmelin, 1791).

In vitro antioxidant activity of different gastropods, bivalves and echinoderm by solvent extraction method was carried out by Pachaiyappan *et al*. (2014). Madhu *et al*. (2014) analyzed the antibacterial and antioxidant activities of the tissue extract of *Perna viridis*(Molluscan:Bivalvia) from Versova Coast, Mumbai. Pawan Kumar *et al*. (2014) observed the pharmacological studies on the venom of the marine snail *Conus lentiginosus* (Reeve, 1844).

Sadhasivam *et al*. (2013) studied *in vitro*antibacterial, alpha-amylase inhibition potential of three nudibranch extracts from South east coast of India. Chakraborty *et al*. (2014) reported the response of pro-inflammatory prostaglandin contents in anti-inflammatory supplements from green mussel *Perna viridis* L. Cahyani *et al*. (2015) evaluated the anti-diabetic potential and secondary metabolites screening of mangrove gastropod *Cerithidea obtuse*.

Makkar and Chakraborty (2016) observed antidiabetic and anti-Inflammatory potential of sulphated polygalactans from red seaweeds *Kappaphycus Alvarezii* and *Gracilaria Opuntia*. Kajal Chakraborty and Minju Joy (2017) evaluated the anti-diabetic and anti-inflammatory activities of commonly available cephalopods.

From the above review it is pronounced that knowledge on the various aspects of isolation of bioactive components and pharmacological activities are lacking for *Lambis truncata*. Considering the importance of this species as a novel bioactive agent for the humans, the present work on *Lambis truncata* is planned and carried out covering all the above countenances.

3. OBJECTIVES

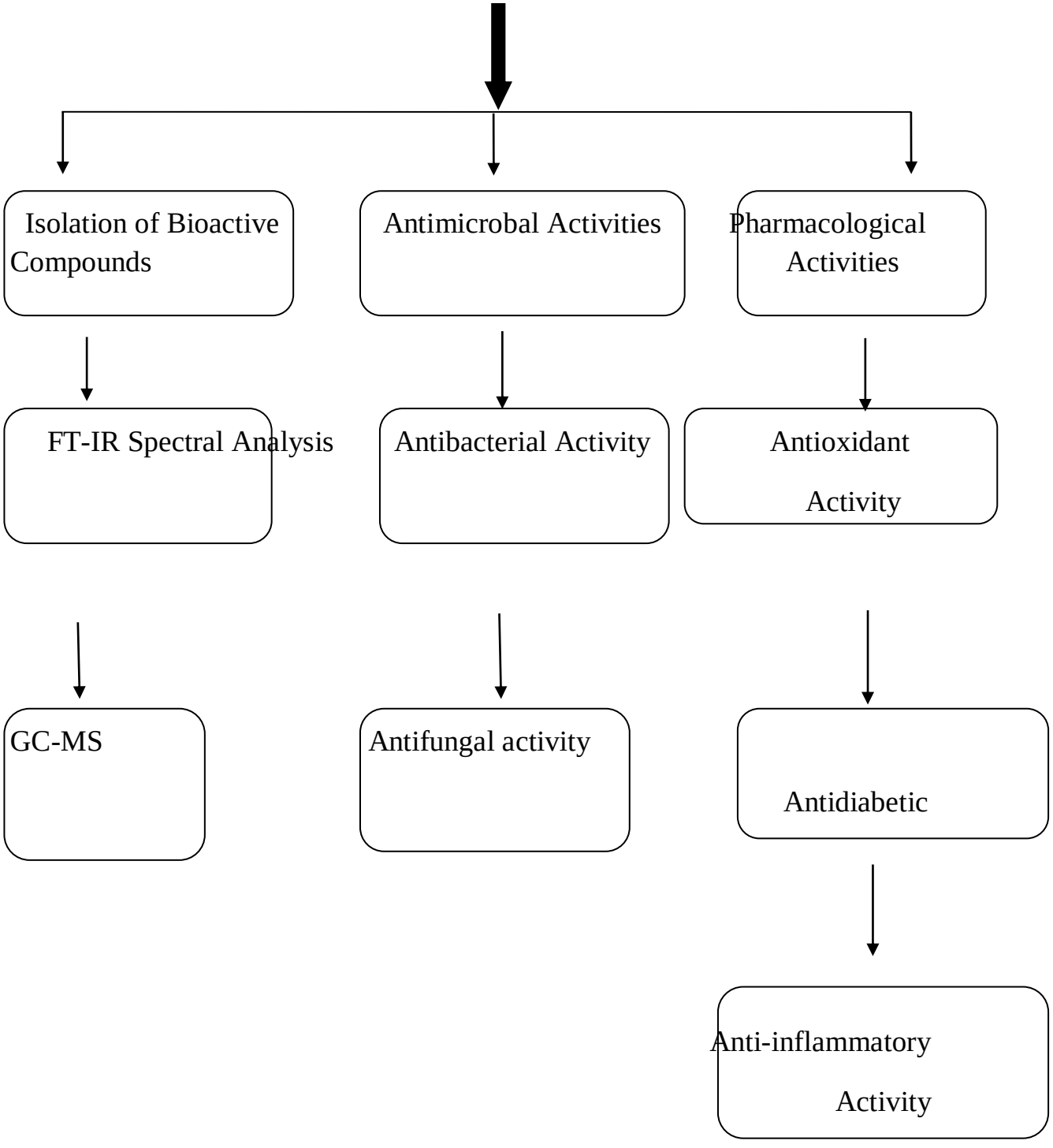
Ocean is known as large biodiversity of fauna and flora. Therefore, the marine environment is an exceptional store house of different shell fish organisms such as oyster, prawn, scallops, squids and octopus. There is good oppurtunity for the discovery of new bioactive substances from marine and estaurine ecosystems. Molluscs in the oceans are common sight and are virtually untapped resource for the discovery of novel compounds. Many studies on bioactive compounds from molluscs exhibiting antitumour, antileukaenic, antibacterial and antiviral activities have been reported worldwide. So, the present study has been carried out with the following objectives:

1. To identify the bioactive compounds in the shell extract of *Lambis truncata* by FT-FR analysis.
2. To elucidate the structure,molecular formula of bioactive constituents of *L.truncata* through GC-MS analysis.
3. To investigate the antibacterial, antifungal, antioxidant, antidiabetic and anti-inflammatory activities of *L.truncata*.

4. EXPERIMENTAL DESIGN

Collection of experimental organism

Lambis truncata from Thoothukudi coastal region



5. MATERIAL AND METHODS

5.1 Description of the study area

The Gulf of Mannar is located between India and Srilanka, stretches from the longitude 78º08' to 79º30' E and along the latitude from 83º5' to 9º25' N. It is a part of the Southward extension of the Bay of Bengal and meets in the Indian Ocean. This geographical area runs from Pamban island including Rameshwaram to Cape Comorin along the Southeast Coast of India to a distance of about 170 nautical miles. The Gulf of Mannar biosphere reserve has an area of about 10,500 km^2 and is considered as 'Biologist's Paradise' for, it has 3600 species of flora and fauna. This coast maintains a rich biological diversity perspective 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 (Figure 1).

5.2 Collection of experimental animals

In the present study the gastropod *Lambistruncata* were collected from the Gulf of Mannar coastal region (Plate 1). The mesogastropod *L.truncata* was collected from the landed by-catch from fishing trawlers operated for crabs and prawns along the Thoothukudi coastal region. These gastropods were collected during the month of November and December

2022. The freshly collected samples were brought to the laboratory, cleaned and washed with fresh sea water to remove all impurities. The shells were broken and then dried in hot air oven at 56ºC for 48 hours and used for further studies.

5.3 Description of experimental animal

5.3.1 Systematic position of *Lambis truncata* (Linnaeus, 1758)

Phylum : Mollusca

Class : Gastropoda

Subclass : Caenogastropoda

Order : Littorinimorpha

Super Family : Stromboidea

Family : Strombidae

Genus : *Lambis*

Species : *truncata*

Lambis truncata is the largest and heaviest of spider shells, up to 40 cm. *Lambis truncata* is similar to *Lambis lambis* but with a more squarish outline. Younger shells are creamy white; columella and lip usually become brown when older. *Lambis truncata* lives on rubble and coarse sand in shallow water *Lambis truncata* is the largest and heaviest of spider shells, up to 40 cm. *Lambis truncata* is similar to *Lambis lambis* but with a more

squarish outline. Younger shells are creamy white; columella and lip usually brown when older.

5.4 Preparation of extract

One gm of the sample was dissolved in 20ml of the methanol solvent and mixed well in conical flask, after mixing the sample was incubated at 40^{o}C, 60-70 rpm in orbital shaker for 24hrs. The extract was filtered through Whatman No.1 filter paper and the extract residue was resuspended in 20 ml of 100% A.R grade methanol. The methanol soluble extracts were dried and solubilized in deionized water. Different concentrations of extracts were prepared and stored at 0^{o}C for further use.

5.5 Fourier Transform Infra-Red spectrum analysis

FT-IR spectroscopy is one of the most powerful analytical techniques used for structural elucidation and identification of compounds. It has been used to examine and provide important data on a wide variety of biological molecules. The functional group present in the methanolic extract of *Lambis truncata* was determined using FT-IR spectroscopy (Bio-read FT-IR 8400s models, USA). Briefly 5 mg of sample was mixed with 100 mg of dried potassium bromide (KBr) and compressed to prepare as a salt disc (10 mm diameter) for reading the spectrum. The absorption was read between 500 and 4000 cm^{-1}. Several spectral values of the isolated functional groups

were compared to published values in the literature to identify the known functional groups.

5.6 GC-MS analysis

GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer System comprising a AOC 20i auto sampler and gas chromatography interfaced to a mass spectrophotometer (GC-MS) instrument employing the following conditions such as Column elite – 5MS fused silica capillary column (30 x 0.25mm ID x 0.25 µm df, composed of 5% Diphenyl 95% Diphenyl Poly Siloxane), operating in electron impact mode at 70eV: Helium (99.999%) was used as a carrier gas at constant flow of 1ml/min and an injection volume 3µl (split ratio of 10:1), injector temperature 250ºC. The oven temperature was programmed from 110ºC (isothermal for 2min), with an increase of 10ºC/min to 200ºC, then 5ºC/min to 280ºC. Mass spectra were taken at 70eV; a scan interval of 0.5s and fragments from 45 to 450 Da.

5.7 Identification of compounds

Interpretation on mass spectrum was conducted using the database of National Institute of Standard Technology (NIST Ver.21), WILEY 8 and FAME having more than 62,000 patterns. The unknown components found in the body tissues of *Lambis truncata* were matched with the spectrum of the

known components stored in NIST, WILEY and FAME, the MS library and predicted from Duke's Ethno Botanical Database.

5.8 Antibacterial Activity

Principle

The antimicrobials present in the given sample were allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

Materials Required

Staphylococcus aureus-902 and *Aeromonas hydrophila* was purchased from MTCC, Chandihar, India. Nutrient Agar medium, Nutrient broth, Gentamicin antibiotic solution was purchased from Himedia, India. Test samples, petri-plates, test tubes, beakers conical flasks were from Borosil, India. Spirit lamp, double distilled water.

Agar-Well Diffusion Method

a. Nutrient Agar Medium

The medium was prepared by dissolving 2.8 g of the commercially available Nutrient Agar Medium (HiMedia) in 100ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes.

The autoclaved medium was mixed well and poured onto 100mm petriplates (25-30ml/plate) while still molten.

b. Nutrient broth

Nutrient broth was prepared by dissolving 2.8 g of commercially available nutrient medium (HiMedia) in 100ml distilled water and boiled to dissolve the medium completely. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.

Procedure

Petri plates containing 20 ml nutrient agar medium were seeded with 24 hr culture of bacterial strains were adjusted to 0.5 OD value according to McFarland standard, (*Staphylococcus aureus*-902 and *Aeromonas hydrophila*). Wells were cut and concentration of sample SV01 (500, 250, 100 and 50 µg/ml) was added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Gentamicin antibiotic was used as a positive control. The values were calculated using Graph Pad Prism 6.0 software (USA) (De Magaldi *et al*., 1997).

5.9 Antifungal Activity

Principle

The anti-fungal agent present in the given sample was allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

Materials Required

Potato dextrose agar medium, Amphotericin B antimycotic solution, test samples, test tubes, beakers conical flask, spirit lamp, double distilled water and petri-plates.

Agar-Well Diffusion Method

Potato Dextrose Agar Medium

The potato dextrose agar medium was prepared by dissolving 20 gm of potato influsion, 2 gm of dextrose and 1.5 gm of agar in 100ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petri plates (25-30 ml/plate) while still molten.

Procedure

Petri plates containing 20ml potato dextrose agar medium was seeded with 72 hr culture of fungal strain (*Aspergillus niger* and *Sporothrix schenckii*) wells were cut and different concentration of sample SV01 (500, 250, 100 and 50 μg/ml) was added. The plates were then incubated at 28°C for 72 hours. The anti-fungal activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Amphotericin B was used as a positive control. The values were calculated using Graph Pad Prism 6.0 software (USA) (De Magaldi *et al*., 1997).

5.10 Antioxidant Activity

5.10.1 Hydrogen Peroxide Scavenging Assay

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate.

Material Required

Hydrogen Peroxide solution and Sodium Phosphate buffer

Procedure

Ability of extracts to scavenge hydrogen peroxide was estimated according to the method reported by Ruch *et al*. (1989) with minor modification. A solution of hydrogen peroxide (43 mM) is prepared in phosphate buffer (1 M pH 7.4). Different concentration of tellurium nanoparticles (50, 100, 250, 500 µg/ml) was added to hydrogen peroxide solution (0.6 ml, 43 mM). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as standard. The free radical scavenging activity was determined by evaluating percentage inhibition (Ruch *et al*., 1989).

Percentage inhibition = [(Control- Test)/Control] ×100

5.10.2 Phosphomolybdenum Assay

The total antioxidant capacity of the extracts was evaluated according to the method described by Prieto *et al*. (1999). An aliquot of 0.5 ml of samples solution (concentrationsranging from 50µg/ml to 500µg/ml)was combined with 4.5 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). In case of blank, 0.5 mL of 45% ethanol was used in place of sample. The tubes were incubated in a boiling water bath at 95°C for 90 min. After the samples were cooled to

room temperature, the absorbance of the aqueous solution of each sample was measured at 695 nm against blank in UV-2450 spectrophotometer (Shimadzu, Japan). The total antioxidant activity was expressed as the absorbance of the sample at 695 nm. The higher absorbance value indicated higher antioxidant activity (Prasad *et al*., 2009).

Percentage inhibition = [(Control- Test)/Control] ×100

5.11 Antidiabetic Activity

5.11.1 α-Amylase Inhibitory Activity

The α-amylase inhibitory activity was determined by the method described by Xiao *et al*. (2006). Samples (40 µl at concentrations ranging from 50µg/ml to 500µg/ml) were mixed in 96-wellmicroplates with 40 µl of amylase solution (100 U/ml in 0.1Msodium phosphate buffer, pH 7.0) and 40 µl of 0.1% starch solution(diluted in the previous buffer). After 10 min at 37°C, 20 µl of 1Mhydrochloric acid (HCl) and 100 µl of iodide solution(5mM iodine (I_2) + 5mM potassium iodide (KI), in distilledwater) were added and the absorbance was measured at 580 nm.Results were expressed as IC_{50} values (µg/ml). Acarbose wasused as the standard.

Percentage inhibition = [(Control- Test)/Control] ×100

5.12 Anti-inflammatory Activity

Preparation of phosphate buffer saline

2.725 g of anhydrous sodium dihydrogen orthophosphate, 0.800 gdisodium hydrogen orthophosphate and 22.500 g sodium chloride were weighed on a Mettler Toledo digital analytical balance(AB204-S, Ohio, USA) and dissolved in distilled water. The solutionwas diluted to the mark with distilled water in a 250 mL volumetricflask. The pH was adjusted to 7.4 using 0.1 N HCI or NaOH.

5.12.1 *In vitro* inhibition of egg albumin denaturation

The anti-inflammatory activity of marine shell extract of *L.truncata*,was determined *in vitro* for inhibition of denaturation of egg albumin (protein) accordingto the method of Mizushima and Kobayashi (1968) with some modifications. 0.2 ml of 1% egg albumin solution, 2 ml of sample extract (concentrations ranging from 50 to 500µg/ml) or standard and 2.8 ml of phosphate buffered saline (pH7.4) were mixed together to form a reaction mixture of total volume5 ml. The control was made by mixing 2 ml of triple distilled water,0.2 ml 1% egg albumin solution and 2.8 ml of phosphate buffered saline to make a total volume of 5 ml. The reaction mixtures were then incubated at 37±2°C for 30 min and heated in a water bath at 70±2°C for 15 min. After cooling, the absorbance was measured at 280 nm by UV/Vis spectrophotometer (Genesys10S, ThermoFisherScientific

Inc., USA) using triple distilled water as the blank. The percentage inhibition was calculated using the relationship:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test sample}}{\text{Absorbance of Control}} \times 100$$

5.12.2 Nitric Oxide Radical Scavenging Assay

This assay was done according to the method of Panda *et al*. (2009). The extracts were prepared and these were then serially diluted with distilled water to make concentrations ranging from 50, 100, 250 and 500µg/ml. The freshly prepared solutions were refrigerated at 4°Cfor later use. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid immediately before use. 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 ml of the sample or standard in ethanol and incubated at 25°C for 180 min. The extract was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extracts or standard but withan equal volume of buffer were prepared in a similar manner as done in the test samples. The absorbance was measured at 546nm using an Ultraviolet–visible (UV/Vis) spectrophotometer (Genesys10S, ThermoFisher Scientific Inc., USA) by using triple distilled water as blank. The percentage inhibition of the extract and standard was calculated and recorded. The percentage nitrite radical

scavenging activity of the sample extracts or standard were calculated using the formula:

$$\% \text{ NO Scavenged} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test Sample}}{\text{Absorbance of Control}} \times 100$$

6. RESULTS

6.1 Fourier Transform Infra Red Spectroscopic Analysis

The IR spectra provided information about the local molecular environment of the organic molecules on the marine molluscan shell. In the present work, FTIR spectral measurements were carried out to identify the potential biomolecules in *L.truncata* extract which is responsible for various biological activities.

The results of FTIR analysis of this study show different stretches of bonds shown at different peaks;3150.50,2872.77,2521.75,1786.92,1683.74,1399.26,1327.90,1193.85,1139.85,1081.99,752.19,712.65,656.72,600.78 cm^{-1}. The wave numbers 3150.50, 2872.77,2521.75, 1786.92 cm^{-1} distinctive of asymmetrical stretching of CH_2 and 1683.74, 1399.26, 1327.90, 1193.85, 1139.85, 1081.99 and 752.19, 712.65, 656.72, 600.78 cm^{-1} positions of the spectrums are the characteristic C=O stretching, C-OH, C-H, C-O and skeletal stretch respectively (Figure 2).

6.4 GC-MS analysis

The sample was subjected to GC-MS analysis. GC-MS analysis from the experimental animal *Lambistruncata* revealed8 compounds that could be

identified as9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester, 1-(3,4-Methylenedioxyphenyl)-2-propanone oxime, methyl ether, 2-Methyl-7-phenylindole, 2-Ethylacridine, 1,4-Bis(trimethylsilyl)benzene, 5,8-Methano-4H-3,1-benzoxazine-2-thione,1,2,4a-rel,5-trans,6,7,8-cis,8a-cis-octahydro, Dodecanoic acid, 1,2,3-propanetriyl ester and Lauric anhydride. The bioactive compounds with their retention time (RT), molecular formula, molecular weight (MW) and concentration (area) are presented in Table 1. The mass spectrum and structures of the compounds identified were present. Chemical component structures present in the crude methanolic extract of *L.truncata* were depicted in figures (3-10) respectively, which could be responsible for various biological activities antibacterial, antifungal, antioxidant, antidiabetic, anti-inflammatory and anticoagulant activities.

6.2 Antibacterial Activity

The antibacterial efficacy of *L. truncata* was investigated against some selected bacterial species *Aeromonas hydrophila* by agar well diffusion method. The shell extract of *L.truncata* developed maximum inhibition zone against all pathogens tested. It has been reported that antibacterial activity was dose dependent.

In *L.truncata* the antibacterial activity showed maximum zone of inhibition against *Aeromonas hydrophila* at the level of 9.5 mm at 500 µg/ml

concentration followed by 5.5 mm at 250 µg/ml, 5.25 mm at 100 µg/ml and 4.25 mm at 50 µg/ml concentration respectively (Figure 11).

6.3 Antifungal Activity

The antifungal efficacy of the shell extract of *L.truncata* was investigated against some selected fungal species *Aspergillus niger* and *Sporothrix schenckii* by agar well diffusion method. The shell extract of *L.truncata* developed maximum inhibition zone against all pathogens tested. It has been reported that antifungal effect was dose dependent.

In *L.truncata* antifungal activity showed maximum zone of inhibition against *Aspergillus niger* at the level of 15.5 mm at 500 µg/ml concentration followed by 5.5 mm at 250 µg/ml concentration. *Sporothrix schenckii* at the level of 6.5 mm at 500 µg/ml concentration respectively (Figure 12).

6.2 Antioxidant Activity

6.2.1 Hydrogen Peroxide Radical Scavenging Activity

The hydrogen peroxide radical scavenging activity of marine molluscan shell extract of *L.truncata* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 87.63 % was observed at 500 µg/ml followed by 75.93% at 250 µg/ml, 66.32% at 100 µg/ml and 35.43% at 50 µg/ml

respectively. The percentage inhibition of 91.73% was found for the standard ascorbic acid. The IC_{50} value of 51.3µg/ml was noted which shows the good antioxidant activity. It has been found that antioxidant activity was dose dependent and the percentage inhibition was found to increase with increase in the concentration respectively (Figure 13).

6.2.2 Phosphomolybdenum Scavenging Assay

The phosphomolybdenum scavenging assay of marine molluscan shell extract of *L.truncata* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 92.73% was observed at 500 µg/ml followed by 75.47% at 250 µg/ml, 63.21% at 100 µg/ml and 40.90% at 50 µg/ml respectively. The percentage inhibition of 89.95% was found for the standard ascorbic acid. The IC_{50} value of 42.1µg/ml was noted which shows the good antioxidant activity. It has been found that antioxidant activity was dose dependent and the percentage inhibition was found to increase with increase in the concentration respectively (Figure 14).

6.3 Antidiabetic Activity

The α-amylase activity of marine molluscan shell extract of *L.truncata* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 72.64% was

observed at 500 µg/ml followed by 54.21% at 250 µg/ml, 43.64% at 100 µg/ml and 28.37% at 50 µg/ml respectively. The IC_{50} value of 52.1 µg/ml was noted which shows the good antidiabetic activity. It has been found that antidiabetic activity was dose dependent and the percentage inhibition was found to increase with increase in the concentration respectively (Figure 15).

6.4 Anti-inflammatory Activity

6.4.1 Egg Albumin Denaturation Activity

The egg albumin denaturation activity of marine molluscan shell extract of *L.truncata* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 91.83% was observed at 500 µg/ml followed by 79.82% at 250 µg/ml, 65.21% at 100 µg/ml and 53.13% at 50 µg/ml respectively. The IC_{50} value of 28.5 µg/ml was noted which shows the good anti-inflammatory activity. It has been found that anti-inflammatory activity was dose dependent and the percentage inhibition was found to increase with increase in the concentration respectively (Figure 16).

6.4.2Nitric Oxide Scavenging Assay

The nitric oxide scavenging assayof marine molluscan shell extract of *L.truncata* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 65.82% was observed at 500 µg/ml followed by 54.73% at 250

µg/ml, 49.64% at 100 µg/ml and 38.67% at 50 µg/ml respectively. The IC_{50} value of 22.4µg/ml was noted which shows the good anti-inflammatory activity. It has been found that anti-inflammatory activity was dose dependent and the percentage inhibition was found to increase with increase in the concentration respectively (Figure 17).

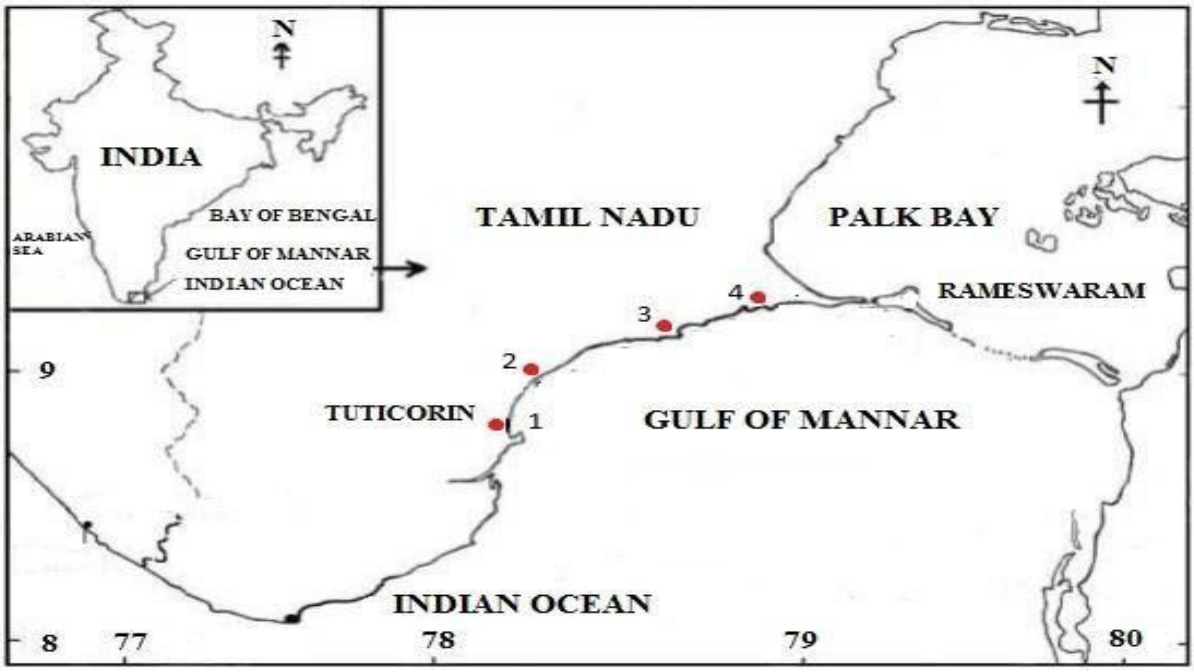


Figure 1: Map showing the study area Gulf of Mannar – Thoothukudi Coastal region

Plate 1: Dorsal and Ventral View of the shell *Lambis truncata*

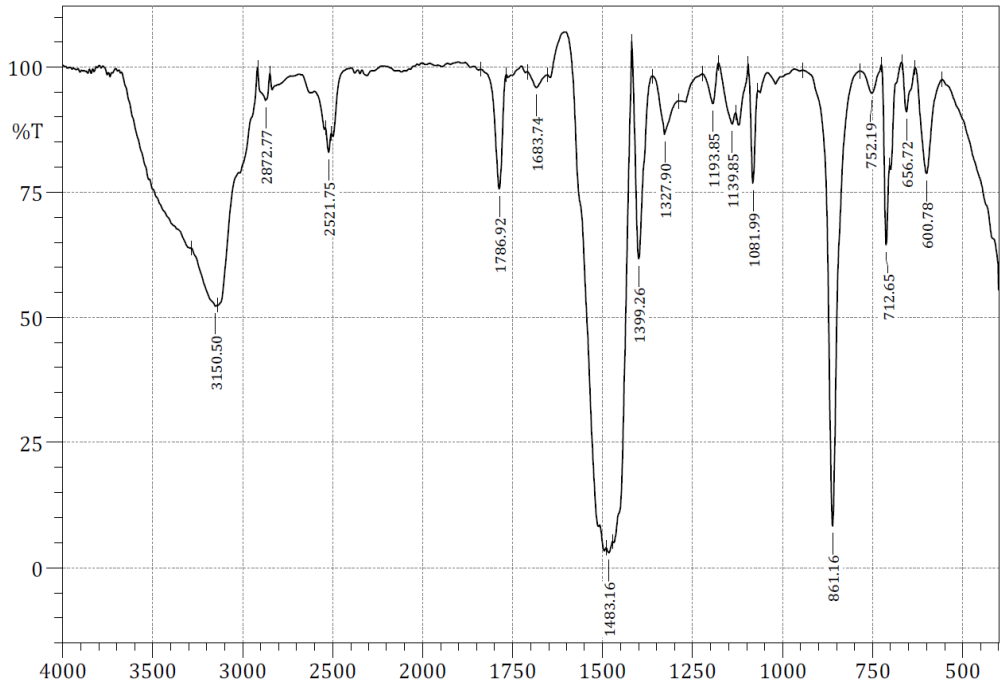


Figure 2: FT-IR Spectroscopy of Molluscan Shell Extract *Lambis truncata*

Table 1: Activity of components identified in the column fractions of *Lambis truncata* by GC-MS

SI. NO.	RT	NAME OF COMPOUND	MOLECULAR FORMULA	MW g/mol	AREA	COMPOUND NATURE	ACTIVITY
1.	13.382	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	$C_{18}H_{34}O_2$	282.5	820542	**Glyceryl monooleate**	Antioxidant
2.	14.994	1-(3,4-Methylenedioxyphenyl)-2-propanone oxime, methyl ether	$C_{19}H_{21}NOS$	311.4	628932	**Oxime methyl ethers**	Anti-inflammatory
3.	14.954	2-Methyl-7-phenylindole	$C_{15}H_{13}N$	207.27	230451	Alkanenitrile	Anticancer, Antioxidant
4.	15.057	2-Ethylacridine	$C_{15}H_{13}N$	207.27	400802	Carboxylic acid ester	Antibacterial, Antifungal
5.	15.091	1,4-Bis(trimethylsilyl) benzene	$C_{12}H_{22}Si_2$	222.47	632785	Carboxylic acid	Antioxidant
6.	17.234	5,8-Methano-4H-3,1-benzoxazine-2-thione, 1,2,4a-rel,5-trans,6,7,8-cis,8a-cis-octahydro183.0	C_8H_7NO	133.15	914297	Carboxylic acid	Antidiabetic
7.	17.320	Dodecanoic acid, 1,2,3-propanetriyl ester	$C_{15}H_{32}O_5$	292.41	197172	Carboxylic acid	Anticoagulant
8.	17.424	Lauric anhydride	$C_{24}H_{46}O_3$	382.6	802742	Aromatic ketone	Antimicrobial

Chromatogram

Figure 3

Name : 9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester

Formula : $C_{18}H_{34}O_2$

MW : 282.5g/mol

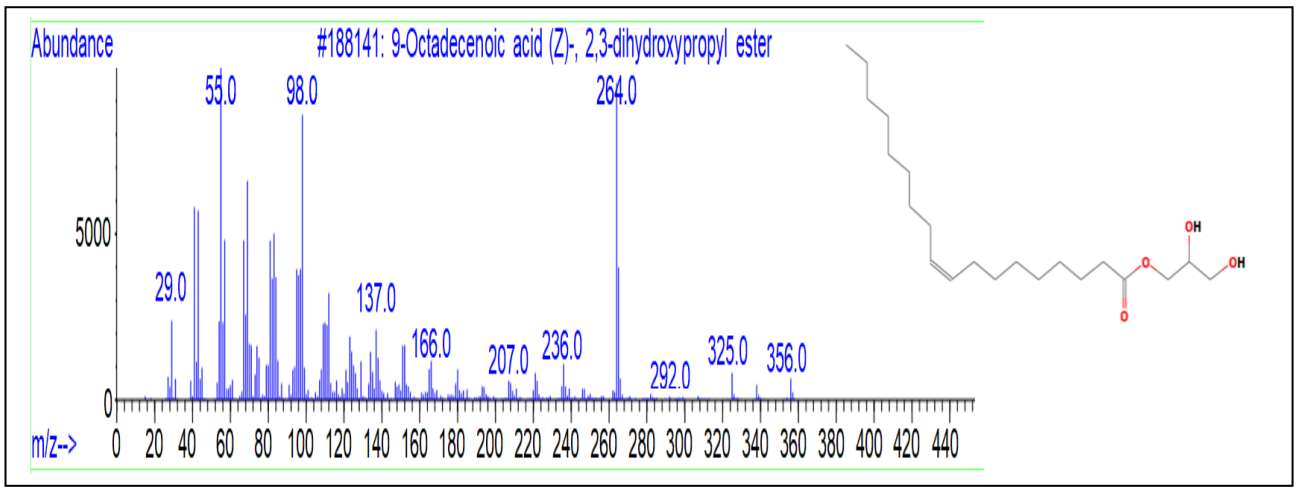


Figure 4

Name : Thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester

Formula : $C_{19}H_{21}NOS$

MW : 311.4g/mol

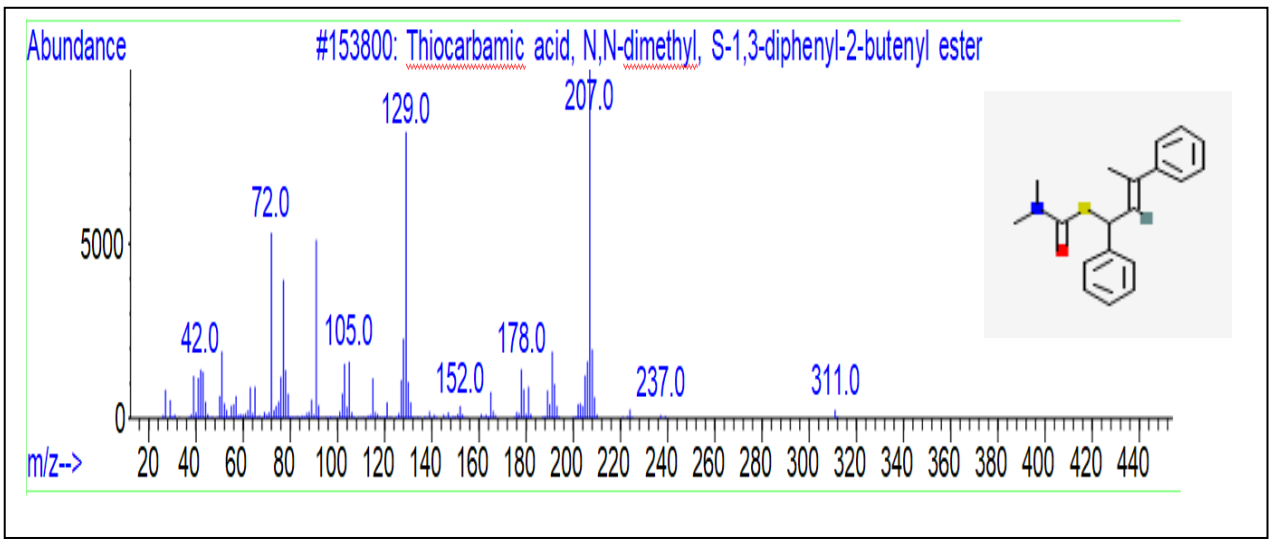


Figure 5

Name : 2-Methyl-7-phenylindole

Formula : $C_{15}H_{13}N$

MW : 207.27g/mol

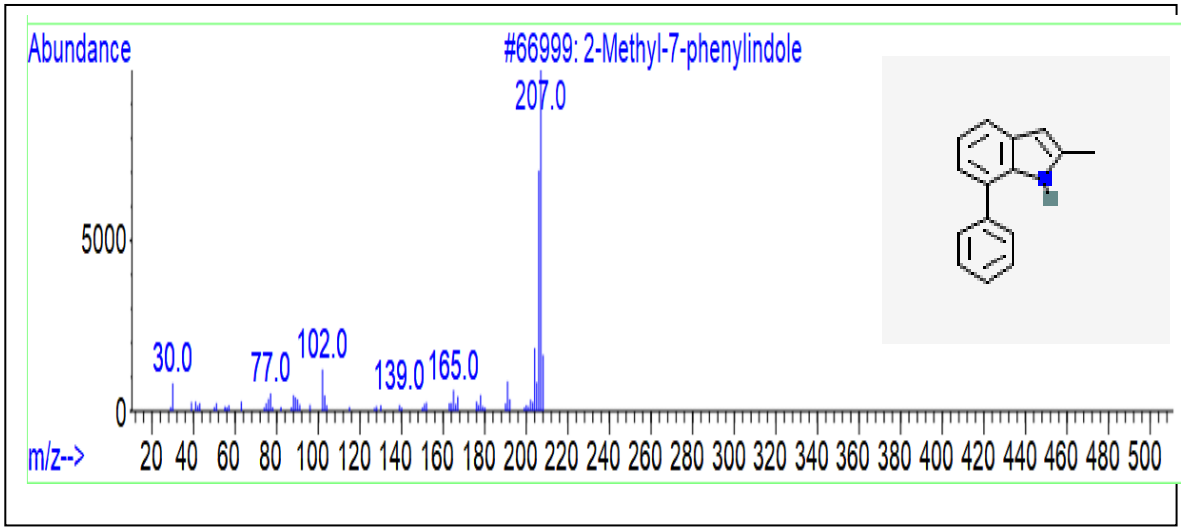


Figure 6

Name : 2-Ethylacridine

Formula : $C_{15}H_{13}N$

MW : 207.27 g/mol

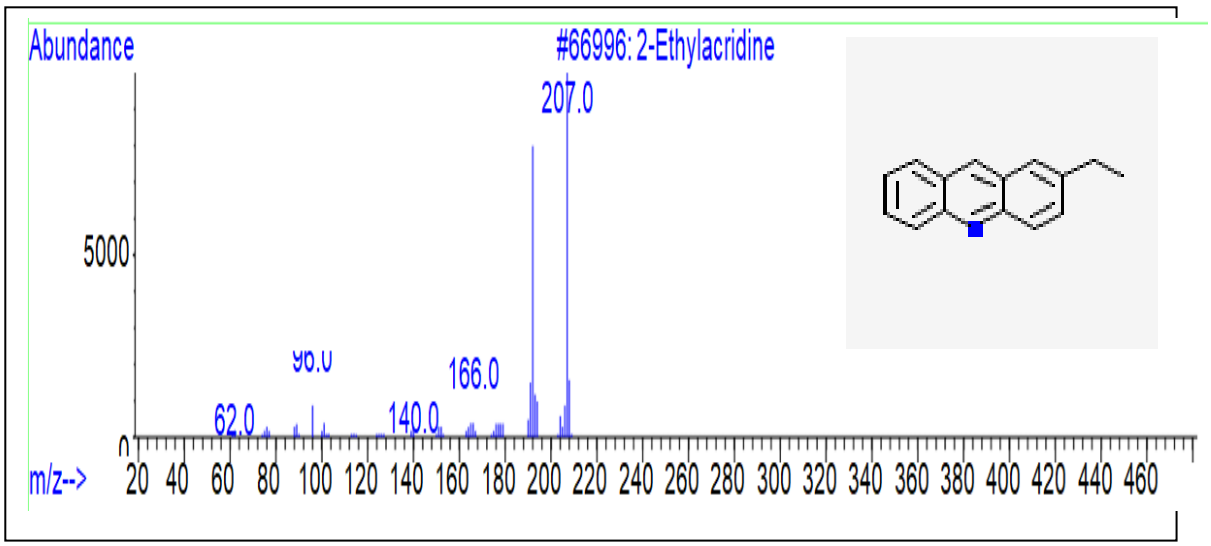


Figure 7

Name : 1,4-Bis(trimethylsilyl)benzene

Formula : $C_{12}H_{22}Si_2$

MW : 222.47 g/mol

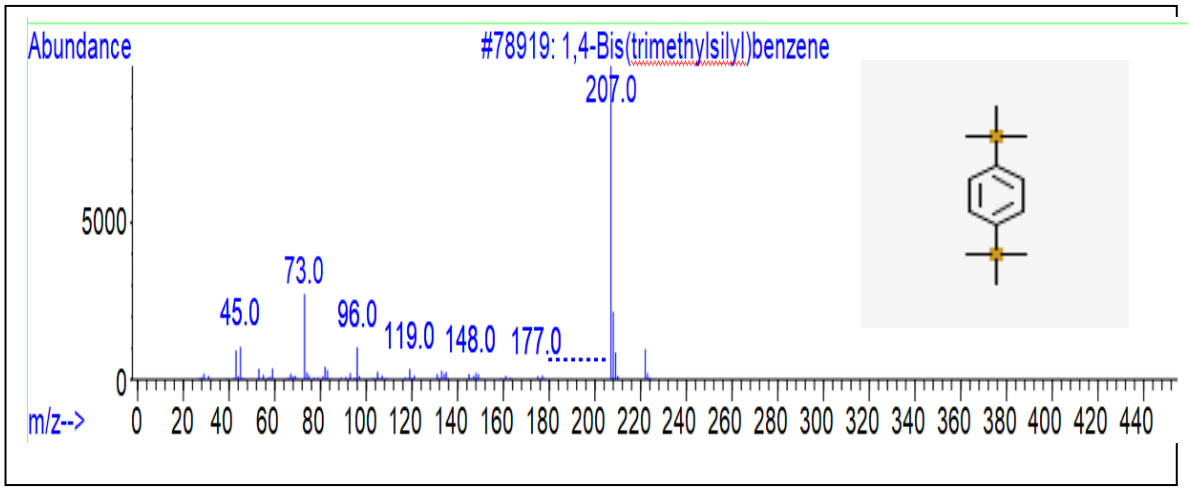


Figure 8

Name : 5,8-Methano-4H-3,1-benzoxazine-2-thione, 1,2,4a-rel,5-trans,6,7,8-cis,8a-cis-octahydro183.0

Formula : $\underline{C_8H_7NO}$

MW : 133.15 g/mol

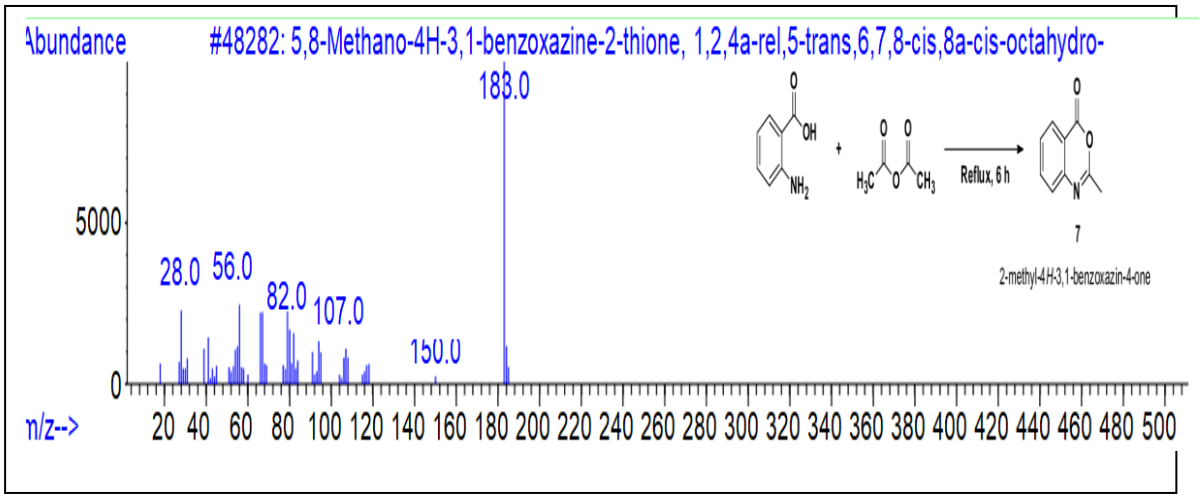


Figure 9

Name : Dodecanoic acid, 1,2,3-propanetriyl ester

Formula : $C_{15}H_{32}O_5$

MW : 292.41 g/mol

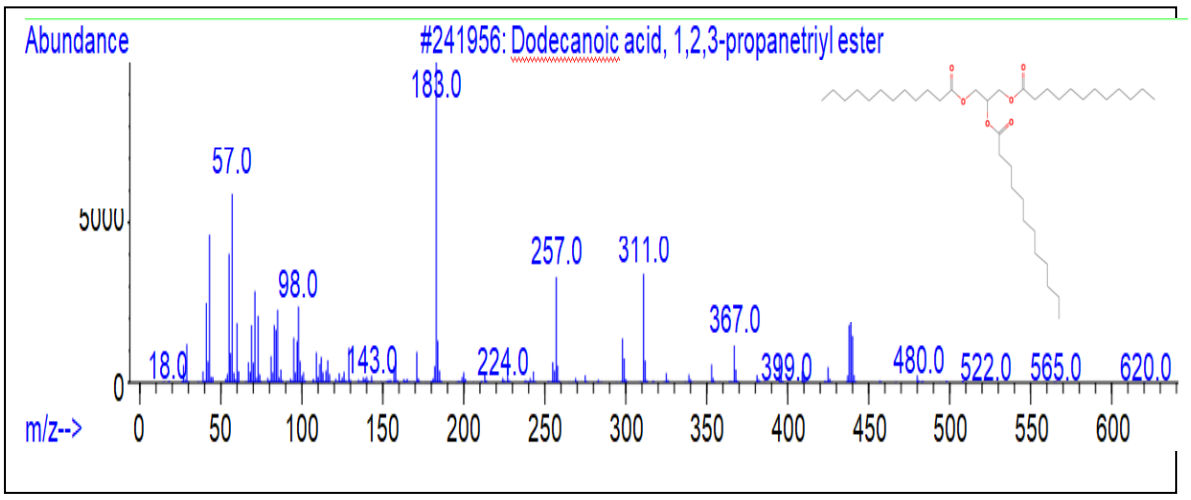
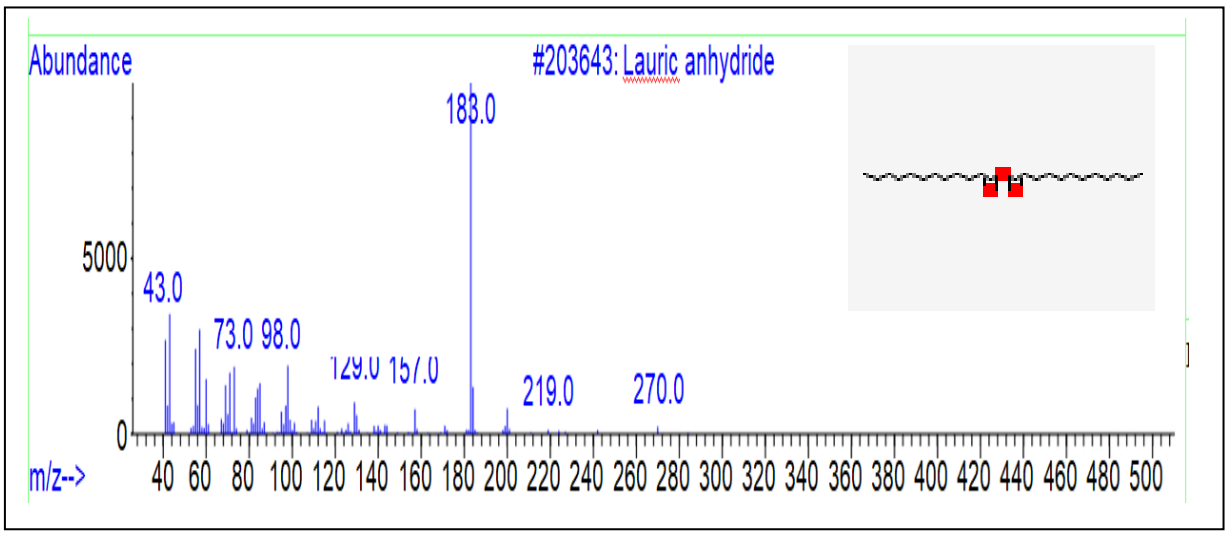


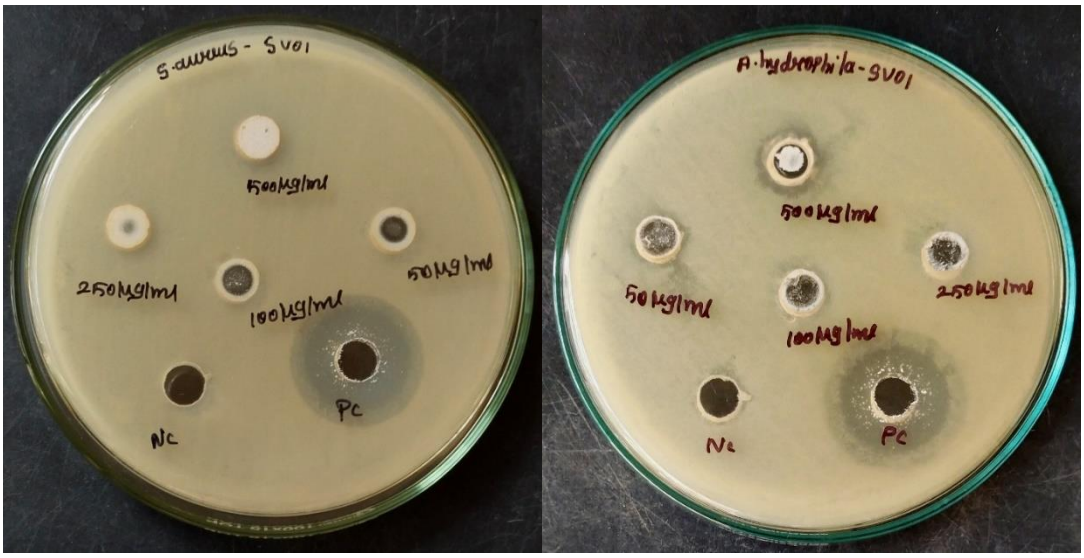
Figure 10

Name : Lauric anhydride

MW : 382.6

Formula : $C_{24}H_{46}O_3$





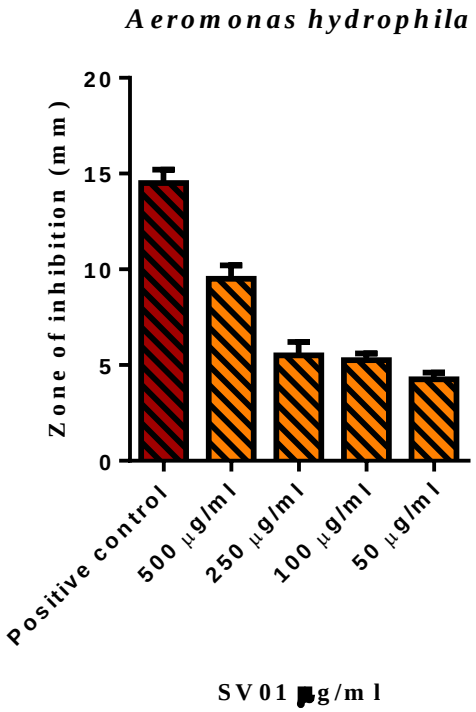


Figure 11: Antibacterial Activity of Molluscan Shell *Lambis truncata* against *Staphylococcus aureus* and *Aeromonas hydrophila*

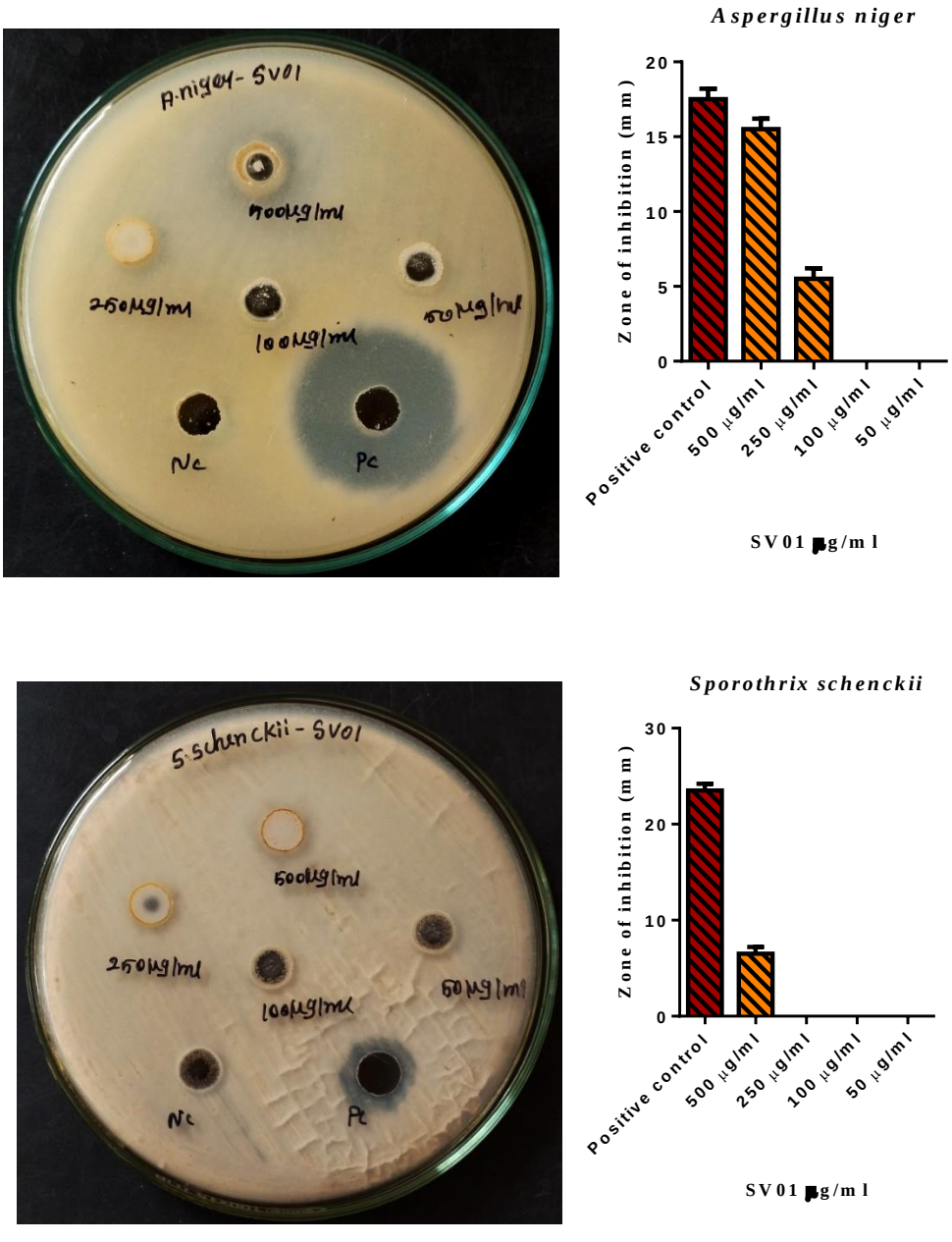


Figure 12: Antifungal Activity of Molluscan Shell *Lambis truncata* against *Aspergillus niger* and *Sporothrix schenckii*

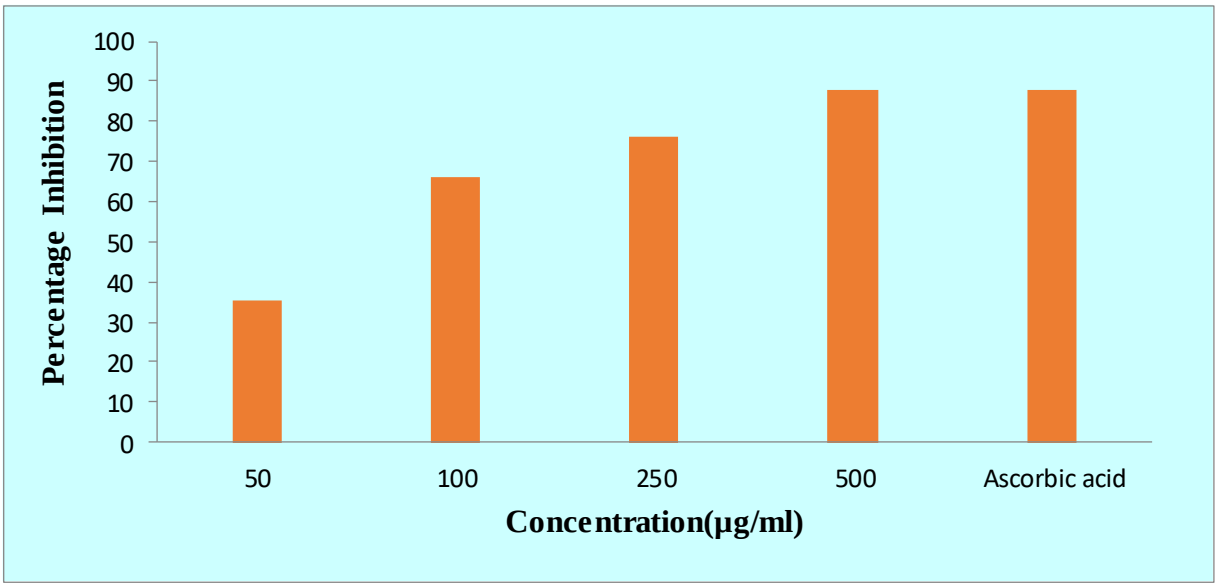


Figure 13: Hydrogen Peroxide Radical Scavenging Activity of Molluscan Shell *Lambis truncata*

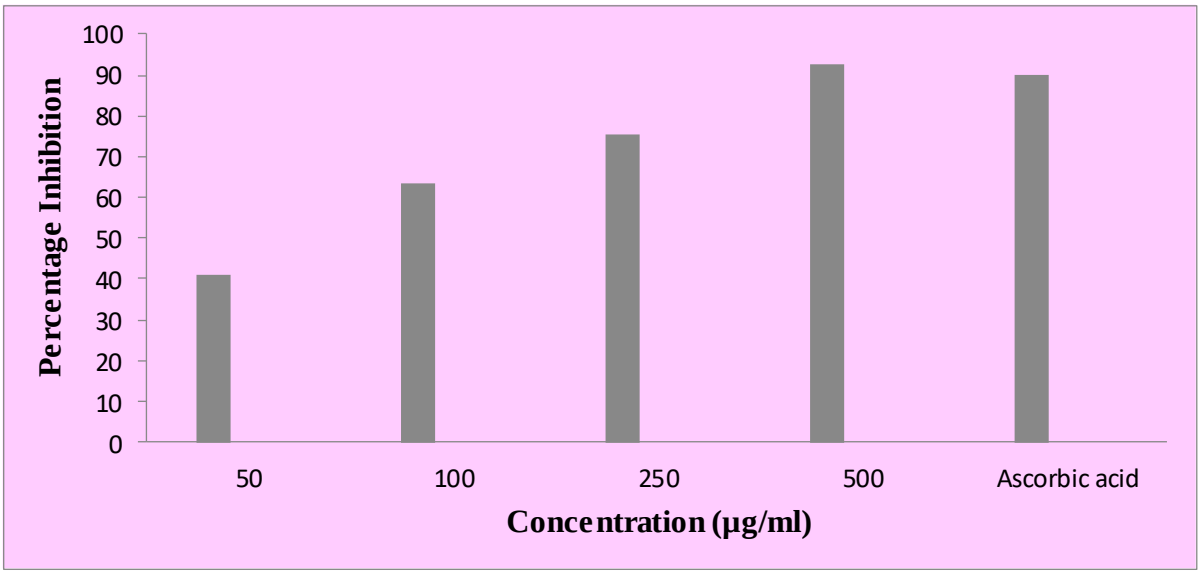


Figure 14: Phosphomolybdenum Scavenging Assay of Molluscan Shell *Lambis truncata*

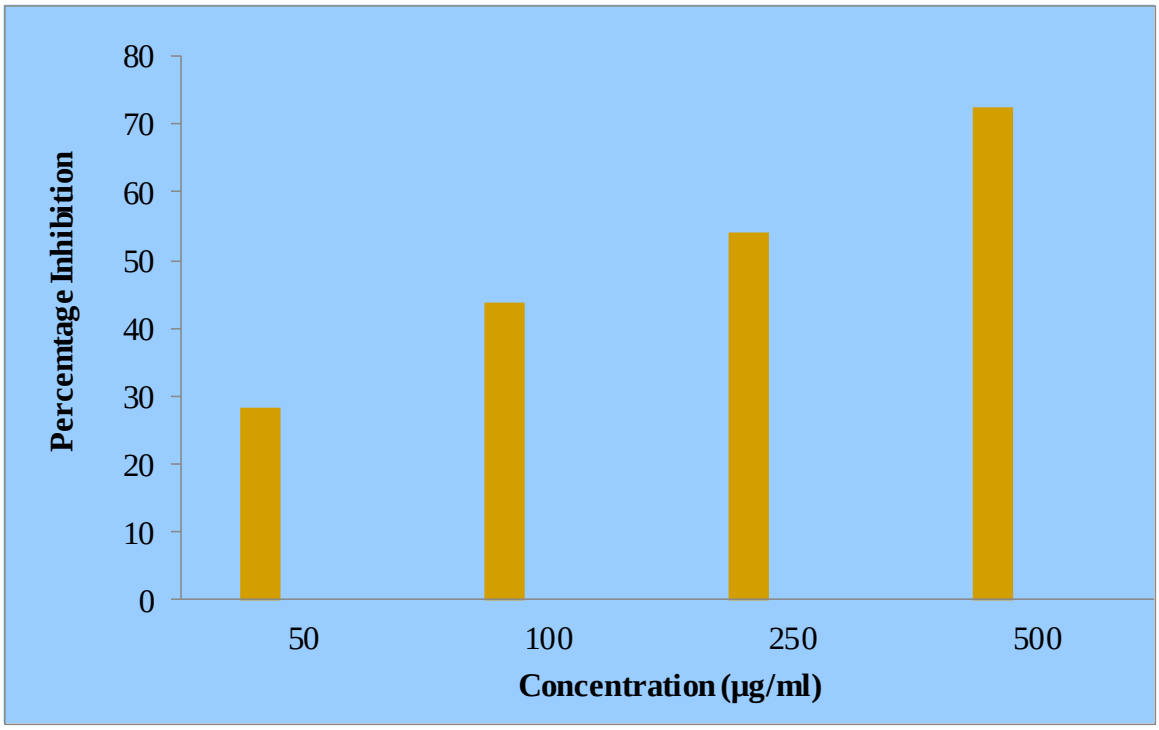


Figure 15: Antidiabetic Activity of Molluscan Shell *Lambis truncata*

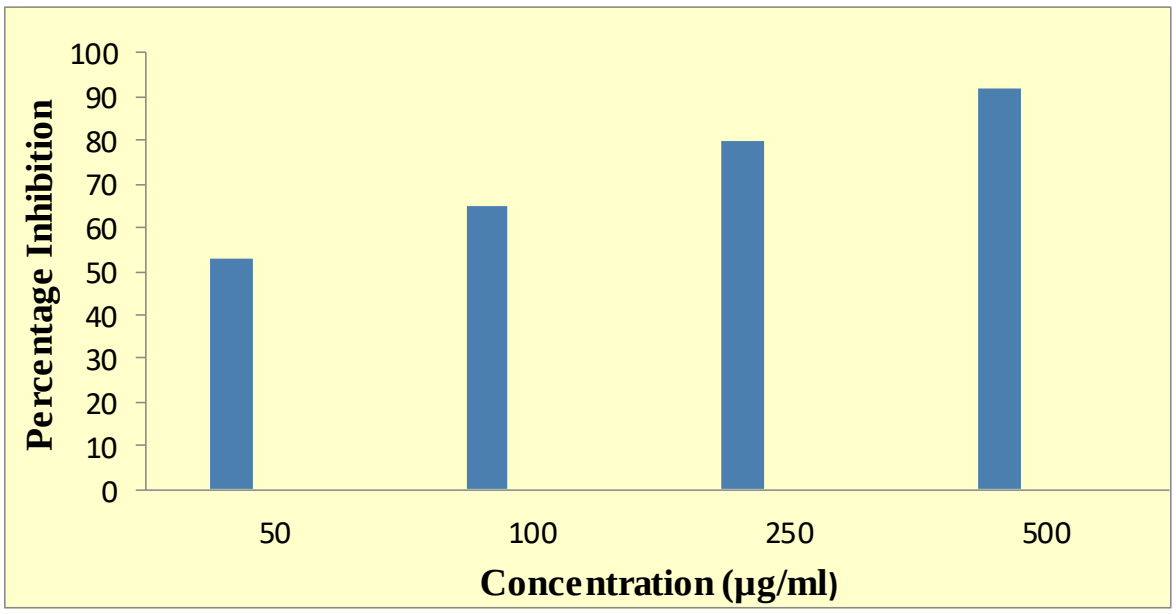


Figure 16: Egg Albumin Denaturation Assay of Molluscan Shell

Lambis truncata

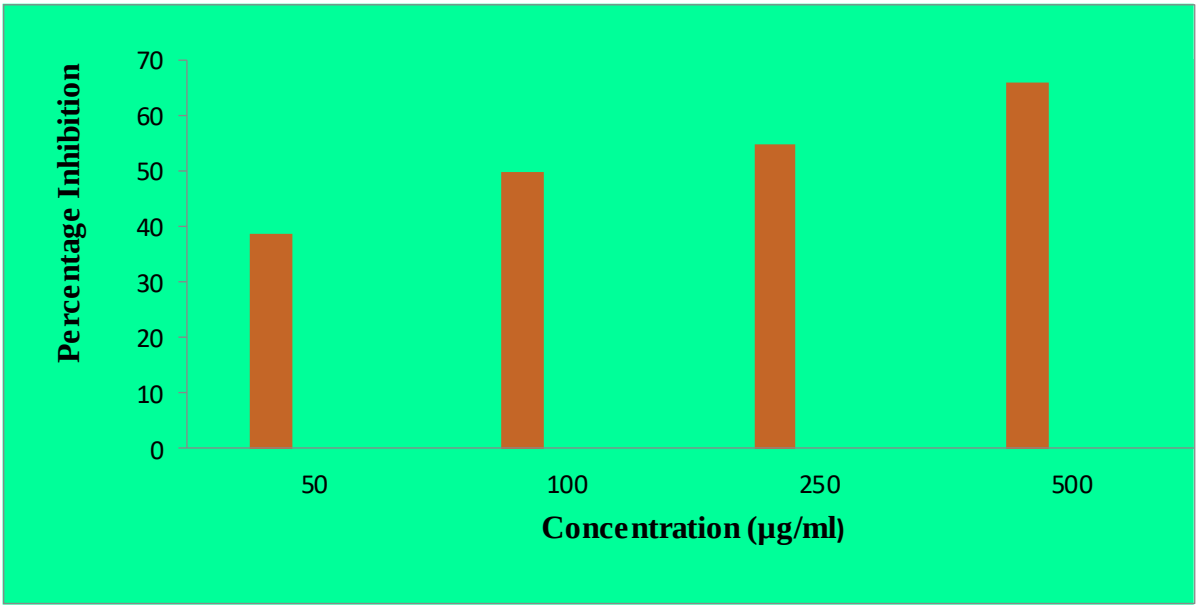


Figure 17: Nitric Oxide Scavenging Assay of Molluscan Shell

Lambis truncata

7. DISCUSSION

Bio-resources have been defined as living entities, which include genetic resources, organisms, and populations of living resources, with actual or potential uses to mankind. Specifically, Coastal ecosystem supports wild bioresources such as Estuaries and lagoons, Pelagic and Benthic ecosystems and marine fisheries.Ocean is known as large biodiversity of fauna and flora (Kamboj, 1999). Therefore, the marine environment is an exceptional store house of different shellfish oranisms such as oyster, prawn, scallops, squids and octopus (Chandran, 2009). So, there is good opportunity for the discovery of new bioactive substances from marine and estuarine ecosystems.

Thousands of bioactive compounds identified in marine organisms reveal that sea creatures constitute a large reservoir for pharmacologically active drug recently reviewed (Mayer *et al.,* 2011). IR spectroscopy is an analytical tool able to detect the characteristics vibrational modes of individual chemical groups and bonds. It has been used to examine and provide important data on a wide variety of biological molecules. The presence of strong bonds above 3000 cm^{-1} indicates the presence of aromatic ring (Sankaravadivu *et al.,* 2000). FTIR analysis reveals the presence of bioactive compounds signals at different ranges.

The results of FTIR analysis of this study show different stretches of bonds shown at different peaks; 3150.50, 2872.77, 2521.75, 1786.92, 1683.74, 1399.26, 1327.90, 1193.85, 1139.85, 1081.99, 752.19, 712.65, 656.72, 600.78 cm^{-1}. The wave numbers 3150.50, 2872.77, 2521.75, 1786.92cm^{-1} distinctive of asymmetrical stretching of $CH_2$and 1683.74, 1399.26, 1327.90, 1193.85, 1139.85, 1081.99and 752.19, 712.65, 656.72, 600.78 cm^{-1} positions of the spectrums are the characteristic C=O stretching, C-OH, C-H, C-O and skeletal stretch respectively (Figure 2).

In contrast to be present study Meenakshi *et al.* (2012) reported the infrared spectrum of the ethanolic extract of *Ectein ascidiavemi* and *Ascidia sydneiensis*. Packiam *et al.* (2013) showed the presence of carbonyl, carboxylic and aromatic ring in ethanolic extract of *Phallusia arabica*. Meenakshi *et al.* (2013) have discussed about the presence of alcohols, phenols, carboxylic acid, aromatic ring, ethers and aliphatic amines in the methanolic extract of *Phallusia nigra* and *Microcosmus exasperates*.

Gayathri *et al.* (2017) analyzed FT-IR spectrum of polysaccharides showing antioxidant activity in *Pila virens*. The present study corroborates well with the above findings. Divya Dharan (2018) showed the presence of aliphatic bromo compounds, phenol, tertiary alcohols, carbonyl compounds and carboxylic acids in ascidian *Aplidium multiplicatum.*

The presence of various bioactive compounds justifies the use of whole animal for various ailments by traditional practitioners. Chemical drugs may lead to adverse effects and recent researchers have focused on pharmacologically active compounds from the natural sources. GC-MS is used to identify the constituents of volatile matter, long chain and branched chain hydrocarbons, alcohols, acids and esters.

In the present study, GC-MS analysis from the experimental animal *Lambis truncata* revealed 8 compounds that could be identified as 9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester, 1-(3,4-Methylenedioxyphenyl)-2-propanone oxime, methyl ether, 2-Methyl-7-phenylindole, 2-Ethylacridine, 1,4-Bis(trimethylsilyl)benzene, 5,8-Methano-4H-3,1-benzoxazine-2-thione, 1,2,4a-rel,5-trans,6,7,8-cis,8a-cis-octahydro, Dodecanoic acid, 1,2,3-propanetriyl ester and Lauric anhydride (Figures 3-10).

Nuzhat Afsar *et al.* (2012) reported the GC-MS analysis of fatty acids of prosobranch gastropod species *Thais carinifera* from Pakistan Coast. Divya Dharan (2018) identified the bioactive compounds from the solvent extracts of *Aplidium multiplicatum* using GC-MS analysis. GC-MS chromatogram of the methanolic extract of *Aplidium multiplicatum* showed 21 peaks which indicates the presence of 21 chemical compounds with various biological activities like anti-microbial, anti-inflammatory, pesticide,

chemoprevention, diuretic and antioxidant. Similar findings was reported by Jemma Hermalin Jesy Diaz *et al.* (2015) in Cephalopods, Subavathy*et al.* (2015) in *Cypraea arabica* and Gayathri *et al.* (2017) in freshwater snail *Pila virens*.

Since antimicrobial resistance is a global public - animal health concern, there is a growing interest in marine ecosystem to find new antimicrobial agents which will be essential drugs for human and animal health and welfare. In the present study, a pronounced antimicrobial activity of the marine molluscan shell extract of *L.truncata*has been observed against some bacterial and fungal strains.

Harekrishna Jana *et al*. (2017) studied maximum antimicrobial activity was observed against *B. subtilis* and *C. albicans* compare to other microorganism. The MIC value of lyophilized body fluid of *S. cucullata* exhibit inhibitory activity mostly against bacterial strain *B. subtilis* and fungal strain *C. albicans* but the value is lower (4.0mg/ml) than standard antibiotics. Grasian Immanuel (2012) prepared solventextracts of the shell powder of *C. moneta* solvent extractand investigated the antibacterial effect against threeopportunistic human pathogens such as *P. vulgaris*, *Micrococcus* sp. and *S. abory* and found that the growth of all the three pathogens was inhibited.

In the present study, In *L.truncata* the antibacterial activity showed maximum zone of inhibition against *Aeromonas hydrophila* at the level of 9.5

mm at 500 µg/ml concentration followed by 5.5 mm at 250 µg/ml, 5.25 mm at 100 µg/ml and 4.25 mm at 50 µg/ml concentration respectively (Figure 11). In *L.truncata* antifungal activity showed maximum zone of inhibition against *Aspergillus niger* at the level of 15.5 mm at 500 µg/ml concentration followed by 5.5 mm at 250 µg/ml concentration. *Sporothrix schenckii* at the level of 6.5 mm at 500 µg/ml concentration respectively (Figure 12). The present study agrees well with the above findings.

Antioxidants are described as a 'substance that when present in low concentrations relative to the oxidisable substrate significantly delayed or reduced oxidation of the substrate' (Halliwell *et al*., 2000). They protect the body by reacting with the ROS to halt the process of oxidation. Therefore, there is a constant need to replenish antioxidant resources endogenously or through supplementation. Many natural and synthetic compounds have been investigated over the decades for their efficacy to protect against oxidative stress (Heo and Jeon, 2009). Antioxidants from natural sources are preferred by consumers about the toxic and carcinogenic effects of synthetic antioxidants.

Numerous antioxidant method and modifications have been proposed to evaluate antioxidant activity and function of antioxidants. Of these, total antioxidant activity, reducing power, DPPH assay, metal chelating, active oxygen species such as H_2O_2, O_2^- and OH quenching assays are most

commonly used for the evaluation of antioxidant activities of extracts (Chang *et al.,* 2002). In the present study antioxidant activity of the shell extract of *L.truncata* was assessed using hydrogen peroxide scavenging activityand phosphomolybdenum assay.

In the current study, hydrogen peroxide scavenging activity showed the highest percentage inhibition of 87.63 % was observed at 500 µg/ml followed by 75.93% at 250 µg/ml, 66.32% at 100 µg/ml and 35.43% at 50 µg/ml respectively.The IC_{50} value of 51.3 µg/ml was noted which shows the good antioxidant activity (Figure 13). In phosphomolybdenum assay, the highest percentage inhibition of 92.73% was observed at 500 µg/ml followed by 75.47% at 250 µg/ml, 63.21% at 100 µg/ml and 40.90% at 50 µg/ml respectively (Figure 14).

Diabetes mellitus is a major global health challenge of current century afflicting over 366 million people world-wide, and by the year 2030, this endocrine disorder is predicted to affect over 552million people, particularly from the middle/low wage countries (Whiting *et al*., 2011). Type 1 diabetes was found to bedue to the autoimmune destruction of insulin-producing islet β-cells of pancreas, while type 2 diabetes (T2D) involves metabolic disorder including insulin resistance and impaired control ofhepatic glucose production (Ozougwu *et al*., 2013). Ravi *et al*. (2012) observed that the methanol extract of gastropod mollusc *Hemifusus pugilinus* exhibited greater

anti-α-glucosidase activity (IC 50 20.27 mg/mL) than the methanol extract of *Natica didyma* (IC50 56.44 mg/mL), although the anti-diabetic properties ofthis group of molluscs were significantly lesser than the EtOAc-MeOH extracts of the cephalopods considered in the present study (IC 90 1.69–5.37 mg/mL; $p < 0.05$).The methanol extract of themarine gastropod mollusc *Cerithidea obtusa* extract was found to posses moderate anti-α-glucosidase inhibitory activity (IC50 36.40 mg/mL) (Cahyani *et al*., 2015).

In the present study, antidiabetic activity showed the highest percentage inhibition of 72.64% was observed at 500 µg/ml followed by 54.21% at 250 µg/ml, 43.64% at 100 µg/ml and 28.37% at 50 µg/ml respectively. The IC_{50} value of 52.1 µg/ml was noted which shows the good antidiabetic activity (Figure 15). The present study corroborates well with the above findings.

Molluscs have been used in ethnomedicine by many traditional cultures to treat different aspects of inflammatory conditions. Notably, the marine molluscs are considered as one of the significant sources to derive bioactivemetabolites exhibiting antitumor, anti-inflammatory,and antioxidant activities. Inflammatory conditions, burns and wounds have been an ongoing concern for human health since the early era of civilisation. Many texts from ancient medicine have recorded the symptoms, signs and treatments for these conditions. Natural treatments are well-documented in traditional European

medicine, Traditional Chinese Medicine (TCM), Siddha and ancient Mediterranean and African traditional medicine and include a surprisingly large number of molluscan species.

Chellaram and Edward (2009a and 2009b) reported the anti-inflammatory activities of the reef-associated molluscs, *Trochus tentorium* and *Drupa margariticola*. In the present study,egg albumin denaturation activity showedthe highest percentage inhibition of 91.83% was observed at 500 µg/ml followed by 79.82% at 250 µg/ml, 65.21% at 100 µg/ml and 53.13% at 50 µg/ml respectively. The IC_{50} value of 28.5 µg/ml was noted which shows the good anti-inflammatory activity (Figure 16).

In nitric oxide scavenging assay, the highest percentage inhibition of 65.82% was observed at 500 µg/ml followed by 54.73% at 250 µg/ml, 49.64% at 100 µg/ml and 38.67% at 50 µg/ml respectively. The IC_{50} value of 22.4 µg/ml was noted which shows the good anti-inflammatory activity (Figure 17). The present study agrees well with the above findings.

Natural products provide important leads for the development of pharmaceuticals, including antimicrobial, antioxidant, antidiabetic and anti-inflammatory agents. Only a small proportion of the molluscan traditional medicines have been tested to confirm their biological activities and most screening studies have tested crude extracts from molluscs without any

chemical characterisation.This highlights the need for further research to strategically identify the bioactive compounds in molluscan medicines to provide leads for novel drugs in the future.

8. SUMMARY

- The results of FTIR analysis of this study show different stretches of bonds shown at different peaks; 3150.50, 2872.77, 2521.75, 1786.92, 1683.74, 1399.26, 1327.90, 1193.85, 1139.85, 1081.99, 752.19, 712.65, 656.72, 600.78 cm^{-1}.
- GC-MS analysis from the experimental animal *Lambis truncata* revealed 8 compounds that could be identified as 9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester, 1-(3,4-Methylenedioxyphenyl)-2-propanone oxime, methyl ether, 2-Methyl-7-phenylindole, 2-Ethylacridine, 1,4-Bis(trimethylsilyl)benzene, 5,8-Methano-4H-3,1-benzoxazine-2-thione,1,2,4a-rel,5-trans,6,7,8-cis,8a-cis-octahydro, Dodecanoic acid, 1,2,3-propanetriyl ester and Lauric anhydride.
- In *L. truncata* the antibacterial activity showed maximum zone of inhibition against *Aeromonas hydrophila* at the level of 9.5 mm at 500 µg/ml concentration followed by 5.5 mm at 250 µg/ml, 5.25 mm at 100 µg/ml and 4.25 mm at 50 µg/ml concentration respectively.
- In *L. truncata* antifungal activity showed maximum zone of inhibition against *Aspergillus niger* at the level of 15.5 mm at 500 µg/ml concentration followed by 5.5 mm at 250 µg/ml concentration. *Sporothrix schenckii* at the level of 6.5 mm at 500 µg/ml concentration respectively.

- The hydrogen peroxide radical scavenging activity of marine molluscan shell extract of *L. truncata* was observed. The highest percentage inhibition of 87.63 % was observed at 500 µg/ml followed by 75.93% at 250 µg/ml, 66.32% at 100 µg/ml and 35.43% at 50 µg/ml respectively.
- The phosphomolybdenum scavenging assay of marine molluscan shell extract of *L. truncata* was observed. The highest percentage inhibition of 92.73% was observed at 500 µg/ml followed by 75.47% at 250 µg/ml, 63.21% at 100 µg/ml and 40.90% at 50 µg/ml respectively.
- The α-amylase activity of marine molluscan shell extract of *L. truncata* was observed. The highest percentage inhibition of 72.64% was observed at 500 µg/ml followed by 54.21% at 250 µg/ml, 43.64% at 100 µg/ml and 28.37% at 50 µg/ml respectively.
- The egg albumin denaturation activity of marine molluscan shell extract of *L. truncata* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 91.83% was observed at 500 µg/ml followed by 79.82% at 250 µg/ml, 65.21% at 100 µg/ml and 53.13% at 50 µg/ml respectively.
- The nitric oxide scavenging assay of marine molluscan shell extract of *L. truncata* was observed at various concentrations of 500 µg/ml, 250

µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 65.82% was observed at 500 µg/ml followed by 54.73% at 250 µg/ml, 49.64% at 100 µg/ml and 38.67% at 50 µg/ml respectively.

9. CONCLUSION AND SUGGESTIONS

Marine molluscs, which are well known as rich sources of diverse and biologically active natural products, have attracted significant attention from researchers due to their chemical and pharmocological properties.chemical studies unveiled a variety of unique scaffolds inspiring leading drugs currently in clinical trials. Thus, extending investigation on unexplored species is expected to reveal novel molecular architectures.

In the present study, the shell extract of marine gastropod *Lambis truncata*_was isolated and characterized for bioactive compounds. GC-MS analysis revealed the presence of 8 compounds responsible for various pharmacological activities viz., antibacterial, antifungal, antioxidant, antidiabetic and anti-inflammatory activities were carried out. The results showed promising effect and development of new drug leads from the marine gastropod *L. truncata*. The success of marine pharmaceuticals development will highly depend upon the intensive interdisciplinary collaboration between biologists, chemists, biotechnologists, phamacists and between universities, hospitals and companies.

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DISTRIBUTION AND ACCUMULATION OF HEAVY METALS IN MOLLUSCS FROM THE TUTICORIN COASTAL REGIONS

A project submitted to

ST. MARY'S COLLEGE (Autonomous), THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

In partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

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THOOTHUKUDI -628 001

April -2023

CERTIFICATE

This is to certify that the project entitled **"DISTRIBUTION AND ACCUMULATION OF HEAVY METALS IN MOLLUSCS FROM THE TUTICORIN COASTAL REGIONS "** is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a project work done during the year 2022-2023 by the following students.

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1. INTRODUCTION

The marine environment is the greatest source of biodiversity covering 71 percent of the Earth's surface and containing 90% of the biosphere. Furthermore, it is a great contributor to economic prosperity, social well-being and quality of life. Oceans offer a large biodiversity of fauna and flora which is estimated to be over 5,00,000 species are more than double of the land (Anand *et al.*,1997). The marine environment is a huge source for yet to be discovered natural products. Apart from the food derived from the marine environment, a wide variety of bioactive substances is being isolated and characterized several with great promise for treatment of human disease (Ramasamy Mariappan and Balasubramanian, 2012).The marine ecosystem plays numerous fundamental environmental functions: regulation of the climate; prevention of erosion; solar energy's accumulation and distribution; carbon dioxide absorption; and maintenance of biological control. (Abdulkadir and Mashood, 2021).

India has been endowed with a vast marine ecosystem and bio-diversity, which sustains a large number of species and the coastal populace is dependent on the resources from this marine eco-system. It has a coastline of about 8000 km adjoining the continental regions and the offshore islands and a very wide range of coastal ecosystems such as estuaries, lagoons, mangroves, backwaters, salt marshes, rocky costs, sandy stretches and coral reefs which are characterized by

unique biotic and abiotic properties and processes. Even though the Indian marine environment is unique for its mega diversity, to date only a fraction of biodiversity has been properly explored for novel bio-active compounds that can be developed as new drugs or agrochemicals (Santhi, 2012).

The Gulf of Mannar, the first Marine Biosphere Reserve situated along the Southeast coast of India, running down south from Rameswaram to Kanyakumari in Tamil Nadu, India is situated between Longitudes 78008 E to 79030 E and along Latitudes from 8035 N to 9025 N, is one of the richest marine ecosystems with nearly 3600 species of living flora and fauna. It has been recognized as a marine biosphere reserve mainly because of its possession of a rich variety of Flora and Fauna. It also has one of the important Marine National Parks of India. The chain of 21 islands within the biosphere and their surroundings with a rich diversity of corals and associated fishes of ornamental and food value gives the Park its importance.

Molluscs in the ocean are a common sight and are virtually untapped resource of novel compounds. There are more than thousands of bioactive compounds discovered in molluscs. They are peptide, depsipeptide, sterols, sesquiterpene, terpenes, polypropionate, macrolides, prostaglandins, fatty acid derivatives, miscellaneous compounds and alkaloids (Blunt *et al.,* 2006).

Pollution is a great and growing threat to human health. It is the largest environmental cause of disease in the world today, responsible for an estimated 9 million premature deaths per year. It causes enormous economic losses, undermines national trajectories of economic development, and impedes attainment of the sustainable development goals (Landrigan *et al*., 2018). Heavy metal toxicity and bioaccumulation are some of the emerging global concerns that affect various life forms, including plants, animals, and human beings. In most developed and developing countries, modern-day developmental activities (industrialization, urbanization, agricultural methods, etc.) have caused serious harm to the land and the environment (23, 31)

Heavy metals are considered the most important form of pollution of the aquatic environment because of their toxicity intrinsic persistence, non-biodegradable nature, and accumulative behaviors (Islam *et al*., 2018). These metals differ from other toxic materials in a way that they are neither created nor destroyed by human. The rapid industrialization, urbanization, population growth, agricultural and other human activities have resulted in severe pollution by heavy metals globally, especially in developing countries. Significant quantities of heavy metals from such activities are discharged into rivers, which can be strongly accumulated, and biomagnified along water, sediment, and aquatic food chain, resulting in sublethal effects or death in local fish population (Tao *et al*., 2004). Some heavy

metals are necessary for life and are called essential elements which are required for a variety of biochemical and physiological functions. However, they can be toxic when present in large amounts (Duffus *et al*., 2002, Wang *et al*., 2001, Tchounwou *et al*., 2008 and Gautam *et al*., 2007).

Cadmium is an inorganic toxicant that belongs to group IIB of the periodic table, and it is an odorless, bluish-white malleable transition metal. It is a non-essential and toxic element for humans mainly affecting kidneys and the skeleton. It is also a carcinogen by inhalation. The presence of cadmium in the environment, increased by activities such as years of coal and fossil-fuel usage and mining is a serious health hazard and its monitoring is essential. It is an ecotoxic heavy metal that adversely affects all biological functioning in plants, animals, and human beings (Suhani et al.,2021).

Lead (Pb) is a non-essential metal naturally present in the environment and often complexed with other elements. Marine ecosystems are sinks of terrestrial contaminations; consequently, lead is detected in oceans and seas. Furthermore, lead is not biodegradable. It remains in soil, atmosphere, and water inducing multiple negative impacts on marine invertebrates (key species in trophic chain) disturbing ecological ecosystems. The primary sources of lead production include mines and

ore smelting, while secondary sources are recycled materials such as batteries and lead pipes (Flora et al.,2006).

Zinc is an element commonly found in the Earth's crust. It is released to the environment from both natural and anthropogenic sources; however, releases from anthropogenic sources are greater than those from natural sources. The primary anthropogenic sources of zinc in the environment (air, water, soil) are related to mining and metallurgic operations involving zinc and use of commercial products containing zinc. Worldwide, releases to soil are probably the greatest source of zinc in the environment. The most important sources of anthropogenic zinc in soil come from discharges of smelter slags and wastes, mine tailings, coal and bottom fly ash, and the use of commercial products such as fertilizers and wood preservatives that contain zinc. Zinc does not volatilize from soil. Although zinc usually remains adsorbed to soil, leaching has been reported at waste disposal sites. Zinc does not volatilize from water but is deposited primarily in sediments through adsorption and precipitation. Severe zinc contamination tends to be confined to areas near emission sources. Large amounts of contaminated soil would need to be ingested in order to reach the registered dietary index value of 3.3–3.8 mg of zinc a day. It is therefore unlikely that the zinc found in the contaminated soil would pose a health risk if ingested (A potential for human exposure)

Copper as a micro-nutrient is essential for all living organisms at lower concentration for their optimal growth and development. But, copper in excess amount than required becomes toxic and the impact is more severe to the aquatic environment. The mechanisms of copper toxicity and storage are diverse as they vary with organisms and mode of uptake. Slightly elevated copper level in natural waters may cause sublethal effects in aquatic organisms such as histological or morphological changes in tissues, suppression of growth and development, poor swimming performance, change in bio-chemistry, change in behavior and change in reproduction (Mahmood Hosasin and Golam Rakkibu, 1999) .

The area under investigation, which is off Tuticorin in the Gulf of Mannar presents great interest because it is an industrial belt consisting of many major industries, involved in the production of chemicals, petrochemicals and plastics. In addition, a major harbour, thermal power plant, heavy water plant and human activities from around Tuticorin to Tiruchendur have altered the ecosystem (Jonathan et al., 2004). Aluminium fluoride, urea, Ammonium chloride and caustic soda manufacturing factories are located in the district of Tuticorin. Except for some of the major industries, the effluents coming out of the small scale industries are disposed off in the coastal area. Tuticorin Municipality generates sewage of 14mld capacity. This sewage is discharged into the sea without any treatment (Tamil Nadu, Fisheries Statistics, 2004).

2. REVIEW OF LITERATURE

The use of marine organism as bioindicators of metal pollution of aquatic environments and suitability for human use from toxicological point has been investigated by many researchers (Amin *et al*. 201I; Mitra *et al*., 2012; Viswanathan *et al*., 2013: Yilmaz *et al*, 2007 and Abdel Salam *et al*., 2011). Humood *et al*., (2013) assessed the heavy metal pollution in the marine environment of the Arabian Gulf and confirmed that heavy metal concentrations in marine organisms were generally within allowable concentrations and pose no threat to public health. Ganesan *et al*. (1995) measured higher concentration of heavy metals like Mn, Fe, Cu and Zn in the seaweeds off Tuticorin. Senthilnathan *et al*., (1998) showed seasonal variation with an increased metal load during monsoon period in the mussel and oysters from the southeast coast of India. Monawwar and Kazi (1998) investigated the concentration and distribution of heavy metals (lead, cadmium, copper, zinc) in Karachi shore and off shore sediments. The measured heavy metal pollution level in the sediment of coastal and offshore area indicated that high concentration of heavy metal were found around Manora channel and Eastern Coastal of Karachi.

Topping (1973) examined Heavy metals in shellfish from Scottish waters. Phillips (1976) studied the common mussel *Mytilus edulis* as an indicator of pollution by zinc, cadmium, lead and copper. Relationship of metals in the mussel

to those discharged by industry. Williamson (1980) studied the variables affecting body burdens of lead, zinc and cadmium in roadside populations of the snail *Cepaeahortensis*. Brugmann (1981) observed heavy metals in the Baltic Sea. White and Rainbow, 1982 studied Regulation and accumulation of copper, zinc, and cadmium by the shrimp palaemon clegans. Neuman (1982) investigated the influence of lead in components of a freshwater ecosystem on molluscan tissue lead concentrations. Stromgren (1982) observed the effect of heavy metals (Zn, Hg, Cu, Cd, Pb, Ni) on the length growth of *Mytilus edulis*. Ikuta, K. (1986) examined correlations between ratios of metals concentration in soft bodies of bivalves.

Palanichamy and Rajendran (2000) examined the heavy metal concentrations in sea water and sediments of Gulf of Mannar and Palk Bay, southeast coast of India. Ke et al., (2001) studied bioaccumulation of Cd, Se and Zn in an estuarine oyster (*Saccrostrea glomerata*). Bu-olayan and Thomas (2001) surveyed the heavy metal accumulation in the gastropod, *Cerithium scabridum* from the Kuwait coast. Cubadda et al.,(2001) analyzed the size dependent concentrations of trace metals in four Mediterranean gastropods. Canli and Atli (2003) examined the relationships between heavy metal (Cd,Cr, Cu, Fe, Pb, Zn) levels and the size of six Mediterranean fish species.

Tomazelli et al.,(2003) studied the biomonitoring of Pb and Cd in two impacted watersheds in southeast Brazil using the freshwater mussel, *Anodontites*

trapesialis as a biological monitor. Jose et al.,(2005) studied the heavy metal concentrations in molluscs from the Atlantic coast of southern Spain. Ezemonye, et.al (2006) observed Bioaccumulation of heavy metals (Cu, Zn, Fe) in freshwater snail from Ikpoba River of South Nigeria. Sidoumou, et.al (2006) examined Heavy metal concentration in molluscs from the Senegal coast. Shanmugam et al. (2007) surveyed bioaccumulation of some trace metals (Mg, Fe, Zn, Cu) from begger's bowl *Cymbium melo*.

Gopinathan and Sobhana Amma, R. (2009) analyzed bioaccumulation of toxic heavy metals in the edible soft tisuues of green mussel (*Perna viridis*) of Mahe Region. Kesavan et al. (2010) elaborated the heavy metal accumulation in three molluscs and Ssediments From Vellar Estuary, Southeast Coast Of India. Palanichamy and Rajendran (2000) indicated high concentration of Cd and Pb in the bottom waters than the surface waters off Tuticorin. 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. Kaladharan *et al*., (2005) determined heavy metal concentrations in sediment, fin fishes and shellfishes in inshore waters of Cochin, southwest coast of India. Bindu *et al*., (2007) studied the trace metal contamination of the marine environment in Palk Bay and Gulf of Mannar. Comparative study of heavy metal concentrations in razor clam (Solenregularis) in Moyan and Serpan, Sarawak by Devagi ., 2008. Monikh *et al*.,

(2011) measured the heavy metal levels in the sediments and ray fish Dasyatis bennettii from Persian Gulf.

Asha *et al* ., (2010) studied the concentration of heavy metals Cd, Cu, Fe, Mn, Ni, Pb and Zn in sea water, sediment and bivalve samples from three stations for one year along Tuticorin coast. The concentration was in the order of Fe>Mn>Zn>Cu>Pb>Cd>Ni. Generally the concentration of Fe was very high in the sediment and bivalves High concentration of Fe, Mn, Cu, Pb and Zn was observed during monsoon season. Lourenco *et al*., (2009); Ayas and Ozogul, (2011) measured concentration of both essential and non essential metals in the digestive gland and mantle of female cephalopods *Sepia officinalis* from two coastal lagoons of Portugal

Fathi Alhashmi Bashir *et al*.,(2012) evaluated of trace metal levels in tissues of two commercial fish species in Kapar and Mersing Coastal Waters, Peninsular Malaysia and the study revealed that the studied metals concentrations are generally low in the tissues of the examined fish in the two study areas. Although the levels of these heavy metals are not high, a potential danger may emerge in the future depending on pollution sources. Yu Wenjin *et al*., (2013) examined the distributional characteristics of heavy metal in Jiangsu Province Shoal Sea and they reported that although there are some heavy metal enrichment and pollution in the core area, no ecological hazard has been produced yet, and

overall environment quality in Xin yang gang core area was excellent. Distribution of heavy metal pollution in Vaipar coastal sediments, southeast coast of India was reported by Abukashim *et al*., (2014).

Jinadasa *et al*., (2015) determined the total mercury, cadmium and lead levels in main export fish of Sri Lanka. Results show that swordfish contained the highest total Hg and Cd levels, whereas yellowfin tuna contained the highest Pb levels. Heavy metal content in marine fish collected from the outlets of Hyderabad and Secunderabad, Andhra Pradesh, India was studied by Rao *et al*., (2014). Krishna *et al*., (2014) studied the Human health risk assessment of heavy metal accumulation through fish consumption from Machilipatnam Coast, Andhra Pradesh, India and reported the concentration of the metals in the fish muscle from Machilipatnam coast pose to health hazards to the consumers. Isacc.,2014 estimated the concentrations of heavy metals Cu, Zn and Pb from Tuticorin, Tamilnadu in marine fishes.

3. OBJECTIVES

Tuticorin coast is situated along the Gulf of Mannar Biosphere reserve. This coast is known for pearl fishing. Now it is flourished with petrochemical industries namely Thermal Power Station, Heavy Water Plant and Tuticorin Alkali Chemical (TAC) pouring heavy metals and other contaminants into the sea. All the wastes from these factories reach the coastal system. As a consequence of these processes, severe damages have already come to light on the Ecosystem along the coast of Gulf of Mannar. In the view of fast deterioration of coastal environment, a careful pollution monitoring is required to stabilize and save the coastal ecosystem before any harmful effects are carried to human beings. So the present study has been carried out to investigate the heavy metal concentration in the commercially important species of marine fishes.

The objectives of the present study are:

- To determine and compare concentration levels of heavy metals (Zn, Pb, Cu, Cd and Hg) in commercially important species of fin and shell fishes.
- To use these organisms as bioindicators of pollution of Gulf of Mannar.
- To ensure the seafood safety from this region.

4. MATERIAL AND METHODS

STUDY AREA

For the present study, different species of mollucs were collected from Gulf of Mannar coastal region. It is situated on the South-east coast of India. This area is remarkable for its richness and variety of fauna and the inshore sea bottom forms an ideal habitat for the growth of the shell fishes which sustain a good fishery. The Indian part of Gulf of Mannar covers approximately an area of 10,500 Km^2 along lat 8°35′ - 9°25′ N and long 78°08′ - 79°30′E (fig.1).

It is a part of the Southward extension of Bay of Bengal, it meets in the Indian ocean.This geographical area runs from Pamban island including Rameshwaram to Cape comarin along the South east coast of India to a distance of about 170 nautical miles. This coast contains a rich biological diversity of flora and fauna largely due to diversified microhabitats such as mangroves, corals, seaweed beds and sea grasses. The faunal diversity is also well pronounced with reference to different molluscan groups. Specimens were collected during low tides from the sea in their natural habitat that is intertidal zone and from reefs by divers.

Two stations namely Threspuram and Tharavaikulam were selected for the study. During December 2022 – February 2023. Molluscs were collected from the fishing nets and washed ashore in shoreline and brought to the laboratory for

identification. The shells were washed in water and with diluted hydrochloric acid to remove the hard outer coat and to reveal the natural colours. Collected specimens were preserved in 90% ethyl alcohol. The shells thus processed have been identified with the available keys, guides and comparing with the collections of Marine Biology Regional Centre, Zoological Survey of India, Chennai, and Center for Advanced Studies in Marine Biology, Porto Novo, and CMFRI Chennai.

ANALYSIS OF HEAVY METAL ACCUMULATION

Systematic Position of Experimental Animal

***Lambis lambis* (Linnaeus, 1758)**

Phylum	:	Mollusca
Class	:	Gastropoda
Super family	:	Stromboidea
Family	:	Strombidae
Genus	:	*Lambis*
Species	:	*Lambis*

Among the various molluscs taken from both the stations, *Lambis lambis* was taken for the analysis of heavy metals such as Cadmium Lead, Copper and Zinc and it was estimated in the whole body tissues of the study animal. Samples were collected from both the stations using trawl nets. The animals were brought to the

laboratory, washed thoroughly with seawater and the shells were broken without damaging the soft body of the animal. The soft tissues were taken, washed well and dried in a hot air oven at a temperature of 105°C for 24 hours. The dried tissues were powdered using a pestle and mortar. Powdered samples were used for metal analysis.

Digestion of Samples

The samples were subjected to acid digestion following the method of FAO (1975). Total Lead, Cadmium, Copper and Zinc were estimated. The samples were digested with a mixture of concentrated nitric acid (HNO_3) and perchloric acid ($HCIO_4$) in the ratio of 1: 2 until the formation of white residue at 100°C in a water bath. The cooled residue was dissolved completely by adding I N HCL and made upto 25ml with distilled water. The content was filtered by cotton wool and the filterate was subjected to metal analysis in Atomic Absorption Spectrophotometer (GBC Avanta Ver 2.02). The instrument was calibrated using standards

5. RESULT

Thoothukudi located in the Gulf of Mannar Biosphere Reserve is one of the most important potential fishing areas along the southeast coast of India. The major fish landing centres are Tharavaikulam, Threspuram and Punnakayil located along the Thoothukudi coast.

A total of 9 species of molluscs were collected and identified and given in Table -1. In both the two stations, maximum species richness of gastropods were available. The following are the distribution of molluscs found in the two stations: *Turbinella pyrum, Tonna dolium, Phalium glaucum, Lambis lambis, Harpulina lapponica, Chicoreus ramosus, Pugilina cochlidium, Babylonia zeylanica, Conus amadis* and *Babylonia spirata.*

The present study revealed the presence of 10 species under 9 families along the Thoothukudi coast. The different of molluscs collected from the two stations are tabulated in Plates 1-10.

The results on the concentration of heavy metals namely cadmium, Lead, Copper and Zinc in *Lambis lambis* from two stations of Gulf of Mannar were determined and the results are represented in the Fig 2, Table 2.

Among the heavy metals estimated in *Lambis lambis*, Zinc was found to be abundant when compared with all the other metals. Zinc reported the highest values

in both the stations. The concentration of zinc varies from 3.11µg/g to 3.50µg/g. Maximum concentration of 3.50µg/g was observed in station 2 and 3.11µg/g was observed in station 1.

The concentration of copper varies from 2.19µg/g to 2.96µg/g in both the stations. *Lambis lambis* showed maximum concentration of 2.96µg/g in station 2 and 2.19µg/g in station 1. Cadmium varied from 1.15µg/g to 1.25µg/g in both the stations. Maximum concentration of 1.25 µg/g was found in station 2 whereas 1.15µg/g was observed in station1. Concentration of lead varies from 0.82µg/g to 0.79µg/g in both the stations. Highest concentration of 0.82µg/g was observed in station 2 and 0.79µg/g concentration was observed in station 1.

TABLE 1 : DIFFERENT MOLLUSCS IN BOTH THE STATIONS

S.NO	MOLLUSCS	STATION 1 THRESPURAM	STATION 2 THARUVAIKULAM
1.	*Harpulina lapponica*	✓	✓
2.	*Chicoreus ramosus*	✓	✓
3.	*Pugilina cochlidium*	✓	✓
4.	*Turbinella pyrum*	✓	✓
5.	*Phalium glaucum*	x	✓
6.	*Lambis lambis*	✓	✓
7.	*Tonna dolium*	✓	x
8.	*Babylonia spirata*	✓	✓
9.	*Babylonia zeylanica*	✓	x
10.	*Conus amadis*	x	✓

PLATE 1 - SYSTEMATIC POSITION OF

Harpulina lapponica (Linnaeus, 1767)

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Gastropoda
Order	Neogastropda
Family	Volutidae
Genus	*Harpulina*
Species	*lapponica*

PLATE 2 - SYSTEMATIC POSITION OF

Chicoreus ramosus (Linnaeus, 1758)

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Gastropoda
Order	Neogastropoda
Family	Muricidae
Genus	***Chicoreus***
Species	***ramosus***

PLATE 3 - SYSTEMATIC POSITION OF

Pugilina cochilidium (Linnaeus, 1758)

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Gastropoda
Order	Neogastropoda
Family	Melongenidae
Genus	***Pugilina***
Species	***Cochilidium***

PLATE 4 - SYSTEMATIC POSITION OF

Phalium glaucum (Linnaeus, 1758)

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Gastropoda
Order	Littorinimorpha
Family	Cassidae
Genus	***Phalium***
Species	***glaucum***

PLATE 5 - SYSTEMATIC POSITION OF

Turbinella pyrum (Linnaeus, 1758)

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Gastropoda
Order	Neogastropoda
Family	Turbinellidae
Genus	***Turbinella***
Species	***pyrum***

PLATE 6 - SYSTEMATIC POSITION OF

Lambis lambis (Linnaeus, 1758)

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Gastropoda
Order	Littorinimorpha
Family	Stromboidae
Genus	***Lambis***
Species	***lambis***

PLATE 7 - SYSTEMATIC POSITION OF

Tonna dolium (Lamarck,1822)

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Gastropoda
Order	Littorinimorpha
Family	Tonnidae
Genus	***Tonna***
Species	***dolium***

PLATE 8 - SYSTEMATIC POSITION OF

Babylonia spirata (Bruguiere,1789)

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Gastropoda
Order	Neogastropoda
Family	Babyloniidae
Genus	***Babylonia***
Species	***spirata***

PLATE 9 - SYSTEMATIC POSITION OF

Babylonia zeylanica (Bruguiere,1789)

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Gastropoda
Order	Neogastropoda
Family	Babyloniidae
Genus	***Babylonia***
Species	***zeylanica***

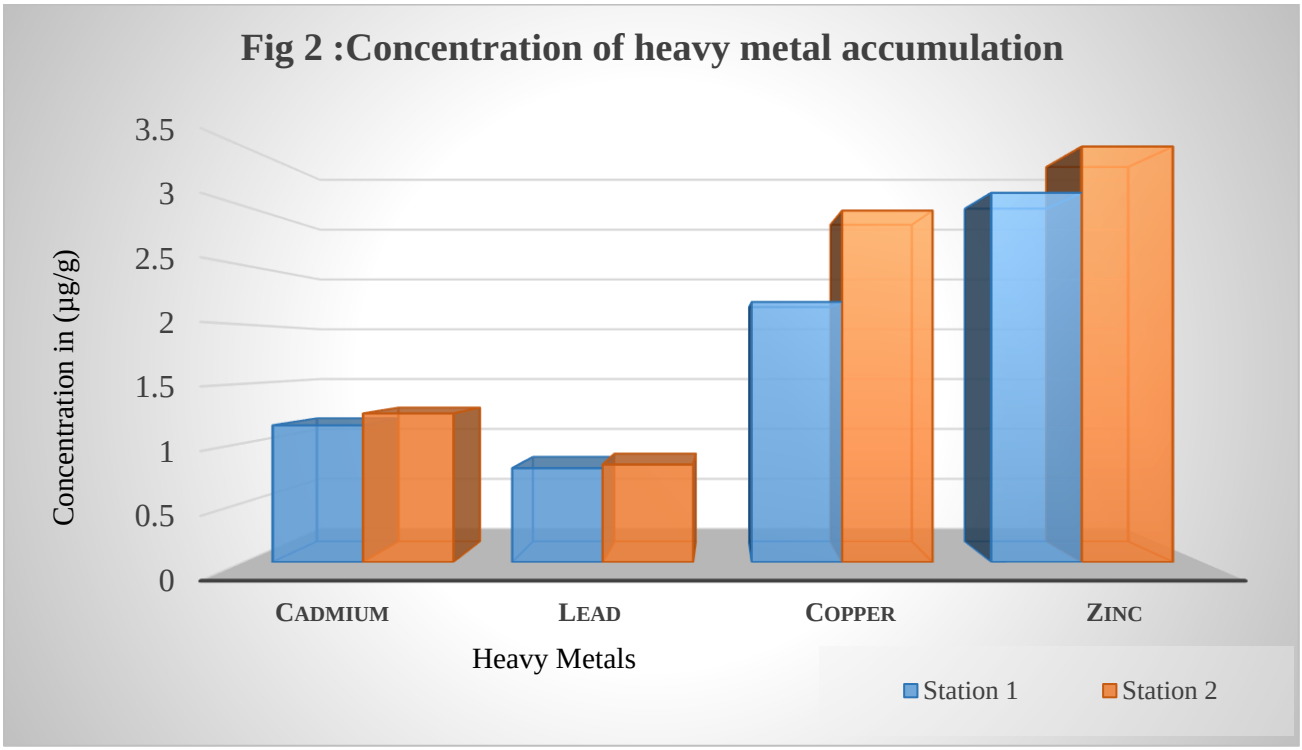
PLATE 10 - SYSTEMATIC POSITION OF

Conus amadis (Gmelin,1791)

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Gastropoda
Order	Neogastropoda
Family	Conidae
Genus	***Conus***
Species	***amadis***

Table 2: Concentration of heavy metal accumulation in the two stations

S.No	Heavy Metal Parameters (µg/g)	Station 1 (Threspuram)	Station 2 (Tharavailullam)
1	Cadmium	1.15	1.25
2	Lead	0.79	0.82
3	Copper	2.19	2.96
4	Zinc	3.11	3.50



6. DISCUSSION

Levels of contaminants in marine animals are of particular interest because of the potential risk to humans who consume them. Accumulation of metals in marine animals is the function of their respective membrane permeability and enzyme system, which is highly species specific and because of this fact different metals accumulated in different orders.

The mollusc`s shells and tissues are good indicator of metal pollution as they are sessile and sedentary and they reflect the heavy metals concentration of that particular area (Brugman 1981). Heavy metal analyzed in the present study showed that *Lambis lambis* Zn contamination level was higher compared to the other metals. This study is almost similar with a previous study (Jamila Patterson et al.,1997) on edible gastropods *Babylonia spirata, Hemifusus pugilinus, Rapana rapiformis, Xancus pyrum* and *Melo melo* at four sites along the southeast coast of India. In the present study, concentration of zinc varies from 3.11µg/g to 3.50µg/g. Maximum concentration of 3.50µg/g was observed in station 2 and 3.11µg/g was observed in station 1

High concentrations of all metals namely Zn, Mn, Fe and Cu were recorded during the rainy monsoon whereas concentrations were low during the dry summer in the studies carried out by most researchers. At Tuticorin and Madras metal concentration were high in all the gastropods confirming that the harbour

as well as the industrial wastes pollute these coastal waters. Similar to the present study Rasyid, 2018 evaluated the concentration of heavy metals lead, cadmium and mercury in the dried marine gastropod *Laevistrombus turturella*. The result showed that the lead content was 0.61 mg/kg and the cadmium 0.23 mg/kg, while the mercury content was not detected.

Primost *et al*.,(2017) reported that cadmium and lead concentration detected in digestive gland and gonad complex of three marine gastropods from Nuevo gulf,namely *Adelomelon ancilla,Buccinanops deformis* and *Trophon geversianus* was 8.02 mg/kg and 1.81 mg/kg, 38.86 mg/kg and 1.95 mg/kg, 104.24 mg/kg and 3.51mg/kg respectively. Habitat and anthropogenic activities near the habitat of marine gastropods are crucial in order to identify the accumulation basis of heavy metals in marine gastropods (Hajeb and Jinap 2009; Zhang *et al.*, 2007). Sharif *et al*., (2016) reported that the weather changes or climate changes could be contributory factors to heavy metal concentration in gastropods.

Mariam *et al*., 2016 investigated the concentration levels of heavy metals in bivalve mollusks Oysters, Mussels and donax at Tudor Creek Mombasa Kenya. Concenteration varied from species to species and size of the bivalves. Fe and Zn were the most abundant heavy metals in all the molluscs while Mn and Cr concentration levels for most of the organisms were very low. *Donax* had the lowest concentration of Fe while oysters and mussels had the highest

concentration levels of Zn. Zn and Fe had the highest concentration levels in all bivalves. The concentration levels for Zn ranged 13.80±3.23 in the *Donax* and 2504.92±6.96 in mussels between 9.15±2.57 µg/g to 440.13±22.39 µg/g.

Cadmium is a very toxic metal and has been responsible for a number of deaths. The most serious situation being the disease called Itai Itai disease. The presence of Cd could be due to the byproduct of the reaction between Zn, Cu andPb. Cd also released from biogenic detritus in order to regenerate phosphate and nitrate. The threshold for acute cadmium toxicity would appear to be a total ingestion of 3–15 mg. Severe toxic symptoms are reported to occur with ingestions of 10– 326 mg. Fatal ingestions of cadmium, producing shock and acute renal failure, occur from ingestions exceeding 350 mg (NAS-NRC, 1982).

Accumulation of cadmium in living organisms is a major ecological concern, especially because of its ability to accumulate very quickly (Besirovic *et al.,* 2010). Furthermore, seafood is the main source of cadmium for people. Cadmium varied from 1.15µg/g to 1.25µg/g in both the stations. *Lethrinus nebulosus* showed the maximum concentration of 4.72µg/g followed by *Babylonia spirata* (3.27 µg/g), 0.23 µg/g (*Sardinella longiceps*) and 0.17 µg/g in *Penaeus indicus* . This study corroborate with the result of Canli and Atli, 2003.

Cadmium content (1.25 mg/kg) in the muscle tissue of the sardine is higher than the recommended limit for cadmium in fish (0.05 mg/kg) according

to the Commission of The European Union (Anonymous 2006) and Turkish Food Codex(Anonymous 2008). But, the maximum allowed cadmium doses for an adult are 0.5 mg/week (Anonymous 1976). The mean level of cadmium was noted in our samples.

Lead constitutes a serious health hazard to both children and adults that may be directly ingested by man or indirectly through aquatic animals like fish and shellfish (Olaifa *et al.,* 2003) and is one of the most important heavy metal pollutant found in the environment, including the aquatic environment (Cigerci *et al.,* 2010). The high blood lead level can cause kidney dysfunction, brain damage, anemia and can inhibits the normal functioning of many enzymes. Pb present in the marine environment through atmospheric deposition and soil erosion as well as vehicle exhaust and industrial discharge. Lead is a potentially toxic chemical.

In the present study, concentration of lead varies from 0.82µg/g to 0.79µg/g in both the stations. Highest concentration of 0.82µg/g was observed in station 2 and 0.79µg/g concentration was observed in station 1. Celik *et al.* (2004) has reported a mean lead concentration of 0.05 mg/kg in the sardine from İzmir Bay (Turkey).

Copper is an essential element. However, it can be potentially toxic to aquatic organisms when in excess in water (Martins *et al.* 2011). In the Turkish Food Codex (Anonymous, 2002) the recommended limit for copper in fish is 20

mg/kg. The concentration of copper varies from 2.19µg/g to 2.96µg/g in both the stations. The mean concentration of copper reported in this study (1.00 mg/kg) was higher than the permissible limits.

Concentration of Zn, Lead and Copper were higher compared with the cadmium and and mercury. Similary, Bhanoo Saulick *et al*.,(2017) confirmed the presence of trace elements primarily of Cu, Ni and Zn and heavy metals Hg and Pb in muscle tissue of the four fish species namely the sky emperor (*Lethrinus mahsena*, Bank), sky emperor(*Lethrinus mahsena*, coastal), blackspot emperor (*Lethrinus harak*) and the spangled emperor (*Lethrinus nebulosus*) in different region of Mauritius. Uptake of heavy metals and trace elements is related to molluscs age, mass and length and aquatic environment. This clearly supports the fact that higher concentration of zinc and copper were recorded and lowest concentration of lead and cadmium.

Several factors may be involved in explaining the differences between our results and those reported by other authors: Intrinsic factors: Fish size, age, sex, reproductive cycle, diet, and metabolic activity. The latter is proportional to heavy metals' accumulation (Kim and Kang, 2015) and extrinsic factors: The environment where molluscs live significantly affects the rate of contaminant accumulation by different organisms, as well as the concentrations of contaminants in the water column of fishing areas, handling, and processing fish

during transportation and storage. The fishing season is also an important factor to consider as well as temperature, salinity, pH, and the presence of ligands in the marine environment (Guner, 2008). The periodical control of heavy metals in the is needed both for the assessment of toxic metal intake from these fish by humans and for generating data for further studies.

7. SUMMARY

- A total of 9 species of molluscs were collected and identified
- In both the two stations, the distribution of molluscs were *Turbinella pyrum, Tonna dolium, Phalium glaucum, Lambis lambis, Harpulina lapponica, Chicoreus ramosus, Pugilina cochlidium, Babylonia zeylanica, Conus amadis* and *Babylonia spirata.*
- Among the heavy metals estimated in *Lambis lambis*, Zinc was found to be abundant when compared with all the other metals. Zinc reported the highest values in both the stations. The concentration of zinc varies from 3.11µg/g to 3.50µg/g. Maximum concentration of 3.50µg/g was observed in station 2 and 3.11µg/g was observed in station 1.
- The concentration of copper varies from 2.19µg/g to 2.96µg/g in both the stations. *Lambis lambis* showed maximum concentration of 2.96µg/g in station 2 and 2.19µg/g in station 1.
- Cadmium varied from 1.15µg/g to 1.25µg/g in both the stations. Maximum concentration of 1.25 µg/g was found in station 2 whereas 1.15µg/g was observed in station1.
- Concentration of lead varies from 0.82µg/g to 0.79µg/g in both the stations. Highest concentration of 0.82µg/g was observed in station 2 and 0.79µg/g concentration was observed in station 1.

8. CONCLUSION & SUGGESTONS

The present study shows that the accumulation of heavy metals was found in could be used as a good indicator organism due to its availability.

Both the stations have diversity of fauna and flora, so stringent pollution abatement measures have to be followed to safeguard the diversified fishery resources of Tuticorin bay. Since Tuticorin bay is a part of the Gulf of Mannar, which is the first Marine Biosphere reserve in the South East Asia, the biodiversity of this region needs to be conserved.

As a measure of conservation of fauna and flora of this bay, the following pollution abatement measures are recommended.

- An effective treatment and measures for industrial effluents and other anthropogenic discharges into the coastal waters so as to reduce the heavy metal.
- Programmes on the protection of Gulf of Mannar biosphere reserve may be periodically broadcast and telecast to raise awareness among the public.

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A STUDY ON FORMULATION AND EVALUATION OF HERBAL HAND SANITIZER

A project submitted to

ST. MARY'S COLLEGE (Autonomous), THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

by

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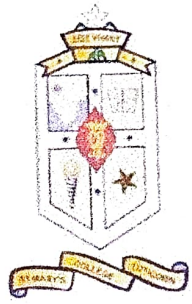

DEPARTMENT OF ZOOLOGY

ST. MARY'S COLLEGE (Autonomous),

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

THOOTHUKUDI -628 001

April -2023

CERTIFICATE

This is to certify that the project entitled **"A Study on Formulation and Evaluation of Herbal Hand Sanitizer"** is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a record of the work done during the year 2022-2023 by the following students.

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CONTENTS

INTRODUCTION

INTRODUCTION

Microorganisms live in all environments on Earth that are occupied by macroscopic organisms, and they are the sole life forms in other environments, such as the deep subsurface and 'extreme' environments **(**Ricardo Cavicchioli, *et al., 2019).* Each day people are exposed to millions of bioaerosols, including whole microorganisms, which can have both beneficial and detrimental effects. As humans carry 10^{12} microorganisms on their epidermis and 10^{14} microorganisms in their alimentary tract, we might be one of the greatest sources of bioaerosols in the built environment (Luckey, 1972; Aaron Prussin and Linsey Marr,2015). Respiration and the shedding of millions of skin cells daily contribute to bioaerosols in the built environment (Adams *et al.,* 2015). Each of us consists of about 40 trillion human cells and about 22,000 human genes, 100 trillion microbial cells (the microbiota) and 2 million microbial genes (the metagenome) (Bianconi *et al*., 2013; Pertea and Salzberg, 2010; Savage, 1977; Turnbaugh, 2007 and Jacques Ravel *et al*., 2014).

A pathogen is defined as an organism causing disease to its host, with the severity of the disease symptoms referred to as virulence. Pathogens are taxonomically widely diverse and comprises of viruses, bacteria as well as unicellular and multicellular eukaryotes (Francois Balloux & Lucy van Dorp

(2017). The ‘pathogenicity’ of a microbe depends on the host as well as on microbial factors. Microbes can be usefully classified into ‘conventional pathogens’, ‘conditional pathogens’ and ‘opportunist pathogens’. Some of the pathogenic diseases are gonorrhea, ophthalmia neonatorum, bacterial meningitis, diphtheria etc. (Shanson *et al*., 1989).

Bacteria are ubiquitous and play an important role in maintaining the environment. Only a small percentage of the world's bacteria cause infection and disease. These bacterial infections have a large impact on public health. In general, bacterial infections are easier to treat than viral infections, since the armamentarium of antimicrobial agents with activity against bacteria is more extensive than with infectious diseases caused by viruses and parasites. Among the top causes of mortality in the world, lower respiratory infection is the third most common and diarrhea is the sixth. Both are often caused by bacteria. Tuberculosis is the seventh most common cause of death (Doron and Gorbach, 2008).

Hands are the first mode of transmission of microbes and infections. Hand hygiene is a key principle and exercise in the prevention, control and reduction of infections. The bacteria resides on hands are classified in two categories namely resident or transient. The resident flora are resides under the stratum corneum and can be found on surface of skin, namely *Staphylococcus*

epidermis, *S. hominis*, Corynebacteria, Propionibacteria, Dermobacteria, Micrococci and fungi *Malassezia spp*. The resident flora protects skin and has antagonistic functions, but cause infections in sterile body cavities, eyes or on non intact skin. Transient flora colonizes the superficial layers of the skin and gets removed by routine hand hygiene, these flora depends on individual profession, habit and skin moisture and sporadically multiply on skin surface. Hands are the source of nosocomial infections if hand hygiene not maintained.

Pathogens cannot be removed by simple washing, therefore hand sanitizing is required. Scientific studies have shown that after hand washing, as many as 80% of individuals retain some pathogenic bacteria on their hands. Moreover, hand washing removes the body's own fatty acids from the skin, which may result in cracked skin that ultimately provides a potential entry portal for pathogens. To overcome the limitations of plain hand washing, hand sanitizers were introduced claiming to be effective against those pathogenic microorganisms as well as to improve skin condition due to the addition of emollients (Andrew Golin *et al.*, 2020). The active mechanism of hand sanitizers against bacteria includes protein denaturation, inhibition of mRNA, and protein synthesis (Lufuno Muleba *et al.*, 2022).

According to the World Health Organization (WHO), sanitizer is defined as “an alcohol-containing preparation (liquid, gel, or foam) designed

for application to the hands to inactivate microorganisms and/or temporarily suppress their growth". Hand sanitizers can be classified as alcohol-based or alcohol-free. Alcohol-based sanitizers comprise between 60 and 95 percent alcohol in the form of ethanol, isopropanol, or n-propanol. Alcohol-free products have a property of disinfectants, such as benzalkonium chloride (BAC), or antimicrobial agents, such as triclosan, which is immediate and purposeful. Several sanitizers comprise emollients such as glycerin that pacify the skin, thickening agents, and provide aroma (Pallavi Singh, *et al.,* 2020).

The use of medicinal plants in traditional medicine has been described in literature several 1000 years back. (Chang *et al.,* 2016; Hanaa Yamani *et al*., 2016). In modern complementary and alternative medical practice, plants are the primary source of therapeutics and each part of the plant, including the seeds, root, stem, leaves, and fruit, potentially contains bioactive components (Jiang *et al.*, 2014, 2015; Mandave *et al*., 2014; Sun *et al.,* 2014; Hanaa Yamani *et al*., 2016). Among the medicinal plants, aromatic herbs are a rich source of biologically active compounds useful both in agriculture and medicine (Mathela, 1991; Cutler and Cutler, 1999; Hanaa Yamani *et al*., 2016). The therapeutic importance of plants has been quoted in the ancient cultures and traditions of many countries and societies and they are believed to be cost effective and safe. Since ancient times, plants and their products have been used

as a culinary preparation or as a remedy in different traditional medicine for many diseases (Rahmani *et al*., 2014; Mohamed Fizur Nagoor Meeran *et al.*, 2017). Turmeric, oregano, thyme, olives and dates have been used extensively for culinary purposes in diets and are also believed to possess beneficial effects against numerous diseases Mohamed Fizur Nagoor Meeran *et al*., 2017).

Biological activities of essential oils range from analgesic, antiseptic, antimicrobial, carminative, diuretic, spasmolytic to hyperaemic and stimulatory (De Groot and Schmidt, 2016; Asja Sarkic and Iris Stappen., 2018) Due to the antimicrobial and antifungal impact of essential oils, preparation of creams, gels and ointments do not necessarily require an additional chemical preservative if they contain an essential oil or a single compound as an active agent (Sticher, *et al.*, 2015; Asja Sarkic and Iris Stappen., 2018).

Ocimum tenuiflorum, also known as *Ocimum sanctum*, Tulsi, or Holy Basil from the family Lamiaceae has been described as the "Queen of plants" and the "mother medicine of nature" due to its perceived medicinal qualities. Oils extracted from the leaves and inflorescence of Tulsi have been claimed to have numerous useful properties, including as expectorants, analgesics, anti-emetics, and antipyretics; stress reducers and inflammation relievers; and as anti-asthmatic, hypoglycemic, hepatoprotective, hypotensive, hypolipidemic, and immunomodulatory agents. (Singh *et al.*, 2010; Hanaa Yamani *et al*.,

2016). Tulsi essential oil could be a valuable topical antimicrobial agent for management of skin infections caused by these organisms or as a wound dressing to prevent infection. (Hanaa Yamani *et al*., 2016)

Greeks, Romans, and Egyptians have used thyme as a preservative, odorant and flavoring agent in foods. Thyme possesses potent antibacterial, antifungal, sedative, antiseptic, antioxidative, expectorant, antispasmodic, antifungal, antivirotic, antihelminthic, carminative and diaphoretic effects (Rustaiyan *et al.,* 2000; Soliman and Badeaa, 2002). Thyme contains an abundant amount of terpenoids, flavonoids, glycosides and phenolic acids (Vila, 2002). Thymol containing herbs is used by the ancient Egyptians for the preservation of mummies (Mohamed Fizur Nagoor Meeran *et al.,* 2017).

Among medicinal plants, mint (*Mentha* species) exhibits multiple health beneficial properties, such as prevention from cancer development and anti-obesity, antimicrobial, anti-inflammatory, anti-diabetic, and cardioprotective effects, as a result of its antioxidant potential, low toxicity and high efficacy. *Mentha* species are widely used in savory dishes, food, beverages, and confectionary products. Phytochemicals derived from mint also showed anticancer activity against different types of human cancers such as cervix, lung, breast and many others. Mint essential oils show a great cytotoxicity potential, induce apoptosis, suppress invasion and migration

potential of cancer cells lines along with cell cycle arrest. Essential oils from mint have also been found to exert antibacterial activities against *Bacillus subtilis, Streptococcus aureus*, *Pseudomonas aeruginosa*, and many others (Majid Tafrihi *et al.*, 2021)

Chaste tree is a large shrub native to the tropical and subtropical regions of the world. The *Vitex* tree, including its leaves and fruits, has been used for herbal remedies in the form of pastes, decoctions, and dried fruits since ancient times. some of the bioactivities exhibited by the *Vitex* genus are anti-inflammatory, analgesic, antihistamine, antimicrobial, antioxidant, and cytotoxic activities against various cancer cell lines (Rani *et al*., 2013 and Nurkhalida Kamal *et al*., 2022). Ex Schauer exhibited both antibacterial and antifungal activities against E*nterobacter aeurogens, Staphylococcus aureus, Candida, and Rhizopus* species, respectively (Kannathasan *et al*., 2011). The paste and juice are made from the leaves for topical application to skin and used orally to treat cellulitis and hives.

Decoctions from the leaves of *V. trifolia* are given orally to ease joint and sciatica pains in Asian countries, while for the treatment of leprosy and skin rashes, the leaves can be ingested together with honey or applied topically. They also added that a mixture of crushed leaves and ghee is

traditionally applied on the area infected with fungus (Kirtikar *et al*., 1935; Nurkhalida Kamal *et al.,* 2022).

Sweet flag (*Acorus calamus*) is commonly known drug in traditional system of medicine. The two most active plants showing potent antifungal activity were *Acorus calamus* and *Piper betel*. α and β asarone is one of the major components of sweet flag possessing strong antibacterial, antifungal, antihelminthic, neuroprotective, antiepileptic activity. The rhizome extract of *A. calamus* exhibited highest antifungal activity inhibiting the mycelial growth completely (100%) against all the 6 test pathogens. The ethanolic extracts of *A. calamus* was active against all the investigated bacterial strains while aqueous extract was totally inactive against the studied gram negative bacterial strains (*E. coli, P. mirabilis and P. aeruginosa)* and showed moderate antibacterial activity against gram positive bacteria *B. subtilis* and *S. aureus* (Hashmat Imam *et al.,* 2013).

The excessive use of chemical based hand sanitizer leads to a number of problems in people having sensitive skin. The drawbacks include skin dryness, irritation, harsh on skin, ocular irritation. In some cases, alcohol may strip the outer layer of skin, which may have negative effects on barrier function of the skin. The side effects and more demand of sanitizer, emphasize on preparation of polyherbal sanitizer. Plants are rich in vast

variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids etc. which have been found to possess in vitro antimicrobial properties. Due to its antimicrobial activities herbs are used in formulation of herb-based hand sanitizers and sanitizers prepared with two or many herbals with multidimensional properties are very effective against the bacteria which are most resistant to the disinfection process and multidrug-resistant pathogens (Chandravanshi *et al*., 2018).

REVIEW OF LITERATURE

Hands are primary mode of transmission of microbes and infections. To defend the skin from harmful microorganism and to avoid spreading of numerous contagious diseases, hand washing is extremely significant precaution. (Kamat *et al.*, 2008; Ahmad *et al*., 2012 ; Johny and Saravanakumar, 2013 and Mounika *et al*., 2017). Hand sanitizer is less effective at killing certain kinds of germs, such as norovirus and *Clostridium difficile*. The sanitizer may be less effective due to incorrectly wiping out hands before sanitizer dries or if concentrations of alcohol too low in sanitizer. Use of plants as source of medicine has been inherited and is an important component of the health care system in India.

Fatima Grace *et al,* (2015) evaluated a poly herbal hand wash from commonly available plants, instead of adopting synthetic preparation. Hand sanitizer is an antiseptic and supplement to the hand washing with soap. Harsha *et al.,* (2016) reported herbal hand sanitizer with bio-active enriched with the goodness of citrus and neem. Sanitizer was evaluated for its bactericidal activity against the specified microorganisms. When tested for 120 seconds of contact time against specified bacterial species resulted in considerable logarithmic reductions of 0.03, 0.125, 0.097 and 0.091 in the bacterial viable counts of *Staphylcoccus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus hirae* respectively.

Olufunke Dawodu (2017) stated that turmeric hand sanitizer will be better than the alcohol rub hand sanitizer because of the powerful anti-inflammatory effect and its antioxidant potential. Rutya Sunil Patankar, *et al.,* (2018) found that the herbal sanitizer was more effective than sterillium. They also showed anti biofilm activity. Antibiotic potentiation activity of sanitizer against pathogens is changing the sensitivity pattern hence can be use in combination with antibiotic in same formulations. Balakrishna, *et al.,* (2018) discovered *Azadirachta indica* are used in many medicinal treatments like skin diseases, antidiabetic, antiviral, ant carcinogenic, immune-modulatory.

Jyotsna singh chandravanshi, *et al.,* (2018) studied the antimicrobial properties of herbs and their potential use as hand sanitizers. Some herbs that are used to prepare herbal hand sanitizers are *Ocimum sanctum* (tulsi leaves), *Eugenia caryophyllus* (clove), *Cymbopogon flexuous* (lemon grass), *Aloe baarbadensis* (aloe), *Mentha arvensis* (mint), *Azadirachata indica* (neem), *Eucalyptus globulus* (eucalyptus).

Balakrishna *et al*., (2018) evaluated the antibacterial efficacy of various herbal oils such as cinnamon oil, eucalyptus oil, menthol oil and lavender oil and found that cinnamon oil showed better antibacterial activity. They also formulated and evaluated poly-herbal hand wash gel containing *Azadirachta indica*, *Ocimum sanctum* and citrus lemon extracts. The anti-

microbial activity of the formulated herbal hand wash gel was tested against *Escherichia coli, Staphylococcus aureus* and *Salmonella typhi*

Nandkishor, *et al*., (2013) prepared herbal hand sanitizer incorporating the leave extracts of *Ocimumcanctum* (Tulsi) and *Eucalyptus globulus* (Nilgiri), the well-known herbal combination with multidimensional activities and evaluated their antimicrobial efficacy and safety of hands. The formulation was evaluated against the specified microorganism such as *E. coli, Pseudomonas aeuroginosa, Staphylococcus aureus, Bacillus subtilis* and *Sacchromyces cerevisiae, Candida albicans* by culture sensitivity test. The significance was found to be more in comparison to the standard reference.

Blessy Jacob *et al*,(2020) prepared the hand sanitizer with the leaves of *Coriandrum sativum* (Coriander), *Azadirachta indica* (Neem), *Ocimum sanctum* (Tulsi), *Syzygium aromaticum* (Clove), *Cymbopogon citratus* (Lemon grass) and *Cinnamomum zeylanicum* (Cinnamon). Combination of these ingredients act as an effective hand sanitizer. The microorganisms such as *Escherichia coli, Staphylococcus aureus, Bacillus subtilis* and *Pseudomonas aeruginosa* were mainly used to test the efficacy of the product. The study revealed that the herbal hand sanitizer was very much efficient in reducing microorganisms from the hand. The formulation

of hand sanitizer was prepared and evaluated for physical properties such as colour, odour and pH etc.

Different formulations are prepared and characterized using eucalyptus oil as an active pharmaceutical ingredient. Among five formulations, F3 is selected based upon its anti-bacterial activity. F3 formulation is evaluated for odour, colour, clarity test, pH and skin irritancy characteristics. The formulation F3 was clear and light orange in colour with mild odour. The pH of herbal hand sanitizer found to be 6.5 with no irritation to skin. Anti-microbial properties evaluated showing good zone of Inhibition in *E.coli* and *S.aureus* are 22mm and 26mm. Vaibhav Rajendra Suryawanshi *et al*, (2020).

Huda Ahmed Alyhamdi (2021) reported the formulations and preparations of hand sanitizers with herbal plants have been proved effective against pathogens and results have also been compared and found effective with alcoholic based formulations of hand sanitizers. These herbal formulations have been considered safe for human health as far as to environment. Ravindra *et al.,* (2021) revealed citrus flavanoid has a large spectrum of biological activity including antibacterial, antifungal anti-diabetic, anticancer and antiviral activities.

Vijaya *et al*, (2021) evaluated herbal hand sanitizer using microorganism suspensions (*E. coli*, *Staphylococcus aureus*), which showed

that herbal hand sanitizer is more efficient than commercial synthetic hand sanitizer in reducing the number of germs on the hands. The increased antibacterial activity and efficacy of these plant extracts can be exploited to create herbal hand sanitizers on a commercial scale. Balakrishna Acharya *et al,* (2021) formulated and evaluated the poly herbal hand wash gel containing *Azadirachta indica, Ocimum sanctum* and *Citrus limon* extracts. The anti-microbial activity of the formulated herbal hand wash gel was tested against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* by spread plate techniques and the results obtained were compared with commercial antibacterial standards.

Arun Kumar *et al,* (2022) prepared herbal hand sanitizer incorporating with the leaves extracts of *Ocimum sanctum* (Tulsi) and *Eucalyptus globulus* (Nilgiri), the well-known herbal combination with multidimensional activities and to evaluate their respective antimicrobial efficacy and safety of hands. The formulation was evaluated against *E. coli*, *P. aeruginosa, K. pneumoniae, S. aureus, M. luteus, S. epidermidis* and *Candida albicans* by culture sensitivity test. Suvarna Bhadane *et al.,* (2022) prepared herbal hand sanitizer incorporating the leaves extracts of Neem (*Azadirachta indica*), Peppermint (*Mentha piperita*), and Aloe vera (*Aloe barbadensis)* the well-known herbal and flavouring combination with multidimensional activities; and evaluated their individual antimicrobial effectiveness and safety of hands.

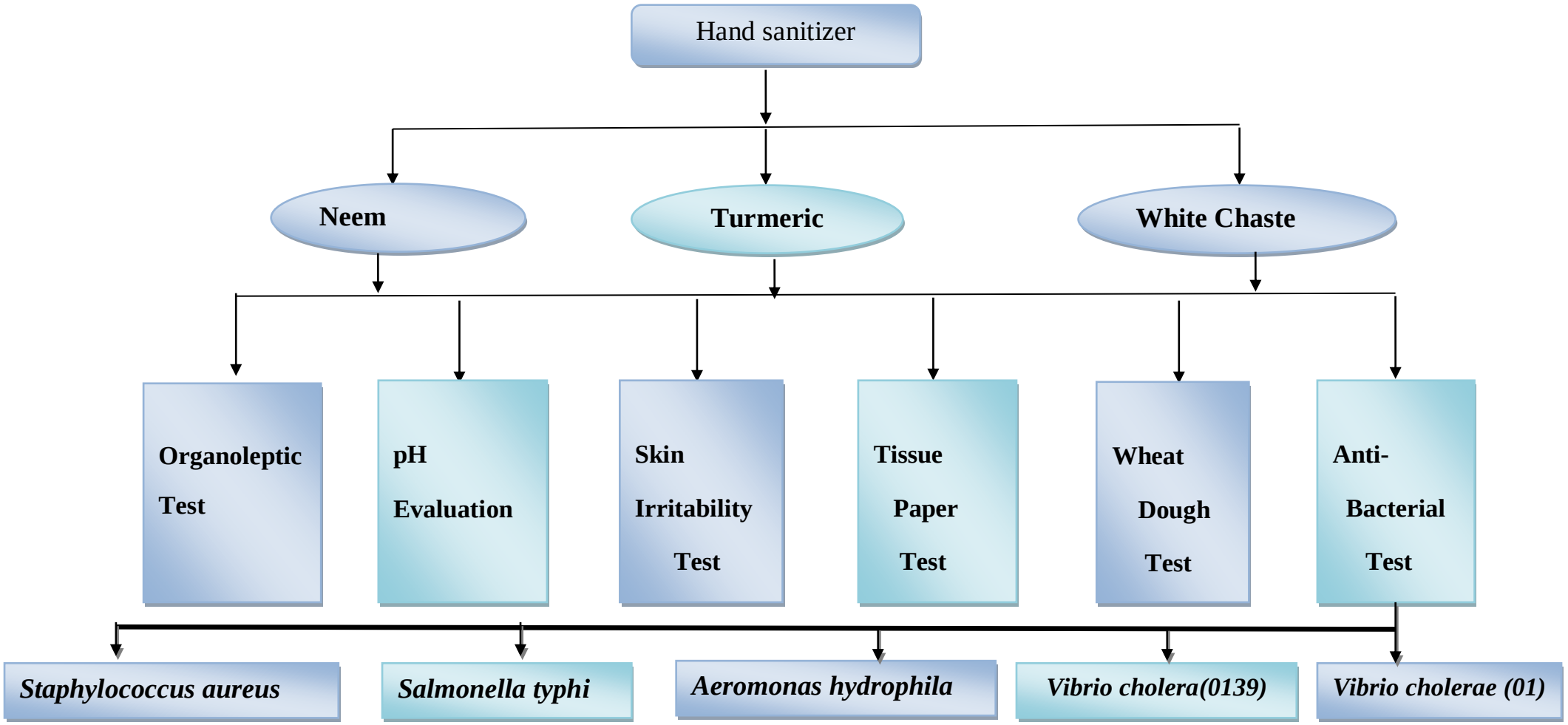
Debgopal Ganguly *et al,* (2022) investigated antimicrobial activity of prepared polyherbal handwash by using different types of herbal plants like *Azadirachta indica* (Neem), *Ocimum tenuiflorum* (Tulsi), *Mentha arvensis* (Pudina), *Syzygium aromaticum* (Clove), *Foeniculum vulgare* (Fennel), *Coriandrum sativum* (Coriander) and *Psidium guajava* (Guava). The formulations were evaluated for appearance,color,odor, pH,viscosity,foam height, and foam retention time. The antimicrobial activity of the six hand wash formulations was tested using the agar plate method against *Staphylococcus aureus* and *Escherichia coli.*

OBJECTIVES

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

- To prepare polyherbal hand sanitizer to maintain hand hygiene.
- To evaluate the organoleptic test of hand sanitizer
- To measure the pH value of the sanitizer
- To analyze the antibacterial activity in the prepared hand sanitizer against five different pathogens.

EXPERIMENTAL DESIGN



MATERIALS AND METHODS

Collection of leaves:

The plant leaves were collected for the preparation of sanitizer from in around Thoothukudi area. The plant selection is based on its potent antimicrobial activity. The plants used for the study were Neem (*Azadirachta indica),* Turmeric (*Curcuma*), Aloe vera, Tulsi (*Ocimum tenuiflorum),* Three-leaved chaste (*Vitex agnus-castus*). The collected plants leaves were cleaned, washed, midrib of the leaves were removed, weighed and shade dried in laboratory. After drying, leaves were grinded in electric grinder machine to get a fine powder.

Preparation of extract for hand sanitizer:

Fine powdered plant leaves 30gm were immersed in the 180ml of ethanol and incubate it for 48 to 72 hours. Extract is prepared by using Soxhlet apparatus and the ethanol should be evaporated by using rotary evaporator, leaving a extracted plant material in the glass bottom flask. The extract is collected and stored it for further use.

Preparation of Hand Sanitizer :

Several natural materials were used to prepare the hand sanitizer in suitable proportions, including aloe vera, glycerin, and vitamin E, in addition to essential oils (Jasmine,white rose), eucalyptus oil, Tulsi powder,

sweet flag powder, menthol crystals, thymol crystals, lemon, soap nut powder. Each formulation was prepared by dispersing glycerin (5% v/v) to aloe vera gel (90% v/v) in a 250 mL beaker and mixed with gentle stirring at ambient temperature. EOs (at 2.5% v/v or 1.25% v/v) were then added dropwise with constant stirring to avoid air bubble formation and to obtain uniform and homogenous gels, followed by adding vitamin E (0.05% v/v). Add the plant extract to this mixture and stir well.

Table 1:

Sample Number	Essential Oils	Aloe Vera	Glycerin	Vitamin E	Distilled Water
Sample 1	2.5% (*v*/*v*)—jasmine	90% (*v*/*v*)	5% (*v*/*v*)	0.05% (*v*/*v*)	2.45% (*v*/*v*)
Sample 2	1.25% (*v*/*v*)—white rose				3.70% (*v*/*v*)
Sample 3	1.25% (*v*/*v*)—jasmine				3.70% (*v*/*v*)

Organoleptic test:

Physical evaluation (colour, odour) was done by sensory and visual inspection.

pH:

One ml of sample of poly herbal hand wash was taken and dissolved in 10ml distilled water. The pH of solution was measured by using digital pH meter.

Skin exposure to sanitizer:

Individual exposure will be assessed by questionnaires. 3ml of the sanitizer prepared was applied to different individuals and feedback was taken. The individuals were asked questions regarding sanitizer odor and experience after using. The skin of hand was examined for redness, irritation and dryness.

Tissue Paper Test:

This test is based upon Paper Chromatography (Chakraborty *et al*., 2021). A circle was drawn by a ball pen on tissue paper and small drops of sanitizer was poured on the tissue paper to test the amount of alcohol present in the sanitizer.

Wheat Dough Test:

Sanitizer was poured into one table spoon of wheat and mixed to test the excess water in the sanitizer. (Chakraborty *et al*., 2021 and Vaishali *et al*., 2022).

Bacterial cultures:

Five bacterial strains namely *Vibrio cholerae* (01), *Vibrio cholerae* (0139), *Aeromonas hydrophila, Salmonella typhi and Staphylococcus aureus* were used for antimicrobial activity. (All the bacterial strains were obtained from Government Medical College, Tuticorin).

Inoculum preparation for bacteria:

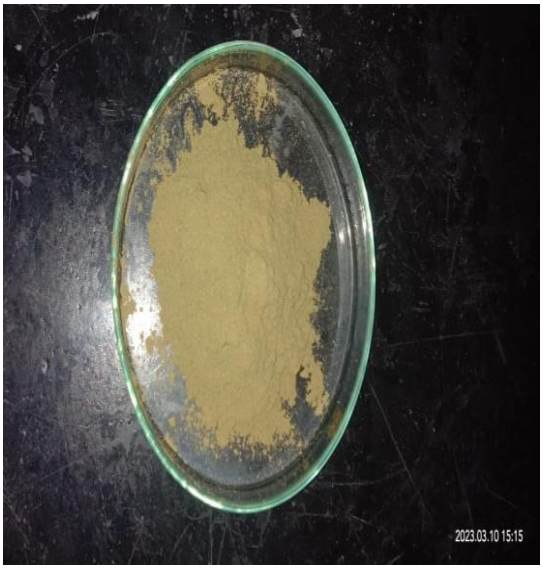
Nutrients broth was prepared and sterilized in an autoclave at 151bs pressure for 15 minutes. All the five bacterial strains were individually inoculated in the sterilized broth and incubated at 37ºC for 24hours. Nutrient agar was prepared, and poured into sterile petridish. The 24 hour old bacterial broth cultures were inoculated in the petridishes using a sterile cotton swab.

Antibacterial activity assay:

The antibacterial activity assay was carried out by disc diffusion method (Kirby & Bauer,1966). Nutrient agar was prepared and poured in sterile petriplates. Bacterial culture was inoculated on the surface of agar plates and spread by using L-rod. The inoculated plates were allowed to dry for five minutes. Sterilized paper disc prepared from whatmann No.1paper disc with 6mm diameter were loaded with sample and placed on the surface of inoculated petri plates along with control using sterile technique. The plate was incubated at 37 ºC for 18-24 hours. The plate was examined for inhibitory zone, the inhibition zones were measured with the outer side of the disc to inner side of the inhibition zone and the zone of inhibition was measured in mm. The experiment was done three times for confirmation of activity.

Plate - 1 INGREDIENTS FOR THE PREPARATION OF SANITIZER

NEEM



TURMERIC

WHITE CHASTE

Plate - 2 **INGREDIENTS ADDED TO THE SANITIZER**

ALOVERA GEL

GLYCERIN

ESSENTIAL OIL – JASMINE & WHITE ROSE

EUCALYPTUS OIL

Plate- 3 **FRESHLY PREPARED SANITIZERS**

NEEM BASED SANIZITER

TURMERIC BASED SANIZITER

WHITE CHASTE BASED SANIZITER

RESULT

In the present study three types of hand sanitizer namely neem based hand sanitizer, turmeric based hand sanitizer and white-chaste based hand sanitizer were prepared. The antimicrobial activity of the sanitizer and its ingredients were evaluated on different bacterial strains and its efficacy was also evaluated. (Plate-3)

a. Organoleptic test:

The organoleptic test of hand sanitizer was conducted to evaluate the physical appearance of the prepared formulations. Visual quality inspection of the prepared hand sanitizer shows that the Neem based sanitizer is green in colour and the sanitizer smells like jasmine. Turmeric based sanitizer is yellow in colour and the sanitizer smells like white rose and White chaste based sanitizer is green in colour, smells like white rose and all the three sanitizer has excellent quality.

b. pH evaluation:

The pH values of the formulated hand sanitizer gels were measured using a digital pH meter. The study was conducted to check the neutralization of different prepared formulations. The ideal standards for a pH value of a topical dosage form should be within the broad pH range of the skin to avoid skin inflammation and irritation. The pH value of neem

based sanitizer is 3.68. It is acidity. White chaste based sanitizer is 2.73 and 3.13 is the pH value of turmeric based sanitizer

c. Skin irritation study:

Skin sensitivity of the sanitizer was checked on different individuals and feedback was collected. The individuals gave positive response with mesmerizing odour and soothing effect after using sanitizer. The individuals were asked to observe redness, irritation, burning sensation and dryness. But no side effects were seen in any individuals after using sanitizer. Like other commercial sanitizer, the prepared sanitizer gave soothing effect and no dryness was observed. (Plate-4)

d. Tissue paper test:

The results of the present study shows that all the three prepared sanitizer contains sufficient amount of alcohol and the ink will be dissolved into sanitizer and starts diffused slowly and moved out the circle (Plate-5)

e. Wheat dough test:

The results of the wheat dough test indicates that there is no excess water in the prepared herbal based sanitizer. It was observed there was no formation of a dough. (Plate-5)

f. Antibacterial activity of hand sanitizer:

The antibacterial activity of the prepared sanitizer and its ingredients were evaluated against five bacterial strains namely *Vibrio cholerae* (01), *Vibrio cholerae* (0139), *Aeromonas hydrophila*, *Salmonella typhi and Staphylococcus aureus*.

The neem based hand sanitizer showed antibacterial activity against all the tested organism. The extract showed activity with the inhibition zone ranging from 5 mm to 10mm. The highest activity of 10mm was recorded against *Aeromonas hydrophila* and lowest activity of 5mm against *Staphylococcus aureus*. The extract also inhibited the growth of *Vibrio cholerae* (01), *Vibrio cholerae* (0139) and *Salmonella typhi* with the inhibition zone of 6mm, 6mm and 8mm respectively. (Plate -6 Fig:1)

The turmeric based hand sanitizer showed antibacterial activity with the maximum inhibition zone of 10mm against *Vibrio cholerae* (0139) and minimum zone of inhibition of 4mm against *Aeromonas hydrophila*. It also shows activity against *Vibrio cholerae* (01) and *Salmonella typhi* with the same inhibition zone of 9mm. 8mm was observed against *Staphylococcus aureus*.

White-chaste inhibited the growth of all five bacterial strains with the inhibition zone of 2mm,3mm,6mm, 7mm and 8mm against *Staphylococcus*

aureus, *Vibrio cholerae* (0139), *Aeromonas hydrophila*, *Vibrio cholerae* (01) and *Salmonella typh*i respectively.

Comparing all the three herbal based sanitizers neem and turmeric based hand sanitizer inhibited the growth of bacteria than white-chaste. A new method of making effective sanitizer with herbal products can be a new approach in future.

Plate - 4

SKIN IRRITATION TEST

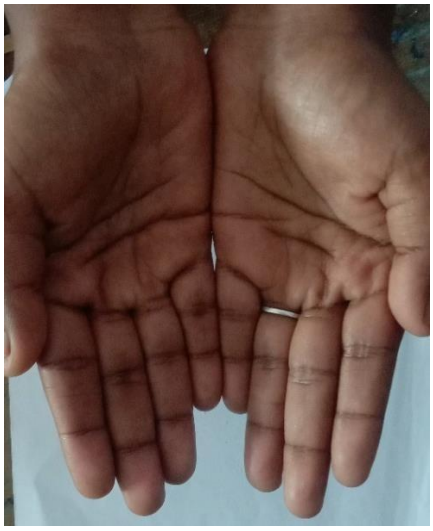
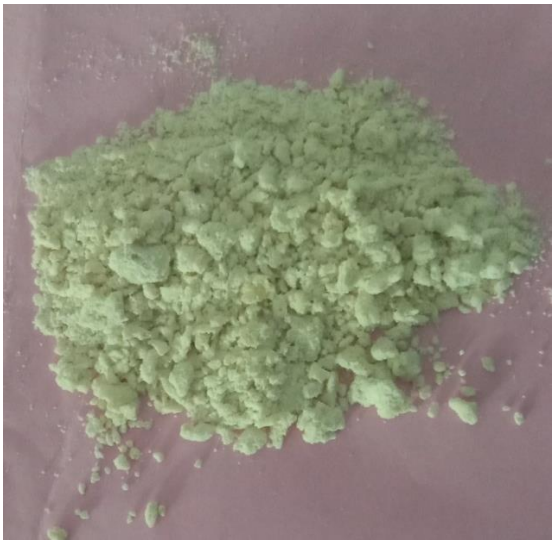


Plate -5

WHEAT DOUGH TEST



TISSUE PAPER TEST

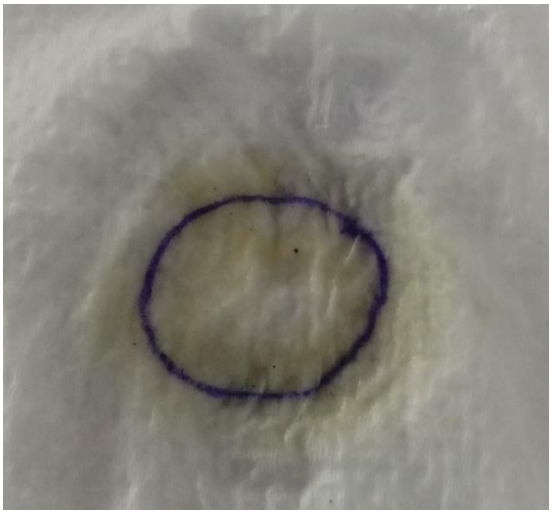
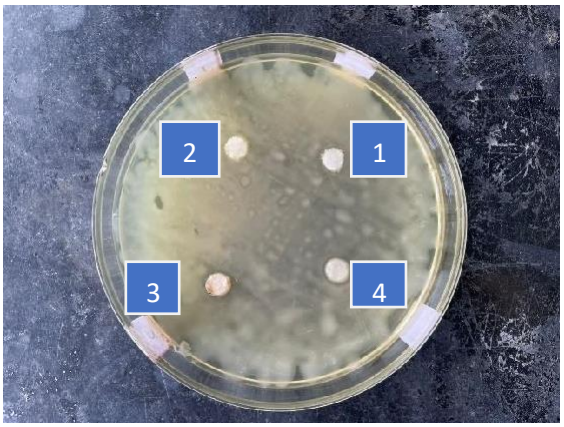
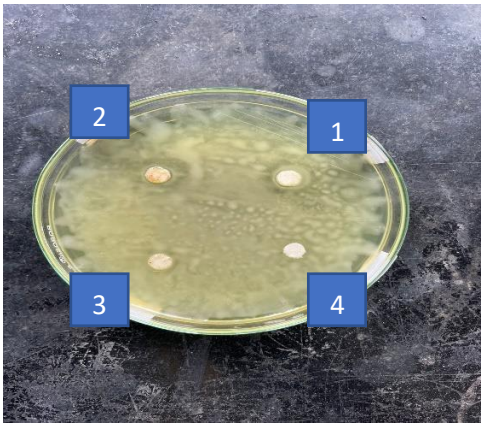


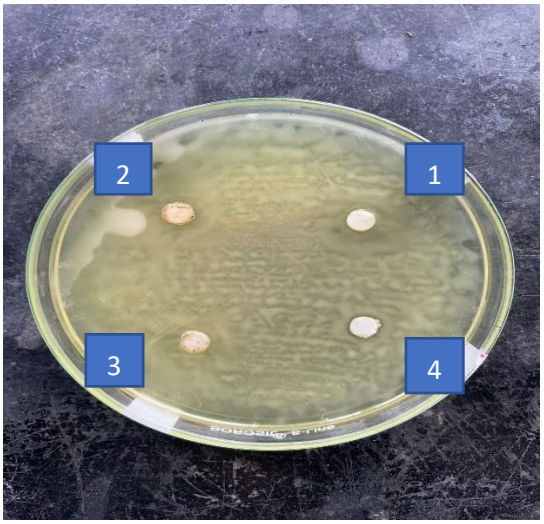
Plate 6: ANTIBACTERIAL STUDY OF HAND-SANITIZER AGAINST VARIOUS PATHOGENS

Vibrio cholerae (01)

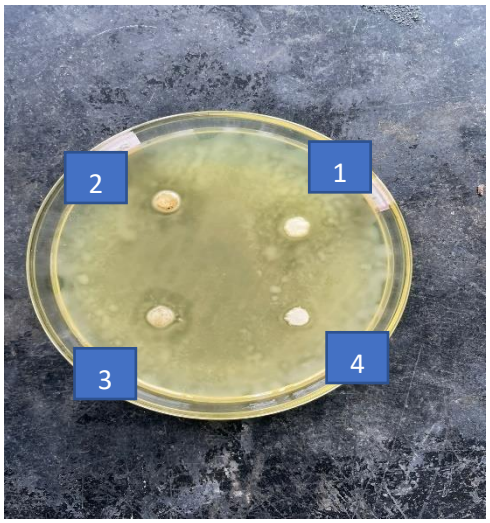


Vibrio cholerae (0139)

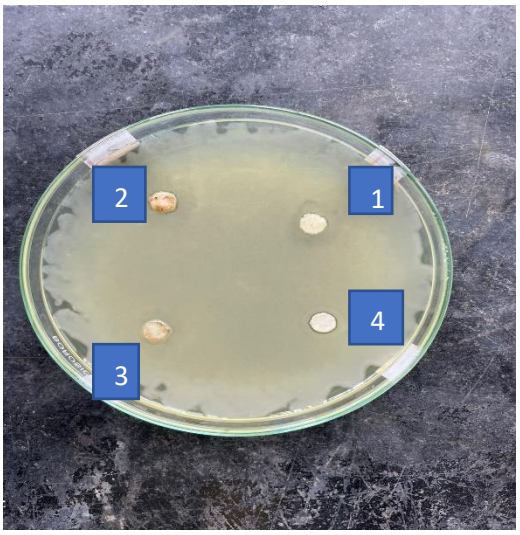




Aeromonas hydrophila



Salmonella typhi



Staphylococcus aureus

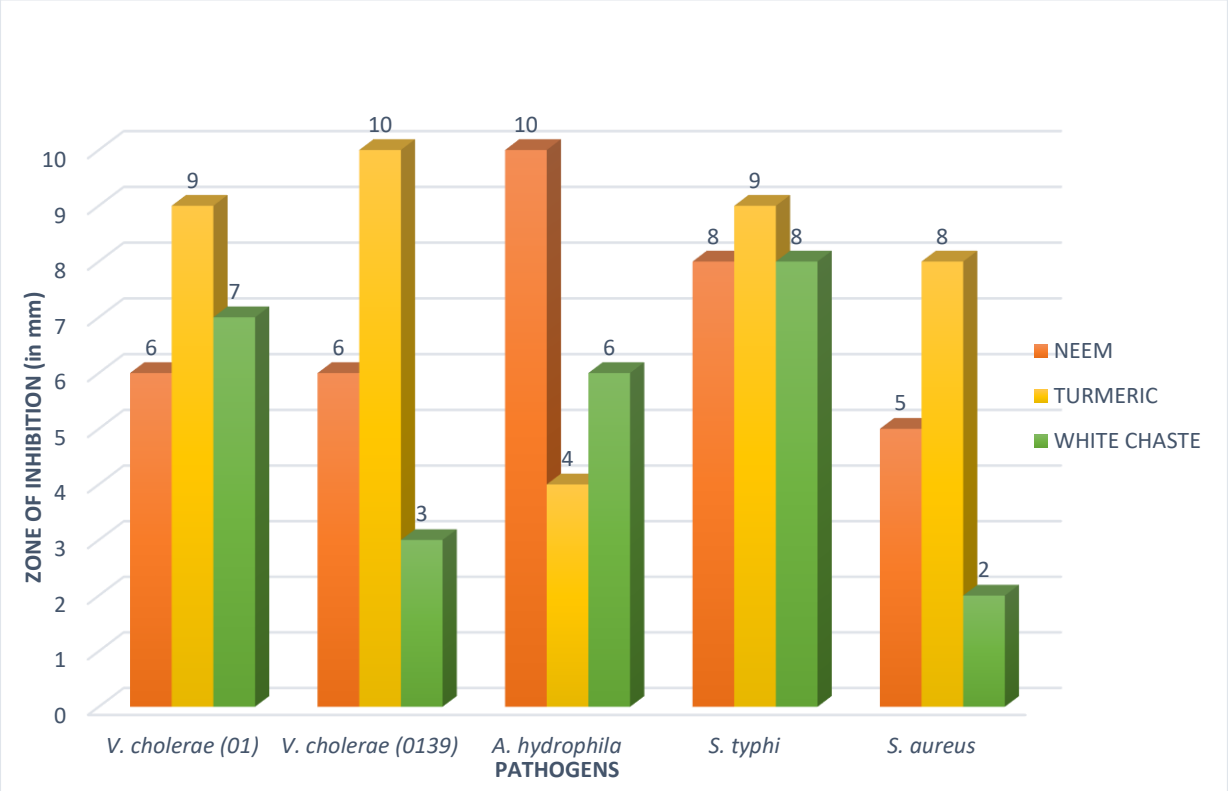
1- Neem based sanitizer

2 - Turmeric based sanitizer

3 - White Chaste

4 - Control

FIGURE 1: ANTIBACTERIAL ACTIVITY OF THE PREPARED HAND SANITIZER



DISCUSSION

Hands are the primary mode of transmission of microbes and infections. Hand hygiene is therefore the most important measure to avoid the transmission of harmful germs and prevent infections. Hand hygiene is the single most important, simplest, and least expensive means of preventing infections. Contaminated hands can serve as vectors for the transmission of microorganisms (Shri Balakrishna Acharya *et al*., 2018).

Hand hygiene is of utmost importance as it may be contaminated easily from direct contact with airborne microorganism droplets from coughs and sneezes. The success of the hand sanitization solely depends on the use of effective hand disinfecting agents formulated in various types and forms such as antimicrobial soaps, water-based or alcohol-based hand sanitizer, with the latter being widely used in hospital settings.

Hand sanitizer can generally be categorized into two groups such as alcohol-based or alcohol-free sanitizer. An alcohol based hand sanitizer may contain one or more types of alcohol, with or without other excipients and humectants, to be applied on the hands to destroy microbes and temporarily suppress their growth. On the other hand, the alcohol-free sanitizer makes use of chemicals with antiseptic properties to exert the antimicrobial effects. These

chemicals have a different mode of action and function according to their chemical functional groups. As they are non-flammable and often used at low concentrations, they are relatively safer to use among children as compared to alcohol-based hand sanitizer (ABHS). To date, most of the effective hand sanitizer products are alcohol-based formulations containing 62%–95% of alcohol as it can denature the proteins of microbes and the ability to inactivate viruses. (Jane Lee Jia Jing *et al.*,2022*).* The liquid herbal hand sanitizer is very easy to prepare, convenient, portable, no side effects and inactivates the microorganisms within few seconds

The use of methanol in hand scrub is not recommended due to its toxic effects and cause severe systemic toxicity, even deaths can occur after oral, pulmonary or skin exposures leads to chronic toxicity (e.g., visual disturbances) if used regularly. Therefore, in place of methanol ethanol, isopropyl alcohol, n-propyl alcohol, or their combinations can be used in alcohol based hand rub (Alan & Thomas, 2018). Therefore, ethanol was selected in the present study to prepare sanitizer.

Hand sanitizers have been heralded as an effective means to reduce bacterial burden and transmission in situations in which soap and water are unavailable to an individual (Chojnacki *et al.,* 2021*).* There is an urgent need and demand of hand sanitizer not only in medical professionals, but also in

common man. Alcohol based sanitizer if used regularly leads dryness of skin and irritation in sensitive people (David *et al*., 2013). So In the present study polyherbal sanitizer was prepared using herbal plant extracts, alcohol, glycerol and camphor. Every ingredient has a specific role in inhibiting the growth of bacteria. Easily available herbal plants used to prepare hand sanitizers with accurate efficacy to reduce microbial load from hands are reported by Huda Ahmed Alghamdi, 2021. Alcohol-based sanitizers are very effective at quickly destroying various pathogens and that too without the need for water, plumbing, and drying facilities (Nina *et al*., 2018). Ravindra Malabadi *et al*., 2021 reported that herbal hand sanitizers with 60% to 70% alcohol were found to be more effective during the recent outbreak of coronavirus disease. (Ravindra Malabadi *et al*., 2021).

Herbal hand sanitizer shows antimicrobial activity against viruses, fungi, pathogens, and bacteria (*E. coli, Pseudomonas aeuroginosa, Staphylococcus aureus,* and *Bacillus subtilis*). Herbal sanitizer prepared from herbal drugs have no side effects as compared to standard. Old drug industry depends on raw materials of medicinal herbs, plant and their extract, which always proved safe (Hagir *et al.,* 2020). The present study has been carried out to find the antibacterial activity of hand sanitizer and found potent as compared to other well-known commercial sanitizer.

In the present study, the herbs selected for the preparation of hand sanitizers are Vitex tree (white chaste tree), *Azadirachta indica (*Neem) and Curcuma (Turmeric) as the main herbs. For the formation of polyherbal hand sanitizer some other herbs are added like tulsi , soapnut, sweet flag, menthol crystal and thymol crystal.Hand sanitizer which was prepared with the help of these herbs were checked anti-bacteial activity against *V.cholerae (01), V.cholerae (0139), A.hydrophila, S.typhi and S.aureus* .

The plants selected for the study were *Ocimum gratissum* (van tulsi), *Ocimum sanctum* (shyama tulsi), *Eucalyptus globules* (niligiri) and *Azadiracta indica* (Neem), *Cuscuta reflexa* (amerbel) *Aloe barbadensis* (Ghritkumari) and *Menthe arvensis* (Mint). The selection of ingredients was on the basis of its medicinal and antibacterial properties reported. The ingredients were also checked for its antibacterial property against bacterial strains *Peudomonas aeroginosa, Bacillus cereus, Streptococcus pyogenes, Klebsiella spp., Escherichia coli, Staphylococcus aureus* and *Dermatophyte* and gave potent activity against all pathogens used for the study (Ganesh *et al.*,2021*)*

Historically, indigenous medicinal plants have been proved and provided the best source of anti-infectious agents. Plants based anti-microbial and antiviral constituents signify a broader source of antiseptics and disinfectants and exhibit the effectiveness against infectious diseases with mitigating a

number of side effects linked with synthetic antiseptic products. Plants with enriched flavonoids and polypeptides have been reported as broad-spectrum antimicrobial and antiviral agents (Abbiw., 1990; Saad *et al*., 2011). Herbal and medicinal plants were used in the present study for the preparation of hand sanitizer and mixture of all extracts showed potent antimicrobial activity against bacterial pathogens.

In the present investigation herbal sanitizer was prepared by using neem, turmeric and white chaste. Sanitizer was tested against five pathogen namely *Vibrio cholerae* (01), *Vibrio cholerae* (0139), *Aeromonas hydrophila, Salmonella typhi and Staphylococcus aureus.* Maximum activity was observed in hand sanitizer prepared with turmeric and neem with the maximum zone of inhibition of 10mm against *Vibrio cholerae (0139*) and *Aeromonas hydrophila.* The minimum zone of 2mm by white chaste against *Staphylococcus aureus.*

Neem also exhibited activity against *Vibrio cholerae (01), Vibrio cholerae (0139), Aeromonas hydrophila, Salmonella typhi and Staphylococcus aureus* of 6mm,6mm,10mm,8mm and 5mm.The zone of inhibition expressed by turmeric was 9mm,10mm,4mm,9mm and 8mm against *V.cholerae(01),V .cholerae(0139), A.hydrophila, S.typhi and S.aureus*. White chaste showed inhibition zone of 7mm,3mm,6mm,8mm and 2mm against *V.cholerae(01), V.cholerae(0139), A.hydrophila, S.typhi and S.aureus* respectively.

In the present investigation, the turmeric-based hand sanitizer is more effective in killing the bacteria than the other sanitizer as it contains more anti-microbial activity when compared to others. The neem and white chaste based hand sanitizer show the average result in destroying the bacteria. The presence of essential oil such as white rose and jasmine helps in enhancing the odor of the hand sanitizer and they also contain some antimicrobial property.

Similar to the present study Mithun *et al.*,2015 observed that the alcohol based herbal hand sanitizer (at concentration 400 μg/ml) showed greater inhibition zones against bacterial species *Escherichia coli*, *Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus*. Kalaivani., 2018 suggested that herbal extracts in mixture giving higher activity than the individual extracts. The combination of the antibacterial compounds from different plant extracts may show synergistic effect enhancing their antimicrobial activity.

The spread of *Staphyloccocus aureus* in various part of the body such as skin, hair and nose causes various infections from minor to fatal diseases. Several studies conducted have also revealed that hand sanitizers containing ethanol or ethyl alcohol produce an impact in reducing microbial growth and are more effective than soap and water (Emma Yaun., 2017)

Vijaya *et al.,* 2021 concluded that herbal hand sanitizer has a significant anti-microbial effect on the specified microorganisms except *P. aeruginosa* and *S. cerevisiae*. Thus, there is immense potential in establishing the use of antimicrobial herbal products as a measure to control multidrug resistant microbes. Rama Sekhara Reddy *et al.,* 2010 reported that chemical analysis of *Sapindus mukurossi* soapnuts revealed the presence of saponins. The soapnut saponin and its derivatives of *Sapindus mukurossi* possess antibacterial and antifungal activity. The saponin showed antibacterial activity against *Bacillus subtilis, Proteus vulgaris* and antifungal activity against *Rhizopus oryzae.*

According to Vijay Kumar Sharma *et al., 2022 Neem*, aloe vera, and tulsi have all been linked to antibacterial action including pathogens *E. coli, P. aeruginosa, K. pneumoniae, S. aureus, M. luteus, S. epidermidis, and C. albicans*. Vaibhav Rajendra Suryawanshi *et al., 2020* demonstrated that herbal hand sanitizer is good in appearance and *Eucalyptus globulus* has an anti-bacterial nature, also Eucalyptus based hand sanitizer has good zone of inhibition. He also mentioned that alcohol-based hand sanitizer having herbal drug can be used effectively in sanitization of hand.

In 2013, Nandkishor *et al.,* formulated and evaluated the herbal sanitizer from the Tulsi leaves extract and niglris leave extract which showed significant anti-microbial effect on the specified microorganisms except *P. aeruginosa* and

S.cerevisiae. Chakraborty *et al*., 2021 prepared the cheap hand sanitizer using aloe vera pulp and guava leaf extract of alcohol based herbal hand sanitizer and its efficiency was tested by tissue paper tests based on paper chromatography and wheat dough test (Chakraborty *et al*., 2021).

The composition (*Azadirachta indica* and *Eucalyptus globulus*) has been attributed with properties like free radical scavenging, antimicrobial, antiinflammatory and analgesic etc. The alcohols have excellent, rapid (within seconds) germicidal activity against vegetative bacteria, fungi, and many viruses and antimicrobial activity is based on protein denaturation of microorganisms. Alcohol sanitizers are highly effective against mycobacteria (the bacteria most resistant to the disinfection process) and multidrug-resistant pathogens. (Rina Maskare *et al.,* 2019*).* Other herbal hand sanitizer, incorporating the leaves extracts of *Ocimum canctum* (Tulsi) and *Eucalyptus globulus* (Nilgiri), the well-known herbal combination with multidimensional activities was formulated and their respective antimicrobial efficacy were studied. The formulation was evaluated against the specified microorganism by culture sensitivity test. The significance was found to be more(Wani *et al.,* 2013*)*

The methanol extracts of *A. citratum*, *C. zeylanicum* and *D. psilurus* showed a synergistic effect with antibiotics inhibiting bacteria. The methanolic

extract of *Acorus calamus* showed the inhibitory action against the bacterial strains of *Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Staphylococcus aureus* (Gilani *et al*., 2006). The third fraction of the crude methanolic extracts has been found to show the highest inhibition against *S. aureus, E. coli* and the fraction is confirmed as β-asarone (Rupali Singh *et al.*, 2011*)*. β-asarone compound of *Acorus calamus* has the highest inhibitory effect against *E. coli* strain at various concentration. The ethanolic and aqueous extract of *Acorus calamus* also showed the inhibitory effect against the above organisms (Umamaheshwari and Rekha., 2018).

SUMMARY

The coronavirus disease 2019 (COVID-19) pandemic has increased dramatically the demand for hand sanitizers. Sanitizers are applied to reduce the pathogenic and spoilage microorganisms. The present investigation evaluated the antibacterial efficacy of herbal based hand sanitizers. This formulation is formulated to maintain hand hygiene. The products showed varying level of inhibition against the test organisms. Different formulations are prepared and characterized using eucalyptus oil as an active pharmaceutical ingredient. S. typhi was the most susceptible to all the products.

The polyherbal hand sanitizer was prepared by adding the ethonolic extract of the herbs .The ethanolic extract was prepared by soaking the grinded herbs in the ethanol for 3-5 days . The ethanolic extract of the herbs was allowed to solidify before adding it to the hand sanitizer . The glycerine and the aloe vera was added as the moisturizer for our skin which is the negative effect by the usage of chemical hand sanitizer.

The hand sanitizer prepared with the help of ethanolic extraction of neem, turmeric and white chaste with aloe vera, glycerin, vitamin E , essential oil and also additional herbs is added like sweetflagpowder,tulsi powder,

soapnut powder, thymol crystal, menthol crystal, lemon and eucalyptus oil shows good anitbacterial property.

The odour of the hand sanitizer is enhanced becausee of the addition of essention oil which provides the pleasant odour.The pH evaluation of the prepared herbal hand sanitizer showed slight acidic which is very good as the surface of the skin is slightly acidic. It can proved by the skin irritability test which was done among our fellow student and it caused no irritation among the students.

The prepared herbal hand sanitizer was found to have greater anti-microbial activity as compared to other hand sanitizers. The sample of turmeric and the neem based herbal sanitizer showed maximum zone of inhibition of 10 mm against *Vibrio cholerae(0139) and Aeromonas hydrophila.*The minimum zone of inhibition was 2mm exibited by white chaste based herbal hand sanitizer against *Staphylococcus aureus.*The turmeric based hand sanitizer showed more effect against all the bacteria.

CONCLUSION AND SUGGESTION

Microbes are mainly transmitted through our hands as they come in contact with the different surfaces. As some microbes are pathogens which may cause life endangering diseases in humans, hand hygiene plays a major role in destroying the microbes present in our hands. Hand hygiene can be maintained through hand washing and also through the usage of hand sanitizer. Hand sanitizer helps in denaturing the protein of the microbes and thereby making the microbes become non-functional.

Hand sanitizer is generally classified into water based and alcohol-based hand sanitizer. Water-based hand sanitizer uses other disinfectants for inactivating the microbes while alcohol helps in denaturing the protein and it is more effective. Hand sanitizer does not require any facilities like hand washing as they require water, soap, towel etc. Hand sanitizer also becomes handy when we are outside or during travel. Continuous usage of chemical hand sanitizer may cause some skin infection, skin irritation and when inhale cause respiratory problems in humans.

Herbal hand sanitizer plays the role of the chemical hand sanitizer in inactivating the microbes and it also plays an important role in keeping our hands free from skin infection and skin irritation. Herbal hand sanitizer can be

easily made with locally available material. It is also eco-friendly and is also very effective against microbes. The essential oil which is used in the herbal hand sanitizer helps in enhancing the odour, give the pleasant feeling to the user and avoid the respiratory problem while inhaling, which is the major default in using the chemical hand sanitizer. Polyherbal hand sanitizer also rich in medicinal properties, and so it provides many benefits to the users like chemical-free and eco-friendly.

The results suggest that the ethanolic extract of neem, turmeric and white-chaste and their combinations with aloe vera, glycerin, and vitamin E, in addition to essential oils (Jasmine,white rose), eucalyptus oil, tulsi powder, sweet flag powder, menthol crystals, thymol crystals, lemon, soap nut powder are capable of giving superior zone of inhibition to protect against the skin pathogens and it can be used as a good hand sanitizer. Thus, there is immense potential in establishing the use of antimicrobial herbal products as a measure to control the multidrug resistant microbes as well as check their spread through hands from one geographical region to another. This herbal hand wash provide an effective and safe alternative to existing marketed synthetic hand wash.

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BIO ACTIVE POTENTIAL OF MARINE PUFFER FISH AROTHRON IMMACULATUS FROM THOOTHUKUDI COAST

A project submitted to

ST. MARY'S COLLEGE (Autonomous), THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

by

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ST. MARY'S COLLEGE (Autonomous),

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

THOOTHUKUDI – 628 001

April - 2023

CERTIFICATE

This is to certify that the project entitled Bioactive potential of marine puffer fish *Arothrone immaculatus* from Thoothukudi coast is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a record of the work done during the year 2022-2023 by the following students.

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CONTENT

INTRODUCTION

1.0 INTRODUCTION

Marine water contains enormous amount of biodiversity, which makes it as a source of huge amount and wide varieties of bioactive components with great therapeutic potential (National research council, 1999). In recent years many bioactive compounds have been extracted from various marine animals, tunicates, sponges, corals and other marine organisms (Jha and Zi-zag 2004). Marine organisms extremely valuable source of functional ingredients such as polysaccharides, vitamins, enzymes, peptides, (Paulo nova *et al.*, 2020). These bioactive molecules possess antibiotic activity. The derived bioactive compounds from seafood helps in building in imbalanced dietary habit and ward off lifestyle related disease (Hosomi *et al.*, 2012). Hence, scientist had an active thirst over the discovery of various drugs from these bioactive compounds. Affirmation is that fish are source of different abundant bioactive compound is a substance that has a biological activity (Najafian *et al.*, 2012).

During respiration of a fish, it generated free radicals and reactive oxygen species. They play a vital role in many diseases including alzheimer's disease, ethanol-induced Steatohepatitis (Chung *et al.*, 2017). The danger of developing chronic ailment such as cardiovascular disease, type 2 diabetes, cancer all leading to mortality are observed to be lowered by increased intake of fish and fish products.

This is due to the presence of bioactive compounds. Fish internal organs constitute approximately 20% of the marine biomass.

The oceans and marine realms with their unique of species including a considerable number of venomous animals are recognized as untrapped reservoirs of molecules and macro molecules that can potentially be converted into highly valuable biopharmaceutical products. These venoms could serve as a research template for development of novel drugs (Morlighem *et al.*, 2019).

Puffer fish belong to the order Tetraodontiformes under the family Tetraodontidae and are variously known as the blow fish, toad fish, globe fish, swell fish and balloon fish (Halstead 1967). These are globally distributed in tropical and temperate sea and fresh waters. They are the most diverse in shallow, warm, tropical and temperate seas with some species entering brackish and freshwater (Alfaro *et al.*, 2007). Puffer fish possess a fatal toxin, this toxin is known as tetrodotoxin (TTX). This is a potent neurotoxin of 100 molecular weight and unique structure determined by three groups in 1964. They have strong toxicity in liver and ovary leading to frequent occurrence of human poisoning. In lower doses, tetrodotoxin has a promising medicinal benefit to treat severe painful diseases.

Now a days the development of resistance by a pathogen to many of the commonly used antibiotics provides an impetus for further attempts to search for new antimicrobial agents to combat infections and overcome problems of resistance and side effects of the currently available antimicrobial agents. Action must be taken to reduce this problem such as controlling the use of antibiotics, carrying out research to investigate drugs from natural resources and also drugs that can either inhibit the growth of pathogen or kill them and have no or least toxicity to the host cell are considered conditions for developing new antimicrobial drugs. The healing of human ailments by using therapeutics that is obtained from animals or ultimately is derived from them. (Kuppulakshmi *et al.*, 2008).

Antibacterial activity is a very complex process that involves living organisms whose every step of life like sustenance, metabolism, respiration and capacity of reproduction could be affected by the presence of toxic substance (Kenawy *et al.,* 2007). The Kirby Bauer method is currently widely used by laboratories and it is a standardized technique for testing pathogen. This testing, measuring the sensitivity of bacteria to antibiotics by culturing bacteria on solid growth media surrounding source of drugs (James bierner, 1973).

Diabetes, correctly termed Diabetes mellitus is a major epidemic of this century which has increased in incidence by 50% over the past 10 years (Shaw *et al.*, 2010). Diabetes is clinically characterized by Hyperglycemia due to chronic or

relative insulin insufficiency (Mathis *et al.*, 2004). Inspite of difficulties in maintaining proper glycemia control with available pharmaceutical approaches, the serious side effects include hypoglycemia, coma, hepatorenal dysfunction (Shanmugam *et al.*, 2011 and Vuksan *et al.*, 2007). Therefore replacing pharmaceutical products with supplementary medicines and diet was of great interest. Marine plants and animals have been widely screened for potentials antihyperglycemic and antidiabetic activities. Derivatives from marine fish have a promising antioxidant and antidiabetic (Bhattacharjee *et al.*, 2014).

Haemolysis in small amount is a normal body process. About 0.8 to 10% of all red cells in the body are haemolysed each day. It is usually balanced by red cell production in the marrow of bones. But sometimes, so many cells breakdown that marrow production is insufficient and anaemia may result. Chemical poison may cause excessive haemolysis. Many biotoxins are known to cause haemolysis of RBC. Haemolytic activity has been observed in many of the tissue products of aquatic organisms including fish, molluscs, algae, etc. Most cytotoxic have considerable potential as anticancer and antiviral agents (Shier, 1988).

The marine environment contains vast array of organisms with unique biological properties but it is one of the most underutilized biological resource. The potential of puffer fish as a source of biologically active products is largely unexplored. Hence a broad based screening of puffer fish for bioactive compounds

is necessary. Thus the present study was intended to analyse the various therapeutic values such as antimicrobial, haemolytic, and antidiabetic activities of pufferfish *A. immaculatus* collected form Thoothukudi coast.

2.0 OBJECTIVES

The objectives of the present study are,

- to study the antibacterial activity of acetic acid extracts of ovary, skin and intestine of marine puffer fish *A. immaculatus* on ten bacterial strains.
- to evaluate the antifungal activity of acetic acid extracts of ovary, skin and intestine of *A. immaculatus* on fungal pathogen *Aspergillus niger*.
- to analyse the haemolytic activity of acetic acid extracts of ovary, skin and intestine of *A. immaculatus* on chicken blood cells.
- to find out the antidiabetic activity of acetic acid extracts of ovary, skin and intestine of *A. immaculatus*.

REVIEW OF LITERATURE

3.0 REVIEW OF LITERATURE

The enormous potential of the sea as a source of energy, food and chemical has led to its being the subject of intense research. Marine natural product represents a vast potential source of new drugs and many interesting biological properties.

Ning-ping Tao (2014) reported that GC-MS analysis of farmed puffer fish (*Takifugu obslurus)* possessed volatile active compounds.

Hye-Jingo *et al.*, (2019) revealed that the skin mucous extract had antibacterial activity against *B.subtilis* , *S.iniae E.coli* and *V.anguillarium.*

Ahamed Alabssawy *et al.*, (2017) focused on the antibacterial activity of pufferfish *L.sceleratus* which showed maximum activity against *E.coli* and maximum antifungal nature against *Aspergillus fumigatus.*

Yi-huan wang *et al.*, (2017) studied that the different tissues of puffer fish had antimicrobial profile against *E.faecelius.*

Tae young him *et al.*, (2017) reported that antibacterial peptide from gills of puffer fish (*Takifugus pardakis*) has potent antibacterial activity against *Bacillus subtilis.*

Indhumathi *et al.*, (2016) reported that *T.oblongus* crude extract exhibited excellent biological properties for the development into novel potential drugs.

Analiza Molina *et al.*, (2016) stated that skin and intestine of *Lagocephalus sapadiceus* exhibited antibacterial activity on pathogen *Staphylaococcus aureus* and *Klebsiella pneumonia.*

Jun-Yan Jin (2012) reported that β – defensin of green puffer fish had antibacterial activity against *Vibrio flurialis.*

Yijia Tang *et al.*, (2017) determined that cultured puffer fish (*Takifugu obscurus*) muscle had fluorquinolones (FQ) which are widely used as antimicrobial agents.

Alifakhri (2016) reported that tetradoxin as a nanocomposite had powerful antibacterial activity against *Staphylococcus aureus* than *E. coli.*

Sasikala *et al.*, (2013) focused on the spine and gonad extract of *Scorpaenopsis venosa* that inhibited the growth of *Rhizoto solani* showing antifungal activity.

Mohana Priya *et al.*, (2013) stated that acetic acid extract of liver, skin, muscle, tissues of *Arothron hispidus* had inhibitory activity against various bacterial and fungal strains.

Manal *et al.*, (2012) determined that the TTX toxin puffer fish of *Lagocephalus sceleratus* has potential use in pharmaceutical industry.

Ramasamy Santharan *et al.*, (2017) reported that the toxins of puffer fish has emerging uses in treatment of pain.

Karunanidhi *et al.*, (2019) demonstrated that *Arothron immaculatus* muscle extract recovered the diabetic rat showing antidiabetic potential.

Rushikesh Sable *et al.*, (2017) studied that peptide from marine sources had potential antibacterial, antifungal and antidiabetic activities.

Bragadieswaran *et al.*, (2010) revealed that pufferfish *Arothron hispidus* had potent haemolytic activity.

Xiao Jun Zhang *et al.*, (2009) determined that *Takifugu rubridus* had antimicrobial nature.

Anguchamy Veeruraj *et al.*, (2016) reported the haemolytic activity of *Arothron stellatus* TTX toxins.

Masilamani Mohan Raj *et al.*, (2015) observed the highest haemolytic activity of *Cychichthys orbicularis* and *Arothron hipidus* in human blood cells.

Sangeetha Subramanian *et al.*, (2008) demonstrated that the mucous of brook trout haddock and hagfish had potential source of novel antimicrobial properties.

Sunil Kumari *et al.*, (2019) evaluated that the *Hypophthalmichthys nobilis*, *Crypinus carpio* exhibited strong antibacterial activity against various pathogenic bacterial strains.

Fatma krichen *et al.*, (2015) studied the antibacterial activities of sulfated polysaccharides from skin of triggerfish with clear zone of growth inhibition.

Eleonor Tendencia *et al.*, (2004) reported that the antibacterial activity of *Tilapia hornorum* against *Vibrio harveyi.*

Boyufu *et al.*, (2020) stated that the mucous and serum of coho salmon, *Oncorhynchus kisutch* inhibited the growth of *Salmonicida* and *Salmonella enterica.*

Kumar *et al.*, (2015) reported that the skin hydrolysate of puffer showed antimicrobial activity against *Staphylococcus aureus, Enterobacter cloacae, E.coli* and *Klebsiella pneumonia.*

Fanghua Wang *et al.*, (2011) reported that serum of rabbit fish (*Siganus oramin*) was inhibited a wide range of Gram negative and Gram positive bacteria.

Dhinakaran *et al.*, (2015) concluded that *Holothuria atria* showed highest activity against *Candida albicans.*

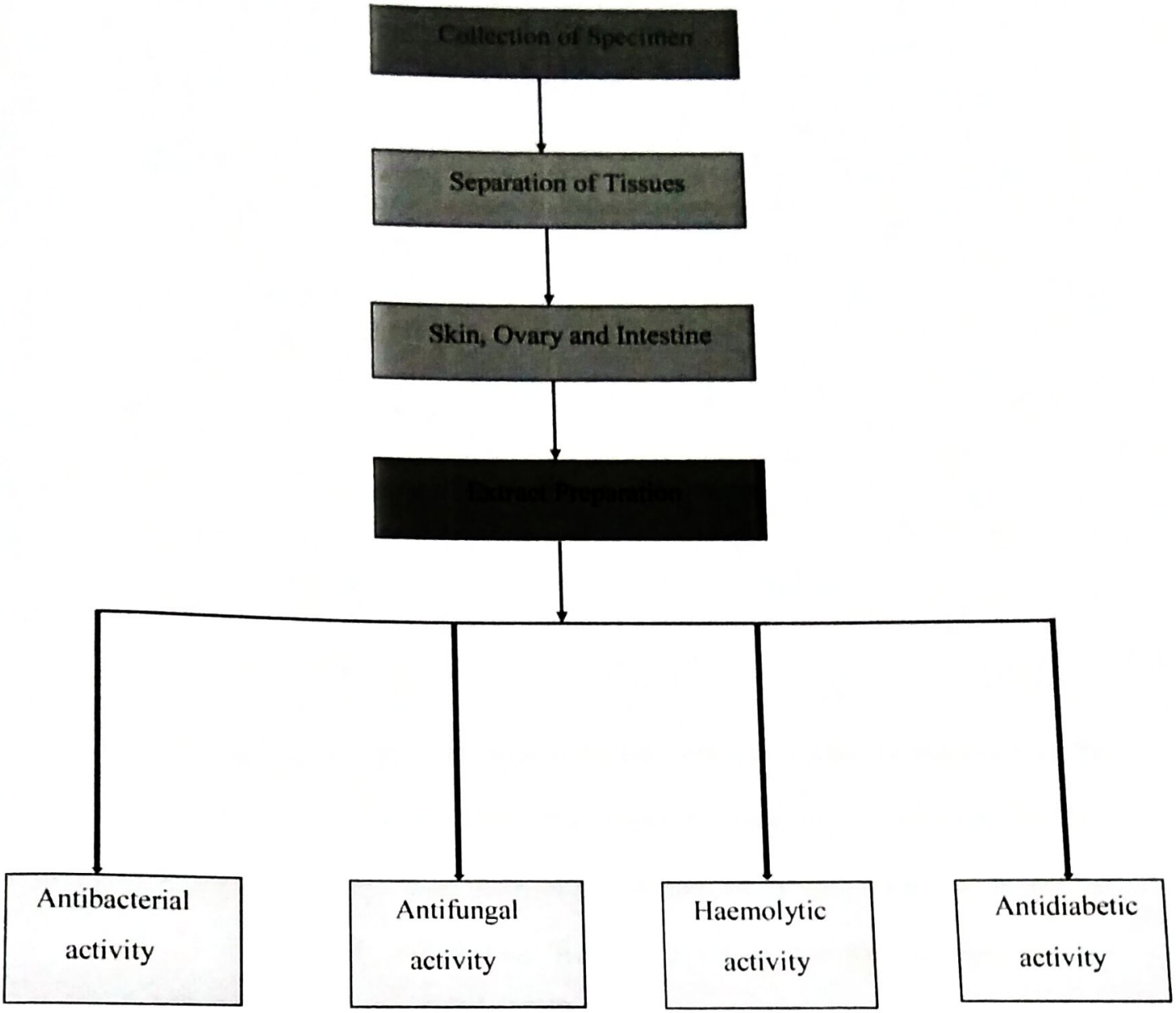
Srikumaran *et al.*, (2011) demonstrated that butanol extract of molluscs *Babylonia spirata* exhibited high zone of inhibition against bacterial pathogen *Proteus mirabilius* and fungal pathogen *Candida albicans*.

Kasitowati *et al.*, (2019) determined the antifungal activity of marine spores *Fascaplysinopsis* and *Haliclona* species against *Candida albicans* and *Aspergillus flavus*.

Rafat *et al.*, (2015) investigated antifungal and antibacterial activities of some marine organisms using disc diffusion method.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN



4.0 MATERIALS AND METHODS

4.1 Systematic Position of Experimental Animal

Kingdom : Animalia

Phylum : Chordata

Class : Actinopterygii

Order : Tetraodontiformes

Family : Tetraodontidae

Genus : Arothron

Species : immaculatus

Class: Actinopterygii

The Actinopterygii or ray-finned fishes constitute a class or sub-class of the bony fishes. The ray-finned fishes are so called because they possess lepidotrichia or "fin rays" , their fins being webs of skin supported by bony or horny spines , as opposed to the fleshy lobed fins. Actinopterygiians are thedominant class of vertebrates comprising nearly 99% of the over 30,000 species of fish. They are ubiquitous throughout freshwater and marine environment from the deep sea to the highest mountain streams. Traditionally they have been divided into the subclass

Chondrostei and Neopterygii. Neopterygii in turn have been divided into infra-class Holostei and Teleostei. Ray-finned fishes constitute a major source of food for millions of people.

Order:-Tetraodontiformes

Tetraodontiformes any member of a group of primarily tropical marine fishes that are closely related to the perciformes (the typical advanced spiny-rayed fishes).It includes the trigger fishes, puffers, filefishes and porcupine fishes. It is notable for a high degree of diversity in anatomical structure and way of life. They are found in temperate and tropical marine waters worldwide. It is associated with coral and rocky reefs and shallow bottoms with a few pelagic species.

Family:-Tetraodontidae

The species that come under this family are well known for its unique and distinctive adaptations that the puffer fish has to defend itself. They are the second most poisonous creature on the planet. The puffer's remarkable ability to expand its body extremely quickly when forced with danger, unavailing its long poisonous spikes that cover its body. They can be found in a variety of colour but can sometimes be hard to identify when they are not inflated. The puffer normally has the appearance of a large tadpole, with bulging eyes and an elongated snout. They are omnivorous and they mainly feed on the algae that grow on the rocks and Corals.

***Arothron immaculatus* Bloch and Schneider, 1801 (Plate 1)**

The immaculatus puffer is a type of puffer fish from the Indo Pacific. It captures the essence of the specialized nature of the puffer that is classic and elegant in design. The immaculatus puffer has a large pillowy body that is predominantly milky white with neutral overtones of varying shades of brown and black. Members of the genus *Arothron* including the immaculatus puffer are sometimes called "Fat puffers" and exemplify the typical puffer design. Though lacking pelvic fins and a hydrodynamic body shape, puffers are extremely maneuverable and are tenacious and capable predators. Like many "fat puffers" the immaculatus puffer displays an unusual degree of intelligence and personality and appear to quickly recognize their owners. The diet of immaculatus puffer should include a variety of meaty food including squid, krill, clams and hard shelled shrimps to help to wear down their ever growing teeth. It reaches the size of 30 cm in length.

4.2 Collection of Specimen

Specimens of puffer fish *Arothron immaculatus* were collected from fish landing centre at fishing harbour, Thoothukudi. Then they were washed with sea water and transported to the laboratory in dry ice and was stored in the deep freezer at-20°C.

4.3 Preparation of acetic acid extract:

Plate – 1

Arothron immaculatus

Specimens of *A.immaculatus* was thawed and dissected into tissues like skin, ovary and intestine. Ten grams of each tissue was homogenised with 50 ml of 0.1% acetic acid and were kept in water bath around 45°C for 10 minutes, cooled and centrifuged off. Then it was stored in the deep freezer at -20° for further use (Kawabata, 1979).

4.4 Antibacterial Activity:

Bacterial Strains:

The reference strains of pathogens used to test antibacterial activity are

Bacillus cereus (BC)

Vibrio cholerae (01) VC (01)

Vibrio cholerae (0139) VC (0139)

Escherichia coli (EC)

Pseudomonas aeruginosa (PA)

Aeromonas hydrophila (AH)

Salmonella typhi (ST)

Shigella flexneri (SF)

Pseudomonas sp (P.sp.)

Staphylococcus aureus (SA)

Broth Culture:

A broth culture is a nutrient solution in which bacteria are being grown.

Broth Medium:

Nutrient broth - 1.3g

Distilled water- 100 ml

2 to 3 ml of sterilized broth medium was taken in a sterilized culture tube. The inoculating loop was flamed and cooled for few minutes. A loopful of each strain was transferred into the individual culture tubes and incubated at room temperature.

Muller-Hinton Agar medium:

Beaf infusion. -30g

Casein acid hydrolysate -17g

Agar -17g

Distilled water - 1000 ml

Muller Hinton agar was suspended in 1000 ml of distilled water and the pH was adjusted to 7.4 ± 0.2. The medium was boiled to dissolve completely and sterilized by autoclaving at 120°C for 15 minutes.

Agar diffusion technique

The antibacterial activity of the acetic acid extracts of ovary, skin and intestine of puffer fish *A.immaculatus* was determined by the standard agar well diffusion assay by using the technique of Perez et al., (1990). Petri plates were prepared by pouring approximately 20 ml of Muller Hinton Agar Medium and allowed to solidify. After solidification, culture of each microbial strain was swabbed to sterile cotton on the surface of medium. Wells of 5 mm diameter were punched using sterilized cork borer. The acetic acid extracts of ovary , skin and intestine of different concentrations (2µl , 4µl , 6µl, 8µl) were loaded in each well . The plates were incubated for 24 hours at 37°C and control was performed in each case. Areas of inhibited microbial growth were observed as clear zone around the well after 24 hours.

4.5 Antifungal Activity

Media preparation

The growth media employed in the present study was potato dextrose agar (PDA).

Potato dextrose agar

Potato – 200 gm

Dextrose – 20 gm

Agar – 15 gms

Distilled water – 1000 mL.

pH – 5.6±2.

The prepared media was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Antifungal activity screening test

The antifungal activity of acetic acid extract of ovary , skin and intestine pufferfish *A. immaculatus* was performed against the fungal strain *Aspergillus niger* by using the agar-well diffusion method.(Magaldi et al.,2004) The PDA medium was poured into sterile petri plates. Sterile cork borer was used to make a well on each of the plates. The 50 µL of extracts,(skin,ovary and intestine) positive and negative controls were poured into each well. The plates were incubated at 28°C for 48-72 hrs. The zone of inhibition was measured after the completion of incubation period and recorded in millimeters (mm).

4.6 Hemolysis assay

The assay was carried out according to Oyedepo and Femurewa (1995) .3 ml of chicken blood was collected in a heparinized tube and centrifuged at 2000rpm for 5minutes. The supernatant was discarded and the cells were resuspended in cold PBS. Aliquot 0.1 ml of blood sample in test tubes. Different concentrations (200 µg/ml,400 µg/ml,600 µg/ml,800µg/ml,1000 µg/ml) of skin , ovary and intestine extracts were added to the test tubes and made it equal volume using PBS. The tubes were heated to 40°C for 10 minutes and cooled . The absorbance was measured at 540 nm.

% Inhibition = Abs Control- Abs sample/Abs Control ×100.

4.7 Alpha amylase inhibition assay- Antidiabetic activity

Antideabetic activity of various tissue extracts of puffer fish *A.immaculatus* was determined following the method of Nickavar and Yousefian(2009).Acetic acid extracts of ovary , skin and intestine of *A. immaculatus* were used to test antidiabetic activity. Different concentration of the extracts (200 µg/ml, 400µg/ml, 600 µg/ml,800 µg/ml and 1000 µg/ml) of samples were taken in separate test tubes. 1ml of 1% starch followed by 0,5ml of amylase enzyme were added to control and test sample tubes and were incubated at 37°C for 30 minutes. After the incubation,

1ml of DNSA reagent was added to all the test tubes and the tubes were heated at 95 °C for 15 minutes. The absorbance was measured at 510 nm.

% Inhibition = Abs Control – Abs sample/ Abs Control ×100.

RESULTS

5.0 RESULTS

5.1 Effect of acetic acid extract of ovary of *Arothron immaculatus* on bacterial strains

All bacterial strains were sensitive to ovary extract of *A. immaculatus* (Plate 2). Clear zone of inhibition with 10.5 mm, 10mm, 6mm and 6mm radius at 8 µl, 6µl, 4µl and 2µl respectively was observed in *Pseudomonas sp. B.cereus* and *S. typhi* were sensitive with zone of inhibition 10mm at 8µl. *E .coli* showed zone of inhibition with 7.5 mm radius at 2µl and 10mm radius at 4µl, 6µl and 8 µl concentrations. The ovary extract produced zone of inhibition ranging from 5 to 8.0 mm radius in *P. aeruginosa. A . hydrophila* and *V. cholerae (01)* showed zone of inhibition with 6mm radius at 4µl ,6µl and 8µl concentrations. *V.cholerae* (0139) produced zone of inhibition with 5mm radius at 4µl and 6µl and 6mm radius at 8 µl concentration. *S.flexneri* was sensitive with zone of inhibition 7mm radius at 2µl and 4µl and 7.5mm radius at 6µl and 8µl concentrations. *S. aureus* was found to be sensitive with zone of inhibition 6mm, 7mm,. 7mm and 7.5 mm radius at 2µl, 4µl , 6µl and 8µl concentrations respectively (Table 1) .The mean zone of inhibition were 5.3 ,4.5 ,4 ,9.4 ,6,7,6.8,7.4,8.2,6.8 in BC, VC (01) ,VC (0139), EC, PA ,AH, ST ,SF ,P sp and SA respectively (Fig.1).

Plate 2: Muller Hinton Agar Plates showing antibacterial activity of acetic acid extract of ovary of *Arothron immaculatus* on bacterial strains

ZI – zone of inhibition , a – 10mg /10 µl , b – 1.0mg/10µl , c – 0.1mg/ 10µl , d – 10 mg /10µl

Table 1: Activity of ovary extract of *Arothron immaculatus* against bacterial strains

Bacterial strains	control	2µl	4µl	6µl	8µl
BC	-	-	+	+	++
VC	-	-	+	+	+
VC(01)	-	-	+	+	+
EC	-	++	++	++	++
PA	-	+	+	+	+
AH	-	+	+	++	++
ST	-	+	+	++	++
SF	-	+	+	++	++
P.sp	-	+	+	++	+++
SA	-	+	+	+	++

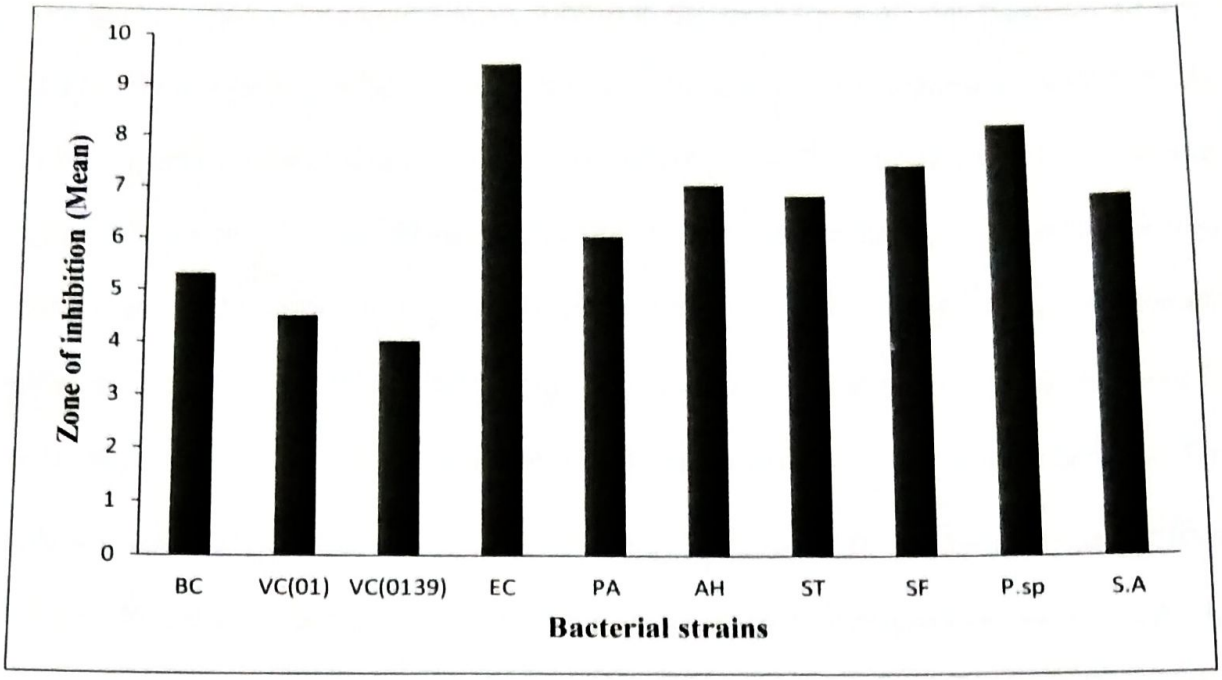
Zone of inhibition (mm) radius

+ - 5 - 7

++ - 7 - 10

+++ - 10 - 13

Figure 1: Antibacterial activity of ovary extract of *Arothron immaculatus*



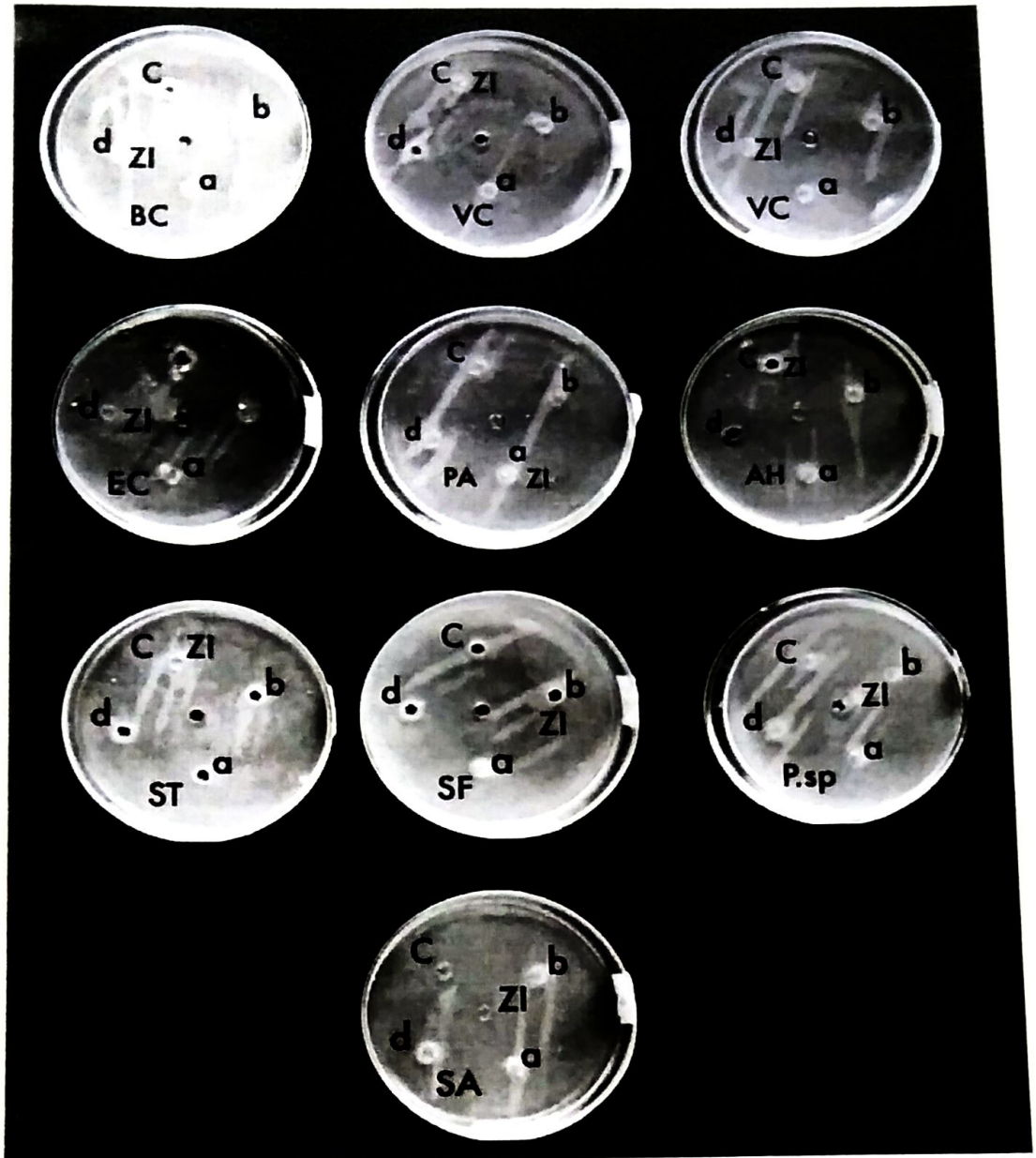
5.2 Effect of acetic acid extract of skin of *Arothron immaculatus* on bacterial strains

The skin extract of *A.immaculatus* inhibited the growth of all the bacterial strains tested (Plate 3) *P.aeruginosa* and *S.aureus* produced zone of inhibition with 6.5mm radius at 8µl concentration. *S .typhi* was sensitive with zone of inhibition ranging from 3.5mm to 6.0 mm radius. The zone of inhibition ranging from 4 to 5.5 mm radius in *V.cholerae (01)* ,*V.cholerae (0139)* and *Pseudomonas sp, S.flexneri* produced zone of inhibition with 5mm radius at all concentrations. *B. cereus* and *E. coli* were sensitive with zone of inhibition 5mm radius at 8µl concentration. The extract inhibited the growth of *A.hydrophila* with zone of inhibition ranging from 3.5mm to 4.0 mm radius (Table 2) .The mean zone of inhibition were 4.4 ,4.6 ,4.7 ,4.2 ,4.6 ,3.8 ,5.2 ,5, 5.4 ,6 in BC , VC (01) , VC (0139) ,BC ,PA ,AH ,ST, SF, P.sp and SA respectively (Fig 2).

5.3 Effect of acetic acid extract of intestine of *Arothron immaculatus* on bacterial strains

The intestine extract of *A.immaculatus* exhibited inhibiting activity against all bacterial strains (Plate 4) *S .flexneri* was sensitive with zone of inhibition 5.0 mm radius at 8µl concentration. The zone of inhibition ranging from 3.5 to 4.5mm radius in *A. hydrophila* and 4 to 4.5 mm radius in *V. cholerae* (01) and *P.aeruginosa*,

Plate 3: Muller Hinton Agar Plates showing antibacterial activity of acetic acid extract of skin of *Arothron immaculatus* on bacterial strains



ZI – zone of inhibition , a – 10mg /10 µl , b – 1.0mg/10µl , c – 0.1mg/ 10µl , d – 10 mg /10µl

Table 2: Activity of skin extract of *Arothron immaculatus* against bacterial strains

Bacterial strains	control	2µl	4µl	6µl	8µl
BC	-	+	+	+	+
VC	-	+	+	+	++
VC(01)	-	+	+	+	++
EC	-	+	+	+	+
PA	-	+	+	+	++
AH	-	+	+	+	+
ST	-	+	+	++	++
SF	-	+	+	+	+
P.sp	-	+	++	++	++
SA	-	+	++	++	++

Zone of inhibition (mm) radius

+ - 3 - 5

++ - 5 - 7

Figure 2: Antibacterial activity of skin extract of *Arothron immaculatus*

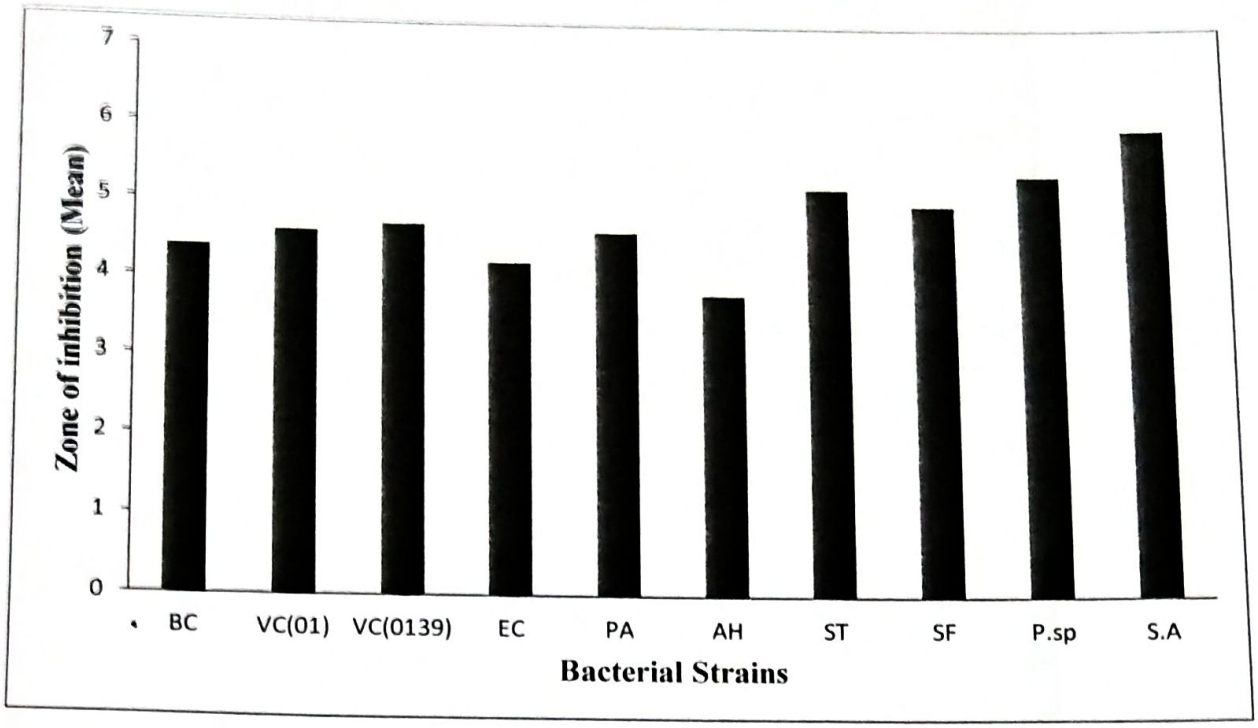
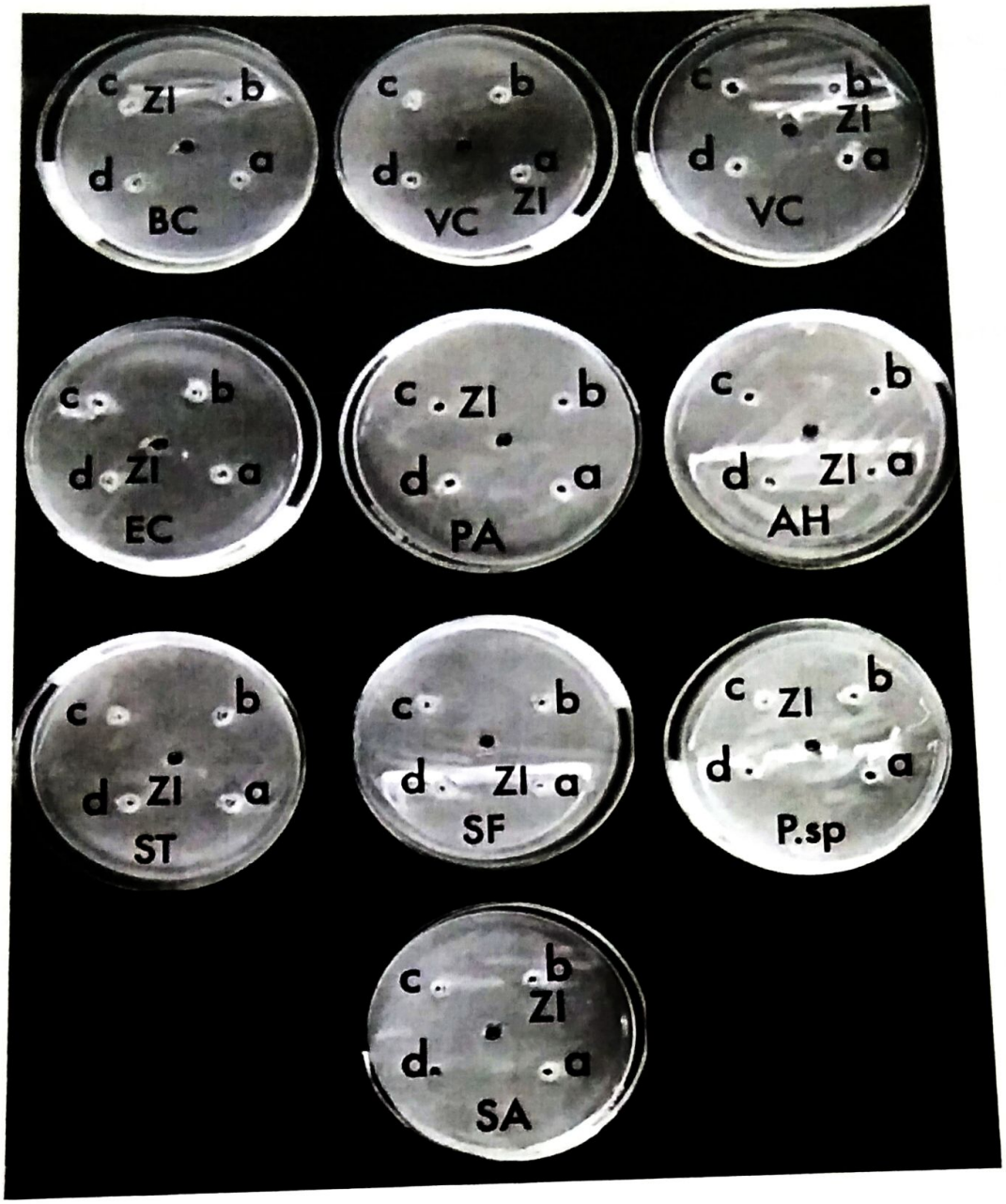


Plate 4: Muller Hinton Agar Plates showing antibacterial activity of acetic acid extract of intestine of *Arothron immaculatus* on bacterial strains



ZI – zone of inhibition , a – 10mg /10 µl , b – 1.0mg/10µl , c – 0.1mg/ 10µl , d – 10 mg /10µl

Pseudomonas sp and *S. aureus* were sensitive with zone of inhibition ranging from 3 to 4 mm radius. The extract produced zone of inhibition with 3.5 mm radius at 4µl and 6µl and 4.0 mm radius at 8 µl concentration. In *V.cholerae* (0139), the zone of inhibition ranging from 3 to 3.5 mm radius in *E.coli* and *S.typhi* (Table 3) .The mean zone of inhibition were 2.6 , 2.2 ,2.7 ,3.3 ,4.2 ,4.2 ,3.3 ,4.4,3.5 ,3.6 in BC ,VC (01) ,VC (0139),EC ,PA ,AH ,ST ,SF ,P.sp and SA respectively (Fig 3).

5.4 Effect of acetic acid extracts of various tissues of *Arothron Immaculatus* on *Aspergillus niger*

Acetic acid extracts of skin, ovary and intestine of *A.immaculatus* were subjected to antifungal activity against *A. niger* at 50µg concentration. The antifungal activity of extracts of various tissues were compared with standard antibiotic Fluconazole (50µg). Acetic acid extracts of skin, ovary and intestine produced zone of inhibition with 7.5mm, 6mm and 5.5 mm radius respectively. (Plate 5, Table 4).

5.5 Hemolytic activity of acetic acid extract of skin of *Arothron immaculatus*

Skin extract showed hemolytic activity of 0.71%, 0.71%, 14.29%,22.14%, and 22.14% at 200 µg/ml,400 µg/ml,600 µg/ml,800 µg/ml and 1000 µg/ml respectively. Intestine and egg extracts unable to lyse the cells. (Plate 6, Fig 4).

Table 3: Activity of Intestine extract of *Arothron immaculatus* against bacterial strains

Bacterial strains	control	2µl	4µl	6µl	8µl
BC	-	-	+	+	+
VC	-	-	-	+	+
VC(01)	-	-	+	+	+
EC	-	+	+	+	+
PA	-	+	+	+	+
AH	-	+	+	+	+
ST	-	+	+	+	+
SF	-	+	+	+	+
P.sp	-	+	+	+	+
SA	-	+	+	+	+

Zone of inhibition (mm) radius

+ - 3 - 5

Figure 3: Antibacterial activity of intestine extract of *Arothron immaculatus*

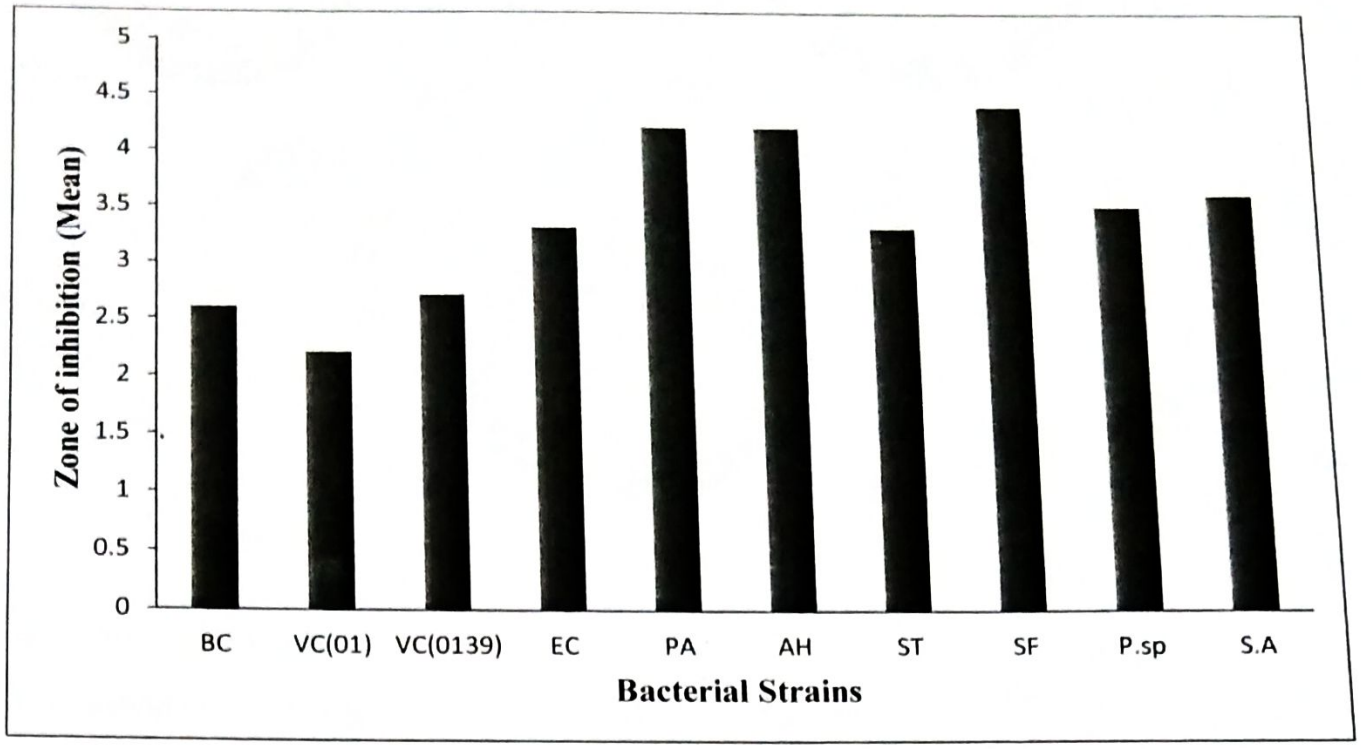
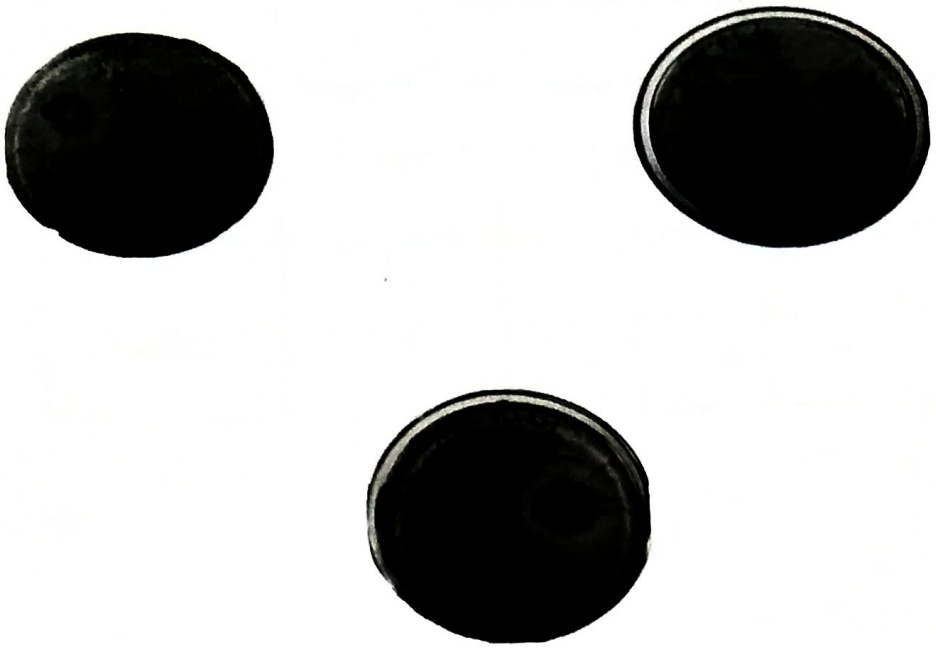


Plate 5 : Activity of skin, ovary and intestine extracts of *Arothron immaculatus* against *Aspergillus niger*



S - skin (50 µg)

I - intestine (50 µg)

E - ovary (50 µg)

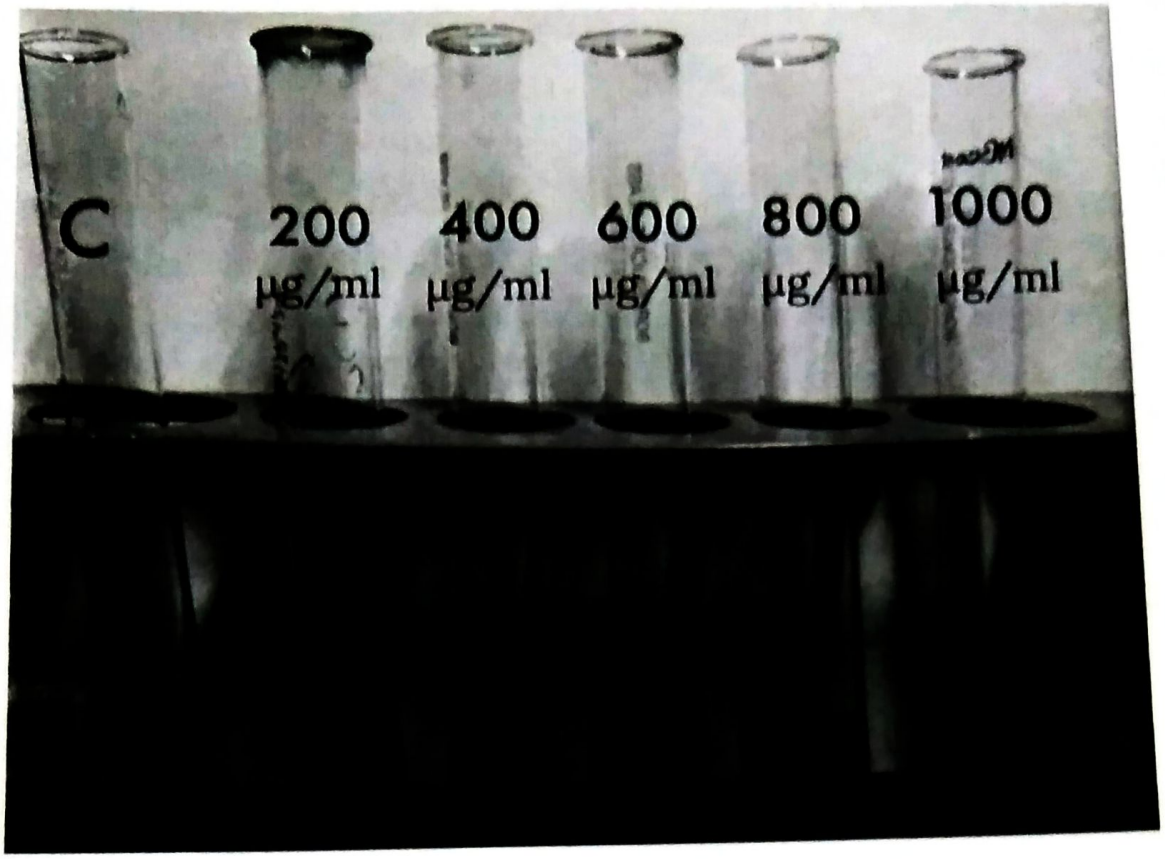
C – Positive control

N – Negative control

Table 4; Antifungal activity of skin , ovary and intestine extracts of *Arothron immaculatus* against *Aspergillus Niger*

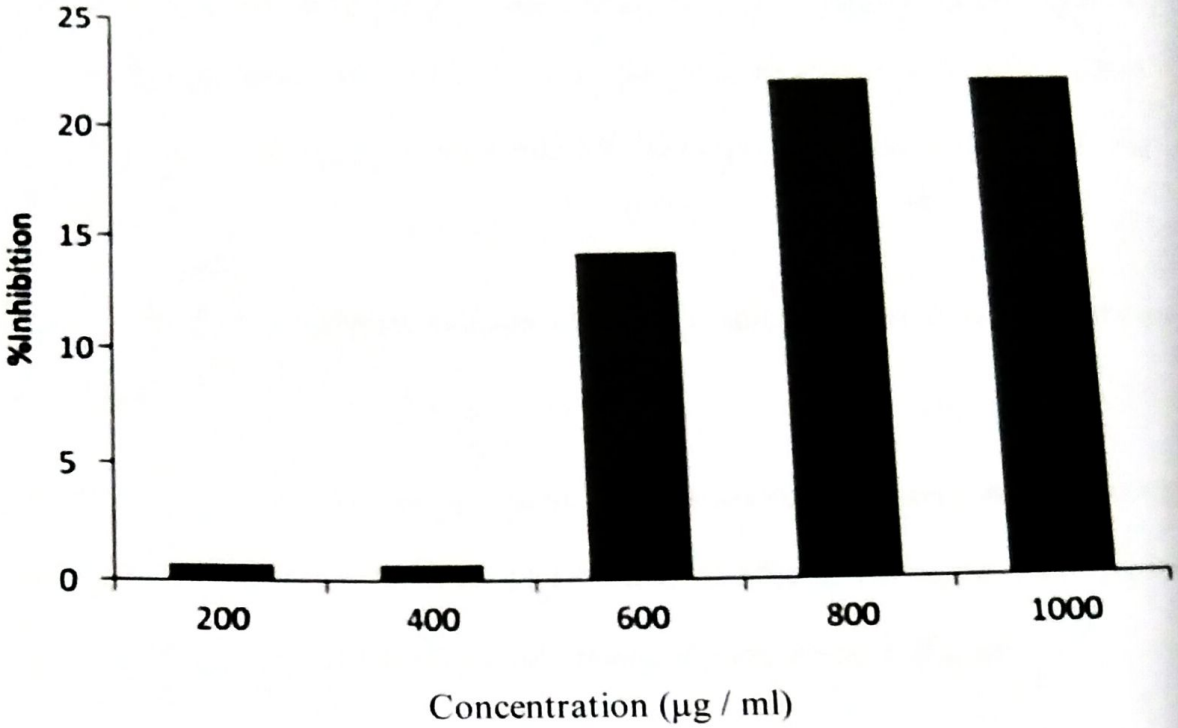
Extracts	Zone of inhibition (mm radius)		
	Sample(50 µg)	**Positive control**	**Negative control**
Skin	7.5	6	-
Ovary	6	8.5	-
Intestine	5.5	8	-

Plate 6 : Photograph showing the effect of acetic acid extract of skin of *Arothron immaculatus* on chicken blood



C - Control

Figure 4 : Percentage of hemolytic activity of acetic acid extrat of skin of *Arothron immaculatus*



5.6 Alpha amylase inhibitory activity of acetic acid extract of ovary of *Arothron immaculatus*

Acetic acid extract of ovary of *A. immaculatus* showed inhibitory activity against alpha amylase enzyme with 9.17%, 23.33%, 34.17%, 49.17%, and 54.17% at 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml,and 1000 µg/ml concentration (Plate7, Fig 5).

5.7 Alpha amylase inhibitory activity of acetic acid extract of skin of *Arothron immaculatus*

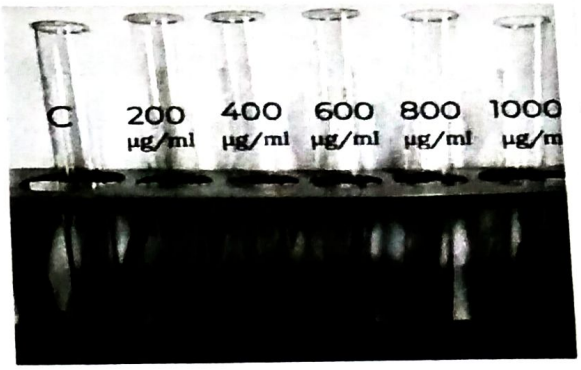
Acetic acid extract of skin of A. immaculatus showed alpha amylase inhibitory activity of 4.16%, 9.17%, 23.33%, 34.17% and 42.50% at 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml, and 1000 µg/ml concentrations (Plate 7, Fig 6).

5.8 Alpha amylase inhibitory activity of acetic acid extract of intestine of *Arothron immaculatus*

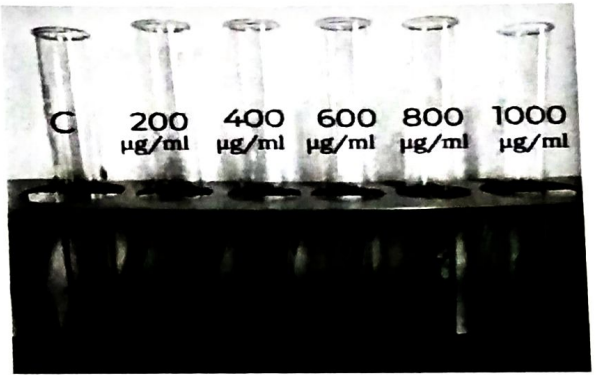
Acetic acid extract of intestine of *A. immaculatus* inhibited alpha amylase enzyme with 9.17%, 16.67%, 23.33%, 34.17% and 43.33% at 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml, and 1000 µg/ml concentrations (Plate 7, Fig 7).

Plate 7 : Effect of acetic acid extract of *Arothron immaclatus* on alpha amylase enzyme

Ovary



Skin



Intestine

C – Control

Figure 5 - Alpha – amylase inhibitory activity of acetic acid extract of ovary of *Arothron immaculatus*

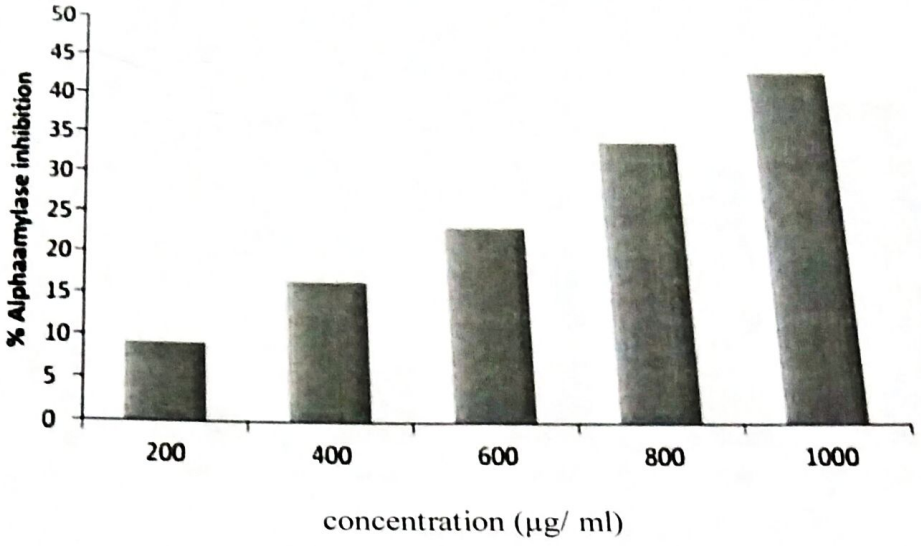


Figure 6- Alpha – amylase inhibitory activity of acetic acid extract of Skin of *Arothron immaculatus*

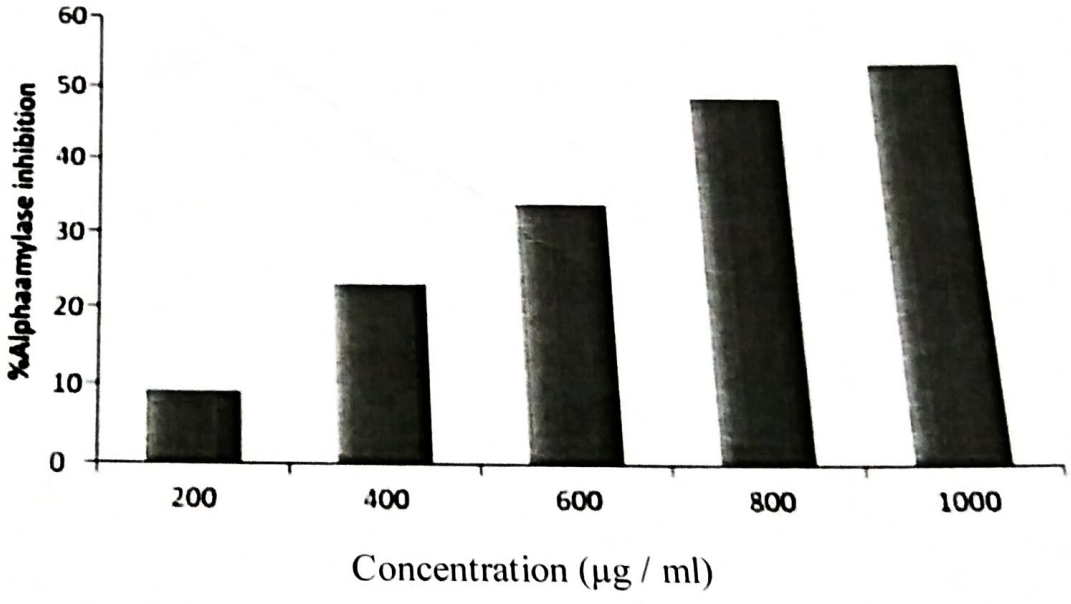
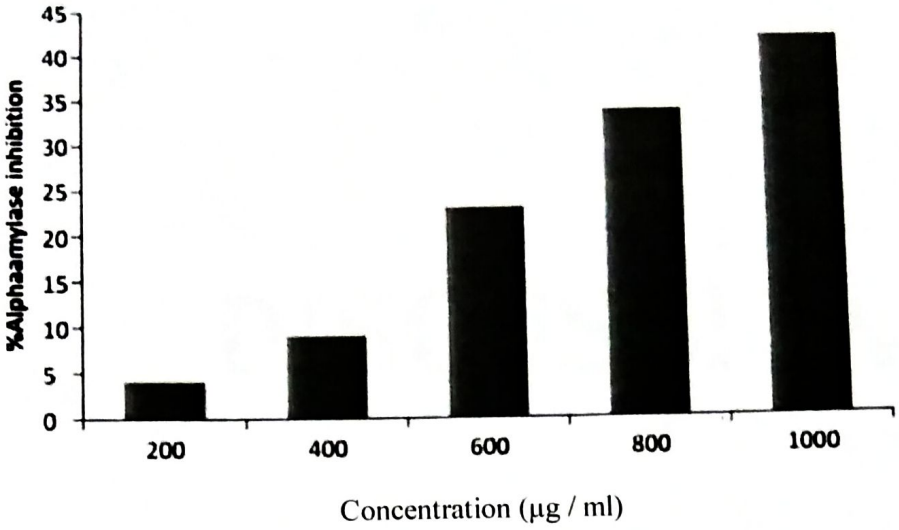


Figure 7 - Alpha – amylase inhibitory activity of acetic acid extract of intestine of *Arothron immaculatus*



DISCUSSION

6.0 DISCUSSION

Marine organisms are yielding more variety with quantity and quality of bioactive compounds over the terrestrial organisms. Among different marine organisms which yield bioactive compounds, fishes have a potential source of marine bioactive compounds.

Antimicrobial compounds from marine fish *A. immaculatus* inhibited the growth of ten bacterial strains . Varying degrees of antimicrobial activity was found in acetic acid extract of various tissues viz . ovary, skin and intestine of puffer fish *A .immaculatus* .Among the various extracts tested , acetic acid extract of ovary exhibited strong anti -bacterial activity. The diameter of inhibition of bacterial growth is regarded as an estimate of strength of the extract . The greatest zone of inhibition was shown by acetic acid extract of ovary of *A.immaculatus* (21 mm diameter) against *K. pneumonia*.. Khora *et al*., (2013) found that the tissue extracts of *Arothron hispidus* shown antimicrobial activity against the bacterial and fungal strains . It showed maximum against *E. coli* by skin extract and minimum was found against *P. vulgari* in liver extract . In the present study , the ovary extract showed maximum antibacterial activity against *Psuedomonas sp* and minimum activity was observed in intestine extract.

In the present study , the acetic acid extracts of ovary, skin and intestine were subjected to antifungal activity on *Aspergellus niger* . The antifungal activity of

the tissue extracts of *A immaculatus* was quite encouraging, as the maximum zone of inhibition produced by skin extract against the fungal pathogen *A.niger*. Mohana Priya *et al.*,(2013) reported that the maximum zone was observed against the *A. niger* in the skin extract of *A. hispidus* and the minimum zone was observed against *Trychophyton viridae* in the liver extract. Reports revealed that the antifungal activity of acetic acid extract of skin of *A.stellatus* has shown activity against fungal organisms. Maximum activity was observed against *A. niger* and *A. flavus* and minimum activity against *T. virdae* (Khora *et al.*, 2014).

In chicken blood cell, the highest hemolytic activity was observed in crude extract of *Cyclichthys orbicularis.* and *A. Inermis* and minimum in *Lagocephalua inermis* (Mohanraj *et al.*, 2015). In the earlier work Chicken blood showed high hemolytic activity on the skin tissue extracts when compared to liver and muscle extract as same as reported by (Bragadeeswaran *et al.*, 2010) has studied the hemolytic potential of tetrodotoxin producing bacteria in *A. hispidus*. In the present study the acetic acid extract of *A. immaculatus* showed hemolytic activity on chicken blood. Skin extract showed maximum hemolytic activity on Chicken blood at 1000 ug/ml concentration.

The crude extracts of liver, intestine muscle and skin of *Tetraoden fahakstrigosus* and *Potamotrygon garouensis* showed hemolytic activity against cow ,sheep ,goat and chicken except the spine of *P.garouensis* which show no

hemolytic activity. The innards of *T.fahakstrigosus* showed the highest hemolytic activity against cow blood (1024HU) while the skin of *T.fahakastrigosus* showed the lowest hemolytic activity against goat blood (16HU) (Abdulkadir *et al*.,2021) In the present observation ,among the three extracts tested for hemolytic activity, the skin extract exhibited hemolytic activity while the ovary and intestine extracts unable to lyse the chicken cells.

a-Amylase enzyme is one of the enzymes responsible for the hydrolysis of α-oriented bond polysaccharides and oligosaccharides such as starch, glycogen and other macromolecules of α-bond linked monosaccharides to disaccharides and finally to glucose (Suganya *et al.*, 2017, IKotowaroo *et al.*, 2006. Abirami *et al.*,2014 and Radhika *et al.*, 2013) *A. immaculatus* muscle extract has greatly reduced the blood glucose level on 14 days of treatment and recovered the animal from diabetes and reverted back the animals to normal (Kaleshkumar *et al.*, 2019). Our results demonstrated that *A. immaculatus* ovary extract has greatly inhibited alpha amylase enzyme. Type 2 Diabetes mellitus- a chronic disorder which requires the constant medical check and proper self-management by a patient for the control of disease and to avoid complications developed through the disease/diabetics.

SUMMARY

7.0 SUMMARY

The present study has been carried out to establish the occurrence ofantibacterial, antifungal, haemolytic, and alpha amylase inhibitory activities of ovary, skin and intestine extracts of puffer fish *Arothron immaculatus*.

Antibacterial activity of acetic acid extract of ovary, skin and intestine of *Arothron immaculatus* against ten bacterial strains. *Bacillus ceseus(BC),Vibrio cholerae[01]VC[01] ,Vibrio cholerae(0139) VC(0139),Escherichia coli(EC), Pseudomonas aerogenosa(PA),Aeromonas hydrophila(AH),Salmonella typhi(ST) ,Shigella flexneri (SF), Pseudomonas sp(P.sp), and Staphylococcus aureas(SA).*

The ovary extract produced maximum zone of inhibition with 10.5 mm in *Pseudomonas sp.* Acetic acid extract of skin exhibited antibacterial activity with zone of inhibition ranging from 3 to 6.5mm radius. In the case of intestine extract, the zone of inhibition ranging from 3 to 5mm radius.

Antifungal activity was assessed using *Aspergillus niger at 50 µg* concentration. The skin extract showed maximum zone of inhibition with 7.5mm radius.

Haemolytic activity of skin showed maximum of 22.14 % at 1000 µg /ml. Acetic acid extract of ovary exhibited maximum alpha amylase inhibitory activity of 54.17 % at 1000 µg/ml concentration.

CONCLUSION AND SUGGESTIONS

8.0 CONCLUSION AND SUGGESTIONS

The drugs obtained from marine source have displayed an exceptional potential in the management of wide array of disease, ranging from acute to chronic disease. Therefore every discovery of metabolites is considered important because it adds to the knowledge base of compounds unique to the marine environment. The present study proved that the ovary extract of puffer fish *Arothron immaculatus* shows significant antibacterial effect. The alpha amylase inhibitory study confirmed that ovary extract significantly inhibited the alpha amylase enzyme. The extracts showed mild antifungal and hemolytic activities. It could be therefore conclude from this study that *Arothron immaculatus* can serve as a therapeutic agent and can be used as a potential source of antibacterial and antidiabetic product. In order to identify the chemical nature and evaluate their novel drug leads further purification of the active sample for the isolation of active compounds is necessary.

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8.0 BIBLIOGRAPHY

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LARVICIDAL, ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF CHOSEN TUNICATE

A project submitted to

ST. MARY'S COLLEGE (Autonomous), **THOOTHUKUDI**

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

In partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

by

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ST. MARY'S COLLEGE (Autonomous),

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

THOOTHUKUDI -628 001

April -2023

CERTIFICATE

This is to certify that the project entitled **"Larvicidal, Antibacterial, And Antifungal Activity of Chosen Tunicate"** is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a project work done during the year 2022-2023 by the following students.

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We do hereby declare that this dissertation entitled, **"Larvicidal, Antibacterial, And Antifungal Activity of Chosen Tunicate"** submitted by us for the award of the degree of Bachelor of Science in Zoology is the result of our 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.

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Date: 03.04.2023

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CONTENTS

Malaria has been a major disease of humankind for thousands of years. It is referred to in numerous biblical passages and in the writings of Hippocrates. Although drugs are available for treatment, malaria is still considered by many to be the most important infectious disease of humans: there are approximately 200 million to 500 million new cases each year in the world, and the disease is the direct cause of 1 million to 2.5 million deaths per year.

Malaria is an acute febrile illness caused by protozoa of the genus *Plasmodium.* Four species cause disease in humans: *P falciparum, P vivax, P ovale* and *P malariae.* In 2008, *P. knowlesi*, a species that used to infect exclusively apes of the genus *Macaque*, was recognized by WHO as the fifth *plasmodium* species that infect humans. Other species of plasmodia infect reptiles, birds and other mammals. Malaria is spread to humans by the bite of female mosquitoes of the genus *Anopheles*. It is preventable and curable.

Clinical Manifestations

The most characteristic symptom of malaria is fever. Other common symptoms include chills, headache, myalgias, nausea, and vomiting. Diarrhea, abdominal pain and cough are occasionally seen. As the disease progresses, some patients may develop the classic malaria paroxysm with bouts of illness alternating with symptom-free periods. The malaria paroxysm comprises three

successive stages. The first is a 15 to 60 minutes cold stage characterized by shivering and a feeling of cold. Next comes the 2 to 6 hour hot stage, in which there is fever, sometimes reaching 41°C, flushed, dry skin, and often headache, nausea, and vomiting. Finally, there is the 2 to 4 hour sweating stage during which the fever drops rapidly and the patient sweats. In all types of malaria the periodic febrile response is caused by rupture of mature schizonts.
In *P.vivax* and *P.ovale* malaria, a brood of schizonts matures every 48 hr, so the periodicity of fever is tertian ("tertian malaria"), whereas in *P malariae* disease, fever occurs every 72 hours ("quartan malaria"). The fever in falciparum malaria may occur every 48 hr, but is usually irregular, showing no distinct periodicity. These classic fever patterns are usually not seen early in the course of malaria, and therefore the absence of periodic, synchronized fevers does not rule out a diagnosis of malaria.

Systematic position of Anopheles mosquito:

Phylum : Arthropoda

Subphylum : Mandibulata

Class : Insecta

Subclass : Pterygota

Order : Diptera

Family : Culicidae

Genus : *Anopheles*

Life cycle of Anopheles mosquito:

It usually takes 10–14 days for an egg to develop into an adult mosquito. Anopheles mosquitoes go through four stages in their life cycle: egg, larva, pupa, and adult. The first three stages are aquatic and last 7-14 days, depending on the species and the ambient temperature.

Eggs:

Eggs are laid singly on the surface of water and are provided with a partial envelope more or less inflated, which acts as a float. The float is characteristic with frilled edges. Adult, female mosquitoes lay 50–200 eggs at a time. Eggs do not tolerate drying out.

Larva:

Larvae live in the water. They hatch from eggs after an incubation period of 2 - 3 days. The larvae are called wrigglers. There are four larval instars, the total larval period being 8 - 10 days. The abdomen of the larvae is 10 segmented but only nine are visible. On the 8th and 9th abdominal segments lie spiracular apparatus with the spiracles opening on the 8th segment, but there is no breathing tube or siphon. The last segment carries four membranous organs called anal gills, besides large number of hairs. Larvae rest just under the surface of water and lie parallel with it. The larvae feed voraciously and grows. The larval skin is moulted four times and after each moult it is called an instar. After the fourth moult, the larva changes into the pupa.

Pupa:

Pupae live in water. They have short breathing trumpets and do not have external mouth parts, so they do not eat during this stage. They exhibit sexual dimorphism, males being smaller than females. An adult mosquito emerges from a pupa and flies away.

Adult:

Adults are dappled winged with characteristic patches on the upper margin of the fore wings. Scutellum is semilunar and the proboscis is straight. Palpi of both male and female are long, jointed, clubbed, equalling or exceeding the proboscis held in a straight line with the body. Adult female mosquitoes bite people and animals. Female mosquitoes need blood to produce eggs. After blood feeding, the female mosquitoes rest for a few days while the blood digests and the eggs develop. After the eggs develop, the female lays them in the water sources.

Life cycle of Plasmodium:

Figure 1 depicts the life cycle of plasmodium. It is almost the same for all the five species that infect humans and follows three stages:

a. Infection of a human with sporozoites
b. Asexual reproduction
c. Sexual reproduction

The two first stages take place exclusively into the human body, while the third one starts in the human body and is completed into the mosquito organism.

The human infection begins when an infected female anopheles mosquito bites a person and injects infected with sporozoites saliva into the blood circulation. That is the first life stage of plasmodium (stage of infection).

The next stage in malaria life cycle is the one of asexual reproduction that is divided into different phases: the pre- erythrocytic (or better, exoerythrocytic) and the erythrocytic phase. Within only 30- 60 minutes after the parasites inoculation, sporozoites find their way through blood circulation to their first target, the liver. The sporozoites enter the liver cells and start dividing leading to schizonts creation in 6- 7 days. Each schizont gives birth to thousands of merozoites (exoerythrocytic schizogony) that are then released into the blood stream marking the end of the exoerythrocytic phase of the asexual reproductive stage.

It is worth mentioning that, concerning *P. vivax* and *P. ovale*, sporozoites may not follow the reproduction step and stay dormant (hypnozoites) in the liver; they may be activated after a long time leading to relapses entering the blood stream (as merozoites) after weeks, months or even years. The exoerythrocytic phase is not pathogenic and does not produce symptoms or signs of the disease. Its duration is not the same for all parasite species.

Merozoites released into the blood stream, are directed towards their second target, the red blood cells (RBCs). As they invade into the cells, they mark the beginning of the erythrocytic phase. The first stage after invasion is a ring stage that evolves into a trophozoite. The trophozoites are not able to digest the

haem so they convert it in haemozoin and digest the globin that is used as a source of aminoacids for their reproduction. The next cellular stage is the erythrocytic schizont (initially immature and then mature schizont). Each mature schizont gives birth to new generation merozoites (erythrocytic schizogony) that, after RBCs rupture, are released in the blood stream in order to invade other RBCs. This is when parasitaemia occurs and clinical manifestations appear. The liver phase occurs only once while the erythrocytic phase undergoes multiple cycles; the merozoites release after each cycle creates the febrile waves.

A second scenario into the RBCs is the parasite differentiation into male and female gametocytes that is a non-pathogenic form of parasite. When a female anopheles mosquito bites an infected person, it takes up these gametocytes with the blood meal (mosquitoes can be infected only if they have a meal during the period that gametocytes circulate in the human's blood). The gametocytes, then, mature and become microgametes (male) and macrogametes (female) during a process known as gametogenesis. The time needed for the gametocytes to mature differs for each plasmodium species: 3- 4 days for *P. vivax* and *P. ovale*, 6- 8 days for *P. malariae* and 8- 10 days for P. falciparum.

In the mosquito gut, the microgamete nucleus divides three times producing eight nuclei; each nucleus fertilizes a macrogamete forming a zygote. The zygote, after the fusion of nuclei and the fertilization, becomes the so- called ookinete. The ookinete, then, penetrates the midgut wall of the mosquito, where it encysts into a formation called oocyst. Inside the oocyst, the ookinete nucleus

divides to produce thousands of sporozoites (sporogony). That is the end of the third stage (stage of sexual reproduction/ sporogony). Sporogony lasts 8- 15 days.

The oocyst ruptures and the sporozoites are released inside the mosquito cavity and find their way to its salivary glands but only few hundreds of sporozoites manage to enter. Thus, when the above mentioned infected mosquito takes a blood meal, it injects its infected saliva into the next victim marking the beginning of a new cycle.

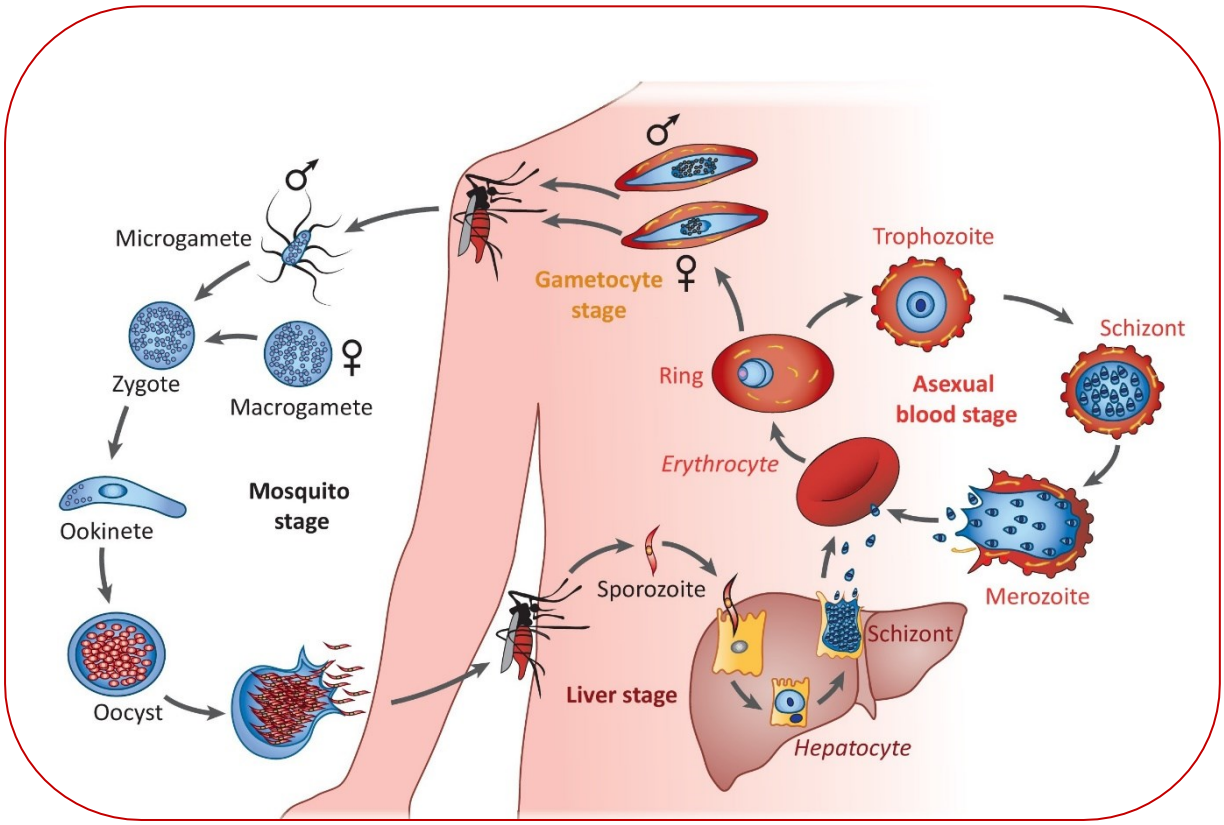


Figure 1. Life cycle of *Plasmodium*

Classification of Malaria:

Malaria is usually classified as asymptomatic, uncomplicated or severe.

Asymptomatic malaria can be caused by all *Plasmodium* species; the patient has circulating parasites but no symptoms.

Uncomplicated malaria can be caused by all *Plasmodium* species. Symptoms generally occur 7-10 days after the initial mosquito bite. Symptoms are non-specific and can include fever, moderate to severe shaking chills, profuse sweating, headache, nausea, vomiting, diarrhoea and anaemia, with no clinical or laboratory findings of severe organ dysfunction.

Severe malaria is usually caused by infection with *Plasmodium falciparum*, though less frequently can also be caused by *Plasmodium vivax* or *Plasmodium knowlesi*. Complications include severe anaemia and end-organ damage, including coma (cerebral malaria), pulmonary complications (for example, oedema and hyperpnoeic syndrome) and hypoglycaemia or acute kidney injury. Severe malaria is often associated with hyper parasitaemia and is associated with increased mortality.

Symptoms:

The first symptoms of malaria usually begin within 10–15 days after the bite from an infected mosquito. Fever, headache and chills are typically experienced, though these symptoms may be mild and difficult to recognize as malaria. In malaria endemic areas, people who have developed partial immunity may become infected but experience no symptoms.

If *Plasmodium falciparum* malaria is not treated within 24 hours, the infection can progress to severe illness and death. Severe malaria can cause multi-organ failure in adults, while children frequently suffer from severe anaemia, respiratory distress or cerebral malaria. Human malaria caused by

other *Plasmodium* species can cause significant illness and occasionally life-threatening disease.

Overall symptoms include fever and chills, sweating, headaches, nausea and vomiting, body aches, weakness, an enlarged liver, mild jaundice and higher breathing rate.

Diagnosis:

Malaria can be diagnosed using the following tests

- Microscopic Diagnosis Using Stained Thin and Thick Peripheral Blood Smears
- QBC Technique (Quantitative Buffy Coat)
- RDTs (Rapid Diagnostic Tests)
- Serological Tests
- Molecular Diagnostic Methods
    - PCR Technique (Polymerase Chain Reaction)
    - LAMP Technique (Loop-mediated isothermal amplification)
    - Microarrays
    - FCM Assay (Flow Cytometry)
    - ACC (automated blood cell counter)
    - LDMS (Laser Desorption Mass Spectrometry)

Treatment:

With early treatment, most people with malaria will make a full recovery. Treatment for individuals with the disease includes:

- Medication to eliminate the parasite from the bloodstream
- Supportive care
- Hospitalization for those with several symptoms
- Intensive care, in some cases

The main antimalarial drugs are:

- Chloroquine
- Hydroxychloroquine
- Primaquine
- Artemisinin-based therapy
- Atovaquone-proguanil

Prevention:

Strategies for preventing malaria include:

- Being aware of the risk
- Preventing mosquito bites
- Taking antimalarial tablets when traveling to an area where malaria occurs
- Getting a prompt diagnosis and treatment if someone thinks they may have the disease
- Administering the vaccine to children who live in places where malaria is endemic

Antimalarial drugs are around 90% effective in preventing malaria.

Side effects of Antimalarials:

i. Chloroquine may cause serious side effects.

Side effects include blurred vision, nausea, vomiting, abdominal cramps, headache, diarrhea, swelling legs/ankles, shortness of breath, pale lips/nails/skin, muscle weakness, easy bruising/bleeding, hearing and mental problems.

ii. Hydroxychloroquine may cause serious side effects such as a seizure, yellowing of your eyes, ringing in your ears, trouble hearing, unusual mood changes, severe muscle weakness, loss of coordination, underactive reflexes, any sudden changes in mood or behaviour, or thoughts about suicide, low blood cell counts, fever, chills, tiredness, sore throat, mouth sores, easy bruising, unusual bleeding, pale skin, cold hands and feet, feeling light-headed or short of breath, low blood sugar - headache, hunger, sweating, irritability, dizziness, fast heart rate, and feeling anxious or shaky etc.

- Primaquine also cause serious side effects like high fever, severe chills, persistent sore throat, signs of a sudden loss of red blood cells (such as severe tiredness, brown urine, pale lips/nails/skin, fast heartbeat or fast breathing with usual activities), or signs of a certain blood problem

- **ANTIBACTERIAL**

STREPTOCOCCUS PYOGENES:

Streptococcus pyogenes infections include pharyngitis (strep throat) and localized skin infection. Strep throat occurs most commonly in children. It can occur anytime and tends to circulate in winter and early spring. Strep bacteria flourish

wherever groups of people are in close contact. It is a mild infection, but it can be very painful. The most common symptoms of strep throat include:

- Pain when swallowing
- Fever
- Red and swollen tonsils, sometimes with white patches or streaks of pus
- Petechiae - on the soft or hard palate (tiny, red spots on the roof of the mouth)
- Swollen lymph nodes in the front of the neck

Other symptoms may include a headache, stomach pain, nausea, or vomiting especially in children.

Strep infection may lead to inflammatory illnesses, including:

- Scarlet fever, a streptococcal infection characterized by a prominent rash
- Inflammation of the kidney (post streptococcal glomerulonephritis)
- Rheumatic fever, a serious inflammatory condition that can affect the heart, joints, nervous system and skin
- Post streptococcal reactive arthritis, a condition that causes inflammation of the joints

Treatment

There are eight different antibiotics recommended by the Centers for Disease Control Trusted Source to treat strep throat. They include:

- Penicillin (oral or intramuscular)
- Amoxicillin (oral)

- Cephalexin (oral)
- Cefadroxil (oral)
- Clindamycin (oral)
- Clarithromycin (oral)
- Azithromycin (oral)

The more common side effects can include:

- nausea
- vomiting
- stomach upset
- diarrhea
- black hairy tongue
- Allergic reaction

Prevention

There's no vaccine available to prevent strep throat. One of the most effective ways to help avoid infection is regularly washing your hands.Don't share drinks or food with someone who has strep throat.

Streptococcus mutans

Streptococcus mutans is facultatively anaerobic, gram-positive coccus commonly found in the human oral cavity and on tooth surfaces and hard to reach areas like pits and fissures - the grooves found in premolars and molars. *Streptococcus mutans* promote decay and the breaking down of tooth enamel. When we eat

sugary foods, the streptococcus bacteria break down the sugar and use it to produce acids that attack tooth enamel.

Signs and Symptoms

Streptococcus mutans can be identified by a chalky white spot on the surface of the tooth indicating an area of demineralization of enamel, which is commonly referred to as a carious lesion. A carious lesion is the earliest diagnosis of tooth decay formation. As the lesion further demineralizes, it can turn brown and will eventually result in a cavity. Before the formation of the cavity, the process is reversible, but once the *Streptococcus mutans* forms the cavity, the tooth structure is lost and cannot be regenerated. A lesion that appears shiny and dark brown suggests that a lesion was once present but the demineralization has stopped leaving a stain. As the enamel and dentin are destroyed, the cavity becomes more noticeable. The affected area of the tooth changes color and becomes sensitive. It also causes dental caries which brings bad breath and foul tastes and gum disease. In highly progressed states, the infection can spread to the soft tissue from the tooth.

Treatment

Fluoride is efficient in preventing tooth decay and making teeth stronger however, it is much less effective if a cavity has already formed. To fix cavities caused by the bacterial strain *Streptococcus mutans*, a dentist will remove the decayed portion of the tooth and fill in the area of decay either with a composite filling or an amalgam filling.

A disadvantage of amalgam fillings is that they cause discoloration, cracks and fractures, allergic reactions, and destroys more tooth structure. These fillings also have poor aesthetics since the silver fillings are not the color of one's natural tooth.

Side Effects:

The chemicals used for the treatment can alter oral microbiota and have undiserable side-effects such as vomiting, diarrhea, tooth staining, tooth decay and it some times leads to oral cancer.

Prevention:

The only prevention is to lessen the impact of the fermentation by product lactic acid. Brushing, flossing and reducing the intake of refined and processed sugars can accomplish this.

ANTIFUNGAL ACTIVITY

Aspergillus ustus:

Aspergillus ustus is a microfungus that belongs to the division Ascomycota. It is a common inhabitant of soil and is also found in indoor environments. It causes *Aspergillosis.* Most people breathe in *Aspergillus* spores every day without getting sick. However, people with weakened immune systems or lung diseases are at a higher risk of developing health problems due to *Aspergillus*. The types of health problems caused by *Aspergillus* include allergic reactions, lung infections, and infections in other organs. *Aspergillus* mold is unavoidable. When mold spores are inhaled, immune system cells surround and destroy them.

But people who have a weakened immune system from illness or immunosuppressant medications have fewer infection-fighting cells. This allows aspergillus to take hold, invading the lungs and, in the most serious cases, other parts of the body. *Aspergillosis* is not contagious from person to person.

Types of *Aspergillosis*:

There are approximately 180 species of *Aspergillus*, but fewer than 40 of them are known to cause infections in humans. The different types of aspergillosis affect different groups of people.

- ❖ Allergic Bronchopulmonary Aspergillosis
- ❖ Chronic Pulmonary Aspergillosis
- ❖ Invasive Aspergillosis
- ❖ Aspergilloma

Symptoms:

The symptoms of **aspergillosis** are similar to asthma symptoms, including:

- Wheezing
- Shortness of breath
- Cough
- Weight loss
- Coughing up blood
- Fatigue
- Stuffiness
- Runny nose
- Headache
- Reduced ability to smell

- Fever is a common symptom of invasive aspergillosis.
- Invasive *aspergillosis* usually occurs in people who are already sick from other medical conditions, so it can be difficult to know which symptoms are related to an *Aspergillus* infection.

The symptoms of invasive aspergillosis in the lungs include:

- Fever
- Chest pain
- Cough
- Coughing up blood
- Shortness of breath

Some people with asthma or cystic fibrosis have an allergic reaction to aspergillus mold. Signs and symptoms of this condition, known as allergic bronchopulmonary aspergillosis, include fever, a cough that may bring up blood or plugs of mucus and Worsening asthma

Treatment:

When possible, immunosuppressive medications should be discontinued or decreased. People with severe cases of aspergillosis may need surgery.

This species shows elevated resistance to antifungal drugs; however, itraconazole, triazole, voriconazole, caspofungin and amphotericin are used. One promising regimen combines voriconazole and caspofungin.

Prevention:

It's difficult to avoid breathing in *Aspergillus* spores because the fungus is common in the environment. For people who have weakened immune systems,

there may be some ways to lower the chances of developing a severe *Aspergillus* infection.

Protect yourself from the environment:

- Try to avoid areas with a lot of dust like construction or excavation sites. If you can't avoid these areas, wear an N95 respirator while you're there.
- Avoid activities that involve close contact to soil or dust, such as yard work or gardening.
- Wear shoes, long pants, and a long-sleeved shirt when doing outdoor activities
- Wear gloves when handling materials such as soil, moss, or manure.
- To reduce the chances of developing a skin infection, clean skin injuries well with soap and water.

Cryptococcus neoformans:

Cryptococcus neoformans is a fungus that lives in the environment throughout the world. People can become infected with *Cryptococcus neoformans* after breathing in the microscopic fungus, although most people who are exposed to the fungus never get sick from it. *Cryptococcus neoformans* infections are rare in people who are otherwise healthy; most cases occur in people who have weakened immune systems, particularly those who have advanced HIV/AIDS. The lungs are the portal of entry for *Cryptococcus*

neoformans, and pulmonary involvement may be minimal if dissemination occurs quickly.

Cryptococcosis may appear in various forms depending on how the infection is acquired. In most cases, the infection begins in the lungs (pulmonary form) and may then spread to the brain, urinary tract, skin, and/or bones (disseminated form). When the infection is limited to the lungs, symptoms may be minimal or apparent at all. Respiratory symptoms may include coughing and chest pain. When the infection spreads, it tends to seek out the central nervous system, especially the brain. In some affected individuals, inflammation of the membranes surrounding the brain and spinal cord (meningitis) may occur as a serious complication.

Symptoms:

Cryptococcus neoformans usually infects the lungs or the central nervous system (the brain and spinal cord), but it can also affect other parts of the body. The symptoms of the infection depend on the parts of the body that are affected.

In the lungs,

- Cough
- Pneumonia

The symptoms are often similar to those of many other illnesses, and can include:

- Shortness of breath
- Chest pain
- Fever

In the brain (*cryptococcal meningitis*)

- Headache
- *Cryptococcal neoformans* is an infection caused by the fungus *Cryptococcus* after it spreads from the lungs to the brain.

The symptoms of *cryptococcal meningitis* include:

- Neck pain
- Nausea and vomiting
- Sensitivity to light
- Confusion or changes in behavior

If you have symptoms that you think may be due to a *Cryptococcus neoformans* infection, please contact your healthcare provider.

Treatment:

People who have *Cryptococcus neoformans* infection need to take prescription antifungal medication for at least 6 months, often longer. The type of treatment usually depends on the severity of the infection and the parts of the body that are affected.

Prevention:

Cryptococcus neoformans infections are rare among people who are otherwise healthy. Most cases of *Cryptococcus neoformans* infection occur in people who have weakened immune systems, such as people who:

- Have advanced HIV/AIDS,
- Have had an organ transplant, or

- Are taking corticosteroids, medications to treat rheumatoid arthritis or other medications that weaken the immune system.

People who have weakened immune systems can avoid areas with a lot of dust.

The objectives of present study were to

- Collect and identify the colonial ascidian *Didemnum psammatode*
- Prepare the crude extract of *Didemnum psammatode* by soxhlet extraction method
- separate the solvent using a rotary evaporator
- study the larvicidal activity of *Didemnum psammatode* against *Anopheles*
- evaluate the antibacterial activity of *Didemnum psammatode* against *Streptococcus mutans* and *Streptococcus pyogenes* by agar well diffusion method
- assess the antifungal activity of *Didemnum psammatode* against *Aspergillus ustus* and *Cryptococcus neoformans* by agar well diffusion method

Larvicidal ACTIVITY

Diaz *et al*., (2022) reported the potential of the Colombian Caribbean flora as a host of bioactive plants against important vectors such as the *A. aegypti* mosquito with potential use in controlled environments. Ochola *et al*., (2022) evaluated the larvicidal activity of *Ocimum kilimandscharicum* oil against third instar mosquito larvae. Manuahe *et al*., (2022) found the combination of clove leaf extract, nutmeg flesh extract and trumpet flower shows the larvicidal activity against the mosquito larvae. Chan et al., (2022) reported that *Ocimum basilicum* has substantial larvicidal activity against *Aedes albopictus* and is a potential source of naturally derived larvicide. Sofi *et al*., (2022) reported the leaf extract of *Artemisia absinthium* shows effective larvicidal activity against *Aedes aegypti*.

Ramkumar *et al*., (2022) analysed that the *C. baccifera* leaf extract-mediated biosynthesis of ZnNPs has the potential to be used as an ideal eco-friendly approach toward the control of mosquito vectors at early stages. Sneha *et al*., (2022) analysed that the possible use of UAE-Essential oils from *Ocimum basilicum*, O.gratissimum, O.tenuiflorum and O.canum in the management of microbial pathogens and mosquito larval control. Abutaha *et al*., (2022) reported that the extracts of *Cinnamomum burmannii* (C.B.) and Syzygium aromaticum (S.A.) were evaluated for larvicidal activity individually and in combination against the *Culex pipiens* larvae.

Njuabe *et al*., (2021) evaluated the extracts of *Momordica foetida*, *Gnidia glauca* and *Vepris soyauxii* showed larvicidal activity against *Anopheles gambiae* and *Anopheles coluzzii*. Aziz *et al*., (2021) evaluated the crude extract of *Vitex ovata* possesses mosquito vector control properties against *Aedes* sps. Gunathilaka et al., (2021) analysed that Zinc oxide nanoparticles can delay the growth of mosquito larvae *Aedes albopictus* and *Anopheles vagus*. Negara *et al*., (2021) reported that Phlorotannins from brown seaweeds show antifungal activity against dermal and plant fungi, and larvicidal activity against mosquitos and marine invertebrate larvae. Silva *et al*., (2021) analysed that the Essential oil of *Eugenia calycina* showed high activity against the *A. aegypti* larvae. The leaves of *E. calycina* are therefore a very promising source of natural larvicidal products.

Nityasree *et al*., (2020) evaluated that methanolic leaf extract of *Solanum lycopersicum* could be an alternative source to control mosquito vectors and further investigation is strongly suggested in order to utilize this source in many disease-endemic areas. Soni and Dhiman *et al*., (2020) evaluated that the synthesis of Zinc oxide nanoparticles and Titanium dioxide nanoparticles using the aqueous stem extract of *Cuscuta reflexa* which is a rapid, eco-safe and this could be a safe and useful technology for mosquito control.

Hung *et al*., (2019) reported the *Erechtites* essential oils may serve as low-cost vector control agents for mosquito-borne infections. Gharsan (2019) analyzed the bioactivity of Plant *Eucalyptus globulus and Ocimum basilium* for larvicidal activity against *Aedes aegypti*. Sofian *et al*., (2019) analysed that the

extract and isolated essential oil from *Zingiber aromaticum* possessed remarkable larvicidal properties. Kasim et al., (2019) studied the aqueous extract of garlic possesses larvicidal activity against the 4th instar of mosquito larval species *Culex* and *Anopheles*. Darsana *et al*., (2019) reported that the possible use of this blend of botanical extract as an ideal ecofriendly, larvicide against *Aedes albopictus*.

Baskar *et al*., (2018) analysed the essential oil from *Atalantia monophylla* could be a valuable source for the development of mosquito repellents and larvicide. Pathak *et al*., (2018) evaluated that out of 108 species of flowering plants, 46 are reported to exhibit mosquitocidal/larvicidal properties against *Aedes.aegyptii*, 35 against *Culex quinquifasciatus*, 16 plant species against *Anopheles stephensi*, respectively. Vargas *et al*., (2018) analyzed the *Bacillus thuringiensis* showed effective larvicidal activity against larvae of *Aedes aegypti*.

Prabakaran *et al*., (2017) evaluated that the essential oils have the potential to provide efficient and can be used as a cheap, eco-friendly, safer for humans and the environment and also efficient alternative to the chemical larvicides. Rawani *et al*., (2017) reported that leaf extract of *Solanum nigrum* has great potential as bio control agent against *Culex vishnui* group and *Anopheles subpictus*. In near future the isolated bioactive phytochemical could be used as a source of an effective mosquitocidal agent.

Tam (2017) evaluated the biocidal potential of *Eucalyptus deglupta* extract against the larvae of *Aedes* sp. Thongwat *et al*., (2017) evaluated that the ethanolic endocarp extract of *Dracaena loureiri* showed effective larvicidal activity. It is

an alternative source for developing a novel larvicide for controlling this mosquito species.

Gandhi *et al*., (2016) found that the Alizarin isolated from roots of *Rubia cordifolia* showed larvicidal activity against *Culex quinquefasciatus* and *Aedes aegypti*. Mukandica *et al*., (2015) analysed that a potential of the incorporation of *Clausena anisate* extracts into the control of mosquito populations. Rocha *et al*., (2015) evaluated that fennel essential oils from both countries (Cape Verde and Portugal) displayed strong larvicidal effect against *Aedes Aegypti*, the Cape Verde essential oils being one of its major constituents, limonene.

Pani *et al*., (2015) reported that different plant parts such as leaf, rhizome, bulb, stem and root bark, whole plant and essential oil showed significant larvicidal properties against different mosquito vectors viz., *An. stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*, *Phlebotomus duboscqi*, *Anopheles aambiae*, *Aedes. fluviatilis etc*. Torres *et al*., (2015) analysed that both hexane and ethanol extracts of *Anacardium occidentale* showed promising potential as an alternative source of a more sustainable, non-toxic, and environmentally friendly solution for the control of dengue vector, *Aedes aegypti*.

Kumar *et al*., (2014) evaluated that the *Cassia occidentalis* (Linn.) has a paramount larvicidal importance and the synthetic chemical can be obtained easily at a very low cost but the use of the plants for larvae control offers a safer alternative too and these extracts are inexpensive, easy to handle, and safer products for the control of mosquito larvae. Chawla *et al*., (2014) reported the

plant *Cassia occidentalis* (Linn.) has a realistic mortality result for larvae of filarial vector. Raveen et al., (2014) evaluated the aqueous extract of Nerium oleander flowers shows the larvicidal activity against the filarial vector, *Culex quinquefasciatus.*

Balakrishnan *et al*., (2013) evaluated the larvicidal actinomycetes as valuable resource for the discovery of novel insecticidal molecules. Ali *et al*., (2013) reported the larvicidal property of *Caulerpa racemosa* might be the prospective alternative source to control the mosquitoes.

Subramaniam *et al*., (2012) analysed the combined effect of *Aleo vera* leaf extract and *Bacillus sphaericus* against *Aedes aegypti.* Govindarajan *et al*., (2012) evaluated the crude extract of *Ervatamia coronaria* and *Caesalpinia pulcherrima* were excellent potential for controlling *Anopheles subpictus* and *Culex tritaeniorhynchus* mosquito larvae.

Kamaraj *et al*., (2011) analysed the role of larvicidal activities of hexane, chloroform, ethyl acetate, acetone and methanol dried leaf and bark extracts of *Annona squamosa* L., *Chrysanthemum indicum* L., and *Tridax procumbens* L. against the fourth instar larvae of malaria vector - *Anopheles subpictus* Grassi and Japanese encephalitis vector - *Culex tritaeniorhynchus* Giles. Kamaraj *et* al., (2011) evaluated that all plant extracts showed moderate effects after 24 h of exposure; however, the highest toxic effect of bark extract of *Annona squamosa*, leaf ethyl acetate extract of *Chrysanthemum indicum* and leaf acetone extract of *T. procumbens* against the larvae of *Anopheles subpictus* (LC_{50} = 93.80, 39.98

and 51.57 mg/l) and bark methanol extract of *A. squamosa*, leaf methanol extract of *C. indicum* and leaf ethyl acetate extract of *T. procumbens* against the larvae of *Culex tritaeniorhynchus* (LC_{50} =104.94, 42.29 and 69.16 mg/l) respectively. Arivoli *et al*., (2011) analysed the application of these extracts to larval habits may lead to promising results in filarial and mosquito management programmes.

Govindarajan *et al*., (2011) analysed that the crude extract of *Ervatamia coronaria* and *Caesalpinia pulcherrima* were excellent potential for controlling *Anopheles subpictus* and *Culex tritaeniorhynchus* mosquito larvae. Perumalsamy *et al*., (2009) evaluated the steam distillate from the root of *Asarum heterotropoides* possess larvicidal activity against third-instar larvae of *Culex pipiens pallens Coquillett*.

Raj Mohan and Ramaswamy (2007) evaluated the leaf extract of *Ageratina adenophora* is more toxic to both *Aedes aegypti* and *Culex quinquefasciatus* and could be effectively used for the control of mosquito larvae. Cheng *et al*., (2003) reported that the leaf bark essential oils of *Camellia japonica* are promising as larvicides against *Aedes aegypti* larvae and could be useful in the search for new natural larvicidal compounds.

Perez *et al*., (2020) emphasize that the utilization of waste materials can be developed as good alternative larvicides that are environmentally safe and inexpensive. Torre *et al*., (2014) analyzed that both the hexane and ethanol extracts of *P. Americana* showed promising potential as an alternative source of a more sustainable, non-toxic and environmentally friendly solution for the

control of dengue vector, *Aedes aegypti.* Paripooranaselvi and Meenakshi (2012) reported that the larvicidal activity of ascidian has two species of ascidians- *Aplidium indicum* and *Phallusia nigra* were investigated against the larvae of *Anopheles stephensi* and *Culex quinquefasciatus*, these extracts were more toxic to Culex quinquefasciatus than to *Anopheles stephensi* and this investigation explores the importance of marine organisms as a valuable resource for the discovery of novel larvicidal molecules.

Antibacterial

Nurhayani and Avianto (2022) evaluated that the turmeric rhizome extract (*Curcuma longa Linn.*) has antibacterial activity against *Staphylococcus aureus* bacteria. Ghetas *et al*., (2022) reported the AgNPs have interesting dose-dependent antimicrobial properties, to limit fish diseases, increase economy and improve human health. Jason *et al.,* (2022) reported the compounds from *Turbinaria* conoides, to determine the bactericidal against *Streptococcus mutans* and *Actinomycetes*. Fialova *et al.,* (2021) analysed the medicinal plants and their active constituents recommended by European Medicines Agency for skin disorders are discussed in terms of their antibacterial effect.

Yuan *et al*., (2021) reported the cell membrane is the main site of flavonoids acting on gram-positive bacteria, and which likely involves the damage of phospholipid bilayers, the inhibition of the respiratory chain or the ATP synthesis. Deepak *et al*., (2020) reported that the methanol crude extracts of

Paphia malabarica and *Crassostrea gryphoides* show activity against both bacterial and fungal strains. Tastan and Sonmex (2020) evaluated that antibacterial effects of fish mucus and fish lectins. Zhu and Zeng (2020) suggested the antibacterial and antibiofilm activities of garlic extract and its potential to be used in the treatment of periprosthetic joint infections.

Siriken *et al*., (2018) analysed that essential oil contents of *Laurus nobilis* are strong antibacterial activity against Gram negative and Gram positive food borne pathogens (*Salmonella*, *Staphylococcus aureus, Esherichia coli, Listeria monocytogenes*), spoilage bacteria (*Pseudomonas aeroginosa*) as well as antifungal effects. Mace *et al*., (2017) analysed 1,2-naphthoquinone and 5-hydroxy-1,4-naphthoquinone inhibit *Streptococcus pyogenes* and should be further investigated as candidates for the management of streptococcal pharyngitis. Chai *et al*., (2016) suggested the essential oil of *Cinnamomum verum* and its bioactive component, cinnamaldehyde, have the potential for application as natural agents for the prevention and treatment of dental caries. Caruso *et al.,* (2014) suggested that the mucus secretions, biological fluids and organs of the examined fish species can be regarded as an interesting source of bioactive compounds with antibacterial and haemolytic properties.

Cavallo *et al.*, (2013) analysed the potential use of seaweed extracts as a source of antibacterial compounds. Divya *et al*., (2013) evaluated the ascidian *Aplidium multiplicatum* is found to have remarkable antimicrobial activies

against isolated microbes. Limsuwan and voravathikunjai (2013) suggested that *Boesenbergia pandurata*, *Eleutherine americana*, and *Rhodomyrtus tomentosa* have great potentials against S. pyogenes.

Prabhu *et al*., (2011) reported the MIC and MBC for methanolic extract tested in the study inferred that the values range between 0.70-0.95 mg/ml and 0.85-1.1 mg/ml respectively. Kumaran *et al*., (2011) reported that the ascidian *Didemnum psammathodes* were found to have remarkable antimicrobial activities against isolated microbes. Chandramathi *et al*., (2011) evaluated the nontoxic extract of ascidian is a genuine source of antibacterial drugs for human use but it needs further investigations. Wei *et al.,* (2010) reported the acidic mucus extracts exhibited bactericidal activity and inhibited the growth of Klebsiella pneumoniae, *Pseudomonas aeruginosa* and *Bacillus subtilis.*

Antifungal

According to Hassanpour *et al*., (2020) the susceptibility of *Cryptococcus neoformans* to fluconazole was increased when combined with eugenol. According to Tomasz and Karpinski (2019)., the marine organisms are rich in nature macrolides and some of these may be used in the future in the treatment of bacterial and fungal infections. According to Eddine *et al.,* (2018)., camel's milk LAB's strains could be selected for application to control spoilage, fungal growth and pathogenic bacteria.

According to Liu *et al*., (2017)., studied the materials derived from *Azadiractha indica, Azadiractha juss* and *Lawsonia inerms* is significant in antifungal activity against a wide range of gram-negative and positive bacteria and fungal species.

Salhi *et al*., (2017)., studied the antifungal properties of *Artemisia herba alba*, *Cotula cinerea*, *Asphodelus tenuifolius and Euphorbia guyoniana*.

Tadesse *et al.,* (2008)., studied the antibacterial and antifungal activity of colonial ascidian *Synoicum pulmonaria* at a lowest concentration concentration of 0.02 mg/ml. Satish *et al*., (2007)., studied the highly significant antifungal activity of *Acacia nilotica*, *Achras zapota*, *Datura stramonium*, *Emblica officinalis*, *Eucalyptus globules*, *Lawsonia inermis*, *Mimusops elengi*, *Peltophorum pterocarpum*, *Polyalthia longifolia*, *Prosopis juliflora*, *Punica granatum* and *Sygigium cumini*. According to Webster *et al*., (2007)., *Fragaria virginiana*, *Epilobium angustifolium* and *potentilla simplex* possess promising antifungal potential. Mohana and Raveesha (2007)., studied the *Decalepis hamiltonii* being an edible one can possible be exploited in the management of seed borne pathogenic fungi and prevention of biodeterioration of grains and mycotoxin elaboration during storage. According to Tuney *et al*., (2006)., the comparison of dried and fresh extract antimicrobial activity. It was found that all test organisms were more sensitive to fresh extracts of the algae. Although fresh extracts of *Galphimia gracilis*, *Dicranopteris linearis*, and *Ectocarpus siliculosus*

are inhibited for the tested bacteria and yeast, their dried extracts had no inhibition activity on either Gram-negative or Gram-positive bacteria.

Collection of Animal Material

Samples of colonial ascidian *Didemnum psammatode* Sluiter, 1895 were collected during the low tide from the intertidal rocky area of Hare Island (Plate: 1). The samples were washed with seawater to remove sand, mud and overgrowing organisms at the site collection and then transported to the laboratory. Identification up to the species level was carried out based on the key to the identification of Indian ascidians by Meenakshi, 1997.

Systematic Position

Didemnum psammatode belongs to

Phylum	:	Chordata
Subphylum	:	Urochordata
Class	:	Ascidiaceae
Order	:	Enterogona
Suborder	:	Aplousobranchia
Family	:	Didemnidae
Genus	:	*Didemnum*
Species	:	*psammatode*

Animal Material

Ascidians commonly called ‘sea squirts’ are an interesting group of marine, sedentary organisms found to occur in abundance in the Tuticorin coast. It is sessile and filter feeding. It lives on plankton that it filters from seawater with a mucous net.

Plate – 2 shows the colony of *Didemnum psammatode*. It is thin and soft. In the test spicules are few, but abundant ovoid faecal pellets are present. Live and preserved colonies are grey in colour.

Preparation of Powder

The specimens were dried under shade. The dried animals were homogenized to get a coarse powder. The dried powders of the tunicate - *Didemnum psammatode* were used.

Preparation of Extract

Soxhlet extraction (Plate: 3)is a method used for the extraction of valuable bioactive compounds from various natural sources. It is used to extract the compound from a solid mixture. It is a simple and convenient method for an infinitely repeated cycle of extraction with a fresh solvent until the complete exhaustion of the solute in the raw material. During extraction with soxhlet, the process of distillation is implicated. It consists of heating a solution up to boiling and then condensing send back to the original flask. 50 g of the *Phallusia nigra* powder was introduced in a thimble. This thimble is then deposited in a distillation flask filled with ethanol solvent. After reaching a submersion level,

a siphon absorbs the solvent in the thimble-holder and then releases it back into the distillation flask. This solution contains the extracted solutes. This process is done continuously until the extraction is completed. (Azmir, 2013). The separation of the extract from the solvent is made by a rotary evaporator. A rotary evaporator (Plate: 4) is an equipment used to remove solvent from a sample through 'evaporation under reduced pressure'. The reduced pressure in the apparatus causes the solvent to boil at a lower temperature than normal. Rotating the round bottom flask increases the liquid's surface area and thus the rate of evaporation. The solvent vapor travels into the cooler water condenser, where it condenses and drips into a separate receiving flask leaving a concentrated compound in the original round bottom flask. After the complete evaporation of ethanol, the crude extract was used for carrying out the experiment. The same procedure was followed for getting the crude extract of ethanolic extract of *Eudistoma viride* also. The excess solvent was evaporated and the dried extracts were kept in a refrigerator at 4ºC for future use.

LARVICIDAL ACTIVITY

Larvae of *Anopheles species* were collected from the stagnant water pools. They were identified by their posture. The larvae that floated horizontally to the surface of the water with the help of the palmate hairs on the abdominal segments were collected. Six Petri plates each with 25 ml water were taken and arranged on a table. Using a filler, 5 larvae of *Anopheles species* were transferred from the stock plastic bowl to each of the 12 bowls. The sets of bowls were labeled as a,

b, c, d, e and f. Bowl labeled ‘f’ acted as the control, without any ascidian extract. 10 ml, 50 ml, 100 ml, 250 ml, and 500 ml of ethanol extract of *Didemnum psammatode* was added to the first set of Petri plate. The experiment was conducted in triplicate and the mean was taken. The larvicidal effect of the extracts was monitored by counting the number of dead larvae at one-hour interval for 24 hours.

ANTIBACTERIAL ACTIVITY

Petri plates containing 20 ml nutrient agar medium were seeded with 24 hr culture of bacterial strains were adjusted to 0.5 OD value according to McFarland standard, (*Streptococcus mutans* and *Streptococcus pyogenes- 1928*) Wells were cut and concentration of sample *Didemnum psammatode* (500, 250, 100 and 50 µg/ml) was added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Gentamicin antibiotic was used as a positive control. The values were calculated using Graph Pad Prism 6.0 software (USA).

ANTIFUNGAL ACTIVITY

Agar- Well Diffusion Method

Potato Dextrose Agar Medium

The potato dextrose agar medium was prepared by dissolving 20 gm of potato influsion, 2 gm of dextrose and 1.5 gm of agar in 100ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes.

The autoclaved medium was mixed well and poured onto 100mm petri plates (25-30 ml/plate) while still molten.

Petri plates containing 20ml potato dextrose agar medium was seeded with 72 hr culture of fungal strain *(Cryptococcus neoformans* and *Aspergillus ustus)* wells were cut and different concentration of sample D2 (500, 250, 100 and 50 µg/ml) was added. The plates were then incubated at 28°C for 72 hours. The anti-fungal activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Amphotericin B was used as a positive control. The values were calculated using Graph Pad Prism 6.0 software (USA).

LARVICIDAL ACTIVITY

In present investigation, ethanolic extract of *Didemnum psammatode* was tested for larvicidal activity using *Anopheles* mosquitoes larvae. Table: 1 and 2; figure: 2; and plate:5 and 6 depict the larvicidal activity of *Didemnum psammatode* against *Anopheles* larva. The highest concentration of the ethanolic extract of *Didemnum psammatode* exhibited highly significant mortality rate. Dose dependent mortality rate was observed. 500, 250, 100, 50 and 10 µg/ml ethanolic extract of *Didemnum psammatode* showed 93, 87, 67, 53 and 27 % mortality rate respectively. In control, no mortality was noticed.

ANTIBACTERIAL ACTIVITY

In the present investigation, ethanolic extract of *Didemnum psammatode* were tested against *Streptococcus mutans* and *Streptococcus pyogenes*. Table: 3 Figure: 3 and 4; Plate: 7 and 8 depict the antibacterial activity of *Didemnum psammatode* against *Streptococcus mutans* and *Streptococcus pyogenes*. The zone of inhibition was 15.5±0.7mm noted at higher concentration (500µg/ml) in *Streptococcus mutants*. In, 250µg/ml, 100µg/ml, 50µg/ml concentration, zone of inhibition was not seen in *Streptococcus mutants*. In control the zone of inhibition was 17.5±0.7.

In *Streptococcus pyogenes*, the Maximum zone of inhibition (14.5±0.7) was seen in higher concentration (500µg/ml), 4.5±0.7 mm zone of inhibition was

observed for 250µg/ml concentration of ethanolic extract of *Didemnum psammatode*. In 100 µg/ml and 50µg/ml no zone of inhibition was observed. In control the zone of inhibition was 17.5±0.7.

ANTIFUNGAL ACTIVITY

In the present investigations, the ethanolic extract of *Didemnum psammatode* was tested for antifungal activity against *Aspergillus ustus* and *Cryptococcus neoformans* (Table: 4; figure: 5 and 6; Plate : 9 and 10). The antifungal activity of *Didemnum psammatode* was higher (13.5±0.7µg/ml) at the highest concentration. The Strongest antifungal activity (14.5±0.7µg/ml) was recorded at 500 µg/ml ethanolic extract of concentration of the extract *Didemnum psammatode* against *Cryptococcus neoformans*. 11.5±0.7 mm zone of inhibition was observed for 250µg/ml concentration of ethanolic extract of *Didemnum psammatode* against *Aspergillus ustus*. 13.5±0.7 mm zone of inhibition was observed for 250µg/ml concentration of ethanolic extract of *Didemnum psammatode* against *Cryptococcus neoformans*. In control and low concentrations no inhibitory zone was noticed.

Table 1: Effect of ethanol extract of *Didemnum psammatode* against *Anopheles* larvae

Concentration of ethanolic extract of *Didemnum psammatode*										Control	
500 µg/ml		250 µg/ml		100 µg/ml		50 µg/ml		10 µg/ml			
D	L	D	L	D	L	D	L	D	L	L	D
5	0	4	1	3	2	2	3	2	3	0	5
4	1	4	1	4	1	3	2	1	4	0	5
5	0	5	0	3	2	3	2	1	4	0	5

Table 2: Effect of ethanol extract of *Didemnum psammatode* on Percentage of mortality

	I	II	III	Mean
500 µg/ml	100	80	100	93
250 µg/ml	80	80	100	87
100 µg/ml	60	80	60	67
50 µg/ml	40	60	60	53
10 µg/ml	40	20	20	27
Control	0	0	0	0

Table3: Antibacterial activity of ethanolic extract of *Didemnum psammatode* against *Streptococcus mutans* and *Streptococcus pyogenes*

Name of the test organism	Zone of inhibition (mm)				
	500 µg/ml	**250µg/ml**	**100 µg/ml**	**50µg/ml**	**PC**
Streptococcus mutans	15.5±0.7	0	0	0	17.5±0.7
Streptococcus pyogenes	14.5±0.7	4.5±0.7	0	0	15.5±0.7

Table: 4. Antifungal activity of ethanolic extract of *Didemnum psammatode* against *Aspergillus ustus* and *Cryptococcus neoformans*

Name of the test organism	Zone of inhibition (mm)				
	500 µg/ml	**250 µg/ml**	**100 µg/ml**	**50 µg/ml**	**PC**
Aspergillus ustus	13.5±0.7	11.5±0.7	0	0	14.5±0.7
Cryptococcus neoformans	14.5±0.7	13.5±0.7	0	0	17.5±0.7

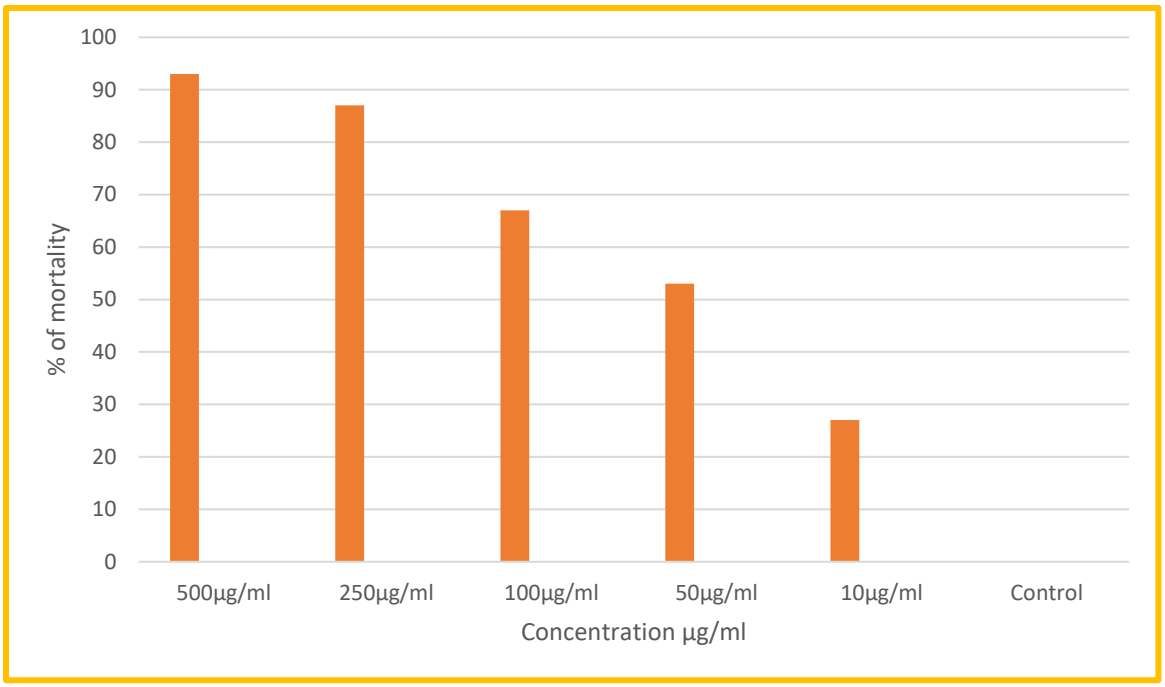


Figure 2: Effect of ethanol extract of *Didemnum psammatode* against *Anopheles* larvae

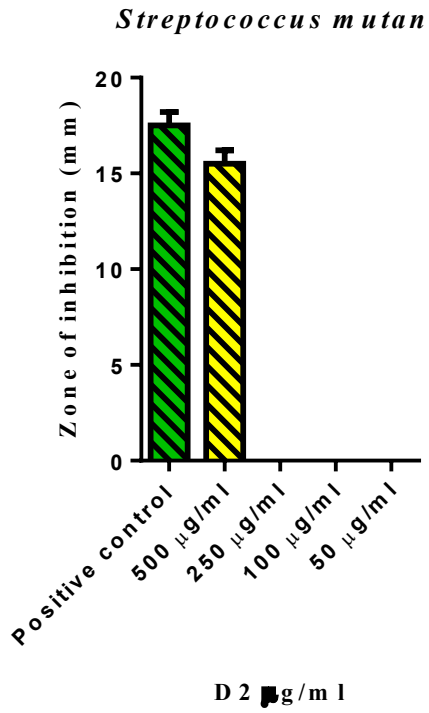


Figure 3: Effect of ethanol extract of *Dp* against *Streptococcus mutans*

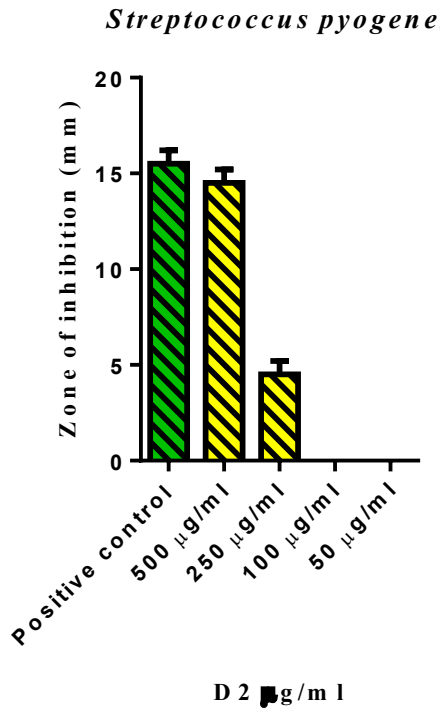
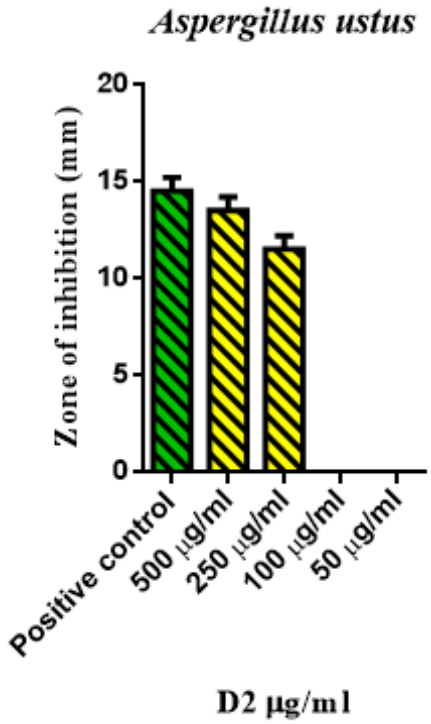


Figure 4: Effect of ethanol extract of *Dp against Streptococcus pyogenessammatode*



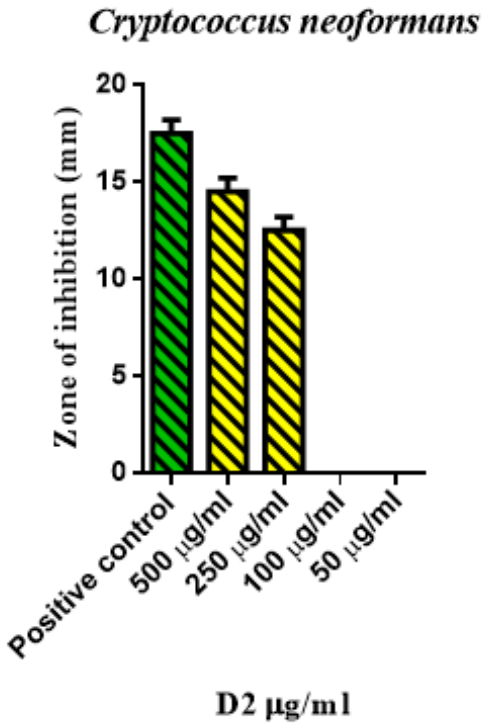


Figure 5: Effect of ethanol extract of *Dp* against *Aspergillus ustus*

Figure 6: Effect of ethanol extract of *Dp against Cryptococcus neoformans*

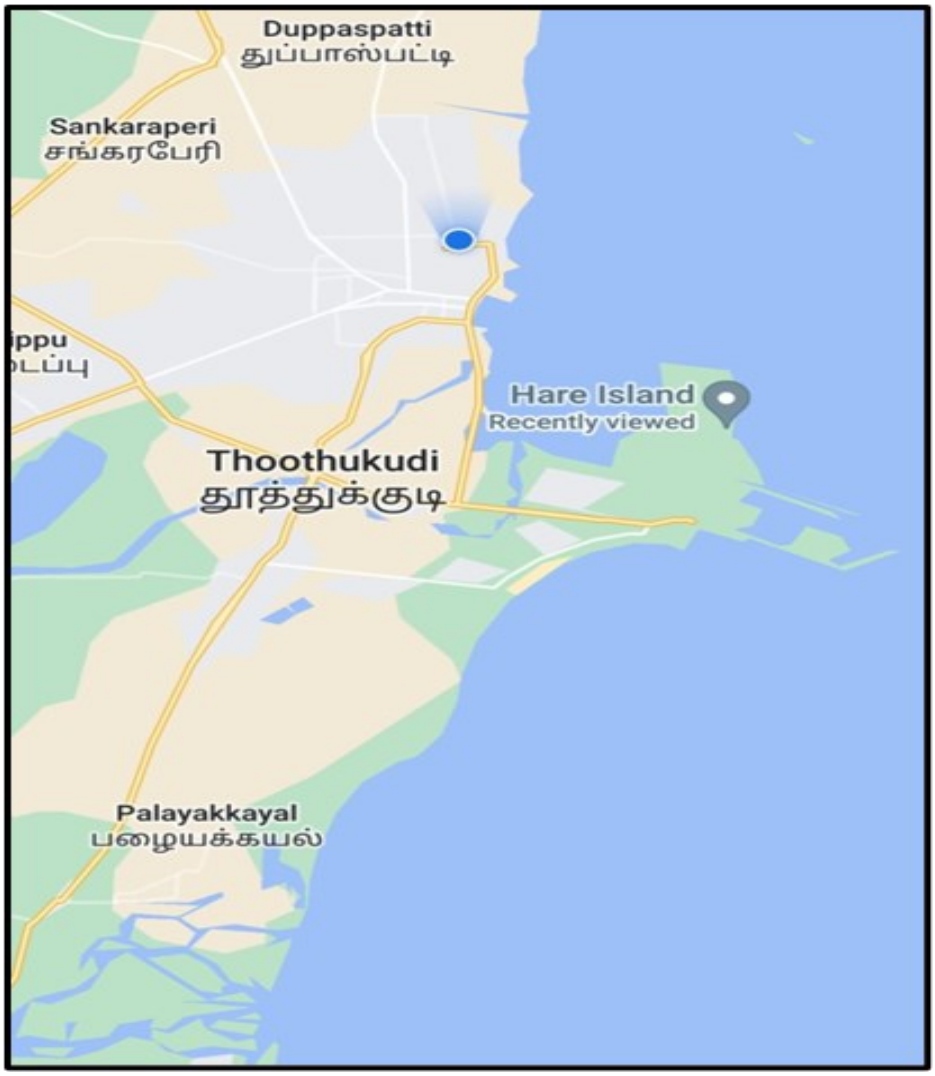


Plate1: Study area – Hare Island

Plate 2: *Didemnum psammatode*

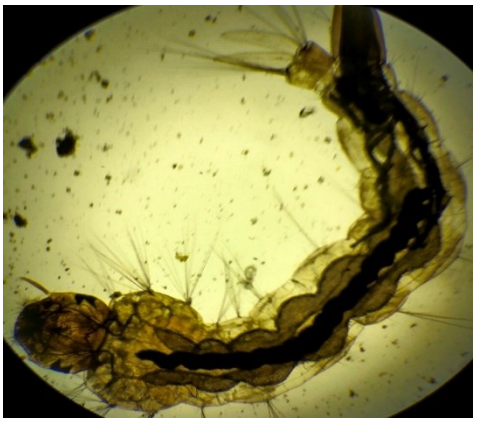
Plate 3: Soxhlet apparatus

Plate 4: Rotary evaporator

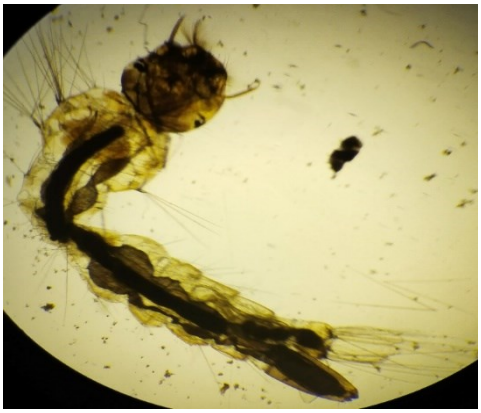
Plate 5: Larvicidal activity of ethanolic extract of *Didemnum psammatode* against *Anopheles* sps.

500 µg/ml

250 µg/ml



100 µg/ml



50 µg/ml

10 µg/ml

Control

Plate 6: Larvicidal activity of ethanolic extract of *Didemnum psammatode* against *AnophelesI* sps.

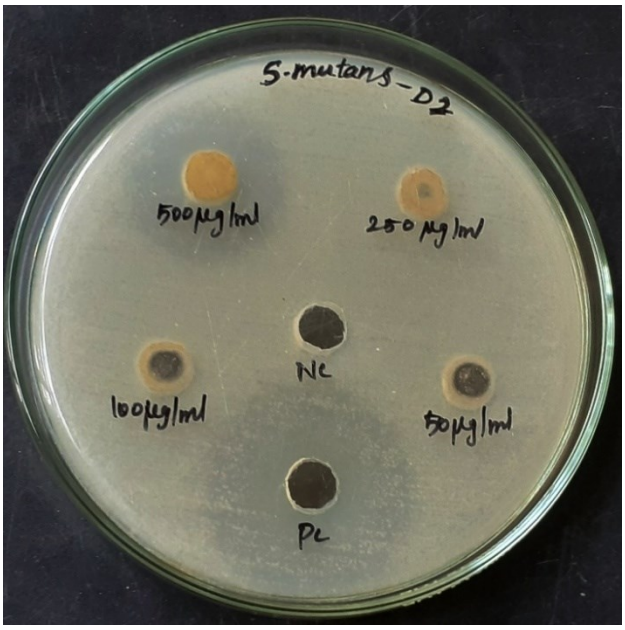


Plate 7: Effect of ethanolic extract of *Didemnum psammatode* against *Streptococcus mutans*

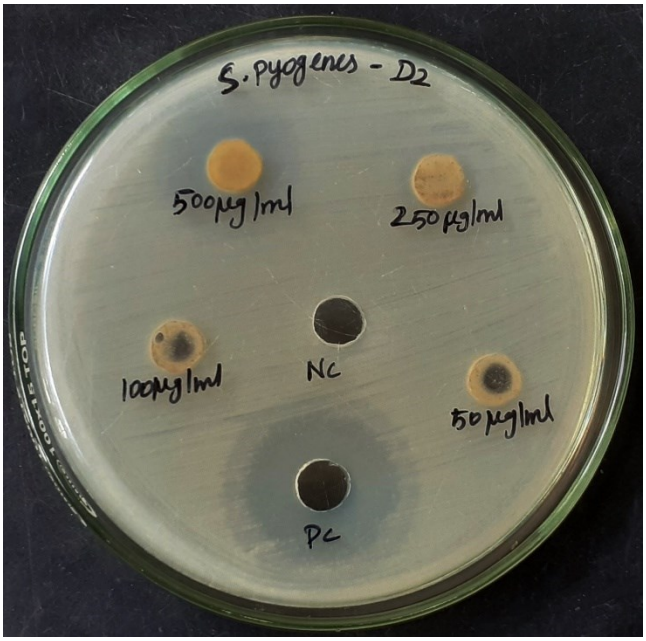


Plate 8: Effect of ethanolic extract of *Didemnum psammatode* against *Streptococcus pyogenes*

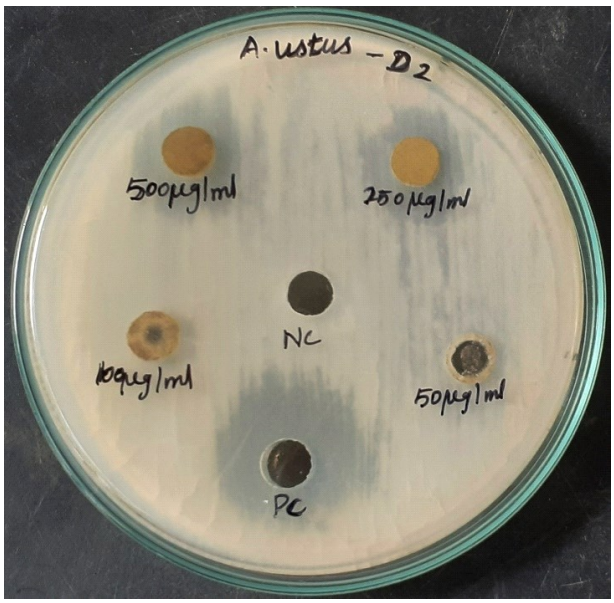


Plate 9: Effect of ethanolic extract of *Didemnum psammatode* against *Aspergillus ustus*

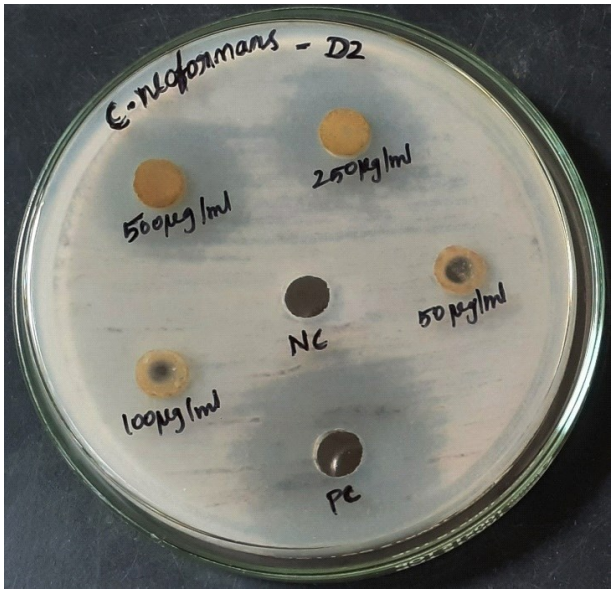


Plate 10: Effect of ethanolic extract of *Didemnum psammatode* against *Cryptococcus neoformans*

LARVICIDAL ACTIVITY

Ethanolic extract of *Didemnum psammatode* was tested for larvicidal activity against *Anopheles* mosquito larvae. Maximum mortality was observed at 500µg/ml of ethanolic extract of *Didemnum psammatode*. According to Silva *et al*., (2021) the larvicidal activity possessed by *Eugenia calycina Cambes* may be due to the presence of spathulenol, aromadendrane-4β, 10α-diol, and 1β-11-dihydroxy-5-eudesmene. The larvicidal activity possessed by *Phallusia nigra* may be due to the presence of alkaloids, terpenoids, saponins as stated by Meenakshi *et al*., 2012. The larvicidal activity possessed by *Cassia occidentalis* may be due to the presence of carbohydrates, glycoside, and tannins (Deepak kumar *et al*., 2014). The extract of *Didemnum bistratum* showed promising anti-Zika vector mosquito larvicidal action than anti-microbial activity, this may be due to the presence of anthraquinone and indole spermidine alkaloids group of compounds (Arumugam *et al*., 2019). According to Thakur *et al*., 2004 the larvicidal activity of Sea Cucumber against *Culex pipiens* may be due to saponins present in the extracts. Acidified Chitosan from marine crab shell shows the larvicidal activity against *Aedes aegypti (S. littoralis)*, this larvicidal activity may be due to the presence of O-acyl chitosan, O-(butyroyl) chitosan, O-(2-methylbutyroyl) chitosan, O-(pentanoyl) chitosan, O-(heptanoyl) chitosan, O-(nonoyl) chitosan and O-(decanoyl) chitosan (Perez *et al*., 2020). The hexane and

ethanol extracts of *Anacardium occidentale* exhibited promising larvicidal activity against *Aedes aegypti*, it is may be due to the presence of unsaturated steroids and triterpenoids, free fatty acids, fats and oils, γ-benzopyrone nucleus (flavonoids), leucoanthocyanins, anthraquinones, and tannins (Torres *et al*., 2015). Rouari *et al*., (2022) reported that rutin, caffeoylquinic acid and chlorogenic acid are responsible for larvicidal activity of acetonic extract of *Oudneya Africana*. Priya et al., 2016 stated that *Eudistoma viride* contains high amount of phenols flavonoids. The larvicidal activity possessed by naturally occurring compounds (Bacteria, Fungi and plants) may be due to the presence of terpenes and phenols (Milugo *et al*., 2021). Since *Eudistoma viride* contains phenols, the larvicidal activity may be due to the presence of phenols.

ANTIBACTERIAL ACTIVITY

Ethanolic extracts of *Didemnum psammatode* was tested for antibacterial activity using *Streptococcus mutants* and *Streptococcus pyogenes*. The zone of inhibition was noted as 15.5±0.7 mm at 500µg/ml concentration in *Streptococcus mutants*. In *Streptococcus pyogenes*, the Maximum zone of inhibition (14.5±0.7) was seen in higher concentration (500µg/ml). The antibacterial activity (*Streptococcus pyogenes*) possessed by medicinal plants may be due to presence of tannins (Nimri *et al.,* 1999). According to Mace *et al*., 2017, 1,2-naphthoquinone and 5-hydroxy-1,4-naphthoquinone inhibit *Streptococcus pyogenes*. The antibacterial activity (*Streptococcus pyogenes*) possessed by the leaf of *Rhodomyrtus tomentosa* may be due to the presence of

rhodomyrtone.(Limsuwan *et al*., 2013). Mint leaves are comprised of menthol, menthone, methyl esters and terpenoids which are responsible for antibacterial effect of *Streptococcus mutans* (Mohanta *et al*., 2007). *Eucalyptus spathulata* twig consists of ketones like juglone, regiolone, sterol, and flavonoid comprising antibacterial potential (Takarada *et al*., 2004). The *Mangifera indica* consists of tannins, bitter gum, and resins . The tannins and resins have astringent effect on mucous membrane; they protect enamel by forming layer on it (Muhammad *et al*., 1981). The active components of aloe vera are aloin and aloe-emodin inhibit protein synthesis by bacterial cells, which explains the antimicrobial activity of aloe vera (Fani *et al*., 2012).Ginger possesses gingerols responsible for the cell membrane rupture, which leads to the direct inhibition of the bacteria (Badreldin *et al*., 2008). The antibacterial activity (*Streptococcus mutans*) possesses by the therapeutic potential of *Ocimum sanctum* has been found to be largely due to eugenol carcacrol, ursolic acid, methyl eugenol, caryophyllene which is responsible for the protein leakage in the bacteria, contributing to its antibacterial effect (Agarwal *et al*., 2010). The antibacterial activity of the crude methanol and ethanol extract of *P. arabica* against human clinical isolates may be due to the presence of alkaloids and peptides (Prabhu *et al*., 2011). The antibacterial activity of ethanolic extracts of *Didemnum psammatode* against *Streptococcus mutants* and *Streptococcus pyogenes* may be due to the presence of alkaloids and peptides.

ANTIFUNGAL ACTIVITY

Ethanolic extracts of *Didemnum psammatode* was tested in Antifungal activity against *Aspergillus ustus* and *Cryptococcus neoformans.* Maximum zone of inhibition was observed in the highest concentration of 500 µg/ml of the extract of *Didemnum psammatode*. According to Kossuga *et al.,* (2004) a new antifungal agent is (2S,3R)-2aminododecan-3-ol (1), has been isolated from the ascidian *Clavelina oblonga* collected in Brazil. According to Pech PD *et al.,* (2022) the antifungal activity displayed by the *Amanita citrina* extract may be due to the presence of agelasidines. In the present study, ethanolic extract of *Didemnum psammatode* exhibited significant antifungal activity against *Aspergillus ustus* and *Cryptococcus neoformans.*

CONCLUSION

Thousands of natural products including alkaloids, cyclic peptides and polyketides *etc* have been isolated from ascidians. Most of these secondary metabolites possess diverse bioactivities, such as antibacterial, antifungal and antitumor activities. The plitidepsin (aplidin) is highly effective against severe acute respiratory syndrome SARS-CoV-2 and it was 27.5 fold more potent than that of remdesivir. YONDELIS (trabectedin) derived from Caribbean tunicate *Ecteinascidia turbinata* indicated for the treatment of metastatic liposarcoma. The animal which are considered as the nuisance and affect the economy by corrosion were used for this study. Such a natural product is good for health and devoid of side effects. The result from the study showed that the ethanolic extract of *Didemnum psammatode* has great potential to be used as eco-friendly larvicide.

SUGGESTION

- As the extract of *Didemnum psammatode* showed larvicidal activity, against *Anopheles species,* other species such as *Aedes species and culex species* can also be tried.
- Similarly, larvicidal activity of other species of ascidians can also be done.
- A further study on isolation, purification, structure determination and subsequent recognition of the novel mechanism of action of the clinically effective agent is suggested.

- Sample of *Didemnum psammatode* Sluiter, 1895 were collected during the low tide from the intertidal rocky area of Hare Island.
- The sample were dried and made into powder. Then the whole body was extracted using Soxhlet extraction method and the solvent was separated by rotary evaporator.
- The ethanolic extract of *Didemnum psammatode* were tested against the larvae of *Anopheles species*. The highest concentration of the ethanolic extract of *Didemnum psammatode* exhibited highly significant mortality rate. Dose dependent mortality rate was observed.
- The ethanolic extract of *Didemnum psammatode* can be used as eco-friendly larvicide.
- Ethanolic extracts of *Didemnum psammatode* was tested for antibacterial activity using *Streptococcus mutants* and *Streptococcus pyogenes*. The zone of inhibition was noted as 15.5±0.7 mm at 500µg/ml concentration in *Streptococcus mutants*. In *Streptococcus pyogenes*, the Maximum zone of inhibition (14.5±0.7) was seen in higher concentration (500µg/ml).
- Ethanolic extracts of *Didemnum psammatode* was tested in Antifungal activity against *Aspergillus ustus* and *Cryptococcus neoformans.* Maximum zone of inhibition was observed in the highest concentration of 500 µg/ml of the extract of *Didemnum psammatode*.

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ANTIOXIDANT AND GC – MS ANALYSIS OF MARINE PUFFER FISH *LAGOCEPHALUS SCELERATUS* FROM THOOTHUKUDI COAST.

A project submitted to

ST. MARY'S COLLEGE (Autonomous), THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

by

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DEPARTMENT OF ZOOLOGY

ST. MARY'S COLLEGE (Autonomous),

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

THOOTHUKUDI – 628 001

April - 2023

CERTIFICATE

This is to certify that the project **entitled Antioxidant and GC-MS Analysis of Marine Puffer Fish *Lagocephalus sceleratus* from Thoothukudi coast** is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a record of the work done during the year 2022-2023 by the following students.

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CONTENTS

INTRODUCTION

1. INTRODUCTION

The Gulf of Mannar has an area of about 10,500 km^2. In this region, totally 3600 species of fauna and flora have been identified. It is one of the most biologically diverse coastal regions in the planet earth (Venkataramani *et al.*, 2007). Ocean offers a large biodiversity of fauna and flora which is estimated to be over 5,00,000 species and more than double that of the land (Anand *et al.*, 1997). This rich diversity of marine organisms assumes a great opportunity for the discovery of new bioactive substances. Thus the marine environment is an exceptional reservoir for bioactive natural products, many of which exhibit structural features that are not found in terrestrial natural products (Johansson and Soderhall, 1985).

Marine organisms are a rich source of structurally novel and biologically active metabolites. Primary and secondary metabolites produced and stored by these organisms may be potential bioactive compounds of interest in the pharmaceutical industries. The number of natural products isolated from marine organism increases rapidly (Faulkner, 2002; Proksch and Muller, 2006). More than 10,000 compounds have been isolated from marine organisms (Proksch *et al.*, 2002) with hundreds of new compounds still being discovered every year.

The rich diversity of marine organisms assumes a great opportunity for the discovery of new bioactive compounds. The bioresources present in the marine ecosystem have potent biomolecules which includes many natural organic product. Many classes of natural products from marine sources exhibiting antitumour, anti-leukaemia, antibacterial and antiviral activities have been reported worldwide (Khora, 2013).

The largest source novel discovery of natural products such as pharmalogical matabolites medicine is the marine biota. There has been an extensive research showing that vast bioactive substances were identified and characterized from marine organisms indeed several of them showed promising results to that human and animal diseases (Grabely and Thieriche, 1999).

Tetraodontidae its diverse with species such as Puffer fish, Balloon fish, Blow fish, bubble fish, Globe fish, Swell fish, Toad fish, Toadies, Honey toads and squab (Mills and Passmore, 1988; Ramaiyan and Senthil Kumar, 1998; Frose and Pauly, 2007). They are commonly distributed in tropics, but are relatively uncommon in temperate regions and completely absent from cold water. There are 189 species of Puffer fishes and 28 genera in the family Tetraodontidae (Oliveira *et al.*, 2006). Puffer fishes are the second most poisonous vertebrate in the world, (Keichii *et al.*,1998). The skin and other certain internal organs of puffer fish to be the common cause of fish poisoning along the Asian coast (Chew *et al.*,1983).

Puffer fish is known to carry tetraodotoxin (TTX) (Bilecenogho *et al.*,2006 , Kasapidis *et al.*,2007); Sabrah *et al.*, 2006). Which is known as non-protein organic compound and one of the strongest marine paralytic toxins today.

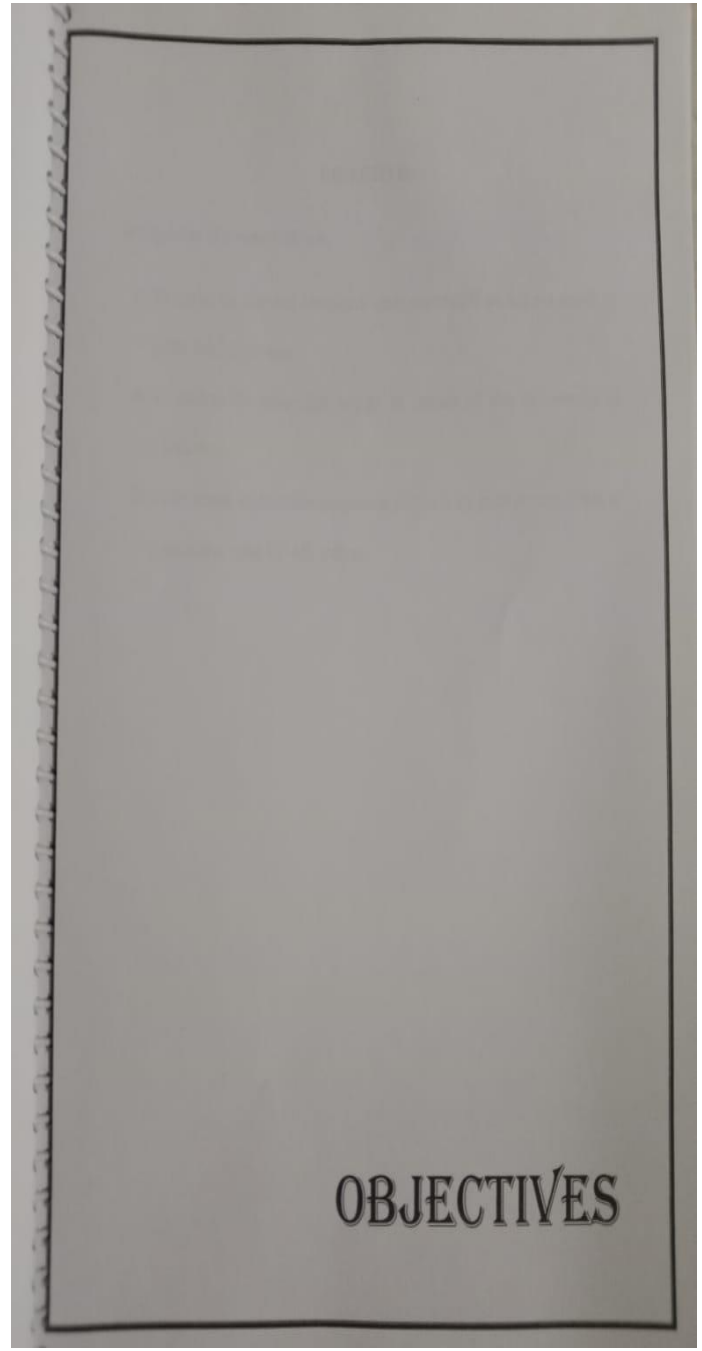
Puffer fish can be lethal if not served properly puffer poisoning usually result from consumption of incorrectly prepared puffer soup or from raw puffer meat. When it is not much more likely to cause death and often causes toxication and induces dizziness of lips. Puffer fish tetrodotoxin deadens the tongue and lips and prickling over the body rapid heart rate, decreased blood pressure and muscle and stops the person who has ingested it from breathing (Helios *et al.*, 2002).

The crude puffer fish extracts have been used for treating migrains and menstrual cramps. Poisoning due to TTX toxicity is recorded from 17^{th} centuary, from the past few years. TTX is studied for its potential for identification of isolation of characterization of voltage - gated sodium channels, in 1992. This specific binding ability of TTX has gained importance in biomedical research. Several reports have been published concerning the effect of TTX on cells of differentiate size, type and shape. A group of scientists performed clinical traits in Canada and China using tetrodotoxin as an analgesic stage of cancer inpatients. The clinical traits indicated that a formulation of TTX, Tetrodotoxin was safe when given in very small doses. It also reported to have potential to alleviate acute heroin withdrawl syndrome in addicts with few side effects.

Antioxidant means "against oxidation". Antioxidants work to protect lipids from peroxidation by radicals. Antioxidants are effective because they are willing to give up their own electrons to free radicals. When a free radical gains the electron from an antioxidant it no longer needs to attack the cell and the chain reaction of oxidation is broken (Dekkers *et al*., 1996). The role of antioxidants has received increased attention during the past decade. However, the use of synthetic antioxidant 8 have potential health hazard (Park *et al*., 2001). Antioxidants can be derived from the daily diet, including fruits, vegetables, nuts and fish (Anderson *et al*., 2001; Pellegrini *et al*., 2006; Rajaram *et al*., 2009). Antioxidants lower the level of low - density lipoprotein cholesterol, thus preventing plaque deposition in the blood vessels. It is beneficial in cancer prevention (Bartlett and Eperjsei, 2003). Many naturally occurring antioxidant compounds in main ingredients used for the preparation of Traditional Chinese medicine have been identified as free radical or active oxygen scavengers (Duh, 1998; Kumaravel *et al*., 2012; Pan *et al*., 2007).

GC/MS is a combination of two different analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS), is used to analyze complex organic and biochemical mixtures. GC can separate volatile and semi-volatile compounds with great resolution, but it cannot identify them. MS can provide detailed structural information on most compounds such that they can be exactly identified and quantified, but it cannot readily separate them. Gas Chromatography

(GC), is a type of chromatography in which the mobile phase is a carrier gas, usually an inert gas such as helium or an un-reactive gas such as nitrogen and the stationary phase is a microscopic layer of liquid or polymer on an inert solid support. A gas chromatography uses a thin capillary fibre known as the column, through which different chemicals pass at different rates depending on various chemical and physical properties. As the chemicals exit at the end of the column, they are detected and identified electronically. The function of the column as to separate and concentrate different components in order to maximize the detection signal. Mass spectrum is an analytical technique, which can provide information concerning the molecular structure of organic and inorganic compounds. It is one of the few methods that can be used as a qualitative analytical tool to characterize different organic substances. With it, one can do analysis of mixtures (gases, liquids and in some cases solids) quantitatively. A mass spectrometer is also useful to investigate reaction mixtures and in tracer work. It is also used in understanding kinetics and mechanisms of unimolecular decomposition reactions (Veerakumari, 2006). The aim of the presents study has been carried out to establish the occurrence of antioxidant activity and GC-MS analysis of *Lagocephalus sceleratus* collected from Thoothukudi coast.


OBJECTIVES

OBJECTIVES

The objectives of present study are,

- To extract the chemical compounds using methanol from skin and muscle of puffer fish *L.sceleratus*.
- To analyze the antioxidant activity of extracts of skin and muscle of *L.sceleratus*.
- To determine the bioactive compounds present in the crude extract of skin of *L.sceleratus* using GC-MS analysis.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Natural products have been used for the treatment of human ailments since the beginning of mankind. Ocean remains as one such treasure for natural products. The oceans cover more than three-quarters of the earth's surface and harbor most of the planet's diversity. But the marine biotope, which is still an unexplored area, can provide us with rich novel natural products. For decades, microbial natural products have been the reservoir for drug discovery, Yet the microorganisms inhabiting the world's oceans have largely been overlooked in this regard (Bollman M, 2010). Natural products once played a major role in drug discovery. The marine environment coves more than 70% of the world's surface. In the past, this has proven to be a rich source of extremely potent compounds, which represent a considerable number of drug candidates (Newman DJ, Cragg GM, 2018). However, to date, the biodiversity of marine microbes and the versatility of their bioactive metabolites have not been fully explored.

Marine sources have played a significant role as an origin for lead molecules ascertained for various pharmacological utilizations in recent times. Interestingly, marine microorganisms remain as the most undiscovered and essential provenience of umpteen bioactive metabolites. From the shallow water in the seashore to the abysmal seaward areas that canvas 70% of the biosphere, microorganisms engross an endurable stretch Pinnaka AK and Tanuka NPS (2019).

From the beginning of humankind, natural products have been a beneficial source as a remedy for various ailments. In worldwide, the available drugs for clinical purpose represents more than 50% are of their natural origin. The drug discovery process from natural products is still ongoing due to synthetic drugs' side effects (Haefner, 2003). The crude product has a significant impact on producing new medicines that bypass infectious diseases (Lahlou M, 2013).

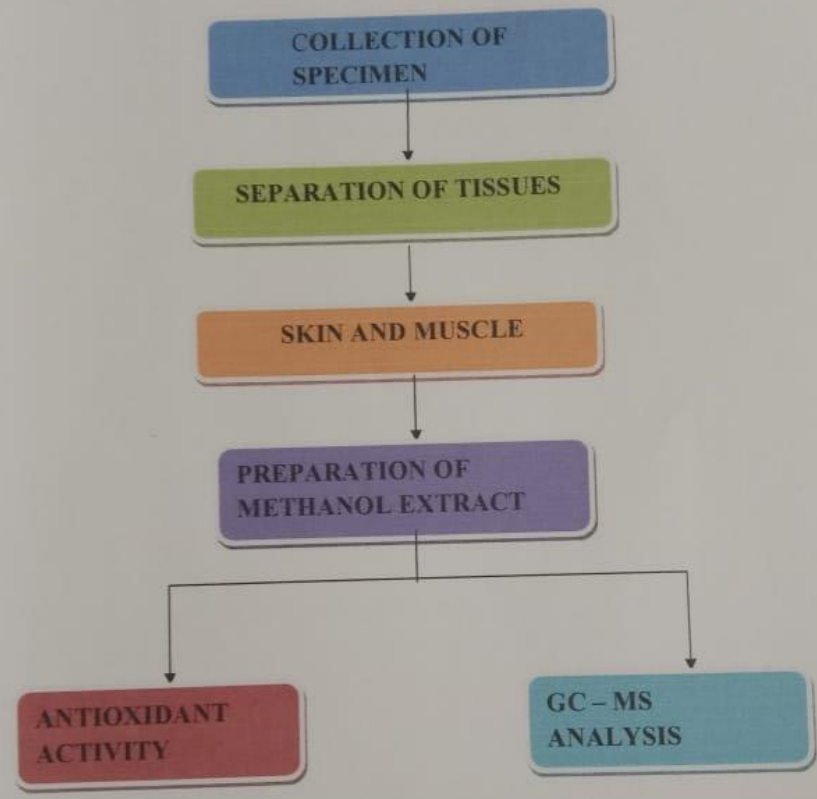
Around 200 species of marine fish, including stingrays, scorpion fish, zebra fish, stone fish, weever fish, toadfish, stargrazers and some species of shark, ratfish, catfish, sugeonfish and blenny which are known or suspected to be venomous (Russell, 1996). The complexity of fish venoms are evident from a number of different components found mainly proteins and peptides (Sivan, 2009) which are responsible for a variety of pharmacological activities. Published literature pertaining to puffer fishes from the Indian region revealed comprehensive taxonomic works during the nineteen century. Hamilton, (1822) and Day (1865) reviewed the taxonomy of the genus Tetraodon through exhaustive morphological descriptions of freshly collected and museum specimens along with review of existing literature and revealed great degree of polymorphism among puffer. Talwar and Jhingran (1991) provided descriptions of eleven puffer species of the Indian coasts along with brief information on their geographical distribution and ecology.

Marine food derived functional ingredients as potential antioxidants in the food industry (Ngo *et al*., 2011).The reducing power of a sample is an indicator of its antioxidant activity. The reducing power assay is used to evaluate the ability of an antioxidant to donate an electron or hydrogen (Yildirim *et al*., 2001). Ali *et al*., (2009) reported that Sardinella with alcalase enzyme has shown a potent reducing power. DPPH is a stable free radical has been used to evaluate the ability of compound as free radical scavengers or hydrogen donars and to evaluate the antioxidant activity. It is a compound with a characteristic absorption at 517 nm, using a Shimadzu UV spectrophotometer (Wu *et al*., 2003).

Protein hydrolysates with antioxidant properties, in particular have become a topic of great interest for the pharmaceutical industries (Alasalvar *et al*., 2002; Hagen and Sandness, 2004). There is also a growing interest in antioxidant from natural sources, which may have lesspotential health hazard compared with synthetic antioxidants. Water soluble proteinous substances may contribute to the scavenging activity of fish. Antioxidative activity of peptides produced from protein hydrolysis has been reported by numerous studies (Srinivas *et al*., 1992; Chen *et al*., 1995; Park *et al*., 2001). Sampath kumar e*t al*., (2012) reported that purification and identification of antioxidant peptide from the skin protein hydrolysate of two marine fishes, horse 21 mackerel (Magalaspis cordyla) and croaker (Otolithes ruber). Studies showed that fish and fishery products have a

EXPERIMENTAL DESIGN

EXPERIMENTAL DESIGN



MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 SYSTEMATIC POSITION OF EXPERIMENTAL ANIMAL:

Kingdom : Animalia

Phylum : Chordata

Class : Actinopterygii

Order : Tetraodontiformes

Family : Tetraodontidae

Genus : *Lagocephalus*

Species : *sceleratus*

3.2 DESCRIPTION OF EXPERIMENTAL ANIMAL:

Class: Actinopterygii

The actinopterygii or ray finned fishes constitute a class of the bony fishes. The ray finned fishes are so called because their fins are webs of skin supported by bony or horny spines ("rays"), as opposed to the fleshy, lobed fins that characterize the ass Sarcopterygii (lobe - finned fish). These actinopterygian fin rays attach directly to the proximal or basal skeletal elements, the radials which represent the link or connection between these fins and the internal skeleton (e.g., pelvic and pectoral girdles). Numerically, actinopterygians are the dominant class vertebrates, comprising nearly 99% of the over 30,000 species of fish. They are ubiquitous throughout freshwater and marine environment from the deep sea to the highest mountain streams. Traditionally they have been divided into the sub-class Chondrostei and Neopterygii. Neopterygii in turn have been divided into two infra

class. Holestei and Teleostei, Ray-finned fishes constitute a major source of food for millions of people (Helfman *et al*., 1997).

Order: Tetraodontiformes

The Tetraodontiformes are an order of highly derived ray – finned fish, also called the Plectognathi. Sometimes these are classified as a suborder of the order Perciformes. The Tetraodontiformes are represented by 10 extant families and at least 349 species overall; most are marine and dwell in and around tropical coral reefs, but a few species are found in freshwater streams and estuaries (Tyler, 1980).

Family: Tetraodontidae

The species that come under this family are well known for its unique and distinctive adaptations that the puffer fish has to defend itself. They are the second most poisonous creature on the planet. The puffer's remarkable ability to expand its body extremely quickly when forced with danger, unavailing its long poisonous spikes that cover its body. They can be found in a variety of colour but can sometimes be hard to identify when they are not inflated. The puffer normally has appearance of a large tadpole with bulging eyes and an elongated snout. They are omnivorous and they mainly feed on the algae that grow on the rocks and corals (Gladfelter *et al*., 1980).

***Lagocephalus sceleratus*: (Plate 1)**

Puffer fish also known as the silver-cheeked toad fish *L.sceleratus* is a widely distributed species which inhabits the tropical Indian and Pacific oceans. This species inhabits sandy or muddy substrate areas near shallow coral reefs at depths reaching 100m.The puffer fish *L.sceleratus* is one of the largest members of its family reaching 110 cm and 7 kg. Scales on the body are absent, while only

very small spinules can be observed on its belly and on its dorsal surface. The lateral lines are easily visible and the body is dark brown with regular blotches on its back, except for the belly which is white.

It has two strong teeth in each jaw which are capable of ripping and damaging fishing nets and long lines giving the family of *L.sceleratus* its name (tetras = four, odontos = tooth) (Plate 1).

3.3 Collection of Specimen:

Specimens of the puffer fish *L.sceleratus* were collected from fishing harbour Thoothukudi. They were kept in ice-box and transported to the laboratory. They were maintained in a deep freezer at - 20°C until use.

3.4 Preparation of methanol extract:

The preparation of methanol extract was followed by Chellaram *et al*., (2014). 10 g of dry powdered tissue was soaked in methanol and kept in an orbital shaker for 72 hours. The extract was filtered through Whatman No.1 filter paper, centrifuged at 15,000 rpm for 30 minutes and the solvent was concentrated by rotary evaporator (VC 100 A Lack Rotavapor at 30°c) with reduced pressure to give a dark brown gummy mass. The resultant residue was stored at 4°C for further analysis.

3.5 Antioxidant activity:

Total Antioxidant Activity (TAA) by Phosphomolybdenum assay:

TAA was estimated by Phosphomolybdenum assay (Prieto *et al*., 1999). Sample of concentration 1000g /ml were taken in individual test tubes and made up 1 ml using distilled water and 2 ml of Molybdate reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium Molybdate). The test tubes

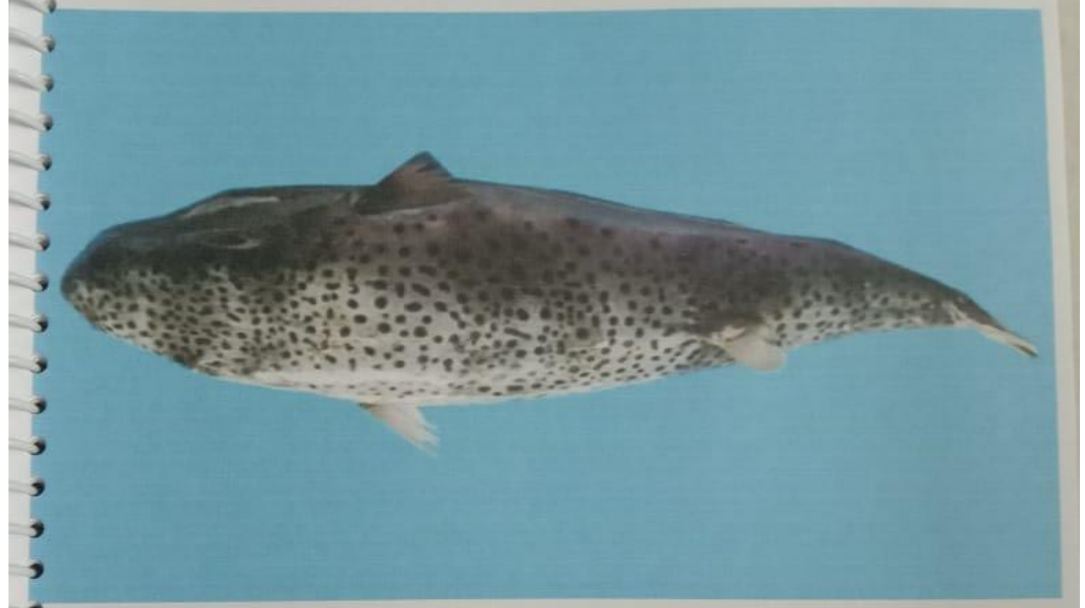


Plate : 1 ***Lagocephalus sceleratus***

were incubated at 95°C for 90 min. After incubation, the tubes were cooled to room temperature for 20 - 30 min and the absorbance of the reaction mixture was measured at 695nm. Ascorbic acid was used as the positive reference standard.

% Antioxidant activity = Abs sample/Abs std * 100

3.6 GC-MS Analysis:

GC-MS analysis of methanol extracts of *L.sceleratus* 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 5ms 30 X 0.25 system) equipped with Elite-1, fused silica capillary column composed of 5% phenyl Arylene 95% Dimethyl poly siloxane. The system comprising a COMBIPAL autosampler set under the following conditions: helium (99.999%) 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°C; the oven temperature was programmed from 100-270°C at the rate of 5°C; total GC running time was 63 minutes. Interpretation on mass spectrum of GCMS was done by using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass 41 spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST, WILEY and FAME-8 library. The name, molecular weight and structure of the components of the test materials were ascertained.

RESULTS

4. RESULTS

4.1 Antioxidant activity:

The antioxidant activity of skin and muscle extracts and positive control (ascorbic acid) was assessed based on their ability to scavenge the TAA phosphomolybdenum assay. The TAA by phosphomolybdenum assay scavenging activity of methanol extracts of skin and muscle of *L.sceleratus* was evaluated. The skin exhibited the strongest antioxidant activity with 16.67 %, 18.94 %, 22.73 %, 25 % and 29.55 % at 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml and 1000 µg/ml respectively (Fig 1). The methanol extract of muscle exhibited antioxidant activity with the percentage of 7.58% at 200 µg/ml, 9.09% at 400µg/ml, 10.61% at 600µg/ml, 12.12% at 800µg/ml and 22.73% at 1000µg/ml concentrations (Fig 2).

4.2 GC-MS Analysis:

Fifteen chemical compounds were identified in the skin extract of *L.sceleratus* through GC - MS study. They are Azulene (RT-4.186), Naphthalene (RT-4.186), 1,2-Cyclohexanediol cyclic sulfate (RT-4.105), Cyclohexylmethyl formate (RT-14.105), trans-4-Methylcyclohexanol (RT-14.105), Hexane, 3-ethyl (RT- 4.662), Azetidine, 1,2-dimethyl (RT-14.662), Heptane, 2,3-dimethyl (RT-14.662), Sulfurous acid, dodecyl 2-propyl (RT-

15.551), 10-Methylnondecane (RT-16.582), Sulfurous acid, butyl decyl ester (RT-16.582), Heptacosane, 1-chloro- (RT-16.582), Tetradecane, 1-fluoro- (RT-17.830), Hexane, 2,3,4-trimethyl (RT-19.362) and Sulfurous acid, nonyl 2-propyl ester (RT-19.362) (Fig 3 – 18).

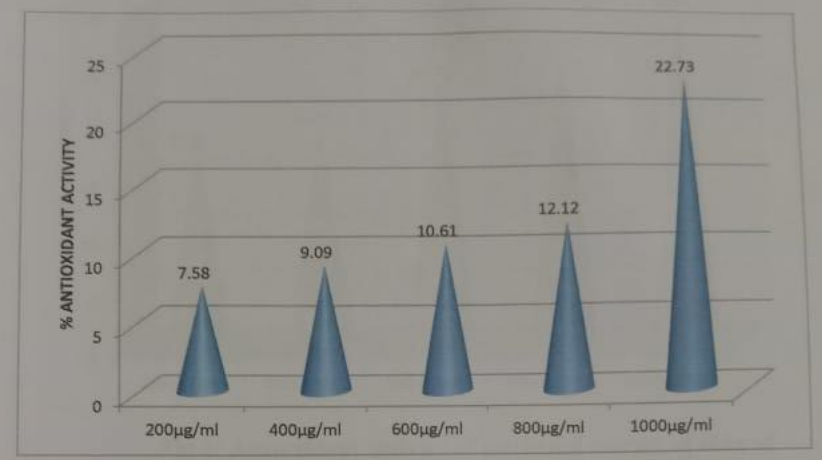


Fig 2: Antioxidant activity of methanol extract of muscle of *Lagocephalus sceleratus*

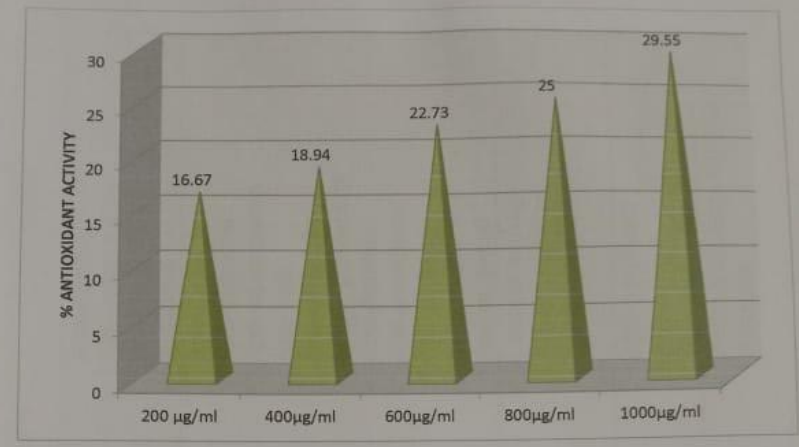


Fig 1: Antioxidant activity of methanol extract of skin of *Lagocephalus sceleratus*

Table: 1 Activity of compounds identified in the skin extract of *Lagocephalus sceleratus*

S NO	RT	Name of the compound	Molecular formula	Molecular weight	Peak area %	Activity
1.	4.186	Azulene	$C_{10}H_8$	128.169	13.58	Anti-allergic Anti-inflammatory Non-steroided Analgestic
2.	4.186	Naphthalene	$C_{10}H_8$	128.169	13.58	Anti-cancer Anti-microbial Anti-inflammatory Anti-vital Anti-tubercular Anti-hypersentivity Anti-diabetic Anti-convulsant Anti-plaletet Aggregation Anti-protozoal
3.	14.105	1,2-cyclohexane diol cyclic Sulfate	$C_6H_{12}O_2$	116.16	12.36	Catalytic activity Allylic Oxidation Epoxidation
4.	14.105	Cyclohexyl methyl cyclohexanol	$C_8H_{14}O_2$	142.2	12.36	Anti-convulsant Anti-seizure Anti-viral
5.	14.105	trans-4-Methyl cyclohexanol	$C_7H_{14}O$	114.19	12.36	Aedes triseriatus oviposition attractant
6.	14.662	Hexane 3-ethyl	$C_{24}H_{25}F_3N_2O_3$	446.5	4.09	Anti-inflammatory Anti-cancer Anti-microbial
7.	14.662	Azetidine	$C_{25}H_{26}N_4O_4S$	478.6	4.09	Anti-cancer

		1,2 dimethyl				Anti-bacterial Anti-microbial Anti-Chizophrenic Anti-malarial Anti-obesity Anti-inflammatory Anti-diabetic Anti-viral Anti-oxidant Analgesic Dopomine antagonist
8.	14.662	Heptane 2,3 dimethyl	$C_{10}H_{15}NO_4$	213.23	4.09	Neurotoxin Anti-microbial
9.	15.551	Sulfurous acid, dodecyl 2-propyl	$C_{15}H_{32}O_3S$	292.5	16.88	Anti-oxidant Anti-chlorination
10.	16.582	10-Methyl non decane	$C_{20}H_{42}$	282.5	20.90	Anti-oxidant Anti-microbial
11.	16.582	Sulfurous acid butyl decyl ester	$C_{14}H_{30}O_3S$	278.45	20.90	Ant- ioxidant
12.	16.582	Hepta cosane1-chloro	$C_{27}H_{55}Cl$	415.2	20.90	Anti-detergents
13.	17.830	Tetradecane 1,-fluro	$C_{14}H_{19}FN_2$	234.31	18.81	Anti-mercaptans Oxofuels Auto motive additive
14.	19.362	Hexane 2,3,4 – trimethyl	C_9H_{20}	128.25	13.38	Anti-microbial Anti-inflammatory Anti-cancer

						Anti-bacterial Anti-oxidant
15.	19.362	Sulfurous acid nonyl 2-propyl ester	$C_{12}H_{26}O_3S$	250.4	13.38	Anti-oxidant Disinfectant Anti-drug Anti-detergent

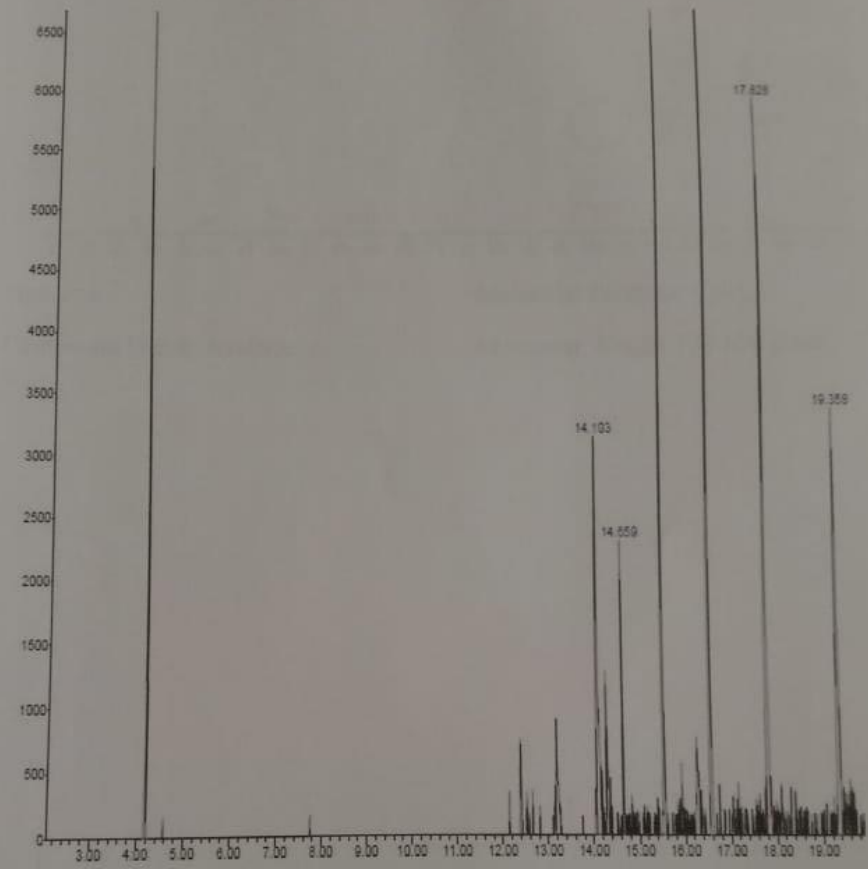


Fig : 3 Chromatogram – Methanol extract of skin of

Lagocephalus sceleratus

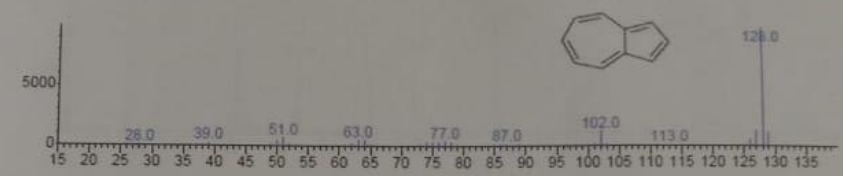


Figure: 4

Molecular Formula: $C_{10}H_8$

Compound Name: Azulene

Molecular Weight:128.169 g/mol

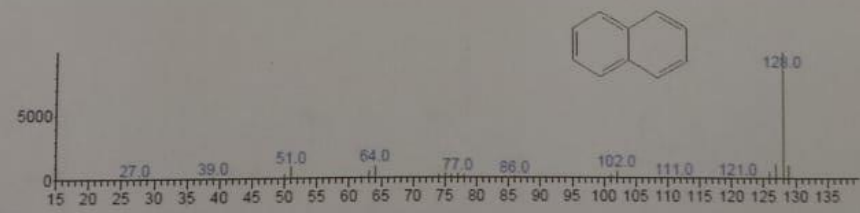


Figure: 5

Molecular Formula: $C_{10}H_8$

Compound Name: Naphthalene

Molecular Weight: 128.169 g/mol

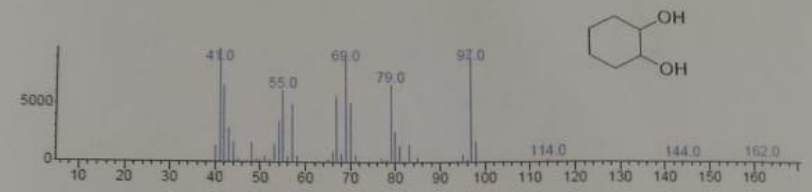


Figure: 6

Molecular Formula: $C_6H_{12}O_2$

Compound Name: 1,2-Cyclohexane diol cyclic sulfate

Molecular Weight: 116.16 g/mol

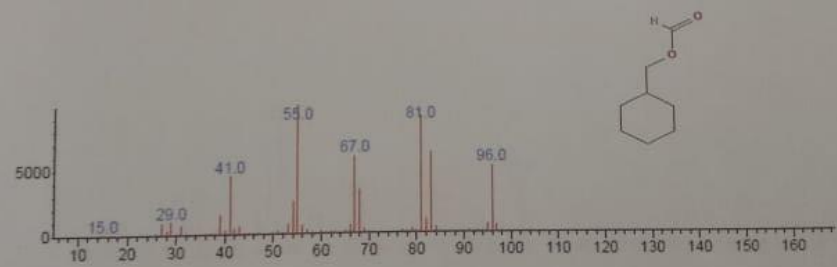


Figure: 7

Molecular Formula: $C_8H_{14}O_2$

Compound Name:Cyclohexyl methyl formate

Molecular Weight: 142.2 g/mol

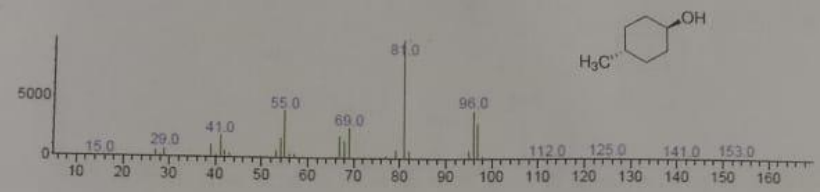


Figure: 8

Molecular Formula: $C_7H_{14}O$

Compound Name: trans-4-methyl cyclohexanol

Molecular Weight: 14.19 g/mol

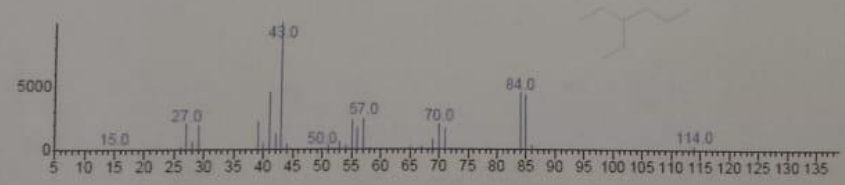


Figure: 9

Molecular formula: $C_{24}H_{25}F_3N_2O_3$

Compound Name: Hexane 3-ethyl

Molecular Weight: 446.5 g/mol

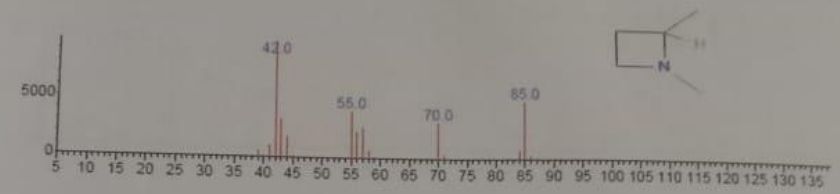


Figure: 10

Compound Name: Azetidine 1,2 dimethyl

Molecular Formula: $C_{25}H_{26}N_4O_4S$

Molecular Weight: 478.6 g/mol

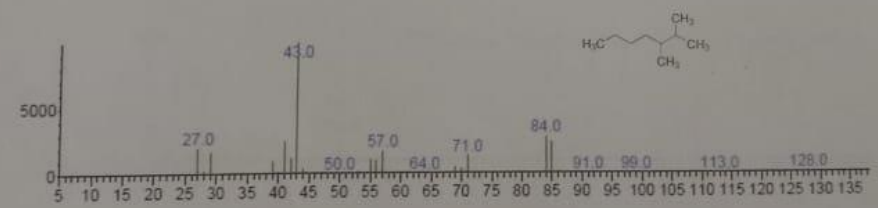


Figure: 11

Compound Name: Heptane 2,3 dimethyl

Molecular Formula: $C_{10}H_{15}NO_4$

Molecular Weight: 213.23 g/mol

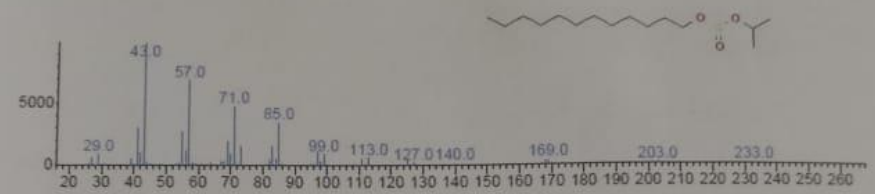


Figure: 12

Molecular Formula: $C_{15}H_{32}O_3S$

Compound Name: Sulfurous acid dodecyl 2 - propyl

Molecular Weight: 292.5 g/mol

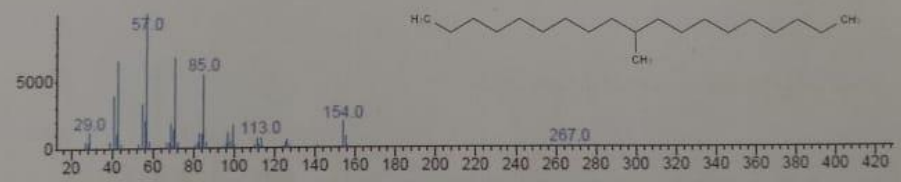


Figure: 13

Molecular Formula: $C_{20}H_{42}$

Compound Name: 10-Methyl non decane

Molecular Weight: 282.5 g/mol

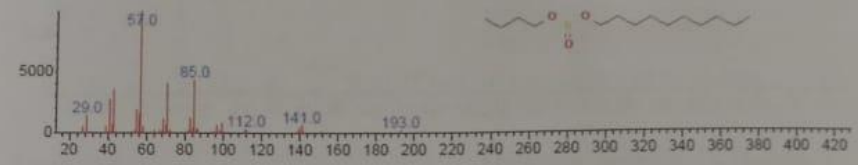


Figure: 14

Molecular Formula: $C_{14}H_{13}O_3S$

Compound Name: Sulfurous acid butyl decyl ester

Molecular Weight: 278.45 g/mol

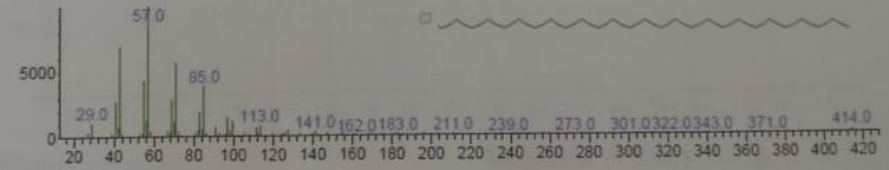


Figure: 15

Molecular Formula: $C_{27}H_{55}Cl$

Compound Name: Hepta cosine 1-chloro

Molecular Weight: 415.2 g/mol

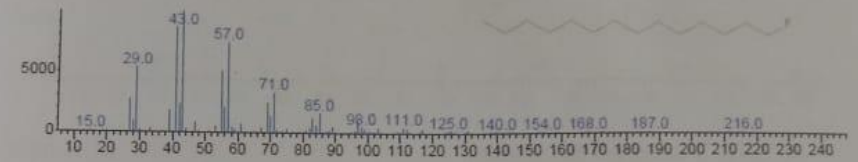


Figure: 16

Molecular Formula: $C_{14}H_{19}FN_2$

Compound Name: Tetredecane 1, -fluro

Molecular Weight: 234.31 g/mol

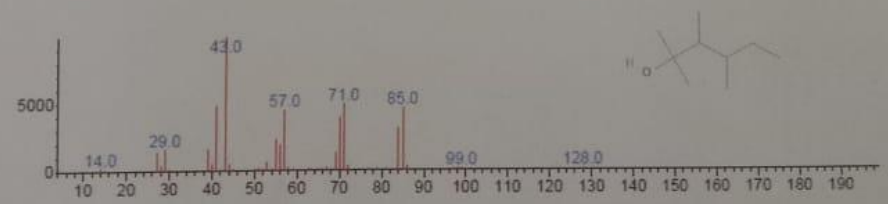


Figure: 17

Molecular Formula: C_9H_{20}

Compound Name: Hexane 2,3,4 – trimethyl

Molecular Weight: 128.25 g/mol

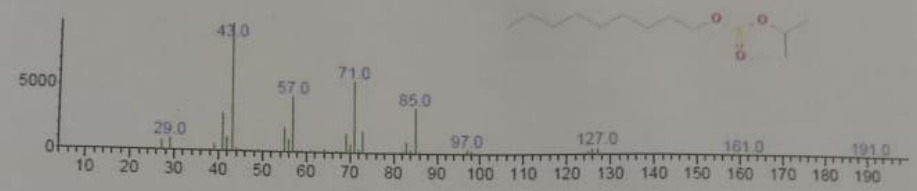


Figure: 18

Molecular Formula: $C_{12}H_{26}O_3S$

Compound Name: Sulfurous acid nonyl 2-propyl ester

Molecular Weight: 250.4 g/mol

DISCUSSION

5. DISCUSSION

The bio resources present in the marine ecosystem have potent biomolecules which includes many natural organic compounds. These compounds are reported to have biological activities like antitumor, antiviral, analgesic etc. (Raja Manikandan *et al*., 2011). Because of so many toxic molecules have been known from marine organisms, it has become evident that ocean is a likely source of pharmaceuticals and interesting biochemicals useful to biotechnology and its researches (Colwell 2002; Rajeev and Xu 2004; Faulkner and Fenical 2005).

Oxidation stress is closely related to all aspects of cancer, from carcinogenesis to the tumor bearing state and from treatment to prevention (Noda and Wakasugi, 2001). Epidemiologic studies have suggested that some antioxidants of dietary constituent exhibit antioxidant properties may be acting as naturally occurring 55 anticancer agents and may explain some of the differences in cancer incidence seen in populations with varying dietary intake (Greenwald *et al*., 2001). The methanol extract of puffer fish *L.sceleratus* was examined for its antioxidant activity using TAA by phosphomolybdenum assay scavenging activity. As Phosphomolybdenum assay has been routinely used to evaluate the antioxidant activity capacities of extracts (Prieto *et* al; 1999). Various extracts of *B.vahilii* were also used to determine their antioxidant capacities by the formation of green phosphomolybdenum complex (Sun Chul Kang *et al.,* 2013). Among the genders,

the activity levels were higher in females than in males (Sanaye *et al*., 2014). Hunget *et al*., (2008) who found relatively higher scavenging activity in belly portions compared to other body portions. Crude extracts of skin and muscle and of *L.sceleratus* were subjected for the evaluation of antioxidant activity using TAA by phosphomolybdenum assay scavenging activity test. Results of the present study showed that the crude methanol extracts of skin and muscle of *L.sceleratus* exhibited antioxidant activity.

These findings are also supporting the present study. In the present study the crude extracts of puffer fish scavenged the TAA by phosphomolybdenum assay in a concentration dependent manner. Among the two tissues tested, skin extract showed strong scavenging activity of TAA by Phosphomloybdenum assay and clearly suggested that the antioxidant activity of skin extract was related to its ability to scavenge TAA by Phosphomolybdenum assay.

GC – MS is an analytical method that combines the features of gas chromatography and mass spectrometry to identify different substances within a test sample (Sparkman *et al*., 2011). GC – MS analysis is an indirect method to detect TTX in a crude extract which is difficult to purify in other advanced analysis method (Yotsu *et al*., 2007). Kirimer *et al*., (2016) determined TTX and fatty acid contents of five specimens of *L. sceleratus* by LC – MS/ MS analysis in intestine, liver, ovary and muscle. The mass spectra of various tissues of *L. sceleratus* was

reported. Detection of tetrodotoxin in puffer fish using GC- MS was reported by Man *et al*., (2010). Sensitive analysis of TTX in human plasma by solid – phase extraction and GC – MS was reported by Kurono *et al*., (2001). Ravi *et al*., (2016) reported the GC – MS analysis of skin, liver, intestine and gonad extracts of puffer fish *Diodon hystrix* to detect the presence of TTX in these organism. Inthumathi and Khora (2017) analysed the presence of tetrodotoxin in the puffer fish *Takifugu oblongus* through GC – MS study.

In the present study totally 15 compounds were identified in skin of *L.sceleratus*. These compunds exhibited the following biological activities such as anti-inflammatory, anti-oxidant, anti-drug, anti-detergent, anti-microbial, anti-bacterial, analgesic, anti-cancer, anti-allergic, anti-malarial, non-steroid, anti-chlorination, anti-protozol, anti-platelet, anti-seizure, anti-chizophrenic, anti-obesity, anti-mercaptans, disinfectant, oxofuels, anti-diabetic, anti-convulsant, and dopamine anatagonist.

SUMMARY

6. SUMMARY

The present study has been carried out to establish the occurrence of anti-oxidant and the characterization of chemical compound from skin and muscle extract of *L sceleratus*.

Methanol extract of skin exhibited maximum TAA by phosphomolybdenum assay - Antioxidant activity with 16.67 %, 18.94 %, 22.73%, 25 % and 29.55 % at 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml and 1000 µg/ml respectively . The methanol extract of muscle exhibited antioxidant activity with the percentage of 7.58% at 200 µg/ml, 9.09% at 400µg/ml, 10.61% at 600µg/ml, 12.12% at 800µg/ml and 22.73% at 1000 µg/ml concentrations respectively.

Chemical compounds present in the skin was characterized through GC-MS analysis. This result showed that there are 15 compounds with anti-microbial, anti-bacterial, analgesic, anti-cancer, anti-allergic, anti-malarial, non-steroid, anti-chlorination, anti-protozol, anti-diabetic, oxofuels, anti-convulsant, anti-mercaptans, anti-seizure, anti-chizophrenic, anti-obesity, and dopamine anatagonist.

CONCLUSION AND SUGGESTIONS

7. CONCLUSION AND SUGGESTIONS

The discovery of new products from natural sources is mainly essential for the development of novel bioactive compound agents. Currently, drugs for medical treatment derived from natural origin exhibit as the most important secondary metabolite source and products without any side effects.

Free radicals are known to play a definite role in a wide variety of pathological manifestations of pain, inflammation, cancer, diabetes, hepatic, damage etc. Anti-oxidants fight against free radicals and protect us from various diseases.

They exert their own action either by scavenging the reactive oxygen by species or protecting the antioxidant defense mechanisms. Among the extracts of skin showed potent scavenging activity on free radical TAA by phosphomolybdenum assay compounds with standard anti-oxidant ascorbic acid.

GC-MS analysis is a useful tool for chemical analysis. Results obtained suggest that, the skin, extract possess compounds with broad spectrum biological activities.

In conclusion, it can be started that the puffer fish *L.sceleratus* extracts have strong anti-oxidant activities. On the basis of these results *L.sceleratus* appears to be good candidate species for the extraction of safe natural anti-microbial, anti-

oxidant, and anti-cancer agents and also could be used in human therapy. Hence this information may help to develop potential purified bioactive compunds from *L.sceleratus* in pharmaceutical industry for the development of drugs.

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8. BIBLIOGRAPHY

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A STUDY ON EFFICACY OF BIOADSORPTION OF ALGAL BIOCHARS

A project submitted to

ST. MARY'S COLLEGE (Autonomous), THOOTHUKUDI

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MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

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THOOTHUKUDI – 628 001

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CERTIFICATE

This is to certify that the project entitled 'A Study On Efficacy Of Bioadsorption Of Algal Biochars' is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a record of the work done during the year 2022-2023 by the following students.

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INTRODUCTION

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INTRODUCTION

The natural and anthropogenic sources of water bodies are contaminated with diverse categories of pollutants such as antibiotics, pharmaceuticals, pesticides, heavy metals, organic compounds, and other industrial chemicals. Depending on the type and the origin of the pollutants, the degree of contamination can be categorized into lower to higher concentrations. Therefore, the removal of hazardous chemicals from the environment is an important aspect. The physical, chemical and biological approaches have been developed and implemented to treat wastewaters. The microbial and algal treatment methods have emerged as a growing field due to their eco-friendly and sustainable approach. Particularly, microalgae emerged as a potential organism for the treatment of contaminated water bodies. The microalgae of the genera *Chlorella, Anabaena, Ankistrodesmus, Aphanizomenon, Arthrospira, Botryococcus, Chlamydomonas, Chlorogloeopsis, Dunaliella, Haematococcus, Isochrysis, Nannochloropsis, Porphyridium, Synechococcus, Scenedesmus,and Spirulina* have been reported as good candidates for the wastewater treatment and biomass production. Microalgae have the potential for adsorption, bioaccumulation, and biodegradation. The microalgal strains can mitigate the hazardous chemicals via their diverse cellular mechanisms. Applications of themicroalgae strains were found to be effective for sustainable developments and circular economy due to the production of biomass with utilization of pollutants.

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Dyes are used in many industries, such as food, pharmaceutical,cosmetic,dyestuffs, textile, paper and plastics. This results in coloured wastewater.It is found that colour is the first contaminant to be recognized in wastewater (Banat et al., 1996). There are more than 8000 chemical products associated with the dyeing process, while over 100000 commercially available dyes exist with over 7×105 metric tons of dye stuff produced annually (Banat et al., 1996).

Even a very small amounts of dyes present in water (less than 1 ppm for some dyes) is highly visible and undesirable (Banat et al., 1996; Robinson et al., 2001). Furthermore, many of the dyes are toxic and carcinogenic and this poses a serious hazard to aquatic living organisms (Vijayaraghavan & Yun, 2008). Dyes also interfere with the transmission of light and upset the biological metabolism processes which cause the destruction of aquatic communities present in ecosystem (Kuo, 1992; Walsh et al., 1980). In addition, the dyes have a tendency to sequester metal and may cause microtoxicity to fish and other organisms (Walsh et al., 1980). Therefore, it is necessary to develop an effective and appropriate treatment technique to remove the dyes from the wastewater before discharging to natural water stream. There might have some difficulties in treating coloured wastewater due to the properties of dyes includes resistance to aerobic digestion, and stability to light, heat and oxidizing agents (Kumar et al., 1988; Sun & Yang, 2003). Conventional methods, such as adsorption,

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coagulation precipitation, filtration and oxidation are not so effective to those recalcitrant dyes and not economical or not so environmental friendly (Senthil et al., 2003).

Several physical, chemical and biological decolourization methods have been developed and reported in the past three decades and some of them have been accepted by the paper and textile industries (Ghoreishi & Haghighi, 2003). In addition, there are other methods including ozonation, irradiation, ion exchange and photo degradation. Some of these techniques may have some limitations, including excess amount of chemical usage, accumulation of concentrated sludge with disposal problems, expensive plant requirements and operational costs, lack of effective colour reduction and sensitivity to a variable wastewater input (Robinson et al., 2001).

Among these numerous techniques, adsorption is a desirable choice because it can be used for bioremediation of different types of colouring materials and can produce a high quality treated effluent (Derbyshire et al., 2001; Ho & McKay, 2003; Jain et al., 2003). Activated carbon is the most commonly used sorbent in commercial system to remove dyes because of its excellent adsorption ability (Crini, 2006). The activated carbon adsorption technique has been cited by the US Environmental Protection Agency as one of the best available control technologies (Derbyshire et al., 2001). However, the

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popularization of activated carbon is limited due to high cost. Therefore, cheaper and effective adsorbents such as

natural materials, biosorbents, and waste materials from industry and agriculture have been studied. The examples of these materials are clay materials (bentonite and kaolinite), zeolites, siliceous material (silica beads, alunite, perlite), agricultural wastes (bagasse pith, maize cob, rice husk, coconut shell), industrial waste products (waste carbon slurries, metal hydroxide sludge), biosorbents (chitosan, peat, biomass) and others (starch, cyclodextrin, cotton) (Crini, 2006).

Application of biosorption for the removal of toxic pollutants or for the recovery of valuable resources from aqueous wastewaters is one of the most recent developments in environmental or bioresource technology. This non-conventional technology has high efficiency, minimum chemical or biological sludges, the ability to regenerate biosorbents, and the possibility of metal recovery following adsorption (Park et al., 2010). Biosorbents for the dye removal are mainly classified into the following categories: bacteria, fungi, algae, industrial wastes, agricultural wastes and other polysaccharide materials. Marine algae, popularly known as seaweeds, are another important biosorbent which has gained momentum in recent years (Vijayaraghavan & Yun, 2008). They are biological resources and available in many parts of the world. The most important is the alginate gel presents in their cell walls offer a convenient basis for the production of biosorbent particles that are suitable for sorption

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process applications (Vieira & Volesky, 2000). Marine algae have been identified as potent metal biosorbents due to the presence of binding sites, such as carboxyl, sulfonate, amine and hydroxyl groups (Davis, Volesky & Mucci, 2003).

Malachite green (MG), a triarylmethane dye, is a dark green and crystalline solid prepared by condensing one part of benzaldehyde with two parts of diemethylaniline in the presence of concentrated sulphuric acid or zinc chloride (Srivastava, Sinha & Roy, 2004). Formally, MG refers to the chloride salt [C6H5C(C6H4N(CH3)2)2]Cl, although the term malachite green is used loosely and often just refers to the coloured cation. MG can also be in oxalate salt form. However, the chloride and oxalate anions do not affect the colour of the dye. Malachite green is most widely used for colouring purpose, amongst all other dyes of its category (Jiang, Sun, Wang & Zhou, 2008). As malachite green is absorbed into the body, it is converted to carbinol form firstly, which is important because it spreads across cell membranes faster. When it is inside the cell, it is then metabolized into a form called leuco-malachite green (LMG). This form is toxic in addition and it is retained in the body for a longer period than the original form of malachite green (Webster's Online Dictionary).

Malachite green is used mainly industrially for leather, wool, cotton, silk, jute, paper and certain fibers. It is also used as a food colouring agent, food additive, a medical disinfectant and anthelminthic (Srivastava, Sinha & Roy,

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2004). On the other hand, it is widely used in aquaculture as parasiticide, fungicide and antiprotozoan to control fungal attacks and protozoan infections (Srivastava, Sinha & Roy, 2004). In African aquaculture, it has been used against infection by bacteria, protozoans, cestodes, trematodes, nematodes, crustaceans, etc. (Hecht and Endemann, 1998). Traditionally, MG has been used to treat fungal infections on fish eggs. Malachite green is used as a biological stain for microscopic analysis of cell and tissue samples. In the Gimenez staining method, basic fuchsin stains bacteria red or magenta, and malachite green is used as a blue-green counterstain. Malachite green can also directly stain endospores within cells; here a safranin counterstain is often used. In addition, MG is rarely used as a saturable absorber in dye lasers, or as a pH indicator between pH 0.2 - 1.8. Leuco-malachite green, the primary metabolite of malachite, is used as a detection method for latent blood in criminalistics. Hemoglobin catalyzes the reaction between LMG and hydrogen peroxide, converting the colourless LMG to the chromatic form of malachite green. Therefore, the appearance of a green colour indicates the presence of blood (Webster's Online Dictionary, 2008).

MG discharged into receiving water bodies, even at low concentrations, will affect the aquatic life and cause detrimental effects in liver, gill, kidney, intestine and gonads. In humans, it may cause irritation to the gastrointestinal tract upon ingestion. Contact of MG with skin causes irritation, redness and pain

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(Daneshvar, Ayazloo, Khataee & Pourhassan, 2007). The toxicity of MG makes it a highly controversial compound. It has been reported to cause carcinogenesis, mutagenesis, chromosomal fractures, teratogenecity and respiratory toxicity (Srivastava, Sinha & Roy, 2004). Even the use of this dye has been banned in several countries and not approved by US Food and Drug Administration (Chang et al., 2001), it is still being used in many parts of the world due to its low cost, ready availability and efficacy (Schnick, 1988). The US Food and Drug Administration have nominated MG as a priority chemical for carcinogenicity testing (Culp & Beland, 1996). A considerable amount of research is being devoted to work out the wide spectrum of biological effects it exerts on different animals and mankind. There is concern about the fate of MG and its reduced form, leuco-malachite green in aquatic and terrestrial ecosystems since they occur as contaminants and are potential human health hazards (Nelson & Hites, 1980; Burchmore & Wilkinson, 1993).

The current study is designed to study the dynamics of algal biochars for biosorption of malachite green dye.

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OBJECTIVES

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AIMS AND OBJECTIVES

In the present study, biochars of *Sargassum and chaetomorpha* was used as the biosorbant to remove basic dyes, which are malachite green a, from a binary aqueous solution. Experiments have been conducted to collect the corresponding data and observe the efficiency of this technique. The objectives of the study are:

1. To investigate the bioadsorption capacity of biochar of *Sargassum and Chaetomorpha* in removal of malachite green from dye solution.

2. To investigate the effects of initial dye concentrations, amount of adsorbant, temperature of pyrolysis and pH on efficacy of biosorption.

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REVIEW OF LITERATURE

REVIEW OF LITERATURE

Bioremediation:

The term of bioremediation has been made of two parts: "bios" means life and refers to living organisms and "to remediate" that means to solve a problem."Bioremediation'' means to use biological organisms to solve an environmentalproblem such as contaminated soil or groundwater. Bioremediation is the use of living microorganisms to degrade environmental pollutants or to prevent pollution. In other words, it is a technology for removing pollutants from the environment thus restoring the original natural surroundings and preventing further pollution (Sasikumar and Papinazath 2003).Bioremediation could simply be defined as a biological process of the decontamination of contaminated environment.

Malachite green (MG, C23H25C1N2) is an organic compound belonging to the class of triaryl methane dyes. Although malachite green has been banned in many developed countries, its illegal use is widespread in some countries due to its low cost and antimicrobial and dying properties. Malachite green is, therefore, in demand in various sectors such as aquaculture, pharmaceuticals, fine chemicals, textiles, and food processing industries in some countries, leading to their production in tonnes on a global scale. Malachite green's solubility and stability in water prevent its degradation by microbes, making it highly persistent. Despite MG's wide-ranging applications, their presence in

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water for a longer duration adversely affects the aquatic ecosystem. Moreover, its toxicity increases with its concentration, exposure time, and environmental temperature. The direct/indirect exposure of humans to MG could result in mutagenesis, respiratory problems, carcinogenesis, and chromosomal fracture. Hence, a sustainable approach that is facile and affordable is urgently needed for the remediation of MG in water bodies.

A variety of treatment methods, such as photocatalysis, microbial fuel cell, advanced oxidation, electrochemical oxidation and adsorption, is available for removing malachite green from contaminated water. Among them, adsorption is one of the most effective and commonly implemented treatment techniques due to its simplicity and efficiency for contaminant removal. Several lignocellulosic and carbonaceous materials (e.g., activated carbon) have been developed as adsorbents. Apart from low cost biological materials, several porous nanostructures from polymers, clays, nanomaterials, and magnetic materials were developed for MG removal. However, the search for cheaper adsorbents with high adsorption and regeneration capacities motivates new research. In past years, the introduction of biochar as an adsorbent has helped overcome some of the limitations (cost and ease of production) of activated carbon. However, not all biochars are effective for wastewater treatment. Therefore, chemical and physical modifications have been developed to enhance the properties of biochars for wastewater remediation. It is evident that

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the pre-and post-treatment/activation of biochar helps achieve desirable characteristics of biochar; yet, it makes the production process more challenging from an economic and environmental perspective. The chemical activation of biochar generally involves using a significant amount of acids, alkali, or metal oxides, which is chemical-intensive and poses questions regarding its environmental sustainability. On the other hand, physical activation does not require chemicals; but the necessity of higher temperature and other conditions makes the process energy- and cost-intensive.

Bioremediation of Wastewater

The bioremediation of wastewater is an important part of bioremediation. The sewage water can be treated by the processes of bioaugmentation and intrinsic bioremediation. The process is done with the help of microorganisms, which can reach any parts of the contaminated places like municipal water tanks. The aerobic microbes are used in these processes, and the water is aerated to provide oxygen for the bacteria to thrive and grow. The bacteria consume the organic contaminants and mould the less soluble parts. The byproduct of this process is nitrogen gas, which is later released into the atmosphere.

It is up to the situation or the availability of the resources that which one fits well. All of them have their unique attributes. Along with these, people have also explored a few more methods such as incineration, landfill burial, treatment

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with the use of chemicals, managing solid waste, managing nuclear waste, and more.

Types of Bioremediation

The bioremediation includes plant-microbe-based remediation (It is defined as phytoremediate and microorganism remediation), which is different in the process/mechanism by which plants/microbes can immobilize, remove or degrade pollutants (khalid et al., 2017).It is includes phytoremediation and microorganism remediation.

Phytoextraction :

It is also know as phytoaccumulation, phytoabsorption or phytosequestration. It is the removal of pollution from the soil or water by the plants roots and translocation, accumulation on the biomass, (Ghosh and Singh, 2005: muthusaranan et al., 2018). Pollutant translocation sutable for cracial biochemical process and it is effective for phytoextraction because the harvestiry root biomass is not feasible (Halim et al., 2003; Mmointyre,2003). The continuous phytoextration used for plants that is accumulate the high level of pollution over the entire life time (Sarwar et al., 2017). The phytoextration generally 5 major type pollutant mobiliazation in the rhizosphore, pollutant uptake by the plant roots, translocation of aerial plant roots, then pollutant sequestration in plant tissues (Memon and Schroder, 2009; Ali et al., 2013).

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The effective phytoexcraction is associated with depth, available for the plant root growth, In the seasonal weather and also climatic conditions (Bharayana et al., 2012).The phytoextration can be improved by the application of the mobilizing agents like that citric acid, ethyenediaminetracetric acid, Nitrilotriacetic acid, aminopolycarboxlic acid and ethylenediaminediscuccinic acid (mahar et al.,2016).

- **Phytofiltration**

The term phytofiltration also know as rhizofiltation. It involves absorption or precipitation of pollution (Khan et al., 2019).In this mechanism related synthesis of chemical, with the roots.

The rhizofiltration straight forward method of bioremediation. It is a method to treat effluents and contaminated water ways, or ground water frame works. The idel plant for rhizofiltration should have rapidly growth roots with ability to remove the contamination from that solution for over long period of time (Dhanam, 2017). It is used for the extensive root architecture and fibrous root. It is helps to draw out the contamination from that ground water and rhizopheric zone (Pilon-Smits, 2005; Ali et al., 2013; Khan et al., 2019).

Phytostabilization

The phytostabilization also know as phytoimmobilizatin. In this process of using plants ability to decrease the mobility (Sarwar et al., 2017; Khan et al.,

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2019). The phytostabilizationcan be divided into 2 characters i) The restoration of a pollutant media aggregated by the roots, absorption into roots. ii) The deployment of plants and plant roots avoid containant movement from the wind and water, draining and dispersion of soil (USEPA, 1999). The phytostailization is not permanent solution to contamination, because the phytostabilization doesn't reduce the pollutants but reduces the contamination near by media (Balan et al., 2011; Khalid et al.,2017).

Phytovalatilization

Phytovalatilization is plant mediated uptake of a contaminants, and transforms volatile compounds and release the compound from the atmosphere (Kumar et al.,2017; Khan et al.,2019). The plant species are extensive root system. It is uptake of contaminants and produce some of specific enzyme or genes (Newman and Refoeld.,2004; Pilon-smits.,2005;Muthusarvanan et al.,2018).

Phytodegradation

The phytodegradation otherwise called as phytotransformation. It is refer to the contaminants and nutrients from the water , sediment ,or soil and chemicals (Bulak et al.,2014; Gomes at al.,2016). Some plants are can be degrade contaminants into the less toxic compounds by the plant's and metabolic process or enzymes(Muthusarvanan et al., 2018).

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Bioremediation although considered as a reliable technique in the middle of present environmental problems, however, it can also be considered problematic because, while additives applied to promote the activity of the particular micro-organism(s) may disrupt other organisms inhabiting same environment when situ. Even if genetically modified microorganisms are released into the environment after a certain point of time it becomes difficult to remove them. Bioremediation is generally very costly, is labor intensive, and can take several months for the remediation to achieve acceptable levels. Another problem regarding the use of insitu and ex situ processes is that it is capable of causing far more damage than the actual pollution itself. Nutrient imbalance can hinder biodegradation. Inadequate provision of nitrogen, phosphorus, potassium, and sulfur (which is probably the most important and the most easily modified of all the factors) could limit the rate of hydrocarbon degradation in the terrestrial environment (McGill and Nyborg, 1975).

Bioadsorption

Bioabsorption is a physiochemical process, that occurs naturally in certain biomass which allows it to passively concentrate and bind contaminants on its cellular structure. Bioadsorption can be defined as the ability of biological materials to accumulate heavy metals from wastewater through metabolically mediated or physico-chemical pathways to uptake the process.

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Bioadsorption is an adsorption process that aims to remove or recover organic solutions using a biological material that may include live or dead microonganism and their components, seaweed, vegetable, industrial waste, agricultural waste, and natural waste as adsorptive medium, named bioadsorbent. The bioadsorpotion process occurs by interactions between the metal and certain active sites (carboxyl, amino, sulfate groups), present in the coatings of the biomaterial. Bioadsorption has been studied as an alternative in extractive metallurgy. Processes for recovery and concertration of High-demand and/or high-added value metals,such as gold, sliver uranium, and also, REMS. In this sense, the use of bioadsorbents is promising, since it is also a cost-effective industrial process compared with the environmental impact of similar technologies.

Biochar Development

Thermochemical conversion methods such as pyrolysis, hydrothermal carbonization (HTC), and gasification are the most commonly used conversion techniques for biochar synthesis . The highest biochar yields were obtained in case of pyrolysis (Wang et al., 2019), and therefore this process was considered to be the most effective and economic in terms of biochar production (Waqas et al., 2020).

In pyrolysis, the biomass was heated to a temperature of 150 ^{0}C - 900^{0}C in an oxygen-free environment to convert biomass into biochar (Ahmad et al.,

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2014). During this thermal degradation, the organic compounds from the composition of biomass feedstock (hemicellulose, cellulose and lignin) are degraded. Thus, the yield of the pyrolysis products (bio-solid,bio-oil and biogas) also varies, depending on the temperature, retention time and heating rate, etc .As a function of these parameters, the pyrolysis processes are classified into slow, intermediate, fast and flash shows the classification of batch production process of biochar.

Slow pyrolysis can be defined as a continuous operation, where oxygen-free feedstock biomass is transferred to a furnace, on the other end, fast pyrolysis depends on very rapid heat transfer, usually to fine particles at less than 650^0C with a heating rate (ca 100^0C -1000^0C). In the gasification chamber, the feedstocks are oxidised at the temperature of about 800^0C and high pressure (Oliver et al., 2013). The conversion of biomass into biochar was carried out by the application of heat and pressure in the presence of water, reaction temperature (160^0C - 800^0C) and the reaction pressure must be maintained at 1 Atm in the liquid form (Vijayaraghavan, 2019). This method is often applied to the wet biomass feedstock for biochar production. Slow pyrolysis and hydrothermal carbonization techniques are the most efficient thermochemical conversion methods to produce the biochar derived from HTC technique was readily biodegradable, whereas biochar derived through slow pyrolysis

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technique was more stable and thus had a distinctly higher potential for carbon sequestraton than HTC (Malghani et al., 2013).

Bioremediation of waste waters contaminated with dye

Biological techniques have been engaged commonly for the treatment of dyes form wastewaters. In recent years, these technologies have gained considerable attention and are currently in the process of commercialization (Vijayaraghavan and Yun 2008). Biosorption is a process that utilizes inactive biological materials to sequester the concentration of pollutants form aqueous solutions .In recent years, biosorption is considered as a rapid, reversible economic, and enivirоment-friendly technology compared to conventional methods (Varshini and Das, 2014; Kucuker et al., 2017). Biosorption is a promising potential alternative to conventional processes for the removal of dyes (Bhagavathi et al.2016). The biobased sorbents used in the dye removal process are usually acquired from various industrial wastes, chemical matter, fungi, polysacchide sorbents bacteria, seaweeds, and agricultural wastes (Vijayarghavan and Yun 2008b). Therefore, finding an effective biosorbent to remove certain contaminants from aqueous media is still a major challenge.

An effictive commercial biosorbent requires the following characteristics; high biosorption capacity (Wang et al., 2017), adequate surface characteristics (Shi et al., 2015), effective and low-cost separation of biosorbent from solutions (Xin et al 2017); Rosales et al., 2017, resilient mechanical strength and thermal

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stability (Wang et al., 2017), availability and cost-effective preparation (Du et al., 2016; Saha et al., 2017) Thus, taking into account these requirements, biochar has been widely used for enironmental bioremediation example the heterogeneous structure of biochar and its potential application in environmental bioremediation.

Thermochemical technologies for algal biochar production:

An exponential increase in demand of algal biochar for various applications has pushed scientists forward for inventing advanced technolgies for converting algal biomass into suitable and sustainable biochar. With respect to this, thermochemical conversion is the commonly know practised route for attaining the desired purpose and it bascally involves methods like pyrolysis, torrefaction. However, the selection of specfic texhnique is highly affected .By numerous factors like desired properties of final product, type of feedstock (either dry or wet), etc. and yield of desired product furture varies with the type method employed. In addition, the process conditions like temperature, heating rate, residence time etc. should also be optimum as they greatly influence the chemical and physical states of final product. Pyrolysis is an often-investigated thermochemical route for converting various feedstock thermochemical route for converting various feedstock biomass into biochar due to its exposed.

To elevated temperature of about 300^0C -650^0C in an oxygen free atmosphere. It basically results in the generation of three major products

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namely. Biochar, bio-oil and non-condesable gases and is further categorized into flash, fast, slow,hydrolytic, catalutic and microwave assisted pyrolysis on the basis of operting parameters. However, in among all these, slow pyrolysis led to maximum solid yield of 25-35%, hence is regarded as major process for biochar production (Kambo and Dulta, 2015), Similary, hydrothermal liquefaction is also a type of theremochemical route which includes exposure of biomass feedstock to moderate temperature of around 200-374c under a high pressure of 5-20 MPa in an inert environment and leads to generation of biochar, biorude oil, gaseous product and aqueous phase. Furthermore, this process involves about more than 70% of carbon conversion from feedstock as biochar or bio-oil and the char yield ranges form 2 to 70% (Ponnusamy et al, 2020).

Hydrothermochemical route that gained attention for hydro-char production due to its cheapness and eco-friendly nature. It involves transformation of carbohydrate in biomass into soild carbon-rich product knows as hydro-char at temperature of around 180-260c Additionally, this specific method occurs in selfgenerated pressure of around <10 bar in water as solvent and results in in maximum yield of product by using short time period and low energy expense. Hence, it offers a benefit to potentially use residue of algal biomass therefore converting it into valuable products (Yu et al, 2017). Moreover, torrefaction is defined as a thermochemical process carried out at

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temperature of around 200^{0}C -500^{0}C in atmospheric pressure under an anerobic atmosphere. In this process, a moderaly degraded soild biomass with maximum carbon content knows as torrefied char is produced. This method involves thermal pre-treatment of biomass leading to removal of volaties by various decomposition reactions thus upgrading quality of biomass. It hs been stated that soild yield of around 51.3-93.9% can be obtained in residue of microalgae after torrefaction at temperature of 200-300c along with residence time between 15 min to 1h (Singh et al., 2021).

Factors affecting the biosorption of dyes on biochar:

Biochar's adsorption capacity is strongly influenced by the biochar characteristics and by several experimental parameters such as solutions pH, biochar dosage, temperature, Initial dye concentration and contact time. In this section, the above-mentioned factors were discussed.

Biochar characteristics:

The biochar produced from different conversion techniques hsas different functional compositions and characteristics, due to the operting conditions. Due to this variation, the biochar synthesis generally adopted the batch reactor. Thus it is very important to understand the current research gap to attain the requirements of adsorptive tendency and uniform quality for large-scale productions. As mentioned earlier, the pyrolysis temperature, reactor residence

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time, the type of thermochemical conversion and nature of feedstock significabtly influence the characteristics of the obtained biochar and have an important effect on the sorption performance of different pollutants.

During the pyrolysis process, the diameter of formed pores varies from nanometres to micrometers. Pore size is one of the most important factors governing the adsorption mechanism. Various researches have shown that the larger pore size is generated at high pyrolytic temperature and causes the surface area to increase. Along with the pyrolysis condition of feedstock biomass also regulate the porosity. For instance, pyrolysis of lignin and cellulose enriched biomaterials produces microporous biochar (Joseph et al., 2007).

The pH of the biochar also varies with pyrolysis temperature and feedstock material type. The pH of the biochar increases at high pyrolytic temperature results in more ash content and degrades the acidic functional groups resulting in high pH. The pH of the solutions greatly affects the surface condition of the biochar. The presence of various functional elements at different proportions in the feedstock decreases the elements ratio of the biochar at varying temperatures. The aromaticity and polarity of the specific biochar are due to these compositions. In general, high-temperature biochar provides a low H/C and O/C ratio compared to low-temperature biochar. The biochar's mineral content is also regulated by the feedstock kind and pyrolysis temperature, along

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MATERIALS AND METHODS

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and warranted that the adsorbent was capable of adsorbing the adsorbate at any particular temperature. The change in temperature with varying uptake capacity was referred to endothemic (absorbs heat) or exothermic (releases heat) process (Karimi et al., 2019; Ofomaja and Ho, 2008).

Initial dye concentration and contact time:

The initial dye concentration of sorbent strongly influences the efficacy of adsorption process. Bustard et al., (1998) showed increasing initial concentration of dyes provided a significant force to cover all mass transfer resistance between the aqueous and solid phases. Also increasing the initial dye concentration increases the number of collisions between dye anions and sorbent. On the other hand, a decrease in decolourization capacity may occur due the accumulation effect of the dye concentration to inadequate biomass concentration for the uptake of higher concentration on the percent of dye removal has a limited effect on the adsorption due to the unavailability of the required number of active surface sites on the biochar. Thus, the removal percent becomes saturated at a particular dye concentration (Abbas -2013).

Malachite green pollution in the aquatic environment results in health hazards such as cancer and respiratory problems. Effective methods need to be developed to remove malachite green from wastewater. Here, we studied the adsorption of malachite green by a low-cost biochar of the algae *Sargassum* and *Chaetomorpha*

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required for maximum removal (Chattoraj et al., 2016). The rate of dye removal percentage was found to be quick in the in initial hours and then decelerated when the biochar dose increased (Dawood et al., 2017).

This rapid rate of percentage removal of dye with adsorbent dosage could be attributed to the availability of the sorption sites on the surface of the adsorbent. Whereas in diminished dosage conditions the dye molecules are more easily accessible thus the effciency of dye removal per unit weight of biochar is higher (Alene et al., 2014; Uddin et al., 2017).

Temperature:

Form the previous research, it is evident that the temperature has a significant influence on the equilibrium of dye uptake. Hence the temperature will be a vital design parameter affecting the sorption capacity (Iftekhar, 2018). Generally, temperature exhibits a strong impact of the adsorption capacity, and it is directly proportional to the rate reaction (Wahab et al., 2017).

The increase in temperature increases physio-sorption and the decrease in temperature will result in a lower rate of physio-sorption. Porous susbtances are better adsorbents as they promote adsorption through an increased surface area. The rate of dye removal increases with the increase in temperature up to a certain limit, after that, there is a limitation in the process. Sathishkumar et al., (2007) recommended that the optimum removal was obtained at 35^0C to 40^0C

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with . The effect of pH on biosorption and the solution pH has a direct influence, not only on the adsorption capacity but also on the surface chemistry of the biochar as well as the accessibility of dye molecules for the binding Sites (Vijayaraghavan and Ashokkumar, 2019). Form most of the previous studies it is inferred that the optimum pH for dyes removal is often neutral or slightly alkaline (Sadhasivam et al., 2007). The percentage of dyes removal is maximum only at optimum condions, and tends to decrease rapidly in acid or alkaline conditions. The solution pH is directly associated with .The competition ability of hydrogen ions with adsorbate ions to active sites on the adsorbent surface (Janaki et al., 2012; Clifford and Noemi, 2010).

Babaei et al., (2016) analysed the role of solution pH on the biosorptive removal of methylene blue dye by biochar derived form agricultural wastes. The inference was observed by varying the solutions pH form 2 to 9. The dye removal efficiency of the biochar was drastically improved, from 40 to 90%. By the pH variation from 2 to 9. The authors also pointed out point of zero charge value (8.5). This upfront sorption behaviour of the biochar was attributed to the negative surface charge of the biochar, which favours electrostatic interactions, during of biosorption process.

Biochar dosage:

Many investigations have been concentrated on adsorbent dosage during dye sequestration techniques to find out an optimum minimum sorbent dose

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MATERIALS AND METHODS

Collection of Sample

Algae sample were collected from the shore line of Mottaigopurambeach in Thoothukudi and from the campus of St. Mary's College (Autonomous), Thoothukudi. Two algal species, *Sargassum* and Chaetomorpha were collected and used for this experiment. (Fig.1)

Sample processing

The algae were cleaned off the dust and it was rinsed with fresh water and shade dried. (Fig. 2)

Pyrolysis of sample

The algal samples were shade dried and crushed into a fine powder. The powder was taken in a China Dish and sealed with a silver foil to maintain oxygen free environment. The sample was then pyrolysed in a muffle furnace for 30 minutes. The pyrolysed sample was taken for further analysis.

Bioadsorption :

50mg of biochar was taken in 100ml of malachite green 50mg /l concentration. The set up was placed in a shaker at room temperature. Samples of dye were taken at regular intervals (5, 10. 15, 20, 25 min). Absorbance at 617 nm was taken in Spectrophotometer. A standard graph for concentration of malachite

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green was plotted by measuring the OD of known concentrations of dye. The concentration of the dye in the experimental sample was obtained from the standard graph.

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Fig. 1 Study area

a.

b.

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Fig. 2 Samples

a. ***Sargassum***

b. ***Chaetomorpha***

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Fig. 3 Biochars of samples

a. *Sargassum*

b. *Chetomorpha*

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Fig .4 Graph representing the percentage adsorption between *Sargassum* and *Chaetomorpha*

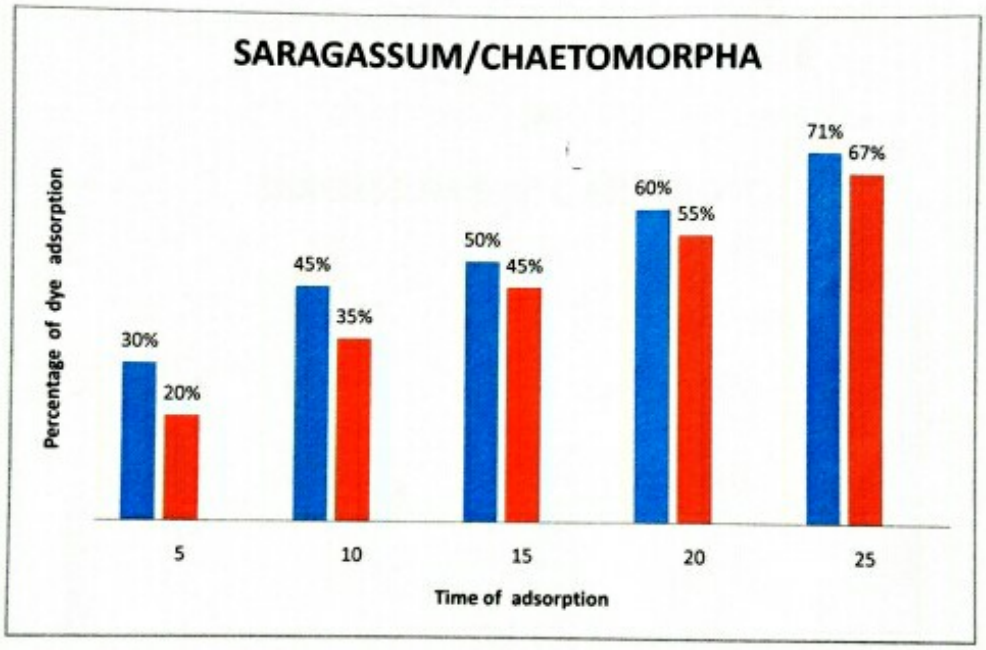

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Fig.5 Graph representing the percentage of bioadsorption between sample pyrolysed at600^{0}C c and 450^{0}C

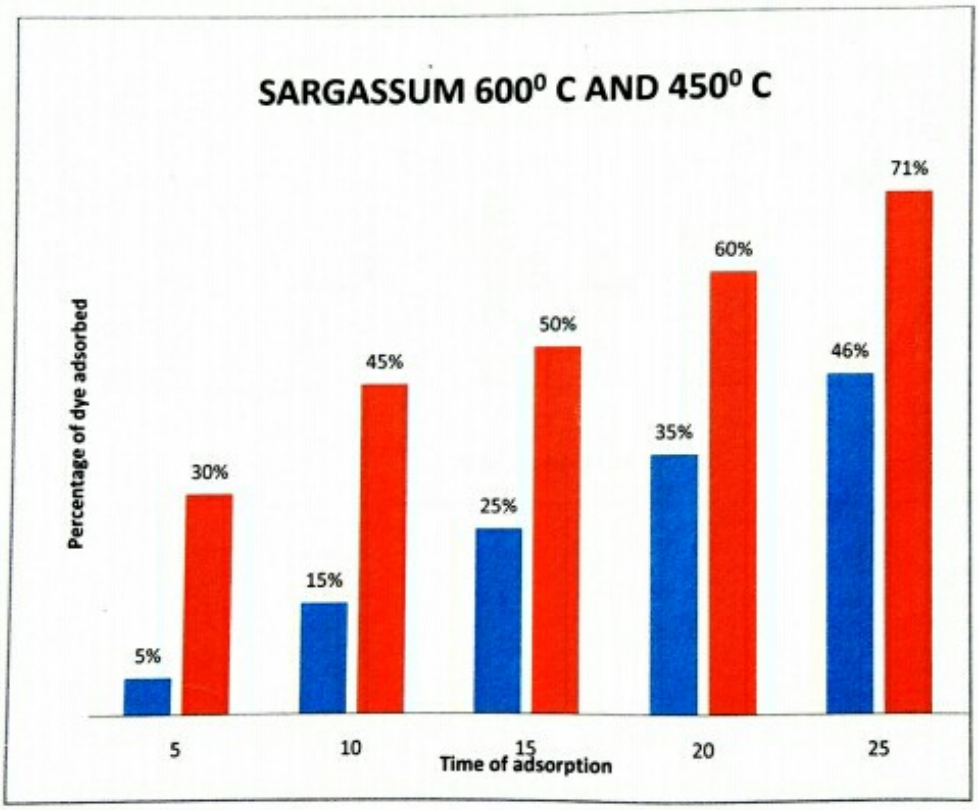

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Fig.6 Graph representing the percentage of bioadsorption pH 7 and pH at 8 *Sargassum*

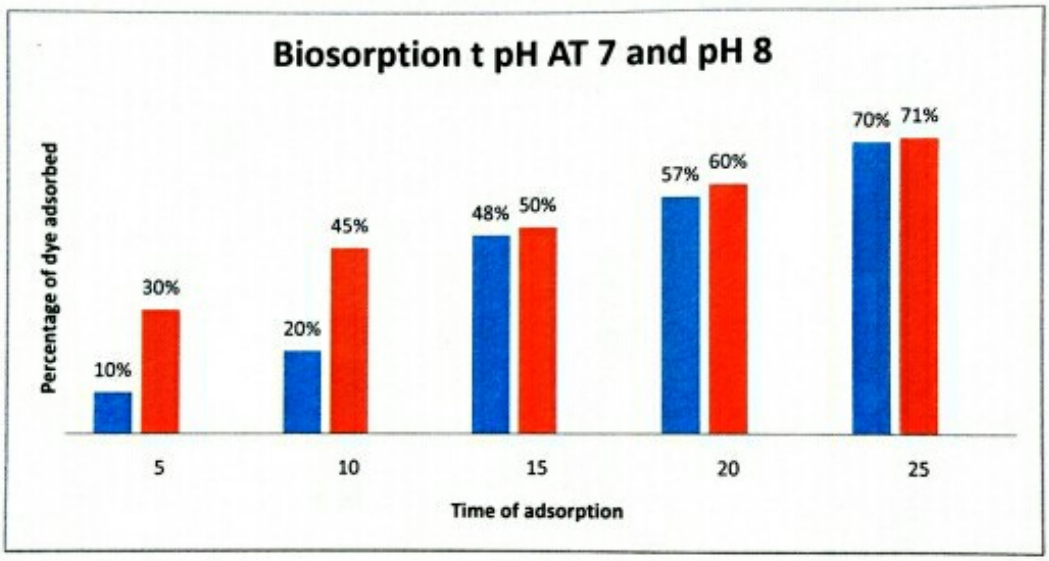

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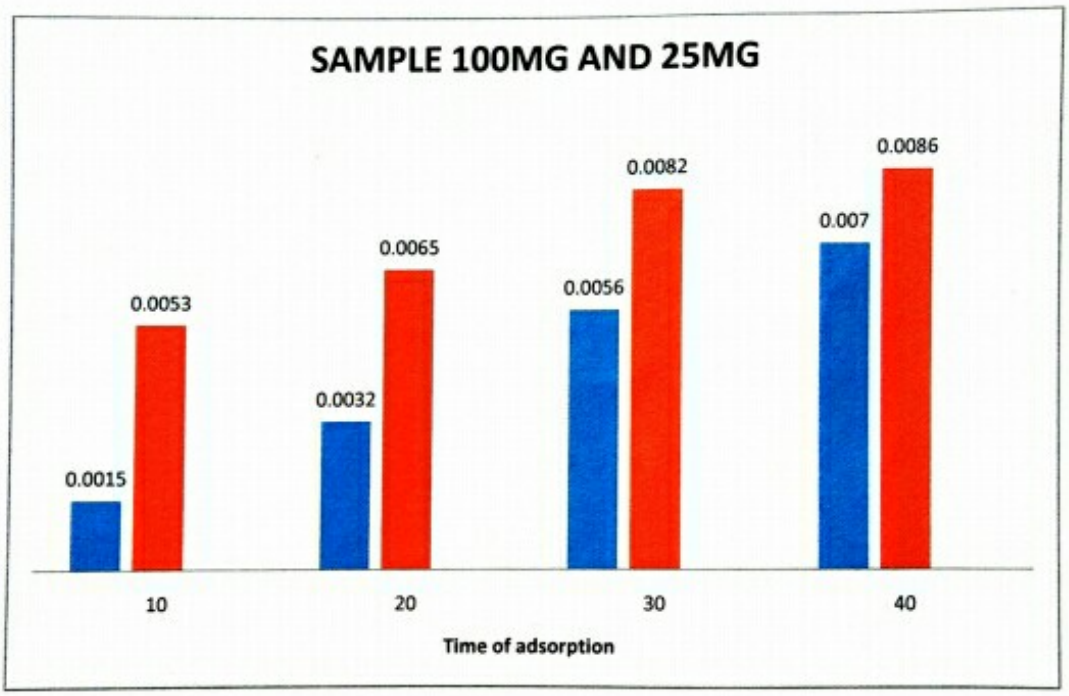
Fig.7 Graph representing the percentage adsorption of sample 100 mg and 25 mg



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RESULTS

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RESULT

Bioadsorption using biochar *Sargassum* and *Chaetomorpha*

Bioadsorption of malachite green dye using *sargassum* and *chaetomorpha* of particle size 0.75 mm were used for bioadsporption of malachite green dye to find the efficiency of their bioadsorption potential. 50 mg of the adsorbent was used for the adsorption of malachite green. Percentage of dye adsorbed by the adsorbent was calculated at time interval of 5 mins, 10 mins, 15 mins, 20 mins and 25 mins and are tabulated in Table 1a & 1b. The comparison of bioadsorption between the sargssum adsorbent and the chaetomorpha adsorbent was done and plotted in a graph (Fig. 4). The *sargassum* sample was found to be more efficient in adsorption when compared to the *chaetomorpha* sample. The functional groups present in the different tissues may be attributed to the difference in bioadsorption potential.

Bioadsorption using Biochar *sargassum* prepared at different pyrolysis temperature 600C and 450C

Biochar of the *Sargassum* was done in a muffle furnace at different temperatures. Pyrolysis was performed at two different temperatures 600^0C and 450 ^{0}C for 30 minutes. 50 mg of the biochar was used for adsorption of malachite green dye. Percentage of dye adsorbed by the adsorbent was calculated at time interval of 5 mins, 10 mins, 15 mins, 20 mins and 25 mins

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and are tabulated in Table 2a & 2b. The comparison of bioadsorption between the biochar prepared at 600^0C and 450^0C was done and the percentage of bioadsorption was plotted in a graph (Fig. 5). Bioadsorption by the samples pyrolysed at 450^0C was more efficient than the adsorbent pyrolysed at 600^0C.

Effect of pH on bioadsorption efficiency

Bioadsorption of malachite green using the biochar of *sargassum* was done at two different pH. The adsorption of malachite green was done at pH 7 and also by adjusting the pH of the reaction to pH 8 with 2N NaOH. Percentage of dye adsorkbed by the adsorbent was calculated at time interval of 5 mins, 10 mins, 15 mins, 20 mins and 25 mins and are tabulated in Table 4a & 4b. The comparison of bioadsorption at pH 7 and pH 8 was done and the percentage of bioadsorption was plotted in a graph (Fig. 6). There was not much difference in efficiency of bioadsorption in pH 7 and pH 8 initially but at 25 minutes the bioadsorption was found to be more efficient at pH 8.

Effect of concentration of adsorbent on bioadsorption efficiency

The efficiency of the adsorbent concentration was studies using sargassum biochar. 100 mg and 25 mg of the adsorbent was used for adsorption of malachite green. Q value for the two samples were calculated at different time points (Table 5a and 5b) and the results were compared (Fig. 7). 100 mg of

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the adsorbent was found to be more efficient in bioadsorption than at lower concentrations

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Table.1 COMPARISON OF % ADSORPTION OF SARGASSUM AND CHAETOMORPHA

a. Percentage adsorption of *Sargassum*

S. No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617 nm	Final concentration of dye mg/l	Amount of dye adsorbed	% of dye adsorption
1.	5	10mg/l	0.785	7	0.3	30%
2.	10		0.625	5.5	0.45	45%
3.	15		0.569	5	0.5	50%
4.	20		0.456	4	0.6	60%
5.	25		0.327	2.9	0.71	71%

b. Percentage adsorption of Chaetomorpha

S. No.	Time of absorption (min)	Initial concentration of dye mg/l	OD value at 617nm	Final concentration of dye mg/l	Amount of dye absorbed	% of dye absorption
1.	5	10mg/l	0.785	7	0.3	30%
2.	10		0.625	5.5	0.45	45%
3.	15		0.569	5	0.5	50%
4.	20		0.456	4	0.6	60%
5.	25		0.327	2.9	0.71	71%

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Table.2 COMPARISON OF% ADSORPTION OF SARGASSUM AT 600^{0}C AND 450^{0}C

a. Percentage adsorption of *Sargassum* pyrolyzed at 600^{0}C

S. No.	Time of absorption (min)	Initial concentration of dye mg/l	OD value at 617nm	Final concentration of dye mg/l	Amount of dye absorbed	% of dye absorption
1.	5	10mg/l	0.785	7	0.3	30%
2.	10		0.625	5.5	0.45	45%
3.	15		0.569	5	0.5	50%
4.	20		0.456	4	0.6	60%
5.	25		0.327	2.9	0.71	71%

b. Percentage adsorption of *Sargassum* pyrolyzed at 450^{0}C

S. No.	Time of adsorption (min)	Initial concentration of dye mg /l	OD value at 617nm	Final concentration of dye mg/l	Amount of dye absorbed	% of dye adsorption
1.	5	10mg/l	0.950	9.5	0.05	5%
2.	10		0.845	8.5	0.15	15%
3.	15		0.737	7.5	0.25	25%
4.	20		0.645	6.5	0.35	35%
5.	25		0.535	5.4	0.46	46%

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Table.3 COMPARSION OF % ADSORPTION OF SARGASSUM AT pH 8 AND PH 7

a. Percentage adsorption of pH at 8

S. No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617 nm	Final concentration of dye mg/l	Amount of dye adsorbed	% of dye adsorption
1.	5	10mg/l	1.045	9	0.1	10%
2.	10		0.837	8	0.2	20%
3.	15		0.556	5.2	0.48	48%
4.	20		0.452	4.3	0.57	57%
5.	25		0.342	3	0.7	70%

b. Percentage adsorption of pH at 7

S. No.	Time of adsorption (min)	Initial concentration of dye mg/l	OD Value at 617nm	Final concentration of dye mg/l	Amount of dye adsorbed	%of dye adsorption
1.	5	10mg/l	0.785	7	0.3	30%
2.	10		0.625	5.5	0.45	45%
3.	15		0.569	5	0.5	50%
4.	20		0.456	4	0.6	60%
5	25		0.327	2.9	0.71	71%

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Table 4 COMPARSION OF % ADSORPTION OF SAMPLE 100MG AND 25 MG

a. Percentage adsorption of sample 25mg

S. No.	Time of adsorption (min)	Initial concentration of dye mg/l	Final concentration of dye mg/l	OD Value at 617 nm	Amount of dye adsorbed	Weight of adsorbent g
1.	10	25mg/l	4.7	0.335	0.53	0.0053
2.	20		3.5	0.255	0.65	0.0065
3.	30		1.8	0.136	0.82	0.0082
4.	40		1.4	0.122	0.86	0.0086

b. Percentage adsorption of sample 100mg

S. No.	Time of adsorption (min)	Initial concentration of dye mg/l	Final concentration of dye mg/l	OD Value at 617 nm	Amount of dye adsorbed	Weight of adsorbent g
1.	10	100mg/l	8.5	0.585	0.15	0.0015
2.	20		6.8	0.479	0.32	0.0032
3.	30		4.4	0.312	0.56	0.0056
4.	40		3	0.219	0.7	0.007

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DISCUSSION

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DISCUSSION

The release of organic and inorganic pollutants into the environment can cause severe changes (contamination in the water, death of trees and grassland, more problems in wildlife, contamination in the food chain, negative health efects on the fora and fauna, severe human health problems etc.) in the atmosphere and generates various types of pollutions (Bhagavathi Pushpa et al., 2019). This is because these pollutants are toxic, mutagenic and carcinogenic, and their presence in ecosystems determines the decrease of the ecosystem quality (Farah et al. 2013). Over the recent past, biological treatment has been one of the cost-efective technologies compared to other processes (Vijayaraghavan and Yun 2008). Compared to other conventional methods, biosorption is considered as one of the most efective treatment techniques, both techno centric and eco centric. Biobased sorbents (or biosorbents) such as fungi, bacteria, polysaccharide sorbents, algae (Sabah et al., 2016; Moghaz Reda and Abdo Sayeda 2018), and agricultural wastes (Vijayaraghavan and Yun 2008) are cheap, available in large quantities, and their performances depend; the type of dye and the experimental conditions (including initial dye concentration, pH, temperature, contact time, etc.). Therefore, biosorbents with a higher potential to bind/take dyes molecules are desirable.

Generally biochars are considered to be good bioadsorbents. In this experiment algal biochar was used forbioadsorptionof malachite green. In the

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present study bisorption was performed using biochars of algae *Sargassum* and *Chaetomorpha*. It was observed that the percentage Adsorbanceof malachile green by the algal biochar increased with increasing time. It was observed that the biosorption efficiency was more for Sargassum biochar when compared to Chaetomorpha biochar. The influence of the pH and Concentration of the adsorbant was studied. It was found that pH 7 was efficient for biosorption than pH 8. The biosorption capacity improved with the increasing amounts of the adsorbant.

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SUMMARY AND CONCLUSION

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SUMMARY AND CONCLUSION

In the present study, biochars of *Sargassum and Chaetomorpha* was used as the biosorbant to remove basic dyes, which are malachite green a, from a binary aqueous solution. Experiments have been conducted to collect the corresponding data and observe the efficiency of this technique. The objectives of the study are:

It was found that *Sargassum* was found to be a better biososrbant when compared to *Chaetomorpha*.

The samples that were pyrolysed at 600°C was found to be more efficient as biosorbants.

The biosorption of solution at neutral pH was found to be better than pH 8.

The biochar of the *Sargassum* can be further studied, so that it can be utilized as biosorption.

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IDENTIFICATION OF BIOACTIVE COMPOUNDS AND ANTIBACTERIAL ACTIVITY OF MARINE ECHINODERM SALMACIS VIRGULATA (l.AGASSIZ AND DESOR,1846) FROM GULF OF MANNAR

A project submitted to

ST.MARY'SCOLLEGE(Autonomous),THOOTHUKUDI

affiliated to

MANONMANIAMSUNDARANARUNIVERSITY,TIRUNELVELI

in partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

by

J.MARIA CLAMENT ANUSHIYA 20AUZO22

A.MARIA PRINCIKA 20AUZO24

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DEPARTMENTOFZOOLOGY

ST. MARY'S COLLEGE
(Autonomous),(Re-accredited with A^+ Grade by NAAC)**THOOTHUKUDI–628001**

April-2023

CERTIFICATE

This is to certify that the project entitled **IDENTIFICATION OF BIOACTIVE COMPOUNDS ANDANTIBACTERIAL ACTIVITY OF MARINE ECHINODERMSALMACIS VIRGULATA (l.AGASSIZ AND DESOR,1846) FROM GULF OF MANNAR** is submitted to **St.Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a record of the work done during the year 2022-2023 by the following students.

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INTRODUCTION

1. INTRODUCTION

The world's oceans, which cover more than 70% of the earth's surface, has been considered as a rich source of compounds possessing novel structures with rich biological activity. The increasing incidence of antibiotic resistance among bacterial pathogens and emerging new disease are posing great challenges to humans. Given the widespread misuse and over prescription of antibiotics by the medical community the antibiotics available today are rapidly becoming less and less effective in the face of emerging multi-drug resistant pathogens of clinical concern.

Microorganisms display interesting competitive mechanisms of which antagonism has been commonly referenced. Microbial antagonism is a biological phenomenon in which certain microorganisms of the normal micro biota that suppresses the growth of other microorganisms through competition for nutrients and the secretion of inhibitory substances. Some microorganisms can inhibit other microorganisms or reduce their growth in medium thanks to their metabolites, indirect (by changing pH, osmotic pressure and surface tension) or direct (by producing toxic component, antimicrobial components, bacteriocin, antibiotics etc.,) this situation is called as antagonistic relation. Microorganisms are not only the cause of infections; they can also produce organic substances that can cure infections. The first marine bacterium based antibiotic was characterized in 1966. As the primary role of antimicrobial

activity can be to antagonize competitors, bacteria also produce antimicrobial compounds when they sense the presence of competing organisms. Many microorganisms contain substances that have antimicrobial antiviral, anticoagulant and cardio active properties few of these substances have unique chemical structures that are unlike any other compounds, may serve as leads to the discovery of new drugs.

The marine environment supports a wide range of microorganisms. Surfaces placed in the sea rapidly absorb bacteria, algae and protozoa. The surfaces appear to provide nutrient sinks which enable diverse microbial communities, to develop and maintain themselves at high population densities (Marshall, 1976). (Baier 1972) and (Leob and Neihof 1975) showed that the first chemical event that takes place when a solid surface is submerged in seawater is the accumulation of organic "Conditioning" film making the surface wet table (Dexter et al., 1975).

(Dexter 1978) The subsequent adsorption of bacteria according to (Marshall 1976) and (Marshall et al., 1971) involves two distinct phases. The first reversible adsorption is an instantaneous attraction, holding bacteria near the surface so that they will still exhibit Brownian motion. The phenomenon is termed reversible because the organisms can be removed easily before substantial contact of all solid surfaces has been made.

Marine invertebrate living in coastal areas may experience substantial fluctuation in environment condition. The effect of biotic and antibiotic factors such as predation, completion, temperature, salinity, food and pollution on the survival, growth, and reproduction of marine invertebrates are well documented by various authors (Thorson 1946, 1966, Kinne 1964, 1971, Foster 1987, Pochenik 1987, Barnes 1989, Boidron - Mentairon 1995. Morgan 1995). However the effect of environmental stress experienced in one developmental stage of the life cycle on the performance of later stages has been examined only in few marine invertebrate species (Bacon 1971, Bayne 1972, Helm et al. 1973, Roller & Stickle 1993, 1994, Hintz & Lawrence 1994, Pechanik et al. 1996a, b. 1998).

A large proportion of natural compounds have been extracted from marine organisms, especially sponges, ascidians, bryozoans and Echinodermss and some of them are currently in clinical trials (Proksch et al., 2002). To date, almost all of the drugs derived from natural sources come from terrestrial organisms. But recently, systematic searches for new drugs have shown that marine animals produce more antibiotic, anti-cancer and anti- inflammatory substances than any group of terrestrial organisms. Particularly promising groups include sponges, tunicates, ascidians, bryozoans, octocorals, annelids, some molluscs, and Echinodermss (Faulkner 2000 and Di Bella et al., 2008).

Most of the pathogens are increasingly resistant to the major classes of the routinely used antibiotic. Many diseases were initially controlled exclusively by the use of antimicrobial drugs. The massive use of antimicrobial for diseases control and growth promotion in animals increases the selective pressure exerted on the natural emergence of bacterial resistance (Riguera, 1997). So there is an urgent need for the discovery of the new and novel antimicrobial drugs to effectively combat not only the drugs resistance but also the new disease producers, hence the search for active drugs from alternative sources including marine environment, obviously becomes imperative. The rich diversity of marine organism assumes a great diversity of the discovery of new bioactive substances. The ocean remains as an untapped source for many drugs and contemporary experimental studies which indicate that, pharmacologically active substances could be isolated from marine organism (Baslow, 1969).

Natural products isolated from marine organisms have increased rapidly and hundreds of new compounds being discovered every year (Faulkner, 2000; Proksch and Muller, 2006). Marine invertebrates offer good source of potential antimicrobial drugs (Bansemir et al., 2006; Mayer et al., 2007: Jayaraj et al., 2008). Studies on antimicrobial mechanisms and compounds of marine invertebrates may provide valuable information for new antibiotic discoveries and give new insight into bioactive compounds in Echinodermss.

The phylum Echinodermsata is the charismatic marine invertebrate and has become a symbol of marine life. Echinodermss contain huge potential of untapped some of bioactive molecules for therapeutic applications in selected fields of cancer research, in the control of bacterial growth as substance with new antibiotic properties, and is also used as antifouling substances. The Echinodermss are of great interest in the field of biotechnology. In the 19th and 20th centuries, great cell biologists used Echinodermss (Sea Urchins) as the model systems to study basic phenomena such as mitosis cell divisions, differentia and organ formation. Today Echinodermss continue to be the model of choice for many cell and molecular biologists offering exciting overtures on the way from molecular to cell biology (Arnone et al., 1997).

Echinodermss are a renewable resource with an economic value to their increasing demand as food and/or source of bioactive molecules exerting antitumor, antiviral anticoagulant, antioxidant, and antimicrobial activities (Loredana et al., 2017). It containing approximately 7,000 living species and is a remarkable economic activity especially in Asia. With the increasing demand for sea urchin roe and trepan (a generic name for sea cucumbers) commercial culture venues have grown in order to maintain the demands for these organisms (Conand 2009 and Furesi et al., 2016).

More ever, recently Echinodermss have received great attention as on exploited source of new bioactive molecules with important

antimicrobial, antiviral, antiprotozoal, antifungal, and antihelminthic anticancer activities suggesting their potential applicability for drug discovery (Layson et al., 2014). The peculiarities of these molecules are stability, activity at low temperature and specificity of action. As invertebrates lacking adaptive immunity, Echinodermss are an excellent model for studying innate immunity. Their defence mechanisms are mediated by cellular and humeral responses (Ramirez and Garcia 2010).

The sea is a source of novel organic bioactive molecules that have much importance in medicine, physiology, pharmacology and biochemistry. The marine environment may contain over 80% of world's plant and animal species although marine compounds are under estimated in current pharmacopeias, it is anticipated that aquatic environment will become an invaluable source of novel compounds in future. Bioactive chemical compounds can be classified as primary metabolites and secondary metabolites from these organisms have recently gained importance as a potential bioactive compound. Like many other marine organisms, Echinoderms have been and continue to be examined as a some of biologically active compound with biomedical applications. (Kelly, 2005).

Sea urchins are small, spiny, globular animal which, with their close skin, such as sand dollars, constitute the class Echinoidea of the

Echinoderms phylum. The shells are known to contain various polyhydroxylated naphtoquinone pigments. (Anderson et al. 1969).

Sea urchins are classic objects of research in different fields of biology, ecology, biodiversity and evolution. At the same time, they are used as raw material to produce foodstuff, in particular, the product of processing gonads, known as "Sea Urchin Roe" (Kaneniwa and Takagi 1986) The Roe of sea urchin is considered to be a prized delicacy due to its tasty qualities in Asian and Mediterranean countries such as Barbados and Chile (Lawrence et al., 1997). The gonads of sea urchins either fresh or in the form of processed food have long been using as luxury foods in Japan (Shinoabokuro.1991).

The sea urchin shells are containing various polyhydroxylated nephtoquinone pigments, spinchromes (Anderson et al., 1969) as well as their analogous compound, Echinochrome of which was shows bactericidal effect was reported by (service et al., 1984).

Japanese demand for sea urchin has risen concerned about overfishing, this making it one of the most valuable sea foods in the world. The population of the Asian pacific region (Hagen, 1996) has also been using it for long time as a remedy for improving general living tone, treatment for a number of diseases and strengthening of the sexual potency of men, especially the middle aged (Ankodinora et al., 1995) Sea urchin fisheries have expanded so greatly in recent years that the natural population of sea urchins in Japan,

France, China the north-eastern united states. The Canadian maritime Provinces and the west coast of North America from California to British Colombia have been overfished to meet the great demand. (Lawrence et al. 2001).

The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural and chemical features not found in terrestrial natural products. The richness of diversity offers a great opportunity for the discovery of new biotic compounds. Modern technologies have opened vast areas of research for the extraction of biomedical compounds from ocean and seas to the treat the deadly disease. (Proksch and Muller, 2006) The secondary metabolites have various functions. It is likely that some of them may be pharmacologically active on humans and useful medicines. (Briskin, 2000).

The bacterial diseases caused series health problem in human, all over the world. More over the bacteria diseases of different species, an instance, *Bacillus subtiles* is accountable for causing food borne gastroenteritis. *Escherichia coli, Staphyloccus aureus* and *Pseudomonasaeruginosa* caused mastitis, abortion and upper respiratory difficulties, whereas *Salmonella sp.* caused diarrhoea and typhoid fever (Jawetz et al, 1995). It becomes a greater problem of giving treatment against resistant pathogenic bacteria (Robert et al., 1999) The emergence of antimicrobial resistance toward a number of conventional antibiotics has stimulated the search for antimicrobial agents from a variety of sources including the marine environments.

Marine organisms represent excellent source for bioactive compounds (Bickmeyer et al., 2005). Bioactive chemical compounds can be classified as primary metabolites, and secondary metabolites. Depending on its biosynthetic origin, biochemical role and genual occurrence.The secondary metabolites, depending on this biopsy synthetic origin, biochemical role and general occurrence. The secondary metabolites have various functions, it is likely that some of them may be pharmacologically active on humans and useful as medicines (Briskin, 2000).

REVIEW OF LITERATURE

2.REVIEW OF LITERATURE

The Phylum Echinodermata composes of approximately 7,000 living species. (Leuckart 1854) successfully established the Echinodermata as a distinct Phylum (Pawson, 2007). About 1,300 echinoderm species of the Indo-Pacific region was recorded by (Pawson 1995). Recent taxonomic research on the holothurians fauna has been conducted by (Thandar 1986-94). Earlier taxonomic studies were conducted by (Clark and Rowe 1971) and (Cherbonnier and Guille 1978) on Madagascar Ophiuroids; (Marshell and Rowe 1981) on Madagascar Crinoids; (Sloan *et al*., 1979) on Seychelles echinoderms; (Cherbonnier 1988) on Madagascar holothurians.

The Class Echinoid consists of free moving echinoderms commonly known as sea urchins and sand dollars. The echinoid body is spherical in shape and flattened along the oral or aboral axis. The body is armed with long movable spines. Sea urchins are brown, black, purple, green, white and red in colour but some are multicoloured (Ruppert and Barnes, 1994). The anus and the madreporiteare situated at the centre of the top on regular sea urchins and the mouth containing the fine toothed chewing mechanism called Aristotle's lantern situated at the centre of the underside. Some species feed on sponges and algae and others are detrital deposit feeders. The sexes are separate and the young ones are formed indirectly by the fusion of sperm and egg, and released into water. There are about 800species of echinoids (James, 2003.)

(Febrina *et a*l; 2000) determined the best body part of sea urchin showing antibacterial activity and the determination of proximate composition, toxicity, bioactive compound and antibacterial activity from the best body part of sea urchin.

(Uma and Parvathavarthini, 1884) elucidates that hexane extract of the sea urchin. Temnopleurus Alexandra has an antibacterial activity of the gram-positive (*Staphylococcus aereus* ATCC 25923, *Bacillus subtitles* MTCC 441, *Enterococcus Faecalis* ATCC 29212) and gram negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeroginosa* ATCC 27853, *Klebsiellla pneumoniae* ATCC 15380, *proteus volgaris* MTCC 1771 bacteria tested, hexane extract showed antibacterial activity for all the bacteria tested except *k.pneumoniae*) it shows that hexane is a potent antibacterial agent and needs further purification for the specific compound, which is responsible for the said activity.

(Parvatha Varthini and Uma 1884) eludictes and aimed at the antibacterial activity of hydroalcoholic extract of the sea urchin, (Temnopleurus Alexandi Bell, 1984).

Bioactive Compounds from the marine habitat have been represented as the greatest under exploited source of potentially active pharmaceutical agents. They produce a variety of metabolites, some of which can be used for drug development (Chellaram and Prem Anand 2010). Echinoderms have

pronounced pharmacological activities or other properties which are useful in the biomedical area.

The methanolic extract of *L. Elongata* showed good antibacterial activity against the clinical pathogens. The present findings on the sea urchin demonstrate that this species may form the basis for the source of active manner of antibacterial potential in future (Maheswaran *et al;* 2016).

Bacteria have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents. Echinoderms have already been reported to contain pharmacologically active compounds with respect to antihistaminic, cytotoxicity (antiangiogenicity, angiogenicity and antibacterial activity (Kelly, 2005). (Haug *et al*., 91.2002) has isolated different extracts from the sea urchin, strongly locentrotus droebachiensis, the sea cucumber cucumaria frondosa and the store fish asterias rubes exhibited antibacterial activity against several strains tested. The crude extract was found to inhibit the growth of several bacteria tested. (Abubakar *et al*; 2012).

(Maheswaran et al; 2015) evaluate the antimicrobial activity of methanolic extract from *L.elongata*. The methanolic extract of *L.elongata* showed good antibacterial activity against the clinical pathogens. The present findings on the sea urchin demonstrate that this species may form the basis for the source of active manner of antibacterial potential in future.

Antibacterial activity has previously been described in a wide range of echinoderm species. The whole body or body walls were tested for

activity. Antimicrobial activity has also been reported in egg extracts of *echinoid Paracentrotus lividus* and the asteroid *Marathasteerias glacialis* (Marimuthu *et al*; 2014).

(Laia Abubakar *et al;*2012)evaluate extracts of the got ,gonad, spines and mouth parts of the sea urchin *Tripneustes gratilla* for antimicrobial and haemolytic activities in vitro. Antibacterial activity has previously been described in a wide range of echinoderm species (Anderson *et al*; 1983, 1989; Bryan *et al*; 1994; Ridzwan *et al*; 1995). In most of the species studied, the whole bodies or body walls were tested for activity.

Sea urchins are common marine organisms belonging to the Phylum echinodermata. Echinodermata is a very small phylum, which includes familiar animals such as sea urchin, sea cucumber and star fish. Sea urchins ubiquitously distributed in the world's oceans, constitute an important part of sub tidal marine communities, and are an important part of fishery resource. Sea urchins have been used as modal organisms over the past century in developmental biology. The phylogenetic position of sea urchins and their importance in studies of embryonic development motivated sequencing the genome of the purple sea urchin (Strongylocantrotus purporatus Tu *et al*; 2012).

(Lola Brasseur et al;2017) reported that biological role in sea urchin physiology, experiments are undertaken on crude extracts from four species and on four isolated spinochromes in order to test their antibacterial,

antioxidant, inflammatory and cytotoxic activities first, the antibacterial arrays shows that the use of crude extracts as representation of antibacterial effects of spinochromes are in accurate.

(Bragadeeswaran *et al*; 2013) elucidates the bioactive potential of aqueous extract of sea urchin Temnopleurus toreumaticus. The antibacterial agents that have been active enough to complete with classical antimicrobial obtained from micro organisms (Rinehart *et al;* 1981)

Pharmacognosy is the scientific study of structural, physical, chemical and biological characteristics of crude drugs. Echinoderms have been poorly studied with reference to their biochemical potential. Chemical composition of gonad extract of sea urchin Strongylocentrotus nudus was assessed by (Hirano*et al.* 1978). The complete amino acid sequence of histone H2B (3) from sperm of the sea urchin Parechinus angulosus was proposed by (Strickland *et al.* 1978). Distribution of fatty acids in lipids of the common Atlantic sea urchin S. droebachiensis was studied by (Takagi *et al*. 1980). (Evans et al. 1983) discovered the protein cyclin in sea urchin embryos. The complete amino acid sequence of echinoidin, a lectin from the coelomic fluid of the sea urchin Anthocidaris crassispina was determined by (Giga *et al*., 1987).(Serrazanetti *et al*. 1995) studied the hydrocarbons, sterols and fatty acids in sea urchin Paracentrotus lividus of the Adriatic Sea.

(Cook *et al*. 2000) assessed the fatty acid compositions of gonadal material and diets of the sea urchin, Psammechinus miliari. (Cruz- Garcia *et al*. 2000) compared the protein, amino acid and fatty acid contents in raw and canned sea urchin *P. lividus*. (Murata and Sata 2000) isolated pulcherrimine, a novel bitter tasting amino acid, from the sea urchin Hemicentrotus pulcherrimus ovaries. (Garcia *et al.* 2000) compared the protein, amino acid and fatty acid contents in raw and canned sea urchin (*P. lividus*) harvested in Galicia (NW Spain).

(Liyana-Pathirana *et al*. 2002) found out the effect of an artificial diet on the biochemical composition of the gonads of S. droebachiensis. Seasonal changes in the biochemical composition of body components of the sea urchin *P. lividus* were studied by (Montero-Torreiro and Garcia-Martinez 2003). The effect of dietary lipids on fatty acid composition and metabolism in juvenile green sea urchin S. droebachiensis was studied by (Castell *et al*., 2004). Variation in gonad fatty acid composition of the echinoid *P. miliaris* was examined by (Hughes *et al*., 2005).

(Amarowicz *et al*., 2012) compared the mineral, protein and pigment contents of shells from red (S. franciscanus) and green (S. droebachiensis) sea urchins. Seasonal variation in the gonad weight and biochemical composition of the sea urchin *P. lividus* was studied by (Arafa *et al*.,2012). (Diniz *et al*.,2012) compared the gross composition and nitrogen to protein conversion factors of

smarine invertebrates belonging to four phyla including porifera, cnidaria, mollusca and echinodermat.

Antimicrobial screening can be used as a rapid and simple preliminary Screening for bioactive compounds during the isolation of natural products. Several drug discovery projects have screened echinoderms for antibiotic activities. An early study by (Ryoyama 1974) reported the biological properties of coelomic fluid preparations from three species of sea urchins A. crassispina, *Pseudocentrotus depressus* and *H. pulcherrimus*. (Wardlaw and Unkles 1978) reported that the coelomic fluid from Echinus esculents possess bactericidal activity against marine Pseudomonas sp. (Rinehart et al. 1981) reported that 43 % of 83 unidentified species of echinoderms (collected from the west coast of Baja California and the Gulf of California) and 58 % of 36 unidentified Caribbean species displayed antimicrobial activities. Early work to document antimicrobial activities of crude extracts from echinoderms showed a wide range of activities against bacterial and fungal isolates (Dybas and Fankboner, 1986). (Service and Wardlaw 1984) reported that Echinochrome A as bactericidal substance in the coelomic fluid of *E. esculentus*. (Service and Wardlaw 1985) evaluated the bactericidal activity of coelomic fluid of

The sea urchin, Echinus esculentus on different marine bacteria. (Canicatti and Roch 1989) studied the antimicrobial proteins from the echinoderm, *H. polii*.

Antimicrobial and anti *staphylococcal* bio film activity from the sea urchin *P. lividus* was assessed by (Schillaci et al. 2010). They also found that sub- MIC concentrations of the 5- kDa peptide fraction of the cytosol from coelomocytes of *P. lividus* were active to inhibit the formation of young and mature staphylococcal biofilm. (Uma and Parvathavarthini 2010b) elucidated that the hexane extract of the sea urchin T. alexandri had antibacterial activity against gram positive and gram negative bacterial strains. They also isolated the bioactive compounds from the sea urchin by GC-MS analysis. Uma and (Parvathavarthini 2010c) tested the antibacterial activity of the ethyl acetate extract of the sea urchin *T. alexandri* against bacterial strains.

(Devi *et al*., 2011) screened the red sea urchin extracts against clinical isolates of bacteria including multi- drug resistant strains and fungi. Antibacterial, antifungal and cytotoxic activities of ethyl acetate, methanol and water- methanol extracts of the cuvierian organ, coelomic fluid and body wall of sea cucumber Bohadschia marmorata from north coastal of Persian Gulf was evaluated by (Mokhlesi *et al*., 2011). (Park *et al*., 2011) investigated the biological activities of Red Sea cucumber Stichopus japonicus collected from Juju Island in South Korea and found that water-soluble fractions possesses good antibacterial effects against *Staphylococcus aurous and S. epidermidis*. (Shankarlal *et al.,*2011) investigated the antimicrobial and antioxidant properties of *S. virgulata* methanolic extract. (Srikumaran *et al.*, 2011)

elucidated the antimicrobial activity of starfishes *Protoreaster lincki and Pentaceraster regulus* against isolated human and fish pathogenic and biofilm microorganisms.

Antibacterial activities of carotenoids in flesh and coelomic fluids of three species of Holothuria (*H. scabra, H. leucospilota and H. atra*) were evaluated by (Abdallah and Ibrahim 2012). (Abubakar *et al*., 2012) evaluated the methanolic and chloroform extracts of the gut, gonad, spines and mouth parts of the sea urchin *T. gratilla* for antimicrobial and haemolytic activities in vitro. (Bjorn *et al*.,2012) isolated antimicrobial peptide centrocin 1 from the green sea urchin

S. droebachiensis and assessed its anti- infectious and anti- inflammatory effects. (Chamundeeswari *et al.,* 2012) evaluated the antimicrobial activity of crude tissue sample of sea star *Astropectenindicus* collected from southeast coast of India against human microbial pathogens viz., *Escherichiacoli, Klebsiella pneumoniae, K. oxytoca, S. aureus, Streptococcus sp.,Pseudomonas aeruginosa, Salmonella paratyphi and S. typhi* using solvent system methanol and ethyl acetate by well diffusion method. According to (Schillaci *et al.,* 2012) the smallest peptide fraction of beta thymosin detected in the coelomocytes of Mediterranean Sea urchin *P. lividus* were able to inhibit biofilm formation against staphylococcal strains.

The antibacterial activity of aqueous extracts from different tissues of sea cucumber Isostichopus badionotus was evaluated by (Moguel-Salazar *et al.,* 2013). (Prabhu and Bragadeeswaran 2013) isolated and characterized the antibacterial compounds from starfish *Stellaster equestris*.(Schillaci *et al.* 2013) suggested that the coelomocytes and immune mediator cells in the echinoderm *H. tubulosa* as an unusual source of antimicrobial and antibiofilm agents. The antifungal activity of the soluble matter and crude saponin extracted from the body wall of sea cucumber (*S. japonicus*) were investigated by (Husni *et al*., 2014).

(Liu *et al.,*2002) studied the hypolipidemic effect of glycosaminoglycans From the sea cucumber Metriatyla sabre in rats fed on cholesterol-supplemented diet.(Kim *et al*.,2002) studied the effects of sea urchin shell on chicken egg quality. Recent reports have shown that the use of sea urchin shells confers certain beneficial advantages, including antioxidant and pharmaceutical effects (Kim *et al*., 2002 and Shankarlal *et al.,* 2011). (Althunibat *et al*., 2009) revealed that the Malaysian sea cucumbers *H. scare H.leucospilota and S. chloronouts* are potential sources of natural antioxidant and anticancer agents. Antioxidant property of polyhydroxylated napthoquinone pigments from shells of purple sea urchin A. crassispina was examined by(Kuwahara et al. 2009).(Hu *et al*. 2010) alleviated orotic acid- induced fatty liver in rats by dietary saponins of sea cucumber Pearsonothuria graeffei. Angiogenic potential of

hexane extract of sea urchin *T. alexandri* was determined by (Uma and Parvathavarthini 2010).

Bioactive Steroidal Glycosides from the Starfish Anasterias Minuta was detected by (Chludil *et al*., 2000). (Diazde-Vivar *et al.,* 2000) isolated two sulphated glycosides and a pentahydroxylated steroid from the Antarctic starfish Labidiaster annulatus. (Beauregard *et al*. 2001) detected and isolated a novel antimicrobial peptide from the echinoderm, (frondosa. Bell *et al*., 2001) synthesized eicosapentaenoic acid in the sea urchin *P. miliaris*.(Borisovets *etal*., 2002) compared the major carotenoid contents in gonads of sea urchins (S. intermedius and *S. nudus*) during maturation.(Kawatake *et al*., 2002) determined the structure of six glucocerebrosides from the starfish *L. maculata*. (Pineiro-Sotela *et al*., 2002) determined the purine bases in sea urchin *P. lividus* gonads by HPLC.

(Shankarlal *et al*., 2011 s) reported that *Salmacis virgulata* is a potent antimicrobial and antioxidant property. The *salmacis virgulata* shows maximum inhibition against the proteus species, so that *salmacis virgulata* will may be utilized for the investigate against the urinary tract infections and the *salmacis vigulata* shows significant activity against the *vibrio chrolerae* and *salmonella typhi* . *salmacis virgulata*(77.51%) showed potent activity at the concentration of 100 µg/ml than compared to standard

ascorbic acid. This study supported, Methonalic extract of *salamisvirgulata* has potential antimicrobial and Antioxidant activity.

(Maoka *et al.*,2014) described the comparison of methanol crude extracted obtained from eggs of farmed and wild specimens reveled a higher bioactivity in farmed individuals with a customized fodder. Several Echinoderms, including sea urchins, are valuable sources of bioactive Compounds but their nutraceutical potential is largely unexplored. In fact, The gonads of some sea urchin species contain antioxidants including Carotenoids and polyhydroxylated naphthoquinones , such as echinochrome A. Astaxanthin is known to have particular bioactivity for the prevention of neurodegenerative disease (Paola cirino*et al.*, 2017)

Antimicrobial substance are widely produced among bacteria.Bacteriocins and bacteriocin like inhibitory substance (SLIS) are ribosomal synthesized antimicrobial peptides produced by a number Of different bacteria that are often effective against closely related species (Riley and wertz 2002,cherif *et al.,* 2003)

The number of natural products isolated from marine organisms increases rapidly, and now exceeds with hundreds of new compounds Being discovered every (Proksch and Muller 2006) the secondary metabolites have various functions. It is likely that some of them may be pharmacologically active on humans and useful as medicines (Briskin 2000). A majority of

pharmacologically active secondary metabolites have been isolated from echinoderms (Caballero *et al.,* 1996)The analogous compound echinochrome A , of which was showed bactericidal effect was reported by service et al (Wardlaw1984)The sea urchin gonads contain Polyhydroxylated naphthoquinone , echinochrome A, which is potential in antioxidant activity.(Lebedev *et al*.,2001) sea urchin gonads are also rich In valuable bioactive fatty acids(PUFAS) and β-carotene (Dincer andcakli 2007).

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OBJECTIVES

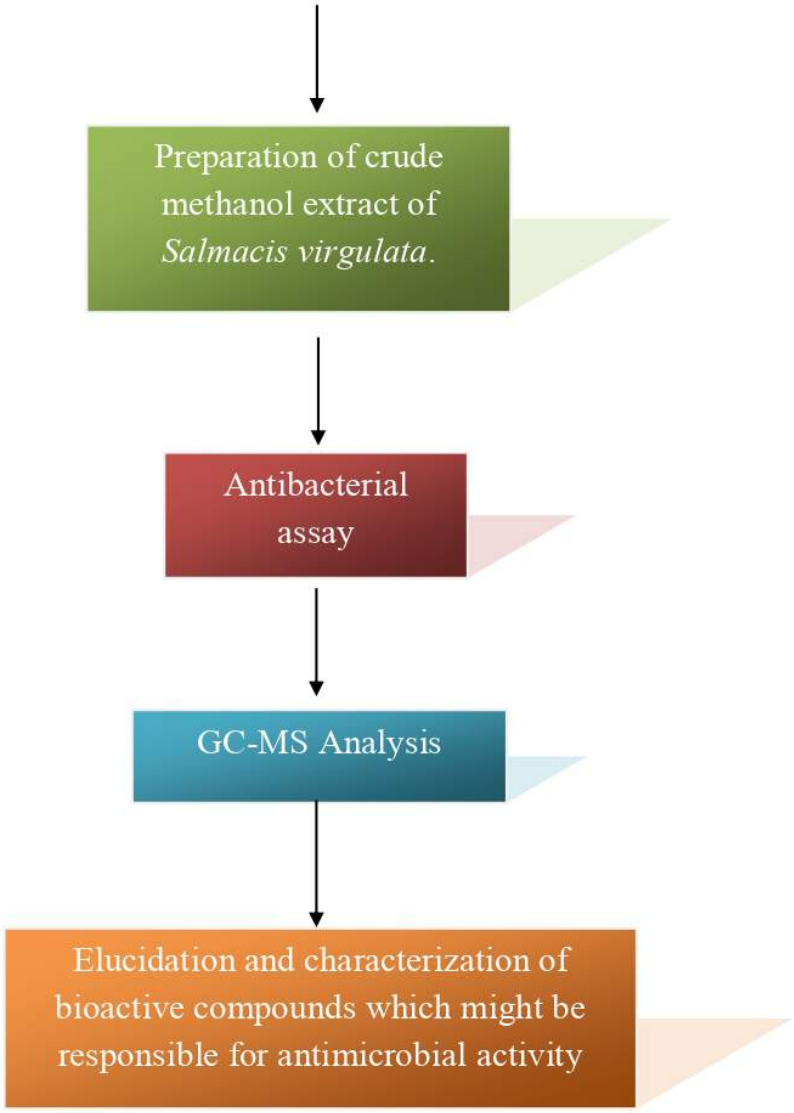
3.OBJECTIVES

Importance of bioactive substance in the present scenario triggered to carry out this present work with the following objectives.

- To collect the probable bioactive extracts from the whole body tissue of *Salmacis virgulata*.
- To test the antibacterial activity against pathogenic bacteria.
- To the potent fraction with superb antibacterial activity.
- To find out the bioactive compounds of the extract by GC.MS analysis.

EXPREMENTAL DESIGNS

Collection of *Salmacis virgulata* from the Gulf of Mannar coastal region of Thoothukudi.



MATERIALS AND METHODS

5. MATERIALS AND METHODS

5.1 SYSTEMATIC POSITION OF EXPERIMENTAL ANIMAL

Salmacis virgulata

Phylum	-	Echinodermsata
Class	-	Echinoidea
Order	-	Echinoida
Family	-	Echinidae
Genus	-	*Salmacis*
Species	-	*S. virgulata*

Salmacis virgulata, sea urchins or urchins are typically spiny, globular animals, Echinodermss in the class *Echinoidea*. Their tests (hard shell) are round and spiny, typically from 3 to 10 cm (1 to 4 in) across; although the largest species can reach up to 36 cm (14 in) they have a rigid, usually spherical body bearing moveable spines. Sea urchins are covered in long thin spikes

where others have a hard shell that is made up of chalky plates. Sea urchin have a round shaped body and with long spines that come off it. The spines of the sea urchin are used for protection, to move about, and trap food particles that are floating around in the water. Sea urchins have five paired rows of tiny tube feet which are found amongst the spines. The feet of the sea urchin have suckers which help the sea urchin to move about, capture food, and to hold onto the ocean floor.

The sea urchins also have little claw-like structure among their spines which the sea urchin uses for protection. These structures (known as pedicellarines) are small stinging structures that are not only used for defence and obtaining food, but are also vital in keeping the body of the sea urchin clean.

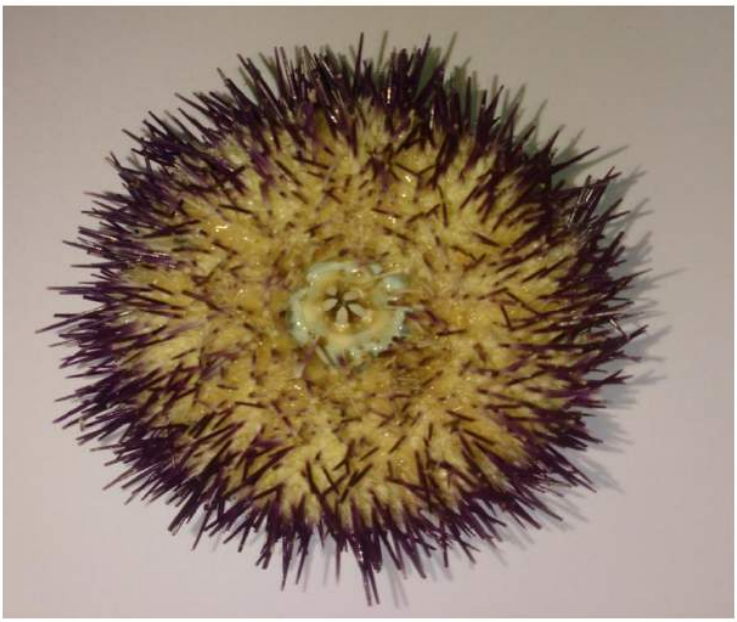
The mouth of the sea urchin (known as the Aristotle's lantern),is found in the middle on the underside of the sea urchins body and has five tooth-like plates for feeding. The anus of the sea urchin is located on the top of the body. As with other Echinodermss, sea urchins do not have a brain and instead rely on their water-vascular system which is like a circulatory system and comprises of water-filled channels that run through the body of the sea urchin.

PLATE -1 ***SALMACIS VIRGULATA***

STUDY ANIMAL

DORASAL VIEW

VENTRAL VIEW



5.2 STUDY AREA

Specimens of *Salmacis virgulata* used in the present study were collected from Gulf of Mannar coastal region. Gulf of Mannar is situated along the south east coast of India. This area is remarkable for its richness and variety of fauna and the inshore sea bottom which forms an ideal habitat for growth of the shell fishes which sustains a good fishery. The Indian part of Gulf of Mannar covers approximately an area of 10,500 km² along lat 8°35- 9°25 N long 78°08 - 79° 30 E. (fig.1)

It is a part of southward extension of Bay of Bengal, it meets in the Indian Ocean. This geographical area runs from Pamban island including Rameshwaram to Cape Comarin to a distance of 170 nautical miles. This coast contains 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 shores etc. The faunal diversity is also well pronounced with reference to different Echinoderms groups.

5.2 STUDY AREA

5.3 ANTIBACTERIAL ACTIVITY

In the present study whole body tissue extract of *Salmacis virgulata* was used for the antibacterial assay. The freshly collected samples were cleaned and washed with fresh seawater to remove all impurities. The shells were removed and the tissues were then dried in hot air oven at 56°c for 48 hours and used for further studies.

a. PREPARATION OF EXTRACT

Methanol extract of the whole body tissues was prepared by following the slightly modified technique. Dried tissue soaked in 100% A.R grade methanol for 10 days at room temperature. After filtration with Whatmann no 1 paper, the methanol extract was reduced by vacuum evaporation. The extract reduce was resuspended in 20ml of 100% A.R crude methanol. The methanol soluble extracts were dried and solubility in deionizer water. Different concentrations of extracts were prepared and stored at 0°c for further use.

b. ANTIBACTERIAL ASSAY:

Antibacterial activity of the extract of *Salmacis virgulata* was determined against three bacterial strains viz, *Pesudomonas , vibirioharvey Aeromonas caviae* these pathogens were obtained from CMFRI Thoothukudi.

C.PREPARATION OF BACTERIAL CULTURE

Nutrient broth medium was prepared and sterilized in an autoclave at 1516 pressure for about 30 minutes. Three bacterial species were inoculated in the nutrient broth and incubated at 28 2°c for 24 hours. Nutrient agar medium was also prepared, autoclaved and transfer aseptically into sterile Petri dishes. On this 24 hours old bacterial broth culture were inoculated by using a sterile cotton swab.

In vitro bacterial assay was carried out by slightly modified disc diffusion Whatman No.1 paper discs with 6mm diameter were impregnated with a known amount of extract of *Salmacis virgulata*.

The impregnated disc along with the control (incorporated with solvent along) was kept at the centre of agar plates, seeded with test bacterial cultures. After incubation at room temperature for 24hrs, the inhibition zones were measured with the outside of the disc to inner side of the inhibition zone. The extracts showing broad spectrum activity were examined for minimum inhibitory concentrations by at different concentration viz. The more potent fraction was characterized to know the functional groups through GC- MS study, Ayya Nadar Janaki Ammal College, Sivakasi.

5.4 GC-MS ANALYSIS:

GC-MS analysis was carried out on a GC Clara's 500 perking Elmer system comprising a AOC 200C auto sample and gas chromatography interfaced a mass spectrophotometer (GC-MS) instrument employing for following conditions such as columnelite-5 MS fused silica capillary column(30 0.25mn id 0.25 composed of 5% diphenyl 95% diphenyl poly giloxane), operating in electron impact mode at 70ev; Helium (99.999%) was used as a carrier gas at constant flow of 1ml/min and an injection volume 3µl (split ratio of 10:1) injector temperature 250^0c. The oven temperature was programmed from 1100c/min to 200^0c, the 5^0c/min to 280^0c.Mass spectra were taken at 700^0c; a scan interval of o.5s and fragments from 45to450Da.

IDENTIFICATION OF COMPOUNDS:

Interpretation on mass spectrum was conducted using the database of National instate of standard technology (NIST ver.21) WILEY 8 and FAME having more than 62,000 patterns. The unknown component found in the body tissues of *Salmacis virgulata*. were matched with the spectrum of the known component stored in NIST, WILEY and FAME the MS library and predicted from Duke's Ethnos Botanica.

RESULTS

6. RESULTS:

6.1 Antibacterial activity of *Salmacis virgulata*

Antibacterial activity of methanol of the whole body tissue of *Salmacis vergula* was tested against three fish bacterial pathogens *Pseudomonos sp, Aeromonas caviae ,Vibrio Harvey* in (fig 2.1 to 2.3). The level of activity was measured by inhibition zones. The extracts developed different zones of inhibitions at different concentrations.

The methanol extract of *Salmacis virgulata* showed activity with the inhibition zones ranging from 0.2 mm to 0.5 mm. The highest activity of 0.5 mm zone was recorded against *Pseudomonas* at 100 mg/ml concentration, whereas a minimum of 0.3 mm inhibition zone was observed against *Pseudomonas* at 50 mg/ml concentration and very negligible activity of 0.2 mm was showed at 10 mg/ml concentration

Methonal extract of *Salmacisvirgulata* showed activity with the inhibition zones ranging from 0.1 mm to 0.4 mm. The highest activity showed 0.4 mm at 100 mg/ml concentration, whereas a minimum activity of 0.2 mm at 50 mg/ml concentration and very negligible activity was showed in 0.1 mm against *Vibrio Harvey*.

Methonal extract of *Salmacis virgulata* showed activity with the inhibition zones ranging from 0.2 mm to 0.4 mm. The highest activity of 0.4 mm at 100

mg/ml concentration against *Aeromonas caviae*, whereas a minimum of 0.3 mm against *Aeromonas caviae* at 50 mg/ml concentration and very lowest activity of 0.2 mm noted against *Aeromonas caviae* at 10 mg/ml concentration.

Of the three concentration of extracts tested in the present study, 100 mg/ml showed more potent activity than the 50 mg/ml and 10 mg/ml. The zone of inhibition increased with increased concentration of all the extracts tested in the present study. Methanol extract showed maximum activities against all the pathogens tested. Hence the methanol extract of Salmacis virgulata was characterised further by GC-MS analysis to know the type of bioactive compounds which could be responsible for antimicrobial activity in the present study.

TABLE-1 ANTIBACTERIAL ACTIVITY OF SALMACIS VIRUGULATA

S.NO	EXTRACT	PATHOGENS	10ug/ml	50ug/ml	100µg/ml
1.	Methanol	*Pseudomonas sp*	0.2mm	0.3mm	0.5mm
2.	Methanol	*Vibrio Harvey*	0.1mm	0.2mm	0.4mm
3.	Methanol	*Aeromonas caviae*	0.2mm	0.3mm	0.4mm

Fig 2 Antibacterial activity of methanol extract of *Salmacis virgulata*

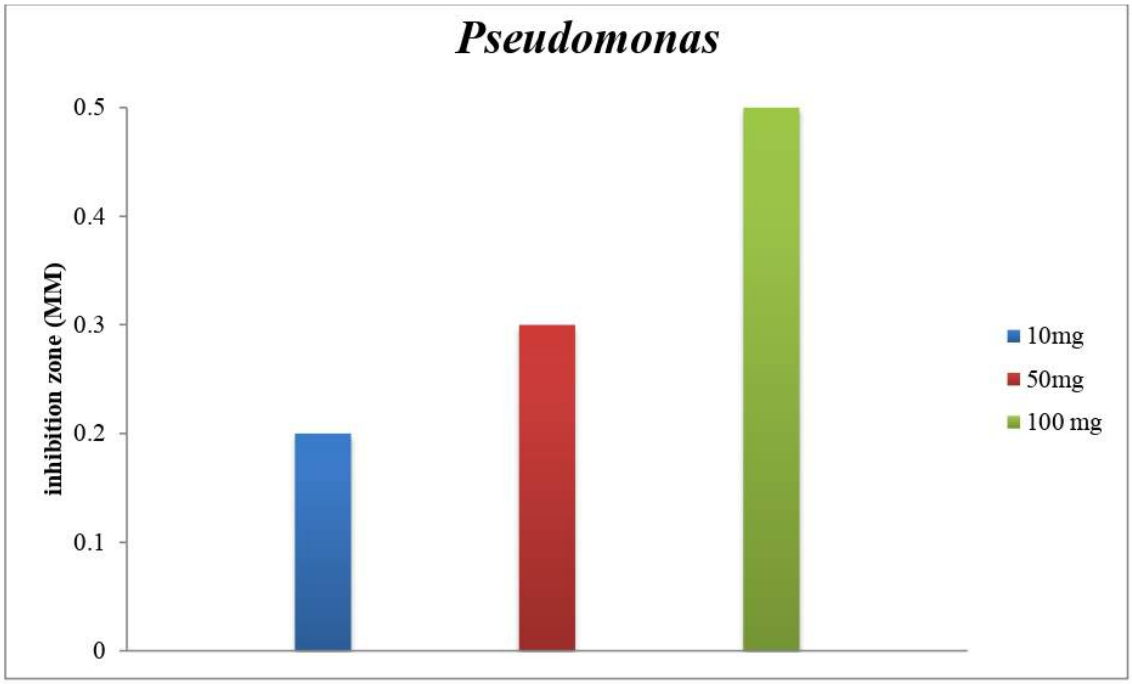


Fig 3; Antibacterial activity of methanol extract of *salmacisvirgulata*

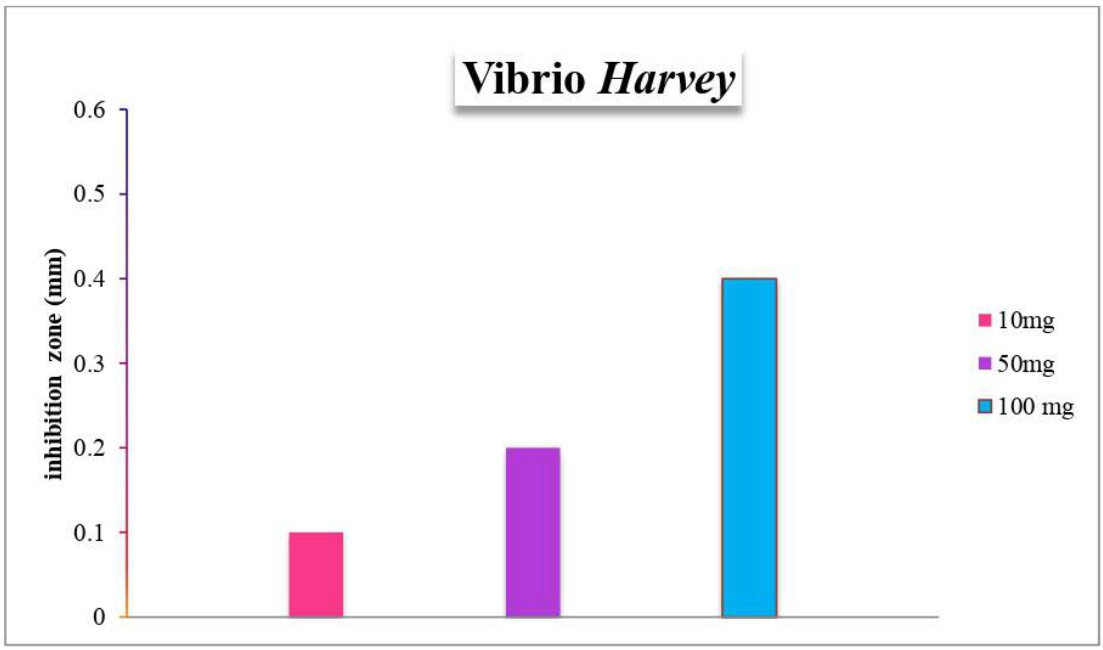


Fig 3 : Antibacterial activity of methonal extract of

Salmacis virgulata

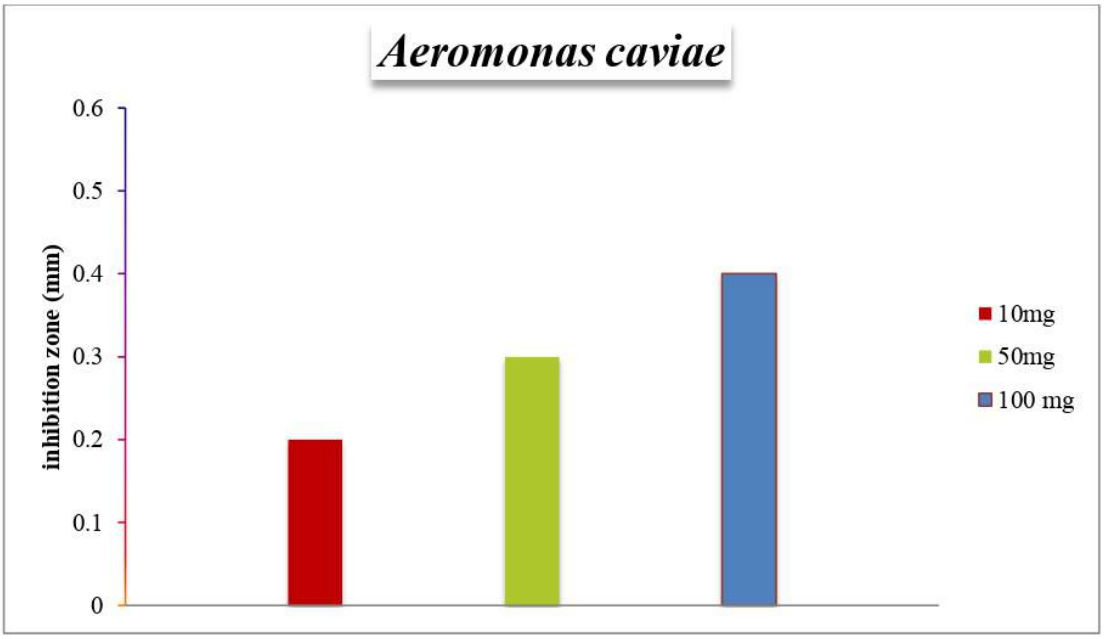


PLATE -2.1 ANTIBACTERIAL ACTIVITY OF METHANOL EXTRACT OF *SALMACIS VIRGULATA*

Pseudomonas

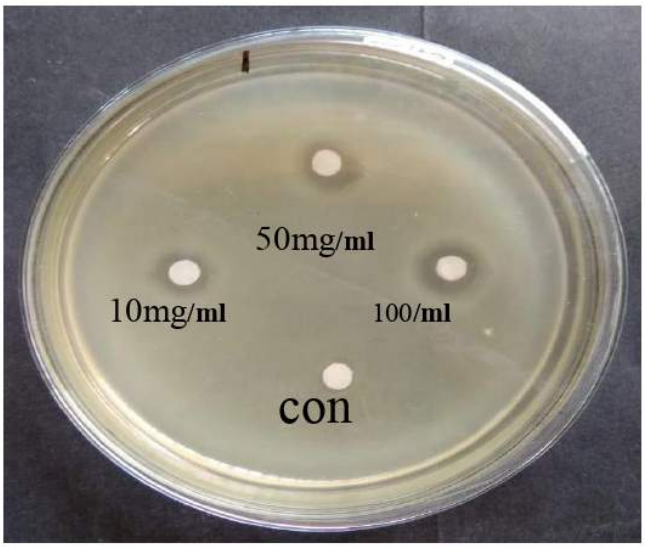


PLATE -2.2 ANTIBACTERIAL ACTIVIY OF METHANOL EXTRACT OF *SALMACIS VIRGULATA*

Vibrioharvey

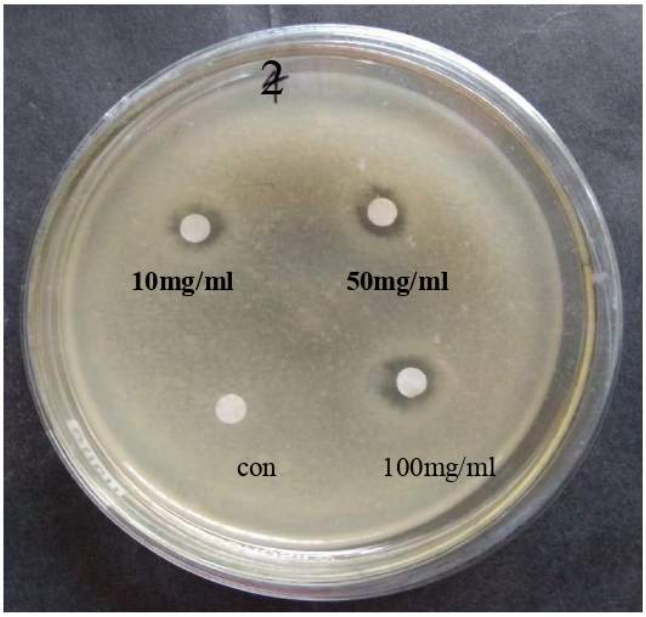
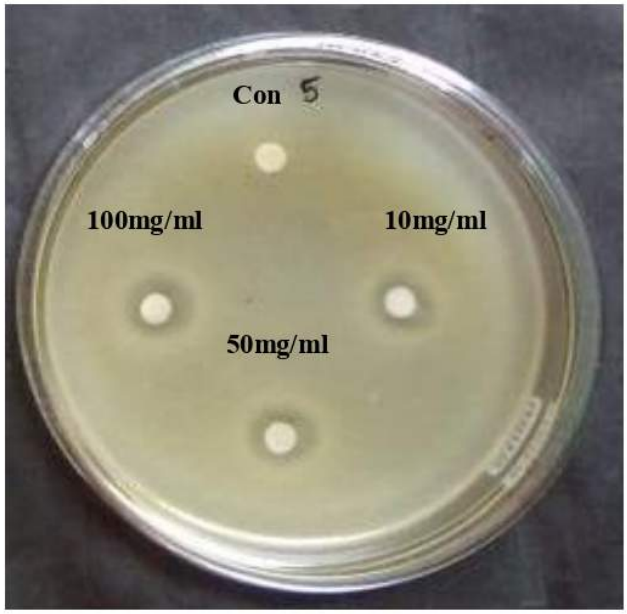


PLATE- 2.3 ANTIBACTERIAL ACTIVITY OF METHANOL EXTRACT OF *SALMACIS VIRGULATA*

Aeromonas caviae



6.2 GC-MS ANALYSIS

Methanol extraction the of the whole body tissue of *Salmacis virgulata* showed significant antimicrobial activity. Hence this fraction was subjected to GC-MS analysis to isolate and characterise the compounds responsible for antimicrobial activities (table 2) .

GC-MS analysis of body tissue of *Salmacis virgulata* exhibited nine peaks, with retention times ranging from 6.039 to 12.027min. All the nine compounds were characterised as 2-Propenoic acid,1-Hexadecanol , Tetradec 11-en-iol acetate , Isopulego1, Nonyl heptafluorobutyrate , Decanoic Acid , Ethinamate,n- hexadecanoic acid , 5-methylcylopent-lenyl methanol.

Among the identified compount as 2-Propenoic acid,1-Hexadecanol , Tetradec 11-en-iol acetate , Isopulego1, Nonyl heptafluorobutyrate , Decanoic Acid , Ethinamate,n- hexadecanoic acid , 5-methylcylopent-lenyl methanol was the most abundant antimicrobial compound present in the methanol tissue extract of *Salmacis virgulata* compound, have the role in fragrance in cosmetics , flavour in foods, anti-inflammatory, antimicrobial, antioxidant, used for vitamin D deficiency, bones diseases, pre-dialysis and diaiysis patients, postmenopausal osteoporosis, reduce cholesterol,Absorption, anticancer, chemo prevention, used in skin diseases compounds, Cure psoriases, effects. These compounds constitute promising novel class of pharmaceuticals

for the treatment of diseases. So it is recommended as a drug. However further studies will need to be undertaken to ascertain its bioactivity.

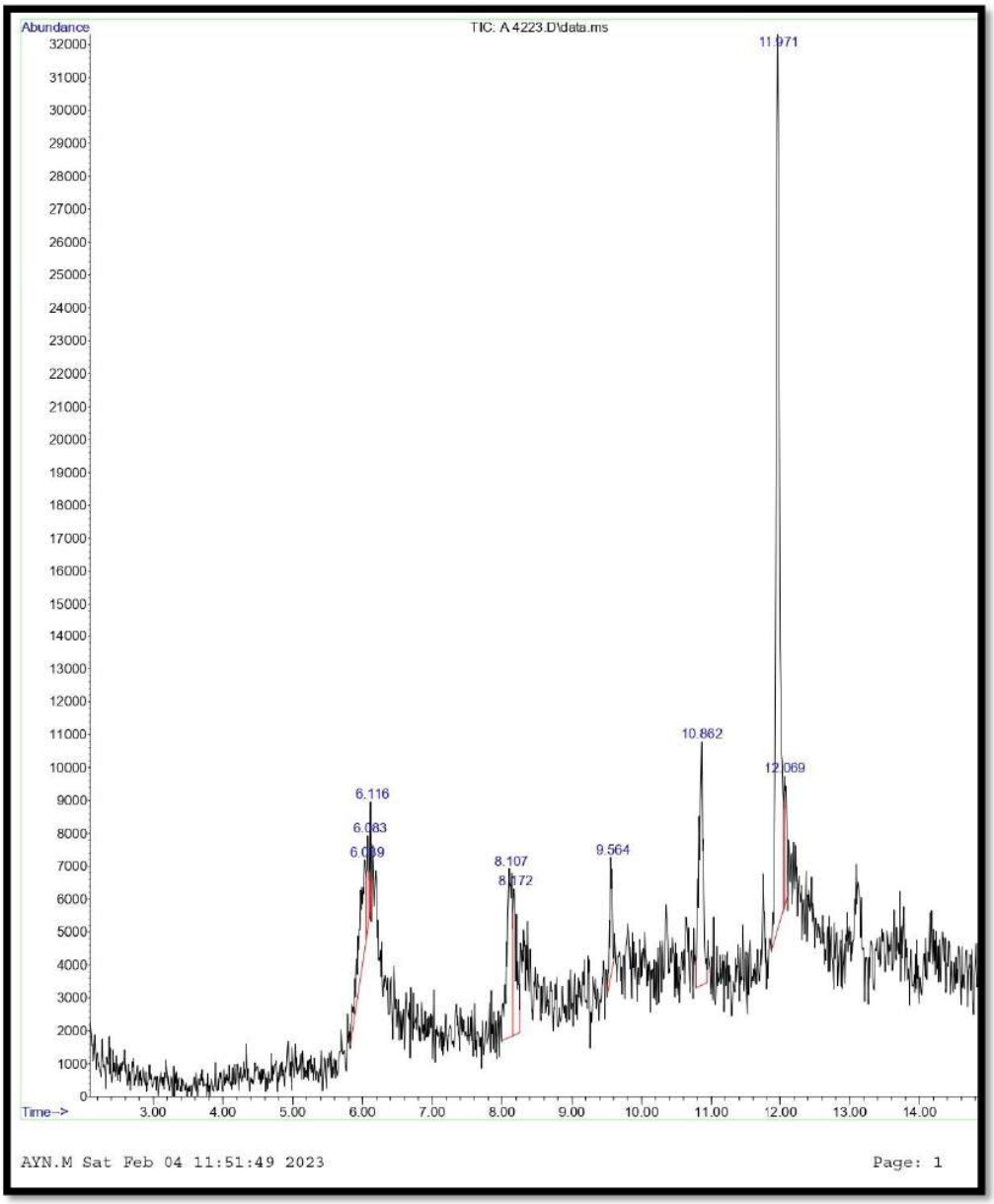
TABLE;2. ACTIVITY OF COMPONENTS IDENTIFIED IN THE METHANOL, EXTRACT OF TISSUE OF *SALMACIS VIRGULATA* BY GC-MS

No.	RT	Compound Name	Molecular Formula	MW	Peak area %	Compound Nature	Activity
1	6.039	2-Propenoic acid	$C_3H_4O_2$	72.06 g/mol	8.71	Carboxylic acid	Preservative fungicide and anti microbial agent.
2	6.086	1-Hexadecanol	$C_{16}H_{34}O$	242.44 g/mol	2.52	Cetyl alcohol	Opacifieu, emulsifier and thickening agent that alter thick ness of liquid and increase and stabilized the foaming capacity.
3	6.115	Tetradec 11-en-iol acetate	$C_{14}H_{26}O_2$	226.35g/mol	1.30	Carboxylic ester	Agrochemicals pesticides fatty acid fatty esters,insect attractants,insecticide
4	8.110	Isopulegol	$C_{10}H_{18}O$	154.25 g/mol	12.13	Aromatic compound	To supplement the natural sources of menthol widely used as a flavouring and in medicinal preparation.
5	8.176	Nonyl eptafluorobutyrate	$C_{13}H_{19}F_7O_2$	340.28 g/mol	7.39	Nonyl ester cheptafluorobutyric acid.	Sequencing synthesis and solubilizing of protein and peptides.
6	9.566	Decanoic Acid	$C_{10}H_{20}O_2$	172.26g/mol	3.83	Carboxylic Acid	To make esters for perfumes, fruit flavours and intermediate for food-grade additive
7	10.861	Ethinamate	$C_9H_{13}NO_2$	167.2g/mol	13.57	Valamin ,valmid	Used to treat insomnia has been replaced by other medicine for treatment insomnia help to produce sleep

8	11.968	n- hexadecanoic acid	$C_{16}H_{32}O_2$	257.42g/mol	46.15	Palmitic acid	Personal care and cosmetic products antioxidants, nematicide.
9	12.027	5-methylcyclopent-1-enyl methanol	$C_7H_{12}O$	112.17 g/mol	4.40	Methyl alcohol	Production of acidic acid and formaldehyde compound also used in anti freeze

FIG; 4 CHROMATOGRAM OF COLUMN EXTRACT OF *SALMACIS VIRUGULATA* BY GC-MS

GC-MS Chromatogram



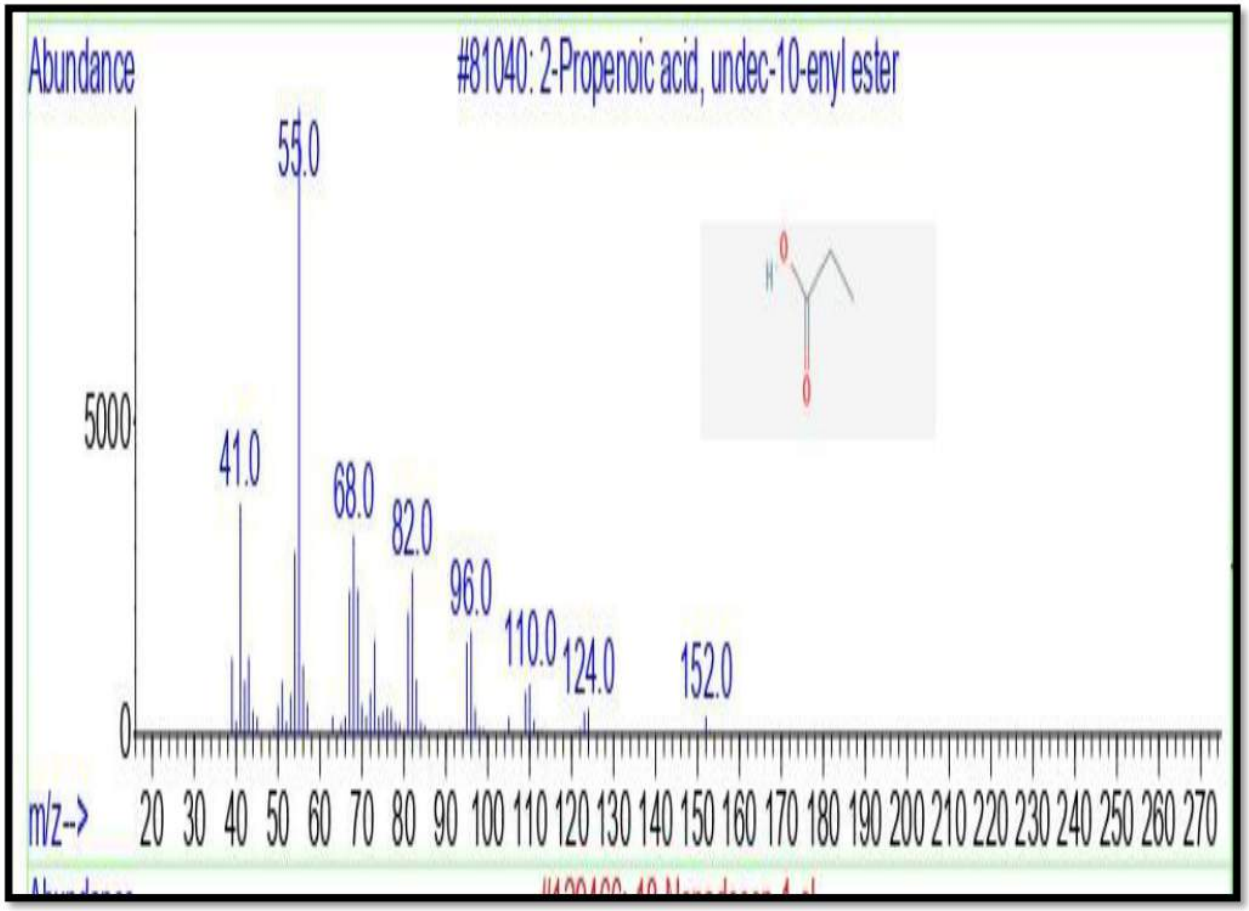
Chromatogram

Fig ; 5

Name;2-Propenoic acid

Formula;$C_3H_4O_2$

MW;72.06g/mol



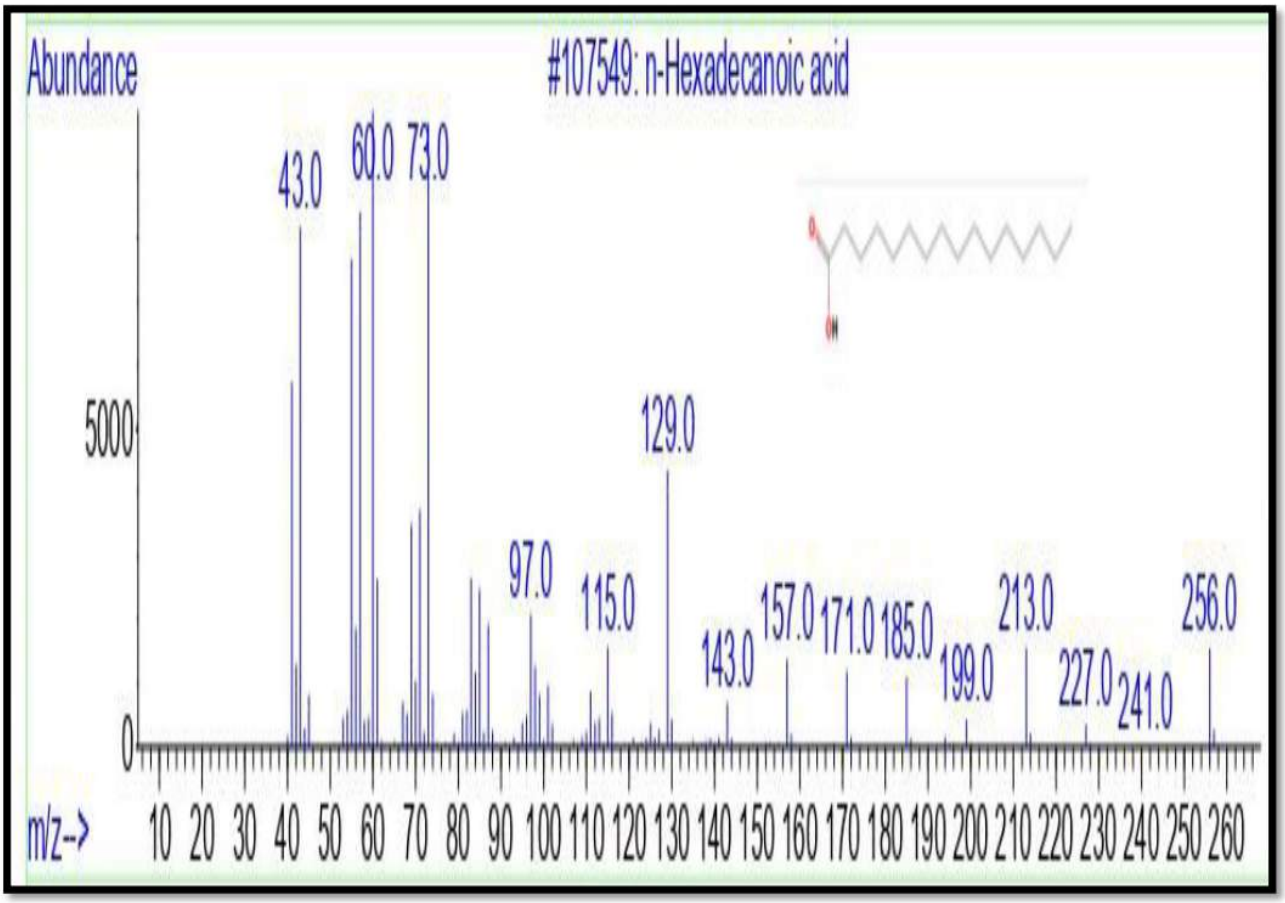
Chromatogram

Fig;6

Name;1-Hexadecanol

Formula;$C_{16}H_{34}O$

Mw;242.44g/mol



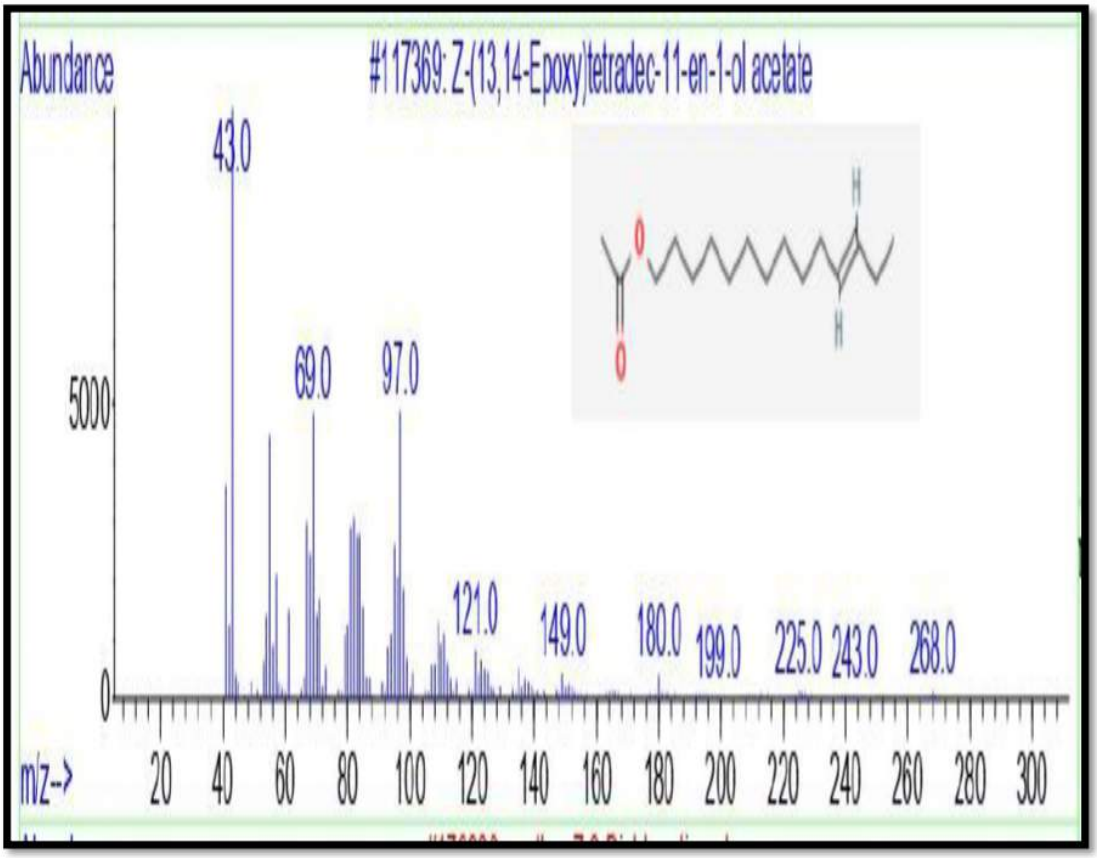
Chromatogram

Fig;7

Name;Tetradec 11-en-iol acetate

Formula;$C_{14}H_{26}O_2$

MW;226.35g/mol



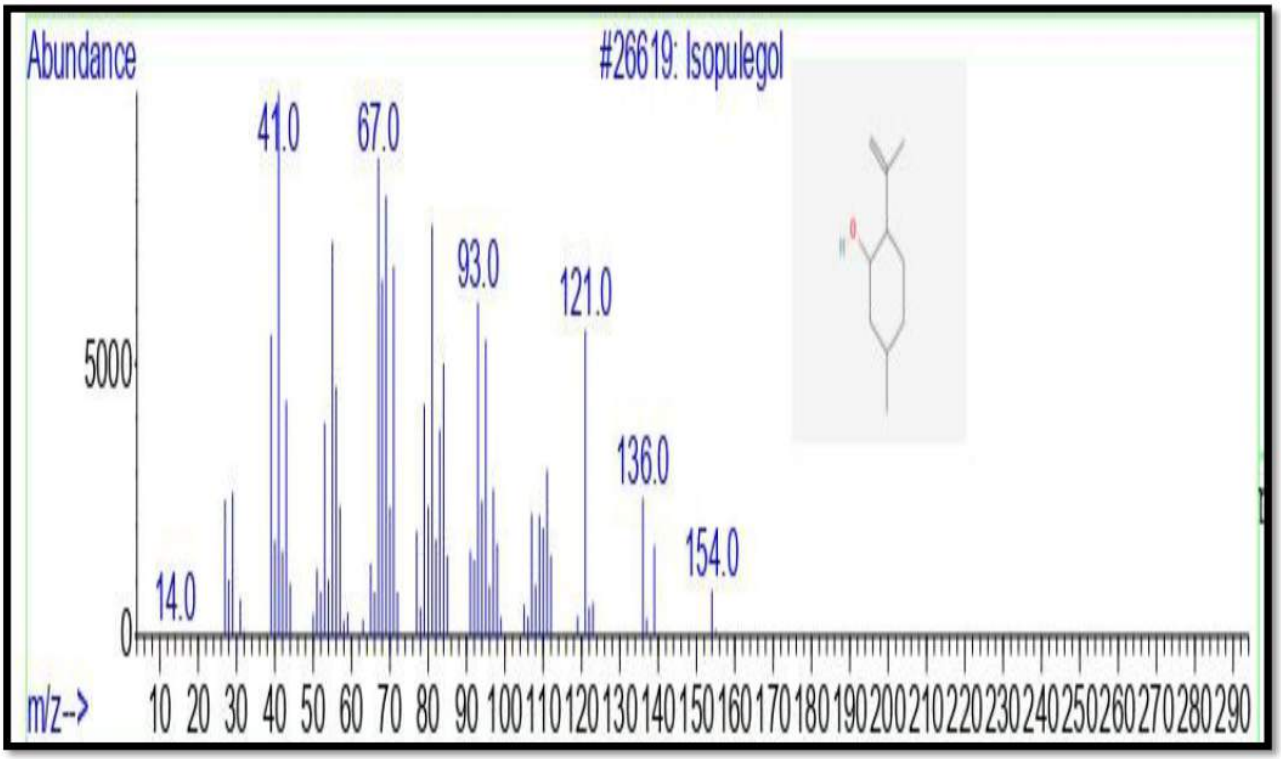
Chromatogram

Fig;8

Name;Isopulegol

Formula;$C_{10}H_{18}O$

MW;154.25g/mol



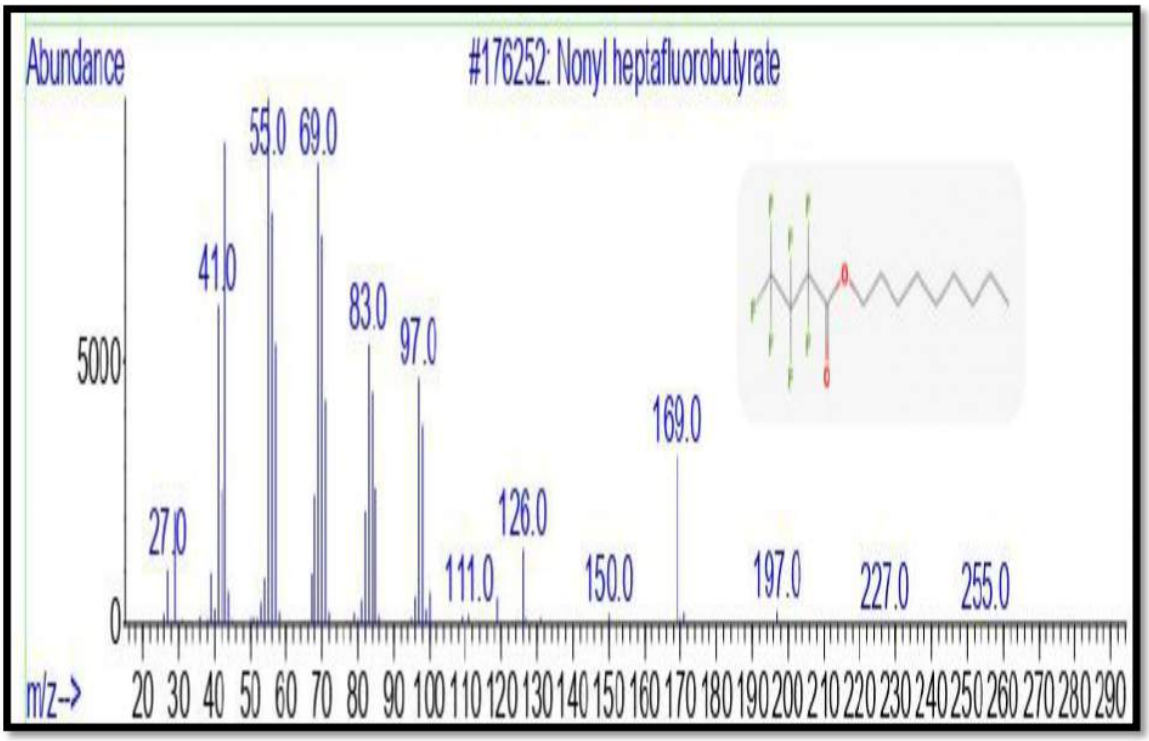
Chromatogram

Fig;9

Name;Nonyl heptaflurobutyrate

Formula;$C_{13}H_{19}F_{17}O_2$

MW;340.28g/mol



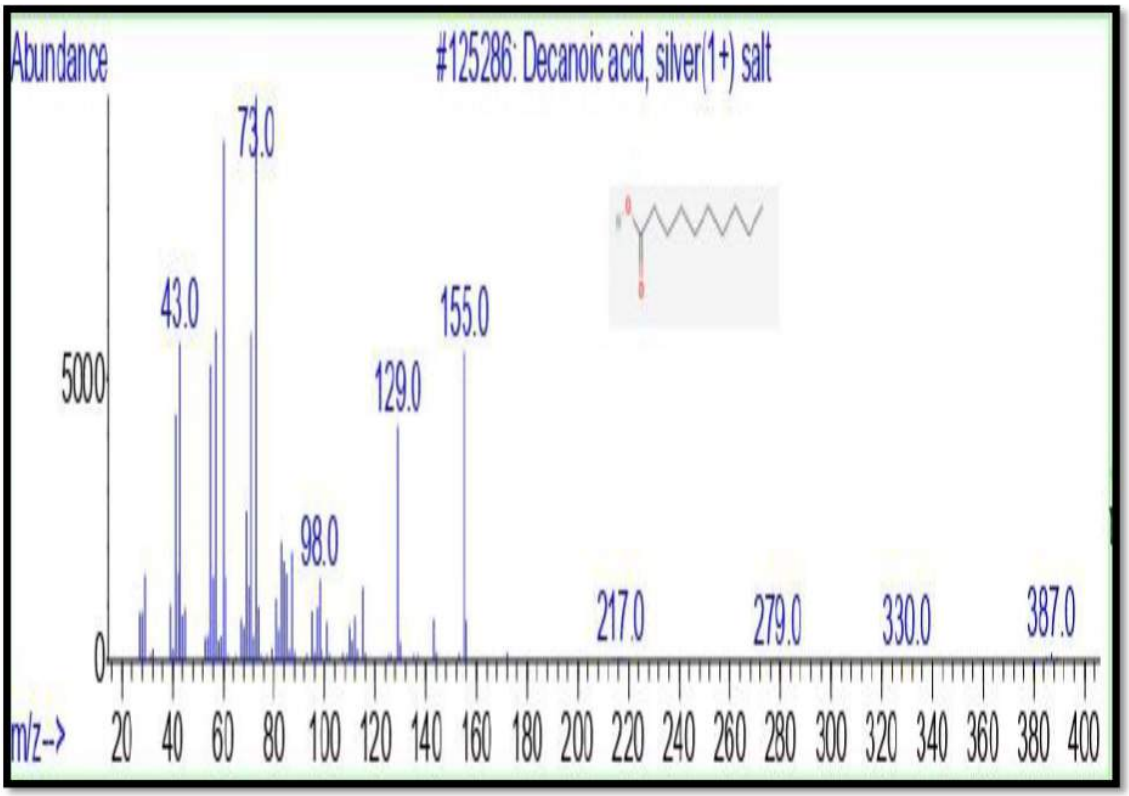
Chromatogram

Fig;10

Name;Decanoic acid

Formula;$C_{10}H_{20}O_2$

MW;172.26g/mol



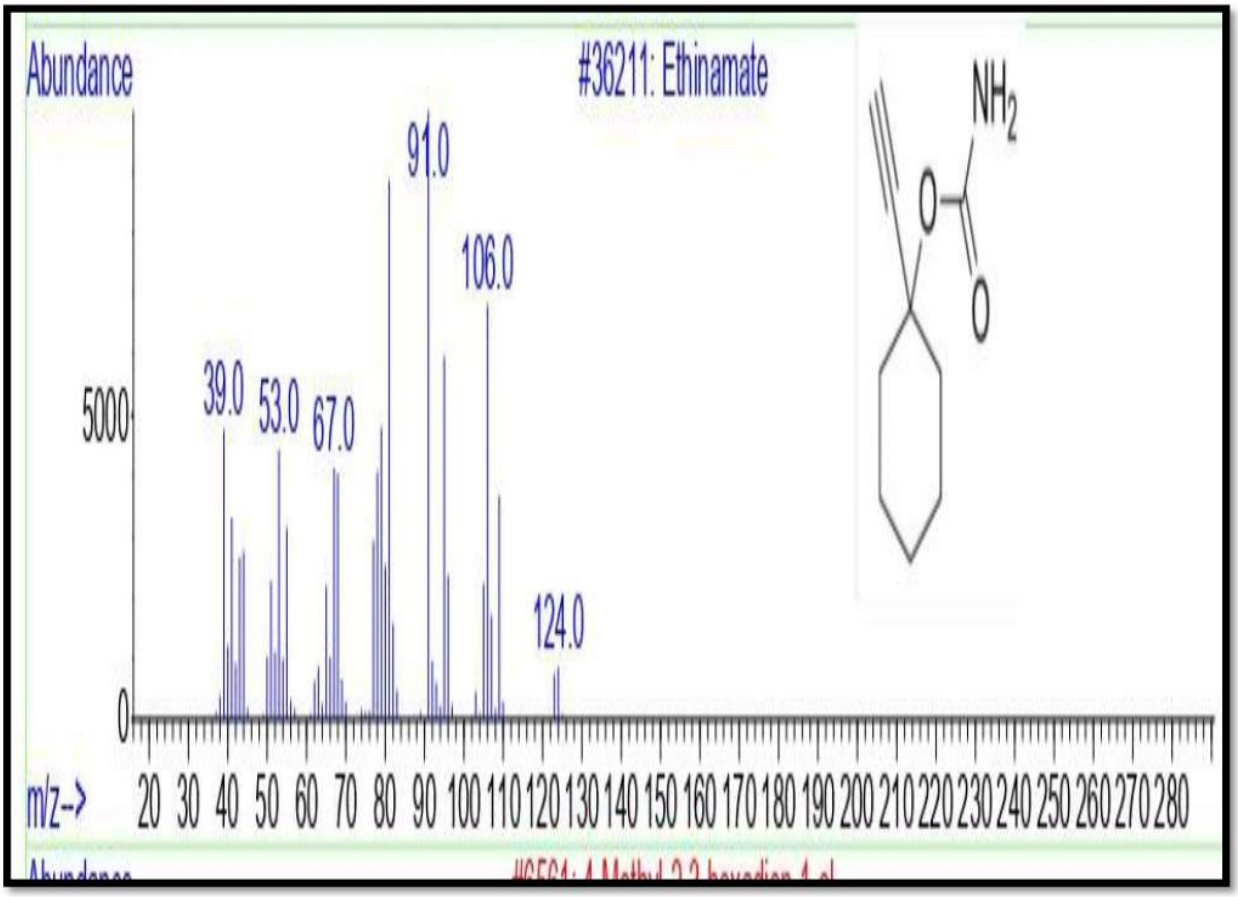
Chromatogram

Fig;11

Name;Ethinamate

Formula;$C_9H_{13}NO_2$

MW;167.2g/mol



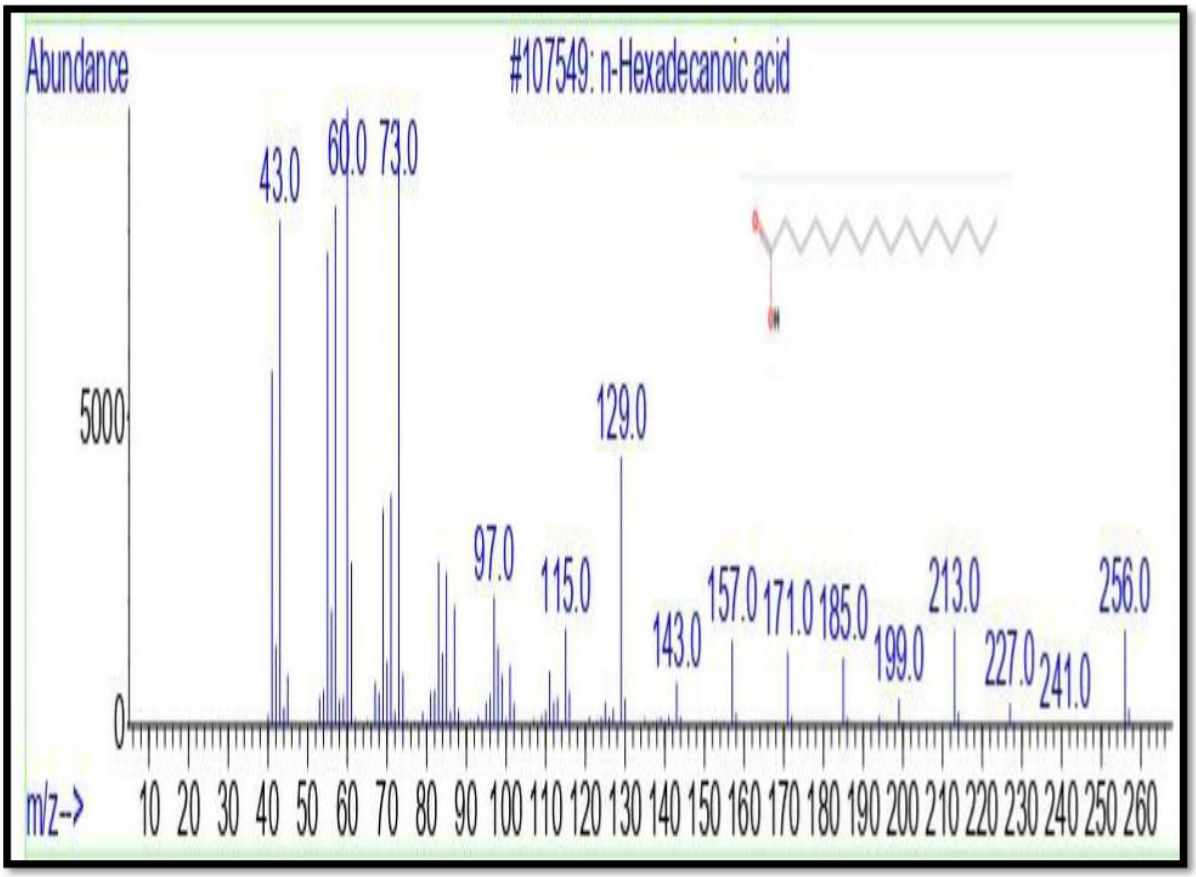
Chromatogram

Fig;12

Name;n-Hexanoic acid

Formula;$C_{16}H_{32}O2$

MW;257.42g/mol



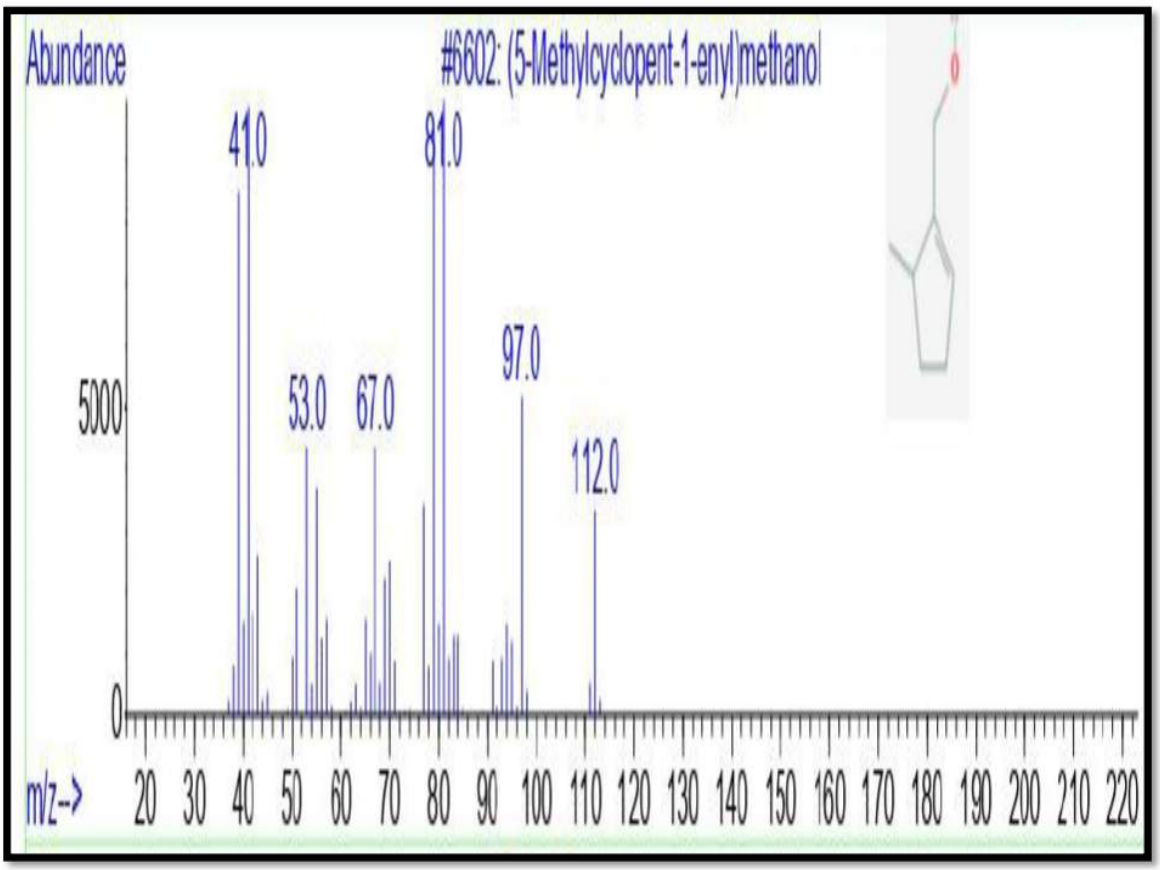
Chromatogram

Fig;13

Name;Methylcyclopent-1-enyl methanol

Formula;$C_7H_{12}O_2$

MW;112.17g/mol



DISCUSSION

7 . DISSCUSSION

The marine environment harbours a wide range of capable of microbes exhibiting bacteriolytic and antibacterial activity. It plays a primary role of the antibiotic substance which could be attributed to ecological competition. The beneficial associations between associated bacteria and their coast have widely reported to produce novel chemical substances, and hence may form the basis of new drugs. The discovery of new classes of antibiotics is necessary due to the increased incidence of multiple resistances, human pathogenic micro organisms to conventional drugs. So, the bacteria associated with barnacle may play a similar role and antibacterial activity could be explained. Similarly, bio film forming marine bacterium D_2 (*Pseudomonas tunicate)* isolated from the surface of the tunicate coined industinalysiswas found to produce a novel protein with bacterial activity against wide variety of marine and medical bacterial isolates as in (zheng *et al.,*2005)..

The antimicrobial activity of sea urchin *T.gratilla* appears to be concentrated mainly in the gut and gonads extracts- little or no activity was observed in the spine and mouth extracts. Further study is required to establish if this observed activity is attributing to proteinaceous (including lysosme-like or non – proteinaceous factors. (Laila *et al*., 2002).

Similarly antifungal activities of various marine organisms and plants have been reported by many workers. (Shanmugam *et al*., 2008) noticed in the

methanol extract of *Sepia aculeate* which exhibited the maximum activity against *A.flavus*. (Bhosale *et al*., 1999) in their study reported that the methanol extract of *polychaete Sabellariaementifera* showed maximum activity against *A.flavus* and minimum against *A.niger* calso support the present study.

Antimicrobial peptides (AMP_S) considered as important immune defence molecules of Echinodermss lacking vertebrate-type adaptive immune system (Smith *et al*., 2010) of Echinodermss has been reviewed earlier (Li *et al*., 2010). AMP_S and lysosome like compounds were recorded in various star fishes *viz., Asterias forbesi, A.rubens* and *Marthasterias glacialis* (Haug *et al*., 2002), (Leonard *et al*.,1990), *canicatti* and (Ancona 1990).

Marine organisms has been a rehabilitated to much natural products especially in therapeutic possible to cure many infectious diseases. (Sreedevi 2014, Minh *et al.,* 2005). Several drug discovery projects have screened Echinodermss for antibiotic activities. The 83 unidentified species of Echinodermss from the Gulf of California showed 43% of antimicrobial activity. (Rinehart *et al*., 1981)In addition, the 36 unidentified species of Echinodermss from Caribbean showed 58% of antimicrobial activity; whereares the 22 species of Echinodermss are collected from the northern Gulf of Mexico showed 80% in antimicrobial activity. (Bryan *et al*., 1994).

Antibacterial activity was detected in extracts of several tissues from the green sea urchin*S.droebachiensis*,the common sea star *Asterias rubens*, and the

sea cucumber *Cucumaria frondosa* (Haug *et al.,*2002). It is an interesting finding that sea urchin being marine animal has the ability to dispose the bacterial boon infection. As the bacterium is a human pathogen, it is important that sea water should not be in the water and the peptides can kill more efficient that the conventional antibiotics. Antibacterial activity from the body wall of several Echinoderms species has been studied by (Billasin and Pomory, 2000). (Stabili *et al.,*1996)have been studied the antibacterial activity in the coelomocytes of the sea urchin *Paracentrotus lividus*.Antimicrobial activity has been found from the eggs of other marine invertebrates as well (Benkendorff *et al.*,2001;Haug *et al.,*2002) and both of these studies showed that at least some of the antibacterial compounds are not proteinaceous.

The composition of valuable components, However, varies greatly among different urchin species and is influence by their natural diet as well as physiological process i.e. reproductive stage (Lawrence 2007, Fernandez, 1998).On the other hand, the high levels of AA and EPA recently dedicated in *Diadema setosum and Salanacis sphaeroides* supported the development of aquaculture of sea urchins (Chen *et al.*, 2010), since PUF as are important for human nutrition (Lawrance 2007).

(Anand and Edward 2001) noted that the crude methanol extracts of *Cypraea errones* exhibited promising results for antibacterial activity. Antibacterial activity of opercular extracts of *Chicoreus ramosus and*

Pleuroplaca trapezium against six bacterial pathogens was reported by (Murugan and Ayyakkannu., 1997). The maximum antibacterial activity against *S.Aureus and E.coli of Trochus radiates* was reported by (Mary Elizabeth, 2003).(Santhana Ramasamy and Murugan., 2003) have reported that the crude methanol extract of *Didemnum psammathodes* inhibited the growth of bacteria. (Mohammed Hussain and Anandhan. 2009) reported that the methanol extract of *D.candidum* exhibited maximum antibacterial activity against *S.typhi, P.aerogenosa and V.cholerae*.

Sea urchins are prone to infestation by the gastropod *Vexillaa vexillum* that can lead to lethal bacterial infections. Out of the four studied sea urchins, only *T.gratilla and E.mathaei* can be infested and develop the disease, the first being 5 times more affected .*D.savignyi and T.pileolus* are probably better production than the two other species thanks to their efficient spines or their pedicellariae toxins .

The Echinodermss have stronger antibacterial effects than Porifera, Mollusks, Bry-zoar annelids (Ridzwan *et al*.,1995). Antibacterial activity has previously been described in some species of Echinodermss (Haug *et al*., 2002- kiani et al 2014- Ridzwan *et al*.,1995). (Haug *et al*.,2002) studied the star fish *Asterias rubens*,and the sea cucucmber *Cucumaria frondose* against gram-positive and negative bacteria. They showed antibacterial activities. The extract of several tissues from *A.rubens and C.frondosa*.the coelomocytes of the sea

urchin paracentrotus lividus showed antibacterial activity against *Vibrio alginolyticus*. (stabili*et al.*,1996). (Rinehart *et al.*, 1981) examine 83 unidentified species of Echinodermss from the west coast of Baja California and the Gulf of California and found 43% of them had antimicrobial activity. In the same study, 58%out of 36 unidentified Caribbean species showed antimicrobial activity. Out of 22 species of Echinodermss collected from the northern Gulf of Mexico, 80% had microbial activity (Bryan *et al.*, 1994).This study demonstrated the presence of antibacterial factors in several tissues such as gonads and test of *E.mathaei*. Whether the same antibacterial factors are responsible for the activity in all organs, is unknown.However, it seen that the antibacterial factors have an important functions as a first line of defense against pathogenic microorganisms.

Antimicrobial activity was observed in both the methanol and the chloroform extracts of the ovary; however the higher inhibition was exhibited by the methanol extracts. This suggested that the antimicrobial components might be present in the sea urchin ovary. The antimicrobial susceptibility showed that, sea urchin ovary extract has the higher zone of inhibition against a few bacteria compared to the conventional antibiotics such as streptomycin, ampicillin, cephalexin and gentamicin. For example, ampicillin showed a very high antibacterial activity against *B.subtilis* and *S.typhi*. however, the methanol extract of sea urchin showed better zone of inhibition against *S.flexneri,*

S.typhimurium, *A.hydrophila, K.pneumoniae, C.freundii, and S.aureus*. *Citobacter freundii* was not inhibited by ampicillin, cephalexin and gentamicin. The methanol extract of sea urchin ovary showed inhibition against these bacteria.

Antibacterial activity by the hexane extract of T.alexndri. Highest activity was observed with the maximum dose of hexane extract and the zone of inhibition was increasing with respect to increasing dose. Echinodermss have ready been reported to contain pharmacologically active compounds with respects to antihistaminic, cytotoxicity and antiangiogenicity. The *ophuroid Ophoplocas januarii* from Argentina contained one new antiviral steroidal sulfate. Similarly, Neothyoside is an antifungal triterpene diglycoside from the Gulf of California holothurians Neothyone gibbons. The major components in the present hexane extract could have been responsible for the antibacterial activity. The biololgical activities of these major bioactive components in relation to parasitism (Paul *et al*, 2002), apoptosis (Jae *et al*., 2008) and antimicrobial activity (Liu *et al*.S, 2010) have already been established, supporting the fact they might have had the antibacterial activity as well. Since antibacterial agents that poses antibacterial activity area of interest in the field of pharmacology, further fractionation, purification, and identification of the exact bioactive compound present in the present hexane extract is of much importance.

In the present study the activity of *Salmacis virgulata* was found to be high which may be due to species specific characteristics more ever the antibacterial activities can be depend upon the nature of solvent and the compounds extracted (Sugesh *et al.,* 2013). The methanol extracts of *Salmacis virgulata* are showing antibacterial properties against the bacterial species tested here. Thus the current studies revealed the presence of potent antimicrobial compounds from Echinodermss Salmacis virgulata of Tuticorin coast.

In the present study GC-MS analysis of *Salmacis virgulata* showed 100 percentage successes in the identification of bioactive compounds responsible for antimicrobial activities. The magnitude of crude extracts of *Salmavis virgulata* possibly reveals the presence of nine antimicrobial compounds 2-Propenoic acid,1-Hexadecanol , Tetradec 11-en-iol acetate , Isopulego1, Nonyl heptafluorobutyrate , Decanoic Acid , Ethinamate, n- hexadecanoic acid , 5-methylcylopent-lenyl methanol . Among the compounds identified n-hexadecanoic acid (Table:2),(Figures:5to 13),identified from GC-MS analysis might be responsible for antimicrobial activity. As the Echinodermss resourcesare rich and varied Indian cost, there exist a great potential for the extraction of bio active compounds of medicinal importance at a lower cost.

SUMMARY

8. SUMMARY

❖The present investigation has been undertaken to find out the antibacterial activities of the marine Echinoderm *Salmacis virgulata*.

❖ Antibacterial activity was tested against three fish bacterial pathogens *Pseudomonas sp , Aeromonas caviae , Vibrio harvey*.

❖The growth of all tested bacterial inhibited by the crude extract of *Salmacis virgulata* and the inhibitory zones varied from 0.1 mm to 0.5mm.

❖ The maximum inhibition zone (0.5mm) was developed against *Pseudomonas*at 100µg/ml concentration and lowest inhibition zone (0.1mm) was recorded against *Vibrio Harvey* at 10µg/ml concentration and methanol is considered to be the most potent fraction.

❖The methanolic extractions of tissue were analyzed by GC-MS to characterize the compound responsible for antimicrobial activities. GC-MS analysis of tissue of *Salmacis virgulata* exhibited nine peaks, with the retention times ranging from 6.039 to 12.027min.

❖ GC-MS study revealed the presence of nine compounds, from the fraction of the nine compounds the following as 2-Propenoic acid,1-Hexadecanol , Tetradec 11-en-iol acetate , Isopulego1, Nonyl heptafluorobutyrate , Decanoic Acid , Ethinamate, n- hexadecanoic acid , 5-

methylcylopent-lenyl methanol . Among the compounds identified n-hexadecanoic acid was the most abundant antimicrobial compounds (46.15%) present in the methanol tissue extract of *Salmacis virgulata*.

❖ The result of the present study showed that the whole body tissues extracts showed potential antimicrobial activity against pathogenic bacterial strains which indicates the presence of potent bioactive substance in them and correct understanding and utilization may lead to its use as antibiotic drugs.

CONCLUSION AND SUGGESTIONS

9. CONCLUSION:

Echinodermss are considerably important as a food source as well in scientific investigation. There is an ever continuous and urgent need to discover new antimicrobial 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 diseases. A further big concern is the development of resistance to the antibiotics in current clinical use.

The present study recommends that the natural bioactive substance have the least quantum of side effects when compared to synthetic products. Although most antibiotic have been derived from the terrestrial products it is the marine world that provide the pharmaceutical industries the next generation of medicines.

GC-MS analysis has aided the evaluation of the major and minor compounds present in methanol extracts of tissues. A novel therapeutic compound from this marine source would be of much use in eradicating the microbial pathogens and it would definitely aid in the control and emergence of drug resistance strain.

10. SUGGESTION

Commercial antibiotics are highly effective to kill the bacterial pathogens involved in common infection. Today people prefer to use pharmaceutical products from natural origin because of their less side effects and nutritive value. So for very few studies have been carried out to show the presence of antimicrobial compounds. This study revealed the presence of bioactive compounds that would inhibit the microbial contaminants in marine or aquaculture field.

The results of the presence study suggest that the Echinoderms of Indian origin with rich bioactive compounds would be used as antimicrobial agents for alternative therapy. New Echinoderms species of Indian waters need to be exposed out with strong antimicrobial agents. The strong antimicrobial activity of *Salmacis virgulata*of Tuticorin coast can be used for future pharmacological research to solve the problems of multi drug resistance in all fields. Studies on the Echinoderms species *Salmacis virgulata* and identification of antimicrobial agents are further needed in the future to solve the problems of multidrug resistance in the microorganisms. Although substantial progress has been made in identifying novel drugs from marine sources, great endeavors are still needed to explore these molecules for clinical applications without altering or disturbing the biodiversity on marine organism. If any animal is found to be suitable candidate species for the exploitation of drugs. The animal can be

cultured by suitable aquaculture practice and thereby we can conserve the fauna as well as not modifying the diversity for the sake of mankind.

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ANTIDIABETIC AND ANTIOXIDANT ACTIVITY OF CHOSEN TUNICATES

A project submitted to

ST. MARY'S COLLEGE (Autonomous), **THOOTHUKUDI**

affiliated to

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In partial fulfilment for the award of the degree of

BACHELOR OF SCIENCE IN ZOOLOGY

1.	**SIVA BHARATHI. S**	**20AUZO40**
2.	**SONALI. T**	**20AUZO43**
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(Re-accredited with 'A^+' Grade by NAAC)

April -2023

CERTIFICATE

This is to certify that the project entitled **"Antidiabetic and Antioxidant Activity of Chosen Tunicates"** is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Bachelor of Science in Zoology** and it is a project work done during the year 2022-2023 by the following students.

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DECLARATION

We do hereby declare that this dissertation entitled, "**Antidiabetic and Antioxidant Activity of Chosen Tunicates**" submitted by us for the award of the degree of Bachelor of Science in Zoology is the result of our 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.

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INTRODUCTION

Health Is Not Valued Till Sickness Comes!

Diabetes Mellitus

Diabetes Mellitus is a chronic metabolic disorder, in which there are high blood sugar levels over a prolonged period. The Briton John Rolle added the term "mellitus" or "from honey" in the late 1700s to separate the condition from diabetes insipidus, which is also associated with frequent urination. Indian physicians around the same time identified the disease and classified it as madhumeha or "honey urine", noting that the urine would attract ants. Galen named the disease "Diarrhea of the urine". It is a fast-growing global problem with huge social, health and economic consequences.

An aging population and obesity are two main reasons for the increase. Furthermore, it has been shown that almost 50% of putative diabetics are not diagnosed until 10 years after the onset of the disease, hence the real prevalence of global diabetes must be astronomically high.

This high blood sugar produces the symptoms of frequent urination, increased thirst and increased hunger. Untreated, diabetes can cause many complications. Acute complications include diabetic ketoacidosis and non ketotic hyperosmolarcoma. Serious long-term complications include heart disease, stroke, kidney failure, foot ulcers and

damage to the eyes. DM can be classified into two main types i.e. type-1 (insulin-dependent) and type-2 (noninsulin-dependent) differ in their pathogenesis, but both have a common symptom of hyperglycemia. Type-1 DM is due to the loss of insulin-secreting beta cells whereas type-2 DM (T2DM) is caused by impaired insulin secretion combined with either a reduction of insulin activity or impairment of maintained activity. According to WHO, T2DM is a major type of diabetes comprising 90% of total diabetic cases globally.

Significant symptoms of diabetes

The classic symptoms of untreated diabetes are weight loss, polyuria (frequent urination), polydipsia (increased thirst), and polyphagia (increased hunger). Symptoms may develop rapidly (weeks or months) in type 1 diabetes, while they usually develop much more slowly and may be subtle or absent in type 2 diabetes.

Several other signs and symptoms can mark the onset of diabetes, although they are not specific to the disease. In addition to the known ones above, they include blurry vision, headache, fatigue, slow healing of cuts, and itchy skin. Prolonged high blood glucose can cause glucose absorption in the lens of the eye, which leads to changes in its shape, resulting in vision changes. A number of skin rashes that can occur in diabetes are collectively known as diabetic dermadromes.

People (usually with type 1 diabetes) may also experience episodes

of diabeticketoacidosis, a type of metabolic problems characterized by nausea, vomiting and abdominal pain, the smell of acetone on the breath, deep breathing known as Kussmaul breathing, and in severe cases a decreased level of consciousness. A rare but equally severe possibility is hyperosmolar nonketotic state, which is more common in type 2 diabetes and is mainly the result of dehydration.

Complications

All forms of diabetes increase the risk of long-term complications. These typically develop after many years (10–20), but may be the first symptom in thosewho have otherwise not received a diagnosis before that time.

The major long-term complications relate to damage to blood vessels. Diabetes doubles the risk of cardiovascular disease and about 75% of deaths in diabetics are due to coronary artery disease. Other "macrovascular" diseases are stroke, and peripheral vascular disease.

The primary micro vascular complications of diabetes include damage to the eyes, kidneys, and nerves. Damage to the eyes, known as diabetic retinopathy, is caused by damage to the blood vessels in the retina of the eye, and can result in gradual vision loss and potentially blindness. Damage to the kidneys, known as diabetic nephropathy, can lead to tissue scarring, urine protein loss, and eventually chronic kidney disease, sometimes requiring dialysis or kidney transplant. Damage to the nerves of

the body, known as diabetic neuropathy, is the most commoncomplication of diabetes. The symptoms can include numbness, tingling, pain, and altered pain sensation, which can lead to damage to the skin. Diabetes-related foot problems (such as diabetic foot ulcers) may occur, and can be difficult to treat, occasionally requiring amputation. Additionally, proximal diabetic neuropathy causes painful muscle wasting and weakness.

There is a link between cognitive deficit and diabetes. Compared to thosewithout diabetes, those with the disease have a 1.2 to 1.5-fold greater rate of declinein cognitive function.

Diagnosis

Diabetes mellitus is characterized by recurrent or persistent hyperglycemia,and is diagnosed by demonstrating any one of the following:

- Fasting plasma glucose level ≥ 7.0 mmol/l (126 mg/dl)
- Plasma glucose ≥ 11.1 mmol/l (200 mg/dl) two hours after a 75 g oral glucoseload as in a glucose tolerance test
- Symptoms of hyperglycemia and casual plasma glucose ≥ 11.1 mmol/l (200 mg/dl)
- Glycated hemoglobin (Hb A1C) ≥ 6.5%.

A positive result, in the absence of unequivocal hyperglycemia, should be confirmed by a repeat of any of the above methods on a different day. It is preferable to measure a fasting glucose level because of the ease of measurement and the considerable time commitment of formal glucose

tolerance testing, which takes two hours to complete and offers no prognostic advantage over the fasting test. According to the current definition, two fasting glucose measurements above 126 mg/dl (7.0 mmol/l) is considered diagnostic for diabetes mellitus.

As per the World Health Organization people with fasting glucose levels from 6.1to 6.9 mmol/l (110 to 125 mg/dl) are considered to have impaired fasting glucose. People with plasma glucose at or above 7.8 mmol/l (140 mg/dl), but not over mmol/l (200 mg/dl), two hours after a 75 g oral glucose load are considered to have impaired glucose tolerance. Of these two prediabetic states, the latter in particular is a major risk factor for progression to full-blown diabetes mellitus, as well as cardiovascular disease. Glycated hemoglobin is better than fasting glucose for determining risks of cardiovascular disease and death from any cause.

The rare disease diabetes insipidus has similar symptoms to diabetes mellitus, but without disturbances in the sugar metabolism (*insipidus* means "without taste" in Latin) and does not involve the same disease mechanisms.

Prevention and Treatment

There is no known preventive measure for type 1 diabetes. Type 2 diabetescan often be prevented by a person being a normal body weight, physical exercise, and following a healthy diet. Dietary changes known to be effective in helping to prevent diabetes include a diet rich in whole grains and fiber, and choosing good fats, such as polyunsaturated fats found in

nuts, vegetable oils, and fish. Limiting sugary beverages and eating less red meat and other sources of saturated fat can also help in the prevention of diabetes. Active smoking is also associated with an increased risk of diabetes, so smoking cessation can be an important preventive measure as well.

Diabetes mellitus is a chronic disease, for which there is no known cure exceptin very specific situations. Management concentrates on keeping blood sugar levels asclose to normal ("euglycemia") as possible, without causing hypoglycemia. This can usually be accomplished with diet, exercise and use of appropriate medications (insulin in the case of type 1 diabetes; oral medications, as well as possibly insulin, in type 2 diabetes).

Learning about the disease and actively participating in the treatment is vital for people with diabetes, since the complications of diabetes are far less common and less severe in people who have well-managed blood sugar levels. The goal of treatment is an HbA1C level of 6.5%, but should not be lower than that, and may be set higher. Attention is also paid to other health problems that may accelerate the deleterious effects of diabetes. These include smoking, elevated cholesterol levels, obesity, high blood pressure, and lack of regular exercise. Specialised footwear is widely used to reduce the risk of ulceration, or re-ulceration, in at-risk diabetic feet.

Lifestyle:

People with diabetes can benefit from education about the disease

and treatment, good nutrition to achieve a normal body weight, and sensible exercise, with the goal of keeping both short-term and long-term blood glucose levels within acceptable bounds. In addition, given the associated higher risks of cardiovascular disease, lifestyle modifications are recommended to control blood pressure.

Medications:

Metformin is generally recommended as a first line treatment for type 2 diabetes, as there is good evidence that it decreases mortality. Routine use of aspirin, however, has not been found to improve outcomes in uncomplicated diabetes. Angiotensin converting enzyme inhibitors improve outcomes in those with DM while the similar medications angiotensin receptor blockers do not.

Type 1 diabetes is typically treated with a combinations of regular and NPH insulin, or synthetic insulin analogs. When insulin is used in type 2 diabetes, a long-acting formulation is usually added initially, while continuing oral medications. Doses of insulin are then increased to effect.

In those with diabetes some recommend blood pressure levels below 120/80 mmHg; however, evidence only supports less than or equal to somewhere between 140/90 mmHg to 160/100 mmHg.

Pancreatic transplantation:

A pancreas transplant is occasionally considered for people with type 1 diabetes who have severe complications of their disease, including end

stage renal disease requiring kidney transplantation.

Diets And Exercise Treatment

Making healthy food choices is very important to help keep your blood glucose level under control. People with diabetes don't need to buy or prepare special foods. The foods that are best for someone with diabetes are excellent choices for everyone: foods that are low in fat, salt, and sugar, and high in fiber, such as beans, fruits, vegetables, and whole grains. These foods help you reach and stay at a weight that's good for your body. Regular physical activity is important for people with diabetes. Being physically active has been shown to improve blood glucose levels in older people whose levels are high. Exercise is especially good for people with diabetes because it helps control weight, helps insulin work better to lower blood glucose, is good for your heart and lungs, gives you more energy, Regular physical activity improves insulin resistance and lipid profile (reduction in triglyceride and increase in high-density lipoprotein (HDL)) and lowers blood pressure (although blood pressure will rise during exercise), the metabolic benefits in type 2 diabetes are lost within 3-10 days of stopping regular exercise, physical activity also protects against the development of type 2 diabetes.

A1C Test

The A1C test is used to detect type 2 diabetes and prediabetes but is not recommended for diagnosis of type 1 diabetes or gestational diabetes.

The A1C test is a blood test that reflects the average of a person's blood glucose levels over the past 3 months and does not show daily fluctuations. The A1C test is more convenient for patients than the traditional glucose tests because it does not require fasting and canbe performed at any time of the day. The A1C test result is reported as a percentage. The higher the percentage, the higher a person's blood glucose levels have been. A normal A1C level is below 5.7 percent. An A1C of 5.7 to 6.4 percent indicates prediabetes. People diagnosed with prediabetes may be retested in 1 year. People withan A1C below 5.7 percent maystill be at risk for diabetes, depending on the presence of other characteristics that put them at risk, also known as risk factors. People withan A1C above 6.0 percent should be considered at very high risk of developing diabetes. A level of 6.5 percent or above means a person has diabetes.

Allopathic medicines are very costly. In contrast, herbal medicines are very cheap. Also, herbal medicines do not have any side effects, as they are free from chemicals. Even though bioactive compounds with cytotoxic activities have been isolated from plants, they are exploited for various reasons.

Marine organisms, especially those that are nuisance to the environment like biofoulers can be screened for antidiabetic and antioxidant activities. Many marine sedentary organisms produce components with unique structural pattern, for their chemical defence which do not occur in terrestrial plants. Tunicates are one of the important source of new active principles for drug development.

ANTIOXIDANT ACTIVITY

Oxygen is an essential element of the aerobic organisms for the production of energy. It is the key element in the human body, capable of combining with every other element leading to the formation of essential components necessary for maintaining its regular metabolic activities. Oxygen regulates about 90% of the body function and plays a pivotal role in the respiration (Severyn, *et al*., 2009), gets absorbed by the blood stream in the lungs, transported to the cells and participates in complex processes of metabolic reactions involving enzymatic and non-enzymatic reactions with organic compounds catalyzed by ionizing radiations resulting in the formation of free radicals. Free radicals have surplus free-floating electrons rather than having harmonized pairs and therefore unstable, but are highly reactive, moves freely through blood stream and in order to attain stability attacks nearby molecules including proteins, carbohydrates and nucleic acids damaging them by stealing their electrons through a process called oxidation. Types of free radicals include the hydroxyl radical, the superoxide radical, the nitric oxide radical and the lipid peroxyl radical. External sources like air pollutants, industrial chemicals, cigarette smoke, alcohol, oxidized poly unsaturated fats and cooked food also contribute to the formation of free radicals leading to irreparable damage to the several organs, causing malfunctions.

The role of free radicals has been implicated in the development of at least 50 diseases. A few of them include arthritis, inflammatory diseases, kidney diseases, cataracts, inflammatory bowel disease, colitis, lung dysfunction,

pancreatitis, drug reactions, skin lesions and aging. Free radicals are also associated with liver damage due to alcohol consumption and the development of emphysema due to cigarette smoking.

Aging is the prime mechanism oriented with the free radical accumulation in the humans as suggested in the Free Radical Theory of Aging . A symptom of aging such as atherosclerosis is considered to be due to oxidation by free radicals. The primary site of free radical damage is the mitochondrial DNA. Damage to the mitochondrial DNA cannot be readily repaired and leads to the shutting down of mitochondria causing cell death and ageing . Bombardment of free radicals with atoms of metals like mercury, lead, cadmium and even pesticides amplifies the production of free radicals several million times resulting in mitochondrial damage. Severe mitochondrial damage in the cells leads to apoptosis occurs due to a cascade initiated by Bcl-2 proteins on the surface of mitochondria. Destruction properties of free radicals will not limit only to the process of aging but also plethora of diseases via various metabolic activities.

Accumulation of free radicals forms cataracts in the human eye. Scavenging of free radicals takes place in the eye, which gets hampered due to age-related insufficient production of antioxidant scavenging systems leading to the formation of an opaque spot on the eye lens causing blindness .

Myocytes are the source of free radical accumulation in the heart. Free radicals damage proteins and calcium pumps on the sarcoplasmic reticulum, resulting in the accumulation of calcium. High levels of calcium cause erratical

contraction of the myocytes causing arrhythmia. Spread of arrhythmia to other cells disrupts heartbeat, causing severe complications.

Free radicals produced due to external sources especially radiation leads to cancer. Most of the radiation energy is taken by the cells, which is absorbed by the water causing one of its oxygen-hydrogen covalent bonds to split and forms free radical. This free radical reacts with another molecule in microseconds of its generation attacks and injures the macro molecules of the cell such as DNA, disrupting its strands and causing mutations in its bases. However, free radicals that are produced during combustion may last little longer in the lungs binding to other air pollutants leading to lung cancer.

Role of antioxidants in promoting Human Health: Antioxidants are the molecules, capable of limiting the macro molecule oxidation of free radicals by terminating the chain reactions, which are the main source of free radical formation in the cell. The critical role of antioxidants in ameliorating the free radicals have been elaborately studied, still it is not clear whether the production of free radicals is the consequence or the cause of a disease. Broadly antioxidants are classified into two types namely enzymatic and non-enzymatic. The non-enzymatic antioxidants are again classified into hydrophilic and hydrophobic. Hydrophilic antioxidants can dissolve into blood and cytosol and react with free radicals. Hydrophobic antioxidants protect the cell membrane from lipid peroxidation, a mechanism by which free radicals degrade the membrane lipids.

The role of antioxidants in scavenging the deleterious effects of free

radicals is complex, which depend on the interactions of various metabolites and enzyme systems having synergistic and interdependent effects on one another. The performance level of antioxidants also depends on the concentration, reactive potentiality with the specific free radical, interaction and function with other antioxidant family members.

Antioxidants may be defined as any substance that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate in a chain reaction. Humans have evolved a highly complicated antioxidant protection system, which involves a variety of endogenous and exogenous compounds that are able to function interactively and synergistically to neutralize free radicals. These include antioxidant enzymes that catalyze free radical quenching reactions, metal binding proteins that sequester free iron and copper ions that are capable of catalyzing oxidative reactions, diet-derived antioxidants, and other low molecular weight compounds such as α-lipoic acid.

Antioxidants have become a popular research topic because they cannot be generated by the human body and hence have to be consumed in the diet. Many fruit and vegetables have been found to be rich sources of antioxidants. Since a large portion of the human diet is based on fruit and vegetables, it is important to understand the biological and biochemical interactions between these dietary antioxidants and living systems. A major benefit from diets rich in fruit and vegetables may be increased consumption of antioxidant vitamins such as

ascorbate (vitamin C) and tocopherol (vitamin E), vitamin like compound-glutathione and pigments such as phenolics-flavonoids and anthocyanin and carotenoids. These compounds along with dissolved sugars, acids, salts, amino acids and some water-soluble pigments are situated in large central vacuoles of parenchyma cells, the main structural unit of edible portion of most fruit and vegetables. They act as major cellular redox buffers that can effectively quench reactive oxygen species (ROS) by donating one or more electrons to ROS. Natural phytochemicals act synergistically to increase their antioxidant capacity such that the total antioxidant effect is greater than the sum of the individual antioxidant activities, and the isolation of one compound will not exactly reflect the overall reaction. Plants have a similar nonenzymatic ROS scavenging system including the ascorbate-glutathione cycle in chloroplasts, glutathione peroxidise cycle in peroxisomes, as well as tocopherol, flavonoids, alkaloids, and carotenoids. Free radicals: Chemically, a free radical is any atom such as oxygen or nitrogen with at least one unpaired electron present and is able to exist independently. Free radicals can easily be formed in three ways:

- by the homolytic cleavage of a covalent bond, generally incurring by high energy input;
- by the loss of a single electron from a normal molecule;
- by addition of a single electron to a normal molecule

These free radicals that are highly reactive molecules can be extremely damaging to the lipids, proteins and cellular DNA, which may lead to many

biological complications, including carcinogenesis, mutagenesis, aging, and atherosclerosis. The oxygen-derived free radical is an important group formed during metabolism. One of these reactions found in biological pathways is the respiratory burst process, which result in free radical products termed ROS. Examples of ROS include superoxide, hydroxyl radicals, and non-oxygen free radical hypo chlorites. Investigations have suggested that ROS are involved in mediating of certain types of inflammatory tissue injury and the most likely sources of these oxidizing agents are produced via phagocytic leukocytes. Activation of phagocytes via interaction of certain pro-inflammatory mediators or bacterial components with specific membrane receptors of leucocytes triggers the assembly of the multicomponent flavoprotein NADPH oxidase which catalyzes the production of superoxide anion radicals. Superoxide will rapidly and spontaneously/enzymatically dismutase to produce hydrogen peroxide and other free radicals. Besides being produced from superoxide, hydrogen peroxide can also be generated by other oxidase enzymes, such as glycollate and monoamine oxidase, or by the peroxisomal pathway that is for β-oxidation of fatty acids. The production of hydrogen peroxide inhuman plasma was found to have involvement of an enzyme activity named xanthine oxidase. The level of xanthine oxidase has been found to increase as a result of tissue injury.

Sources of free radicals: Free radicals emanate from the environment, from other free radicals in chain reactions and from many normal biological processes *in-vivo*. Free radical reactions are initiated continuously in cells and tissues in the

body from both enzymatic and non-enzymatic reactions. Enzymatic reactions serving as sources of free radical reactions include those involved in phagocytosis, prostaglandin biosynthesis and in the Cytochrome P450 system. Free radicals also arise in the non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing radiation.

Sources of free radicals from the environment include tobacco smoke, ozone derived from air pollution, automobile exhaust emissions, excessive radiation, pesticides, deep fried foods, hydrogenated oils and toxic metals which we inhale or digest. The destructive free radical nitrogen dioxide for example, which is the result of a reaction between nitric oxide and oxygen, is formed in cigarette smoke and vehicle exhaust and has been implicated in respiratory illnesses and irreversible lung damage.

Cancer is the uncontrolled virtually autonomous growth of abnormal cells that can arise in any organ or tissues of the body. A transformed neoplastic or cancerous cell is simply a once-normal cell which continues to grow and multiply without limitation. This maybe due to a multiplicity of endogenous and exogenous factors, including oxidative stress, which initiate a change in the cell's DNA, resulting in a tumour. Only transformed cells that escape detection by the immune system have the opportunity to become tumours. The immune system usually isolates and destroys the abnormal cells before they proliferate enough to be noticeable as a tumour. Free radicals can compromise immune cell function reducing immune responses which can allow the abnormal cells to continue

growing. The question of why the risk of cancer increases with age is an interesting one. Harman, (1993) suggests that this increase is probably due in part to the increased level of endogenous free radical reactions with advancing age and inadequate antioxidant defences resulting in an increased rate of mutations in proto-oncogenes (involved in normal cell growth and development) and tumour-suppressing genes coupled with the progressive diminishing capacity of the immune system to eliminate transformed cells.

The role of ROS in carcinogenesis

- Carcinogenesis is a complex process with the *in vivo* generation of ROS leading to oxidative DNA damage being a significant contributory factor. critical factor in mutagenesis is cell division. Cancer is rare in non structural alterations in DNA such as gene sequence amplification;
- translocations and base pair mutations the oxidised form of guanine has altered base-pairing properties;
- activation or inhibition of signal transduction pathways - over expression of a growth factor receptor is commonly involved in the majority of squamous cell carcinomas of the lung;
- abnormal cell-to-cell communication that allows unrestricted cell proliferation;
- interference with genes that modulate cell growth preventing programmed cell death by apoptosis or necrosis;

- damage to proteins such as DNA repair enzymes compromising repair of a mutation once it has occurred.

The immune system keeps an ever-present vigil to protect us from invading organisms and remove damaged, aged or altered cells which have the potential to cause cancer. White blood cell membranes, like all cell membranes, are composed of lipids, which are highly susceptible to free radical attack. Numerous links have now been established between free radical reactions and altered immune cell function.

ROS can decrease the membrane fluidity of white blood cells, significantly reducing their function. Loss of membrane fluidity has been directly related to the decreased ability of lymphocytes to respond to challenges of the immune system. Free radicals can also damage the DNA of immune cells resulting in mutations and reduced cell function. Ironically, free radical damage forms the basis of some chemotherapy drugs and radiation used in cancer treatment. Well-documented side effects like hair loss, reduced immunity and gastro-intestinal disturbances result from the barrage of free radicals that indiscriminately destroy healthy cells as well as malignant ones.

Antioxidants and cancer: Epidemiological data provides strong evidence of a cancer prophylactic effect of high intakes of vegetables, fruits and whole grains containing high levels of antioxidant micronutrients and phytochemicals. Some naturally-occurring phytochemicals such as phenolic/polyphenolic compounds, from green tea, curcumin from turmeric, genestein from soy and red

clover and silymarin from milk thistle, may reduce cancer risk according to initial trials.

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. 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 defense, like sessile organisms, are believed to have evolved chemical defense 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 screened for antioxidant activity.

Many marine sedentary organisms produce components with unique structural pattern, for their chemical defense which do not occur in terrestrial plants. Sponges, bryozoans and tunicates are important source of new active principles for drug development. The marine environment is an excellent source of novel chemicals, not found in terrestrial sources. Marine organisms such as ascidians, sponges and soft corals containing symbiotic microorganisms are a rich source of bioactive compounds. The compounds derived from marine organisms have antioxidant properties and anti-cancer activities, but they are largely unexplored.

Since the few last decades, marine environment have been recognized to be a rich source of bioactive metabolites with varied biological and pharmacological activities. Bioactive peptides with novel structures have also been shown in ascidians. Sac-like sea squirts inhabiting the sea floor produce complex anti-tumor compound which is hundreds to studies on antioxidant property of ascidians especially in *Phallusia nigra* and *Eudistoma viride* are lacking. As ascidians are available along the Tuticorin coast an attempt has been made to assess their *in vitro* antidiabetic and antioxidant activity.

OBJECTIVES

The objectives of the present study are to

- ✓ collect and identify the two species of ascidians
- ✓ prepare crude extract by Soxhlet extraction
- ✓ assess the *In-vitro* antidiabetic activity of *Phallusia nigra* by α-amylase inhibition assay
- ✓ evaluate the *In-vitro* antidiabetic activity of *Eudistoma viride* by α-amylase inhibition assay
- ✓ determine the antioxidant activity of *Phallusia nigra* by superoxide scavenging assay
- ✓ study the antioxidant activity of *Eudistoma viride* by superoxide scavenging assay
- ✓ compare antidiabetic activity of *Phallusia nigra* and *Eudistoma viride*
- ✓ compare antioxidant activity of *Phallusia nigra* and *Eudistoma viride*

Antidiabetic Activity

Kumar *et al*., 2008 stated that the compound 'Mycaminose', isolated from *Syzygium cumini* possess anti-diabetic effects against Streptozotocin induced diabetic rats. Udayakumar, *et al*., 2009 studied the *Withania somanifera* root and leaf possess antidiabetic and antihyperlipidemic activities in alloxan-induced diabetic rats. Meenakshi *et al.,* (2012) analysed a dose dependent antidiabetic effect with 200 mg/kg bodyweight possessing significant activity without any toxic effect on liver and kidney.

Tziveleka *et al.,* (2021) analysed the antioxidant potential and biogenetic origin of 301 macroalgal metabolites, categorized according to their chemical classes, highlighting the mechanisms of antioxidative action. Marhamati *et al.,* (2021) reported that tunichrome of *Phallusia nigra* has excellent biological effects as a bioactive compound for food fortification. Biswal *et al*., (2021) reported study mainly focusses on the current research of different source of antioxidants in edible cooking oil. Rani *et al*., (2021) analysed the present review highlighted the potential of various microorganism to produce antioxidants and their importance as innovative sources of natural bioactive molecules.

Putra *et al.,* (2020) evaluated the active compounds from the extract of the *Fusarium* showed antibacterial and antioxidant activities. Sankaravadivu *et al.,* (2000) analysed the reducing power of the extracts increase with increase in

concentration. Roselin *et al.,* (2018) reported that the extracts of a colonial ascidian *Didemnum psammatodes* possess strong antioxidant activity when compared with standard ascorbic acid. Elya *et al.,* (2018) reported the methanol extract of the ascidian *Didemnum* sp. exhibited antioxidant activity. Kumaran and Bragadeeswaran (2017) evaluated ascidian represent a promising biological resource for derivation of new compounds with antioxidant potential. Priya *et al.,* (2016) evaluated the ethanolic extract of *Eudistoma viride* a promising antioxidant potential against free radical induced oxidative damage. Ivette Palma *et al*., (2016) analysed the antioxidant activity of aqueous and methanol extracts of Pleurotus ostreatus in different growth stages.

Packiam *et al.,* (2015) reported that commonly available ascidian has active biochemical potential possessing antioxidant, anti-inflammatory and antimicrobial properties for curing various ailments. Balakrishnan *et al*., (2014) reported associated bacteria had good antioxidant activity which needs extensive attention in terms of drug discovery. Saeed *et al*., (2012) analysed that *Torilis leptophylla* act as an antioxidant agent due to its free radical scavenging and cytoprotective activity. *Jiang Ai-li* and *Chang-hai* (2006) reported the components extracted from *Salvia plebeia* should preserve the quality of ascidian oil from ascidian oxidative deterioration. Krishnaiah *et al*., (2004) analysed the antioxidant properties of lamellarin γ, lamellarin γ-monoacetate, lamellarins K, U, and I, and lamellarin C-diacetate.

Palakkal and Ganesan 2005 tested antioxidant activity of the extract of

Macaranga peltata and the correlation between the total phenolic content and antioxidant activity. Kumaran *et al*., 2007 observed a statistical correlation between the antioxidant properties and phenolic contents of methanolic extracts of stem barks, root bark, leaves and fruits from *Morus alba*. According to Bhaskar and Balakrishnan, 2009 the extracts of the roots of *Carissa carandas* and *Pergularia daemia* possess antioxidant properties and could serve as free radical inhibitors or scavengers.

Mahdi-Pour *et al*., 2012 observed the antioxidant activity of methanolic extracts of various parts of *Lantana camara*. Nahid *et al*., 2017, evaluated the antioxidant, antimicrobial and phytochemical constituents of the methanol extract of *Artemisia indica*. The powerful antioxidant activity is attributed to the greater amount of total phenol and flavonoid compound in the ethanolic leaf extract of *Memecylon umbellatum* as stated by Anbukkarasi *et al.,* 2017.

Trad *et al*., 2018 observed the butanolic extract of *Ephedra alte* had high phenolic contents and exhibited high antioxidant activity both *in-vitro* and *in-vivo*. Keerthana and Visweswaran 2018 observed that the drug Seeraga choornam has promising therapeutic antioxidant activity when compared with the standard drug. This research work can help for medical practitioners to use this poly herbal compound for the treatment of cancer.

Sharma *et al.,* 2019 determined *in-vitro* antiradical activity and *in-vivo* metabolism of polyphenol. Tekin and Küçükbay, 2020 the extracts of flowers of *Punica granatum* L. had high content of flavonoids and other phenolics with

antioxidant activity. Arshan *et al*., 2020 observed the phytochemical analysis of extracts *Annona squamosa* linn leaves had glycosides, saponins, tannins, flavonoids, phenols, etc. In-vitro antioxidant activities clearly suggest that methanol extract has higher antioxidant activity than the other extract due to a higher presence of phenolic and flavonodal constituents in the methanol extract.

Zhang *et al*., 2010 observed antioxidant activity of five polysaccharides extracted from five algae including one brown alga *Laminaria japonica*, one red alga *Porphyra haitanensis* and three green algae *Ulva pertusa*, *Enteromorpha linza* and *Bryopsis plumose.* Revathi *et al*., 2015 observed the maximum antioxidant activity in the methanol extract of *Hypnea valentiae* and antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. Priya *et al*., 2016 stated that the ethanolic extract of colonial ascidian, *Eudistoma viride* by DPPH method reveals a promising antioxidant potential against free radical induced oxidative damage.

Chirag *et al*., (2013) reported, dietary plants contain variable chemical families and amounts of antioxidants. It has been hypothesized that plant antioxidants may contribute to the beneficial health effects of dietary plants. Komes *et al*., (2011) observed the extraction efficiency of phenolics, as well as the antioxidant capacity of plant extracts, was affected by both prolonged extraction and hydrolysis. Skrovankova *et al*., (2012) reported medicinal plants are traditionally used in folk medicine as natural healing remedies with therapeutic effects such as prevention of cardiovascular diseases, inflammation

disorders, or reducing the risk of cancer. Scartezzini and Speroni (2000) was evaluated a lot of medicinal plants, traditionally used for thousands of years possess antioxidant activities. Michalaka *et al*., (2022) evaluated, highlights the beneficial applications of sea-weeds and their extracted compounds, which have antioxidant properties as feed additivesand impact animal health and production.

Collection of Animal Material

Samples of simple ascidian *Phallusia nigra* Savigny, 1816 were collected from the under surface of the barges of Tuticorin harbor by SCUBA diving. *Eudistoma viride* Tokioka, 1955 were collected during the low tide from the intertidal rocky area of Hare island.

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.

Systematic Position

Phallusia nigra belongs to

Phylum	:	Chordata
Subphylum	:	Urochordata
Class	:	Ascidiacea
Order	:	Enterogona
Suborder	:	Phlebobranchia
Family	:	Ascidiidae
Genus	:	*Phallusia*
Species	:	*Nigra*

Eudistoma viride belongs to

Phylum	:	Chordata
Subphylum	:	Urochordata
Class	:	Ascidiacea
Order	:	Enterogona
Family	:	Polycitoridae
Genus	:	*Eudistoma*
Species	:	*Viride*

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* (Plate – 1) 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.

Plate – 2 depicts *Eudistoma viride.* It is characterized due to the three rows of pharyngeal slits, long esophagus, flat stomach in the posterior region of the abdomen, very conspicuous longitudinal muscles extending from the pharynx to the end of the abdomen and larvae that are incubated in the atrial cavity.

Preparation of Powder

The specimens were dried under shade. The dried animals were homogenized to get a coarse powder. The dried powders of the tunicates - *Phallusia nigra* and *Eudistoma viride* were used.

Preparation of Extract

Soxhlet extraction is a method used for the extraction of valuable bioactive compounds from various natural sources. It is used to extract the compound from a solid mixture. It is a simple and convenient method for

infinitely repeated cycle of extraction with a fresh solvent until complete exhaustion of the solute in the raw material. During extraction with soxhlet, the process of distillation is implicated. It consists of heating a solution up to boiling and then condensed send back to the original flask. 50 g of the *Phallusia nigra* powder was introduced in a thimble. This thimble is then deposited in a distillation flask filled with ethanol solvent. After reaching to a submersion level, a siphon absorbs the solvent in the thimble-holder and then release it back into the distillation flask. This solution contains the extracted solutes. This process is done continuously until the extraction is completed. (Azmir, 2013). The separation of the extract from the solvent is made by rotary evaporator. A rotary evaporator is an equipment used to remove solvent from a sample through 'evaporation under reduced pressure'. The reduced pressure in the apparatus causes the solvent to boil at a lower temperature than normal. Rotating the round bottom flask increases the liquid's surface area and thus the rate of evaporation. The solvent vapour travels into the cooler water condenser, where it condenses and drips into a separate receiving flask leaving a concentrated compound in the original round bottom flask. After the complete evaporation of ethanol, the crude extract was used for carrying out the experiment. The same procedure was followed for getting the crude extract of ethanolic extract of *Eudistoma viride* also. The excess solvent was evaporated and the dried extracts were kept in refrigerator at 4ºC for the future use.

α-amylase inhibition activity

The assay was carried out following the standard protocol with slight modifications (Hansawasdi, 2000). Starch azure (2 mg) was suspended in 0.2 mL of 0.5M Tris–HCl buffer (pH 6.9) containing 0.01 M $CaCl_2$ (substrate solution). The tubes containing substrate solution were boiled for 5 min and then preincubated at 37°C for 5 min. Ethanol extract of *Phallusia nigra* was dissolved in DMSO in order to obtain concentrations of 1000, 800, 600, 400, and 200 µg/mL. Then, 0.2 mL of ascidian extract of particular concentration was added to the tube containing the substrate solution. In addition, 0.1 mL of porcine pancreatic amylase in Tris–HCl buffer (2 units/mL) was added to the tube containing the ascidian extract and substrate solution. The reaction was carried out at 37°C for 10 min. The reaction was stopped by adding 0.5 mL of 50% acetic acid in each tube. The reaction mixture was centrifuged at 3000 rpm for 5 min at 4°C. The absorbance of resulting supernatant was measured at 595 nm using spectrophotometer (Perkin Elmer Lambda 25 UV–VIS spectrophotometer). Same procedure was followed for ethanolic extract of *Eudistoma viride* to test its α-amylase inhibitory effects. Acarbose, a known α-amylase inhibitor was used as a standard drug. The experiments were repeated thrice. The α-amylase inhibitory activity was calculated by using following formula:

$$\% \text{ inhibition} = \frac{Absorbance\ of\ Control - Absorbance\ of\ Sample}{Absorbance\ of\ Control} X\ 100$$

Superoxide radical scavenging

This activity was measured by the reduction of nitro blue tetrazolium according to a previously reported method (Fontana, 2001). The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce (NBT) to a purple formazan. One ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 μM), NBT (50 μM), PMS (15 μM) and various concentrations (0–20 μg/ml) of sample solution. After incubation for 5 min at ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated.

The percentage inhibition was calculated by using following formula:

$$\% \text{ inhibition} = \frac{Absorbance\ of\ Control - Absorbance\ of\ Sample}{Absorbance\ of\ Control} X\ 100$$

Antidiabetic activity:

In the present investigation, ethanolic extract of *Phallusia nigra* and *Eudistoma viride* were tested for antidiabetic activity. Table: 1 and Figure: 1 depict the antidiabetic activity of *Phallusia nigra*. The percentage of inhibition observed was 54.73, 52.63, 42.11, 13.16 and 5.26 at 1000, 88, 600, 400 and 200µg/ml concentration of ethanolic extract of *Phallusia nigra* respectively. The percentage of inhibition increased gradually from lowest to highest concentration ethanolic extract of *Phallusia nigra*.

Table: 2 and Figure: 2 depict the antidiabetic activity of *Eudistoma viride*. The percentage of inhibition observed was 48.95, 47.37, 34.22, 10.53 and 1.50 at 1000, 88, 600, 400 and 200µg/ml concentration of ethanolic extract of *Eudistoma viride* respectively. The ethanolic extract of *Eudistoma viride* exhibited progressive increase in percentage of inhibition with concentration. Antidiabetic activity of *Eudistoma viride* was higher than *Phallusia nigra* at the same concentration.

Antioxidant Activity:

In the present investigation, ethanolic extract of *Phallusia nigra* and *Eudistoma viride* were tested for antioxidant activity. Table: 3 and 4 and Figure: 3 and 4 depicts the antioxidant activity of *Phallusia nigra* and *Eudistoma viride.*

Percentage of inhibition observed was 37.50, 48.50, 53.0, 65.0 and 68.75

at 200, 400, 600, 800 and 1000 µg/ml concentration of ethanol extract of *Phallusia nigra* respectively. In control % of inhibition was not observed. In *Eudistoma viride*, the maximum % of inhibition was observed at higher concentration. Percentage of inhibition observed was 43.75, 54.68, 62.56, 71.88 and 75 at 200, 400, 600, 800 and 1000 µg/ml concentration of ethanol extract of *Eudistoma viride* respectively. No % of inhibition was not observed in control. Antioxidant activity of *Eudistoma viride* was higher than *Phallusia nigra* at the same concentration.

Table 1: Antidiabetic activity of ethanolic extract of *Phallusia nigra*

Concentration µg/ml	Absorbance at 510 nm	% Inhibition
Control	1.900	--
200	1.800	5.26
400	1.650	13.16
600	1.100	42.11
800	0.900	52.63
1000	0.860	54.73

Table 2: Antidiabetic activity of ethanolic extract of *Eudistoma viride*

Concentration (µg/ml)	Absorbance at 510 nm	% Inhibition
Control	1.900	--
200	1.870	1.50
400	1.700	10.53
600	1.250	34.22
800	1.000	47.37
1000	0.970	48.95

Table 3: Antioxidant activity of ethanolic extract of *Phallusia nigra*

Concentration (µg/ml)	Absorbance at 560 nm	% Inhibition
Control	1.600	-
200	1.000	37.50
400	0.824	48.50
600	0.752	53
800	0.560	65
1000	0.500	68.75

Table 4: Antioxidant activity of ethanolic extract of *Eudistoma viride*

Concentration (µg/ml)	Absorbance at 560 nm	% Inhibition
Control	1.600	-
200	0.900	43.75
400	0.725	54.68
600	0.599	62.56
800	0.450	71.88
1000	0.400	75

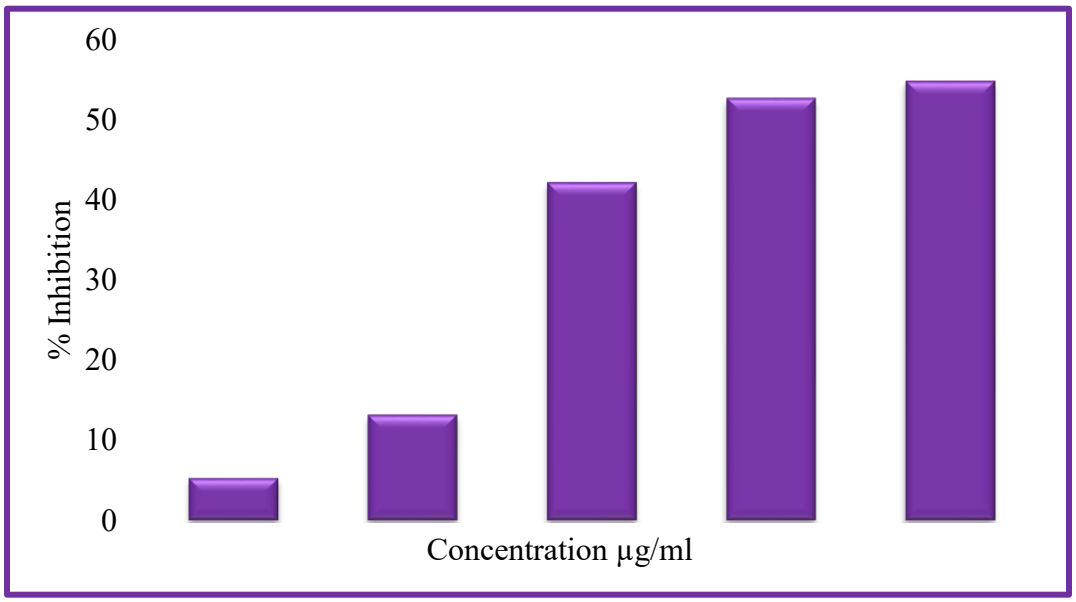

Figure -1: Antidiabetic activity of ethanolic extract of *Phallusia nigra*

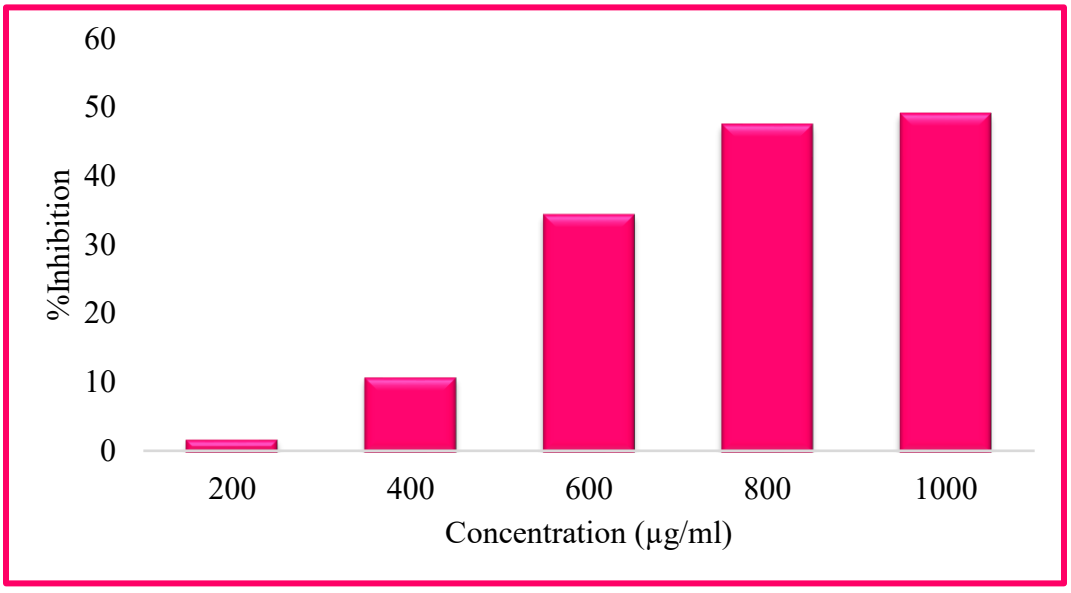

Figure -2: Antidiabetic activity of ethanolic extract of *Eudistoma viride*

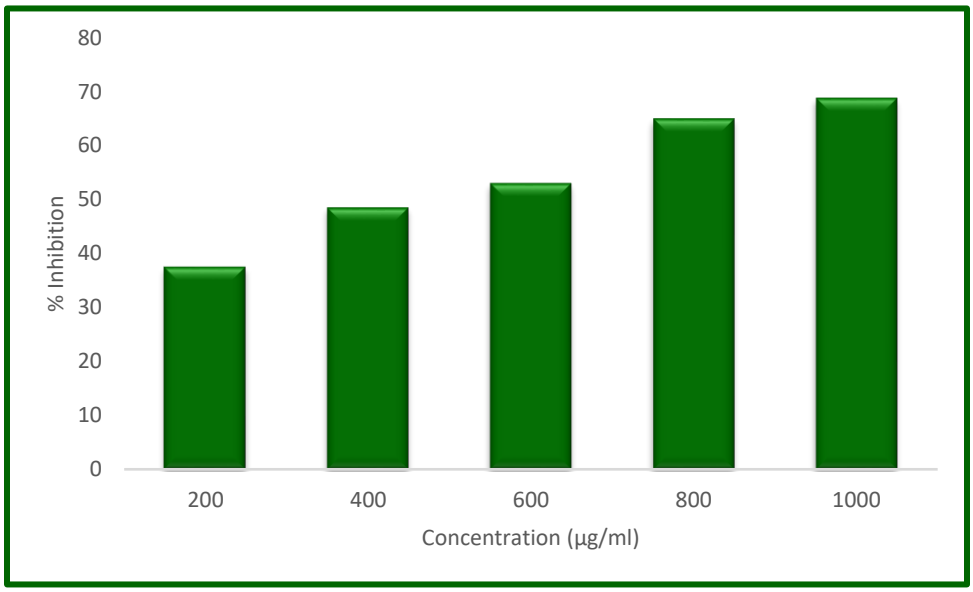

Figure -3: Antioxidant activity of ethanolic extract of *Phallusia nigra*

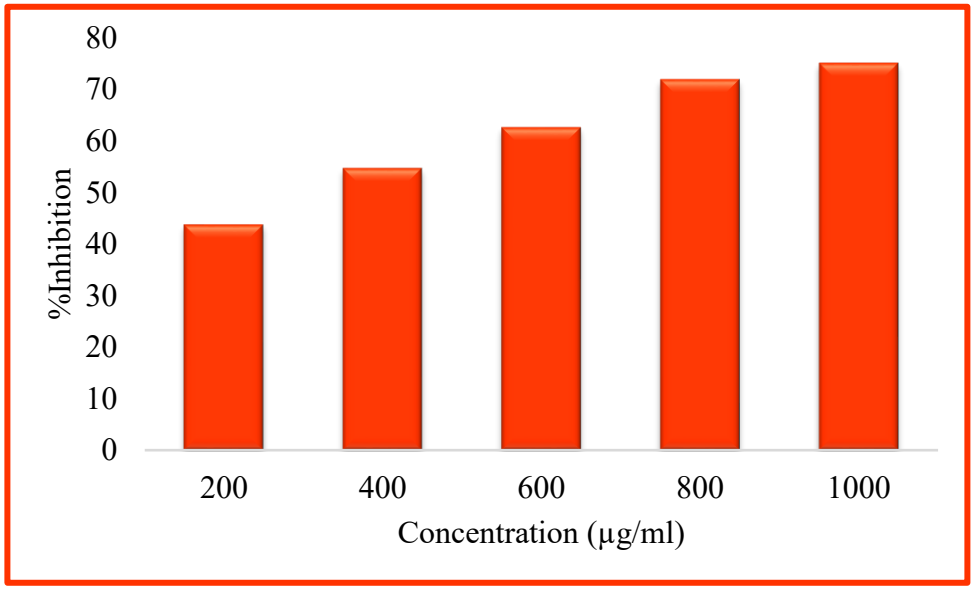

Figure -4: Antioxidant activity of ethanolic extract of *Eudistoma viride*

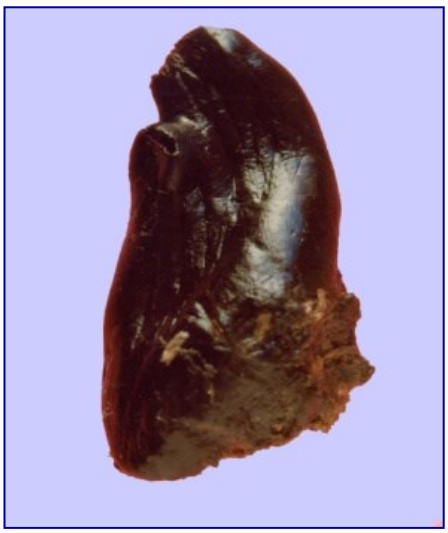

Plate: 1 ***Phallusia nigra***

Plate: 2 ***Eudistoma viride***

DISCUSSION

The principle of *In-vitro* α- amylase inhibition assay, enzyme that plays a role in digestion of starch and glycogen are considered a strategy for the treatment of disorders in carbohydrate uptake, such as diabetes. 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. As per the above mechanism, the extract showed concentration dependent affinity towards the inhibition of α-amylase. The ethanolic extract of *Phallusia nigra* was observed as more active extract. Myricetin, a flavonoid compound, was documented to have antioxidant and antidiabetic activities. Myricetin facilitated the metabolic action of insulin by stimulating phosphatidylinositol 3-kinase (PI3K) and its effectors (Saltiel, 2001).

Ethanolic extract of *Phallusia nigra* and *Eudistoma viride* were tested for antidiabetic activity. In *Phallusia nigra*, the maximum percentage of inhibition was observed as 54.73% in higher concentration (1000µg/ml). Maximum percentage of inhibition observed was 48.95 in higher concentration (1000µg/ml) of ethanolic extract of *Eudistoma viride*. The antidiabetic activity of *Myrica gale* and rose root extract may be due to the presence the polyphenols (Lood and Rupasinghe 2019). The antidiabetic activity possessd by aloe vera may be due to the presence of Alkaloids, anthraquinones, and enthrones. (Fatemeh Haghani *et al* 2022). The antidiabetic activity possessed by many plants, fruits, vegetables

and leaves may be due to flavonoids, Leucoanthocyanidins, terpenoids and sterols. (Asad Ullah *et al* 2020). Antidiabetic activity of medicinal plants may be due to Quercetin, oleanolic acid, kaempferol, ursolic acid, rutin, β-sitosterol, and mangiferin. The antidiabetic activity of marine brown algae may be due to Phlorotannins (Thilina *et al* 2020). The presence of antidiabetic activity possessed by *Musa* may be due to presence of flavonoids, tannins, phlobatannins, alkaloids, glycosides and terpenoids (Tong Li *et al* 2005). The leaves of *A. remota* extracts had flavonoids, tannins, saponins, phenolic compounds and steroids, where some are considered as bioactive constituents in the management of diabetes (Tafesse *et al.,* 2017). Phenolics and flavonoids found in *E. hirta* such as quercetin, quercitrin, and rutin have been proved to be effective inhibitors of mammalian α-amylase (Ngan Tran et al 2020).

The high content of astaxanthin in *H. roretzi* indicates its potential roles and functions in anti-diabetes activity. (Zhu *et al* 2022). Flavonoids are present in *Phallusia nigra* (Priya *et.al* 2019). The antidiabetic activity of *Phallusia nigra* may be due to presence of flavonoids.

The ethanolic extract of *Phallusia nigra* and *Eudistoma viride* were tested for antioxidant activity. The ethanolic extract of *Phallusia nigra* and *Eudistoma viride* exhibited 68.75 and 75 % of inhibition at their highest concentrations respectively. Antioxidants in different parts of plants such as ascorbic acid, vitamin E and phenolic compounds possess the ability to reduce the oxidative damage associated with many diseases including cancer, cardiovascular diseases,

cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and ageing (Bharti *et al*., 2012). The antioxidant activity possessed by ascidian Didemnum obscurum may be due to pressence of lamellarin alkaloids, lamellarins γ , α , and ε , lamellarins M , K , K-diacetate , K-triacetate , U , I , C-diacetate , and X-triacetate (Krishnaiah *et al*., 2004).The antioxidant activity possessed by ascidian *Didemnum* sp may be due to presence of alkaloids, steroids, saponins and glycosides (Berna Elya et al., 2018). The antioxidant activity possessed by ascidian *Eudistoma viride* may be due to presence of any of these chemical constituent such as flavonoids and phenolic (Priya *et al*., 2018). The antioxidant activity possessed by ascidian *Didemnum psammathodes* may be due to presence of flavonoids and phenolic (Roselin *et al*., 2018). The significant antioxidant activity of the *Eudistoma viride* extracts may be due to the presence of flavonoids and phenolic compounds. The antioxidant activity of *Eudistoma* sp may be due to presence of alkaloids, triterpenoids, tannins, flavonoids and phenolics.

CONCLUSION

The *invitro* antidiabetic study have been performed based on the α-amylase inhibition assay. Our study demonstrates that the ethanolic extract of *Phallusia nigra* the highest percentage (54.73) reduction in blood glucose levels and the ability of *the* extracts in reducing blood glucose levels presumably due to the presence of antioxidant constituents such as flavonoids. The ethanolic extract of *Phallusia nigra* and *Eudistoma viride* possess substantial antioxidant activities. The antioxidant potential of the extract may be attributed to its phenolic content as well as the presence of the flavonoids, Thus, the free radical scavenging ability of *Phallusia nigra* and *Eudistoma viride* could provide health benefits to humans by protection against oxidative stress.

- Antidiabetic and antioxidant activity can be determined using other assays also.
- A further study on isolation, purification, structure determination and subsequent recognition of the novel mechanism of action of the clinically effective agent is suggested.
- Chemotherapeutic and spectroscopic techniques can lead to the development of new drugs.
- As the extract of *Phallusia nigra* and *Eudistoma viride* showed antidiabetic and antioxidant activity, other species of ascidians can also be tried.

* Diabetes Mellitus is a chronic metabolic disorder, in which there are high blood sugar levels over a prolonged period. This high blood sugar produces the symptoms of frequent urination, increased thirst and increased hunger. Untreated, diabetes can cause manycomplications.
* Antioxidants are the molecules, capable of limiting the macro molecule oxidation of free radicals by terminating the chain reactions, which are the main source of free radical formation in the cell. The role of free radicals has been implicated in the development of at least 50 diseases. A few of them include arthritis, inflammatory diseases, kidney diseases, cataracts, inflammatory bowel disease, colitis, lung dysfunction pancreatitis drug reactions, skin lesions and aging.
* The marine environment is an excellent source of novel chemicals, not found in terrestrial sources. In the present study, *in vitro* antidiabetic and antioxidant activity of ethanolic extract of *Phallusia nigra* and *Eudistoma viride* was assessed. Samples of ascidians were collected and identification up to the species level was carried out based on the key to identification of Indian ascidians by Meenakshi, 1997.
* The specimen was dried under shade and was homogenized to get a coarse powder. Soxhlet extraction is a method used for the extraction of valuable bioactive compounds from various natural sources. Antidiabetic activity

was assessed by alpha amylase inhibition assay. The study of free radical in the antioxidant component is assayed by Superoxide radical scavenging activity (Blois, 1958). Percentage of inhibition was calculated.

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BIOGENIC SYNTHESIS, CHARACTERIZATION AND APPLICATIONS OF CALCIUM NANOPARTICLES FROM *HARPULINA LAPPONICA* (Linnaeus, 1767)

A project submitted to

ST. MARY'S COLLEGE (Autonomous), THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

Bachelor of Science in Zoology

by

M. MANIMALA	20AUZO21
C. MASHA	20AUZO25
J. THANGA ROSELIN	20AUZO46

DEPARTMENT OF ZOOLOGY

ST. MARY'S COLLEGE (Autonomous)

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

THOOTHUKUDI-628001

April - 2023

CERTIFICATE

This is to certify that the project entitled **Biogenic Synthesis, Characterization and Applications of Calcium Nanoparticles from *Harpulina lapponica* (Linnaeus, 1767)** is submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfillment for the award of the degree of **Bachelor of Science in Zoology** and it is a record of the work done during the year 2022-2023 by the following students.

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CONTENTS

1. INTRODUCTION

More than 70% of the surface of the globe is covered by the ocean, which also contains more than 30,000 different species of flora and animals and 34 of the 36 known phyla. Over 80% of all plant and animal species in the world are known to exist in the maritime environment. Many bioactive substances have recently been isolated from a variety of marine plants, animals, and microorganisms. Due to the extensive exploration and exploitation of land resources, researchers increasingly turn to the ocean (Bhimba *et al*., 2010).

For approximately three decades, marine creatures have been a rich source of novel chemical compounds that have accelerated the development of marine natural product chemistry. The animal kingdom's most diversified group, the mollusca, has the second-highest number of species (after arthropods), but a significantly wider range of body types. The seven living classes of molluscs range from bivalves (oysters, mussels, etc.) which are typically immobile and have a relatively small central nervous system, to the largest and most sophisticated vertebrates, the swiftly moving squids and octopuses that hunt. About 540 million years ago, during the early to middle Cambrian period, the phylum first evolved. Because most molluscs have tough calcareous shells, they are widespread. There are as many fossil species known as extant ones. Most molluscs are marine, but two groups, the gastropoda and bivalvia make it into

fresh water, and the gastropods are important on land, as snails and slugs (Brooks, 2013).

Molluscs are characterized by the presence of a calcareous shell (in various forms) and sheet of tissue called a mantle, which secretes the shell and encloses a mantle cavity with gills or lungs, division of the body into a ventral muscular foot used for locomotion and a visceral mass protected by the shell, a special toothed structure called a radula used in feeding. This is a pretty clear set of features, and one could imagine an ancestor of all classes looking a bit like a limpet, living on the sea floor and grazing algae. All of these feature are either lost or very substantially changed in one or more of the classes (Dunstan and Hodgson, 2014).

Nano is a metric measure of one billionth of a meter and covers a width of 10 atoms. In terms of comparison with real objects, an example that hair is 150,000 nanometers may be given. The rapidly developing nanotechnology is the inter-disciplinary research and development field of biology, chemistry, physics, food, medicine, electronics, aerospace, medicine, etc., which examines the design, manufacture, assembly, characterization of materials that are smaller than 100 nanometers in scale, as well as the application of miniature functional systems derived from these materials. It represents the whole of development activities. As for the nano biotechnology, on the other hand, it is

the result of a combination of biotechnology and nanotechnology branches with a common combined functioning. (Guzman *et al*., 2012).

Nanotechnology has begun to break free from the constraints of the laboratory and conquer new applications to transform our way of life. In comparison to massive bulk materials, these nanoparticles' enhanced surfaces are gives them various chemical, optical, mechanical, and magnetic capabilities. Due to incredible properties nanoparticles have become significant in many fields in the recent years, such as energy, health care, environment, agriculture, etc. The preparation of nanoparticles are carried out either by (i) nanoparticles synthesis or by (ii) processing of nanomaterials into nanostructure particles. The increased surfaces of these nanoparticles are responsible for their different chemical, optical, mechanical, megnatic properties as compared to conforms of laboratory and conquering the new application to change our lives (Murphy *et al*., 2010).

Nanotechnology is crucial because it has a head start on understanding, using, and controlling matter at magnitudes of a minute scale, similar to nearing atomic levels, to create new materials, tools, and structures. In contemporary nanotechnology, the synthesis of nanocrystals is in the spotlight. In numerous ways, including imaging, sensing, targeted drug administration, gene delivery

systems, and artificial implants, the biosynthesis of nanoparticles by plant extract is now used as a tool to explore the most uncharted territory in the medical sciences (Huang, 2007). Nanotechnology has emerged as a key research area in health and medicine in recent years. Nanomedicine has had a rapid and widespread impact on health care, opening up a wide range of opportunities in many sectors and scientific efforts (Kandasamy *et al*., 2021b; Narayanan *et al*., 2021d; Nassar *et al*., 2020).

The synthesis of nanoparticles and their functionalization to effectively utilize them in biological applications including drug delivery is currently a challenge. Calcium carbonate among many other inorganic nanosized particles offers promising results for biomedical applications (Biradar, 2011). Calcium (Ca) is one among the major essential nutrients for plant growth and development. It activates enzymes, nitrate uptake, plant biomass ratio, the rate of photosynthesis and increase metabolisms (Kabali Vijai Anand., 2020).

Calcium is a basic material broadly conveyed in the earth. It is the fifth most bountiful element (by mass), typically found in sedimentary rocks in the mineral types of calcite, dolomite and gypsum (Savithramma *et al*., 2007). Calcium is found in upwards of 80 compounds some of the time called calcium salts, for example, calcium carbonate (lime).

Calcium is an essential part of nursery lime, otherwise called agrarian lime, which is utilized to improve the dirt quality by expanding pH and water holding limit of acidic soils. Calcium carbonate sources, for example, limestone and chalk, along side other synthetic compounds are utilized in the readiness of agrarian lime, when added to the dirt goes about as a calcium hotspot for plants. Calcium carbonate happens in three primary precious stone polymorphs, for example, calcite, aragonite and vaterite. Of these, calcite find in nature is more and is the most thermo dynamically stable under surrounding conditions (Sabriye, 2012).

Calcium carbonate is an inorganic calcium salt which can be derived from shelled molluscs, limestone, coccolithophores, plant ashes, chalk, and marble. It is considered to be a primary source of CaO, which has been identified as a biological constituent of human bone. $CaCO_3$ nanoparticles with desired sizes, shapes, and morphologies has been extensively researched for their vast application in industrial, electronic, and agricultural fields (Abbas Ibrahim Hussein, 2020).

Calcium nanoparticles are abundant inorganic biomaterials with different morphological structures that have attracted the interest of researchers in different fields. This interest is due to the wider application of these

nanoparticles in many industries, such as the paint, rubber, and plastics industries. With the present focus of interest in nanotechnology, calcium carbonate nanoparticles have been observed to be biocompatible for use in medicine, pharmaceutical industries, and drug delivery systems. The most important aspect with respect to the synthesis of nanoparticles is control of the particle size, polymorphism, and morphology of the desired material. Control of this parameter has led to the development of new materials with unique properties that differ from those in the bulk material. Many studies have been conducted to mimic nature in the synthesis of nanomaterial with the aim of analysing the biogenic materials and identifying how nature controls the morphology, size, and polymorphism in organisms (Abdullahi Shafiu Kamba, 2013).

After synthesis, precise particle characterization is necessary, because the physicochemical properties of a particle could have a significant impact on their biological properties. In order to address the safety issue to use the full potential of any nano material in the purpose of human welfare, in nanomedicines, or in the health care industry, etc it is necessary to characterize the prepared nanoparticles before application. The characteristic feature of nanomaterials, such as size, shape, size distribution, surface area, shape, solubility, aggregation, etc. need to be evaluated before assessing toxicity or biocompatibility. To

evaluate the synthesized nanomaterials, many analytical techniques have been used, including ultraviolet visible spectroscopy (UV-vis spectroscopy),Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), atomic force microscopy (AFM), and so on. Even though several studies are concerned with the synthesis of Ca/CaO nanoparticles by using biological routes in micro organisms (Long *et al*., 2009.The green synthesis of Ca/CaO nanoparticles by animal material is not carried so far.

Widespread use of UV-visible spectroscopy in the investigation of nanomaterials as a marker for the creation of nanoparticles. UV-visible spectroscopy is frequently used method of choice to measure affinity labeling's response in a nanoparticle-based analysis. The spectroscopic features of an indication of a nanoparticle's size can be found in them dispersion by adjusting the surface plasmon's position SPR to a straight forward wavelength function (Bendayan *et al*., 2012).

In past years, nanoparticles had many biomedical applications such as antimicrobial, antioxidant, anti-inflammatory, antiviral, cytotoxic, anticancer, antidiabetic, anti-HIV, and so on (Abdel-Aziz *et al*., 2014). Presently, several metallic nanomaterials are being synthesized using copper, zinc, titanium, magnesium, gold, alginate, and silver. Nanoparticles are being used for several

purposes, from medical treatments, using different branches of industrial production such as solar and oxide fuel batteries for energy storage, to wide incorporation into diverse materials of everyday use such as cosmetics or clothes (Dubchak *et al*., 2010).

Green synthesized nanoparticles have an incredible aptitude against diabetes and regulate the functioning of diabetes by α-amylase release from pancreas, colonic, α-glucosidase, insulin levels, glycemic absorption and other histochemistry characteristics during *in vivo* and *in vitro* studies. Metal-based nanoparticles were discovered to have antioxidant capabilities, scavenging free radicals and lowering reactive oxygen species (ROS) production. The exact chemical pathways that determine metal nanoparticles' antioxidant properties are yet unknown. The large surface area and electrical configuration act as catalysts for oxidant-reduction reaction characteristics, and oxygen point defects in these NPs are thought to be responsible for their antioxidant capacity. Therapy with nano-based developed medicines, in comparison to essential medication, may be a potent clinical alternative for people with significant disorders due to such features (Thanh *et al*., 2021).

Antioxidants can protect the human body from free radicals and ROS effects. They retard the progress of many chronic diseases as well as lipid

peroxidation. The commercially used synthetic antioxidants BHA and BHT have been suspected of being responsible for liver damage and carcinogenesis. Hence, a need for identifying alternative natural and safe sources of antioxidants has been created, and the search for natural antioxidants, especially of marine origin, has notably increased in recent years (Pachiyappan *et al*., 2014).

Diabetes triggers the production of reactive oxygen species (ROS) (Savu *et al*., 2012 and Son, 2012). Consequently, agents exhibiting radical-scavenging activity can abolish ROS-induced oxidative damage (Pietta, 2000 and Kucharska *et al*., 2004). It was suggested that the antidiabetic activityof nanoparticle is related to the efficient inhibitory action of carbohydrate digestive enzymes such as α-amylase and α-glucosidase (Kannan *et al*., 2016).

Inflammation and diabetes mellitus is the most prevalent clinical issues worldwide (Bornstein *et al*., 2020 and Thanh *et al*., 2021). Fortunately, there is inflammation that plays an essential role in the healing system and maintains the regular functionality of cells. Nevertheless, two types of inflammations are recognized as problematic, namely acute and chronic inflammation (Dunnill *et al*., 2017 and Narayanan *et al*., 2021a). Acute inflammation is familiar among people with the symptoms of redness, swelling, pain around tissues and joints (Corti, 2014). Inflammation can be caused by several factors such as microbial

infections, physical hazards, and chemical agents (Furman *et al*., 2019 and Narayanan *et al*., 2021b).

Diabetes mellitus, a growing metabolic disorder, poses a substantial burden on healthcare systems (Egbuna *et al*., 2021; Estes *et al*., 2018 and Narayanan *et al*., 2021c). The World Health Organization (WHO) has recognized Non-Communicable Diseases (NCD) as a major global health risk, and diabetes is one of four major NCDs that require immediate global attention (Alleyne *et al*., 2013).

As per the International Diabetes Federation (IDF), there are 415 million diabetics worldwide, with 75 percent living in low and middle-income countries. According to current trends, the world will have 642 million diabetics by 2040 (Ogurtsova *et al*., 2017). With the world's second-largest diabetes population (69 million as of 2015), India is an influential centre for the worldwide diabetes epidemic. If current trends continue, India will have 123.5 million diabetics by 2040 (Zheng *et al*., 2018). After the United States (84,100), India (70,200) has the second highest number of children (15 years) with type I diabetes in the world (Ibrahim, 2017). As a result, finding excellent, side-effect-free or low-risk, eco-friendly, and cost-effective remedies to treat these health complications is critical.

Inflammation is the reaction process of living tissues to stimuli elicited by inflammatory agents like physical injuries, heat, microbial infections, and pestilent chemical irritations. The response of cells toward inflammation can cause certain pathological manifestations characterised by redness, heat, swelling, and pain with even impaired physiological functions. Inflammation has been involved within the pathological process of the many diseases including arthritis, stroke, and cancer. Protein denaturation has been correlate with the prevalence of the inflammatory response and ends up in numerous inflammatory diseases including arthritis. Tissue injury throughout life may well be referable to denaturation of the protein constituents of cells or of ground substance. Hence, the flexibility of a substance to inhibit the denaturation of protein signifies apparent potential for anti inflammatory activity (Aditya Jain, 2015).

Further more, the development of simple and environmentally friendly processes for the preparation of nanoparticles using non-toxic reagents and the cost-effective procedure becomes a priority. In this context, green synthesis of nanoparticles using microbes, marine organisms, and plant extracts has become popular as these methods are biologically compatible, environmental and economically friendly (Quaresma *et al*., 2009). Hence, the biological

approaches, i.e. using biomass of plant, microbes, and so on to produce nanoparticles, receive more attention among the researchers.

So, the present study has been carried out with a view to investigate the biosynthesis of calcium nanoparticles using the marine molluscan shell extract *H.lapponica*. The nature of the synthesized nanoparticles can be characterized by standard scientific techniques such UV–visible spectrophotometer, and functional groups involved in the reduction, capping, stabilization of nanoparticles can be studied by Fourier-Transform Infrared Spectroscopy (FTIR). This is the first approach to fabricating the calcium nanoparticles using the extracts derived from *Harpulina lapponica* and evaluating their antioxidant, anti-diabetic and anti-inflammatory activities by *invitro* approach.

2. REVIEW OF LITERATURE

Nanoparticle synthesis using biological organisms by green synthesis technology is biologically safe, cost-effective, and environment-friendly. Plants

and microorganisms have established the power to devour and accumulate inorganic metal ions from their neighboring niche. The biological entities are known to synthesize nanoparticles both extra and intracellularly. The capability of a living system to utilize its intrinsic organic chemistry processes in remodeling inorganic metal ions into nanoparticles has opened up an undiscovered area of biochemical analysis (Dan Zhang *et al*., 2020).

Preetha Devaraj *et al*. (2013) synthesized and studied the characterization of silver nanoparticles using cannonball leaves and their cytotoxic activity against MCF-7 Cell line. Sri Ramkumar Vijayan *et al*. (2014) studied the synthesis and characterization of silver and gold nanoparticles using aqueous extract of seaweed, *Turbinaria conoides* and their anti microfouling activity. Monaliben Shah *et al*. (2015) elucidated metallic nanoparticles via biological entities.

Safaa Ali *et al*. (2015) investigated the applications of biosynthesized silver nanoparticles for the control of land snail *Eobania vermiculata* and some plant pathogenic fungi. Suresh Sagadevan and Koteeswari (2015) analysed the structure, surface morphology, optical and electrical properties of copper nanoparticles. Maria Benelmekki (2015) gave an introduction to nanoparticles and nanotechnology.

Hamed Barbadi *et al*. (2015) synthesized and characterized the biogenic tellurium nanoparticles by using *Penicillium chrysogenum.* Aruna Jyothi Kora and Lora Rashtogi (2015) studied the synthesis of palladium nanoparticles using gum ghatti (*Anogeissus latifolia*) and studied their applications as an antioxidant and catalyst. Yu Ioni *et al*. (2016) reported the synthesis of graphene with noble metals nanoparticles on its surface.

Khwaja Salahuddin Siddiqui and Azamal Husen (2016) studied the green synthesis and investigated the characterization and uses of palladium and platinum nanoparticles. Dmitri Talapin and Elena Shevchenko (2016) gave an introduction to nanoparticle chemistry. Kashinath *et al*. (2017) synthesized and studied the structure of graphene nanoparticles.

Rakhi Majumdar *et al*. (2017) synthesized palladium nanoparticles with green leaf extract of *Chrysophyllum caimito* (star apple) and studied their applications as efficient catalyst for C-C coupling and reduction reactions. Alpaslan *et al*. (2017) studied the synthesis and characterization of selenium nanoparticles lysozyme nanohybrid system with synergistic antibacterial properties. Results of this efforts highlighted the nanohybrid systems with synergistic antibacterial properties to overcome the emerging antibiotic resistance as well as to define fruitful applications in biomedicine.

Stefanos Mourdikondis *et al.* (2018) studied the characterization techniques for nanoparticles and compared the complemented the properties of nanoparticles. Mostafa Abo Elsoud *et al*. (2018) synthesized and investigated tellurium nanoparticles. David Medina Cruz *et al*. (2019) synthesized citric juice mediated tellurium nanoparticles with antimicrobial and anticancer properties.

Andrew Smith *et al.* (2019) investigated the synthesis, properties and applications of graphene oxide or reduced graphene oxide and their nanocomposites. Asep Bayu Dani Nandiyanto *et al*. (2019) interpreted the FTIR spectroscopy of organic material. Qaisar Abbas (2019) discussed the UV-Vis spectroscopy technique for nanoparticles.

Kabali Vijai Anand *et al*. (2020) investigated the preparation and characterization of calcium oxide nanoparticles from marine molluscan shell waste as nutrient source for plant growth. Joanna Jagiello *et al*. (2020) studied the synthesis and characterization of graphene oxide and reduced graphene oxide composites with inorganic nanoparticles for biomedical applications.

Tannia Velazquez-Urbina *et al*. (2021) synthesized and characterized the silver nanoparticles supported on bivalve mollusc shell for catalytic degradation of commercial dyes. Hojat Veisi *et al*. (2021) showed the synthesis of palladium nanoparticles fabricated magnetic Fe_3O_4 nanocomposites over *Fritillaria*

imperialis flower extract as an efficient recyclable catalyst for the reduction of nitroarenes. Koduru Mallikarjuna *et al*. (2021) synthesized the reduced graphene oxide supported palladium nanoparticles by *Coleus amboinicus* and its enhanced catalytic efficiency and antibacterial activity.

From the above review, it was reported that the marine molluscan shell extract has not been used to synthesize and study the applications of nanoparticles. So, the current study has been carried out to elucidate the synthesis and applications of calcium nanoparticles using the marine molluscan shell extract of *H.lapponica.*

3. OBJECTIVES

Over the past few years, biogenic methods for designing nanoparticles are in limelight due to the ability to generate semi-healthcare and para-pharmaceutical consumer goods. Conseqently, sustained efforts are being made

to develop clean, green and eco-friendly processes for synthesizing metallic nanoparticles in industrially viable setting. The major advantage of using biological materials is the availability of secondary metabolites, amino acids, proteins which are routinely used in the synthetic steps of nanoparticles. The present study reports the eco-friendly synthesis of calcium nanoparticles from the marine gastropod *Harpulina lapponica* by the facile, clean and easily scalable method. The present study has been carried out with the following objectives:

- To synthesize the green nanoparticles through UV-Vis spectroscopy.
- To characterize the synthesized nanoparticles using FT – IR.
- To analyze the antioxidant, antidiabetic and anti - inflammatory properties of synthesized calcium nanoparticles using the marine gastropod shell extract *H.lapponica.*

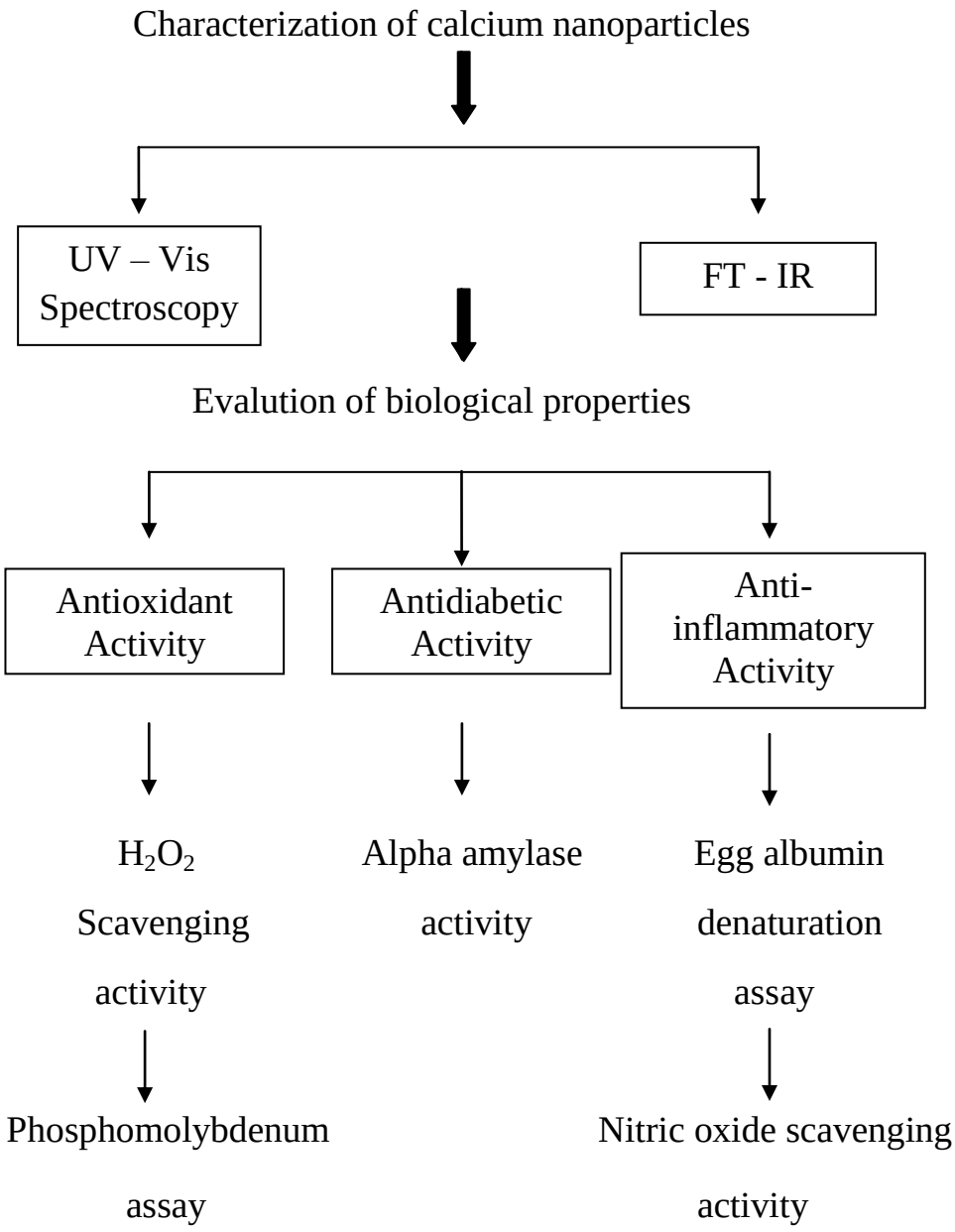
4. EXPERIMENTAL DESIGN

Collection of experimental organism
Harpulina lapponica from Thoothukudi coastal region – Vellappatti coast

↓

Synthesis of calcium nanoparticles

↓



5. MATERIALS AND METHODS

5.1 Description of the study area

The Gulf of Mannar is located between India and Srilanka, stretches from the longitude 78°08' to 79°30' E and along the latitude from 83°5' to 9°25' N. It is a part of the Southward extension of the Bay of Bengal and meets in the Indian Ocean. This geographical area runs from Pamban island including Rameshwaram to Cape Comorin along the Southeast Coast of India to a distance of about 170 nautical miles. The Gulf of Mannar biosphere reserve has an area of about 10,500km^2 and is considered as 'Biologist's Paradise' for, it has 3600 species of flora and fauna. This coast maintains a rich biological diversity perspective 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 (Figure 1).

5.2 Collection of experimental animal

In the present study the gastropod *Harpulina lapponica* was collected from the Vellappatti, Thoothukudi coastal region (Plate 1). The neogastropod *Harpulina lapponica* was collected from the landed by-catch from fishing trawlers operated for crabs and prawns along the Vellappatti village, Thoothukudi coastal region. The gastropods were collected during the month of December 2022. The freshly collected samples were brought to the laboratory,

cleaned and washed with fresh sea water to remove all impurities. The shells were broken and then dried in hot air oven at 56^{o}C for 48 hours and used for further studies.

5.3 Description of experimental animal

5.3.1 Systematic position of *Harpulina lapponica* (Linnaeus, 1767)

Phylum	:	Mollusca
Class	:	Gastropoda
Subclass	:	Caenogastropoda
Order	:	Neogastropoda
Super family	:	Volutoidea
Family	:	Volutidae
Genus	:	*Harpulina*
Species	:	*lapponica*

A heavy shell with a short spire and inflated body whorl. The apex is bulbous; the succeeding two or three whorls have low vertical ribs; the remaining whorls are smooth. The suture is shallow; the columella has seven or eight folds. Cream, with brown blotches (arranged in three bands on body whorl) and many spiral rows of dashes. Shell size 60-103 mm. This marine species is native to the Indian Ocean, particularly South India and Sri Lanka.

5.4 Synthesis of Calcium Nanoparticles

Synthesis of calcium nanoparticles is carried out using 0.01M Calcium chloride in double-distilled water using *Harpulina lapponica*. Calcium chloride and the shell extract of *Harpulina lapponica* were mixed together in a ratio of (9:1, 8:2, 7:3, 6:4, and 5:5). In this different ratio concentration, a 5:5 ratio concentration was selected for the bulk preparation because it shows a higher production than other ratios stirred at 800 rpm using a magnetic stirrer. The mixture turned into yellowish – green color. The whole reaction was carried out in the dark. The obtained suspension was centrifuged at 15,000 rpm for 15 min. The pellet containing calcium nanoparticles was washed 3–4 times with deionized water to remove impurities. The precipitated nanoparticles were lyophilized. Lyophilized nanoparticles were stored in a cool, dry and dark place and further characterization was carried out.

5.5 UV – Vis Spectral Analysis

Ultraviolet–visible spectroscopy (UV) refers to absorption spectroscopy or reflectance spectroscopy in part of the ultraviolet and the full, adjacent visible spectral regions. Molecules containing bonding and non-bonding electrons (n-electrons) can absorb energy in the form of ultraviolet or visible light to excite these electrons to higher antibonding molecular orbitals. UV-absorption spectra

of synthesized calcium nanoparticles by using *Harpulina lapponica* were measured using UV- Visible spectrometer (Shimadzu UV-2700).

5.6 Fourier Transform Infra Red Spectroscopy (FTIR)

An infrared spectrophotometer is an instrument that passes infrared light through an organic molecule and produces a spectrum that contains a plot of the amount of light transmitted on the vertical axis against the wavelength of infrared radiation on the horizontal axis. The functional group present in the synthesized calcium nanoparticles was determined using FTIR spectroscopy (Bio-read FTIR 8400S models, USA).

5.7 Antioxidant Activity

5.7.1 Hydrogen Peroxide Scavenging Assay

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate.

Material Required

Hydrogen Peroxide solution and Sodium Phosphate buffer

Procedure

Ability of extracts to scavenge hydrogen peroxide was estimated according to the method reported by Ruch *et al*., (1989) with minor modification. A solution of hydrogen peroxide (43 mM) is prepared in phosphate buffer (1 M pH 7.4). Different concentration of calcium nanoparticles (50, 100, 250, 500 µg/ml) was added to hydrogen peroxide solution (0.6 ml, 43 mM). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as standard. The free radical scavenging activity was determined by evaluating percentage inhibition (Ruch *et al*., 1989).

Percentage inhibition = [(Control- Test)/Control] ×100

5.7.2 Phosphomolybdenum Assay

The total antioxidant capacity of the extracts was evaluated according to the method described by Prieto *et al*. (1999). An aliquot of 0.5 ml of samples solution (concentrations ranging from 50µg/ml to 500µg/ml) was combined with 4.5 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). In case of blank, 0.5 mL of 45% ethanol was used

in place of sample. The tubes were incubated in a boiling water bath at 95°C for 90 min. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each sample was measured at 695 nm against blank in UV-2450 spectrophotometer (Shimadzu, Japan). The total antioxidant activity was expressed as the absorbance of the sample at 695 nm. The higher absorbance value indicated higher antioxidant activity (Prasad *et al*., 2009).

5.8 Antidiabetic Activity

5.8.1 α-Amylase Inhibitory Activity

The α-amylase inhibitory activity was determined by the method described by Xiao *et al.* (2006). Samples (50, 100, 250, 500 µg/ml) were mixed in 96-well microplates with 40 µl of amylase solution (100 U/mL in 0.1Msodium phosphate buffer, pH 7.0) and 40 µl of 0.1% starch solution (diluted in the previous buffer). After 10 min at 37°C, 20 µl of 1M hydrochloric acid (HCl) and 100 µl of iodide solution (5mM iodine (I_2) + 5mM potassium iodide (KI), in distilled water) were added and the absorbance was measured at 580 nm. Results were expressed as IC_{50} values (µg/ml).

5.9 Anti-inflammatory Activity

Preparation of Phosphate Buffer Saline

2.725 g of anhydrous sodium dihydrogen orthophosphate, 0.800 g disodium hydrogen orthophosphate and 22.500 g sodium chloride were weighed on a Mettler Toledo digital analytical balance(AB204-S, Ohio, USA) and dissolved in distilled water. The solution was diluted to the mark with distilled water in a 250 ml volumetric flask. The pH was adjusted to 7.4 using 0.1 N HCI or NaOH.

5.9.1 *In vitro* Inhibition of Egg Albumin Denaturation

The anti-inflammatory activity of biosynthesized calcium nanoparticles of *H. lapponica*, was determined *in vitro* for inhibition of denaturation of egg albumin (protein) according to the method of Mizushima and Kobayashi (1968) with some modifications. 0.2 mL of 1% egg albumin solution, different concentrations (50, 100, 250, 500 µg/ml) of sample extract or standard and 2.8 mL of phosphate buffered saline (pH 7.4) were mixed together to form a reaction mixture of total volume5 mL. The control was made by mixing 2 mL of triple distilled water, 0.2 mL 1% egg albumin solution and 2.8 mL of phosphate buffered saline to make a total volume of 5 mL. The reaction mixtures were then incubated at 37±2°C for 30 min and heated in a water bath at 70±2°C for 15 min. After cooling, the absorbance was measured at 280 nm by UV/Vis spectrophotometer (Genesys10S, Thermo Fisher Scientific Inc., USA) using

triple distilled water as the blank. The percentage inhibition was calculated using the relationship:

$$\text{Percentage Inhibition} = \frac{\text{Absorbance of Control - Absorbance of Test sample}}{\text{Absorbance of Control}} \times 100$$

5.9.2 Nitric Oxide Radical Scavenging Assay

This assay was done according to the method of Panda *et al.* (2009). The extracts were prepared and these were then serially diluted with distilled water to make different concentrations from 50, 100, 250, 500 µg/ml. The freshly prepared solutions were refrigerated at 4°C for later use. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1%naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid immediately before use. 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the sample or standard in ethanol and incubated at 25°C for 180 min. The extract was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extracts or standard but with an equal volume of buffer were prepared in a similar manner as done in the test samples. The absorbance was measured at 546nm using a Ultraviolet–visible (UV/Vis) spectrophotometer (Genesys10S, Thermo Fisher Scientific Inc., USA) by using triple distilled water as blank. The percentage inhibition of the extract and standard was calculated and recorded. The percentage nitrite radical

scavenging activity of the sample extracts or standard were calculated using the formula:

$$\% \text{ NO Scavenged} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test sample}}{\text{Absorbance of Control}} \times 100$$

6. RESULTS

6.1 Synthesis and Characterization of Calcium Nanoparticles

6.1.1 UV-Visible Spectroscopic Analysis

UV-Visible spectroscopic analysis confirmed the formation of the biosynthesized calcium nanoparticles using the marine molluscan shell extract *Harpulina lapponica* (Figure 2 and 3). The above solutions were subjected to optical measurements by UV-Visible spectrophotometer. In *H.lapponica*, the wavelength obtained around 260 nm suggested the presence of calcium nanoparticles in the solution (Figure 4). This is the specific wavelength which indicates synthesized calcium nanoparticles. The maximum absorption was obtained around 260 nm. The occurrence peak at absorption intensity between 200 to 900 nm indicated the presence of surface plasmon resonance.

6.1.2 Fourier Transform Infra Red Spectroscopic Analysis

The IR spectra provided information about the local molecular environment of the organic molecules on the surface of nanoparticle. In the present work, FTIR spectral measurements were carried out to identify the potential biomolecules in *H.lapponica* shell extract which is responsible for reducing and capping the bioreduced calcium nanoparticles. FTIR measurements were carried out to identify the possible biomolecules responsible for capping and efficient stabilization of the metal nanoparticles synthesized by marine molluscan shell extract.

The results of FTIR analysis of this study show different stretches of bonds shown at different peaks; 3751.03, 3333.64, 2079.46, 1637.55, 1400.38, 1196.82, 1155.96, 1082.41, 1024.53, 927.13, 534.43cm^{-1}. The image shows a strong absorption peak around 3751.03cm^{-1} to 1400.38cm^{-1} which shows the presence of C-H stretching vibration. A peak around 500cm^{-1} to 1100 cm^{-1} shows the presence of C-O stretching frequency. A peak around finger print region confirms the presence of calcium nanoparticles (Figure 5).

6.2 Antioxidant Activity

6.2.1 Hydrogen Peroxide Radical Scavenging Activity

The hydrogen peroxide radical scavenging activity of marine molluscan shell extract of *H.lapponica* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 97.31% was observed at 500 µg/ml followed by 79.15% at 250 µg/ml, 70.14% at 100 µg/ml and 31.12% at 50 µg/ml respectively. The percentage inhibition of 87.73% was found for the standard ascorbic acid. The IC_{50} value of 65.1 µg/ml was noted which shows the good antioxidant activity. It has been found that antioxidant activity was dose dependent and the percentage inhibition was found to increase with increase in the concentration respectively (Figure 6).

6.2.2 Phosphomolybdenum Scavenging Assay

The phosphomolybdenum scavenging assay of marine molluscan shell extract of *H.lapponica* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 93.97% was observed at 500 µg/ml followed by 81.87% at 250 µg/ml, 56.41% at 100 µg/ml and 38.90% at 50 µg/ml respectively. The percentage inhibition of 89.95% was found for the standard ascorbic acid. The IC_{50} value of 56.3 µg/ml was noted which shows the good antioxidant activity. It has been found that antioxidant activity was dose dependent and the percentage inhibition was found to increase with increase in the concentration respectively (Figure 7).

6.3 Antidiabetic Activity

The α-amylase activity of marine molluscan shell extract of *H.lapponica* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 69.71% was observed at 500 µg/ml followed by 45.28% at 250 µg/ml, 38.45% at 100 µg/ml and 19.48% at 50 µg/ml respectively. The IC_{50} value of 29.3 µg/ml was noted which shows the good antidiabetic activity. It has been found that antidiabetic activity was dose dependent and the percentage inhibition was found to increase with increase in the concentration respectively (Figure 8).

6.4 Anti-inflammatory Activity

6.4.1 Egg Albumin Denaturation Activity

The egg albumin denaturation activity of marine molluscan shell extract of *H.lapponica* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 93.68% was observed at 500 µg/ml followed by 90.61% at 250 µg/ml, 87.19% at 100 µg/ml and 83.78% at 50 µg/ml respectively. The IC_{50} value of 21.9 µg/ml was noted which shows the good anti-inflammatory activity. It has been found that anti-inflammatory activity was dose dependent and the percentage inhibition was found to increase with increase in the concentration respectively (Figure 9).

6.4.2 Nitric Oxide Scavenging Assay

The nitric oxide scavenging assay of marine molluscan shell extract of *H.lapponica* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 61.29% was observed at 500 µg/ml followed by 46.67% at 250 µg/ml, 44.49% at 100 µg/ml and 34.48% at 50 µg/ml respectively. The IC_{50} value of 28.9 µg/ml was noted which shows the good anti-inflammatory activity. It has been found that anti-inflammatory activity was dose dependent and the percentage inhibition

was found to increase with increase in the concentration respectively (Figure 10).

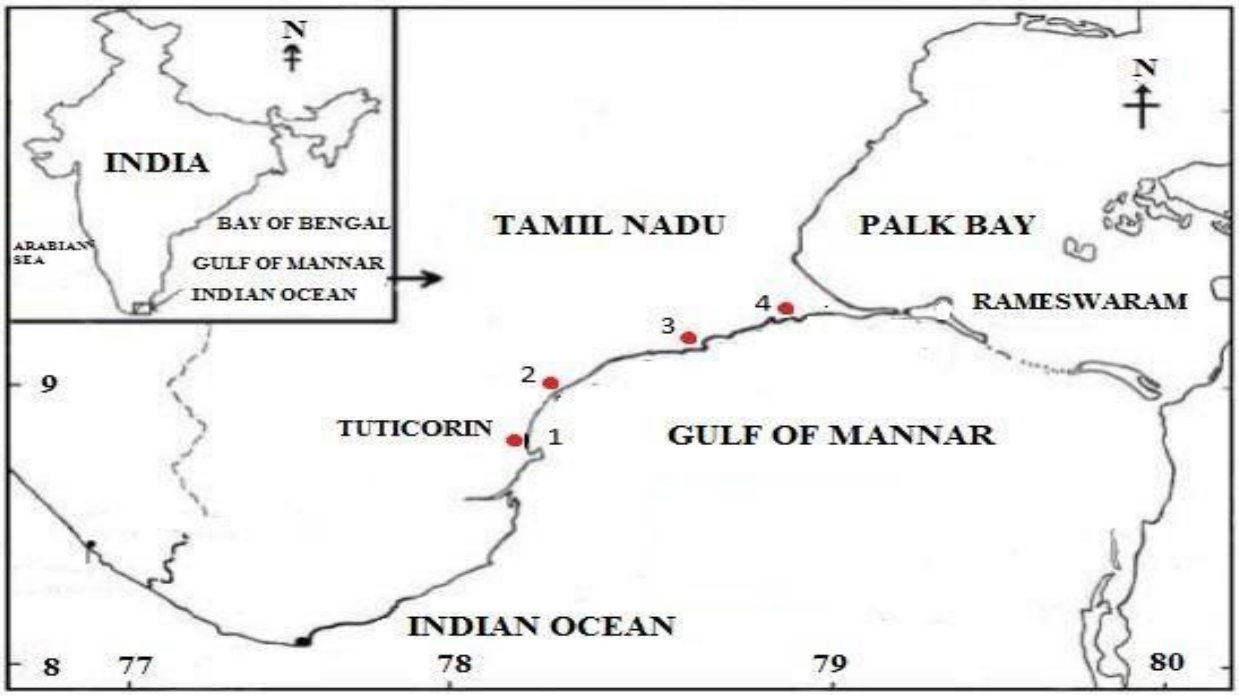


Figure 1: Map showing the study area Gulf of Mannar - Thoothukudi Coastal region

PLATE 1

Dorsal and Ventral view of the shell –*Harpulina lapponica*

Figure 2: Synthesis of Calcium Nanoparticles using *Harpulina lapponica*

Figure 3: Synthesized Calcium Nanoparticles using the Shell Extract of *Harpulina lapponica*

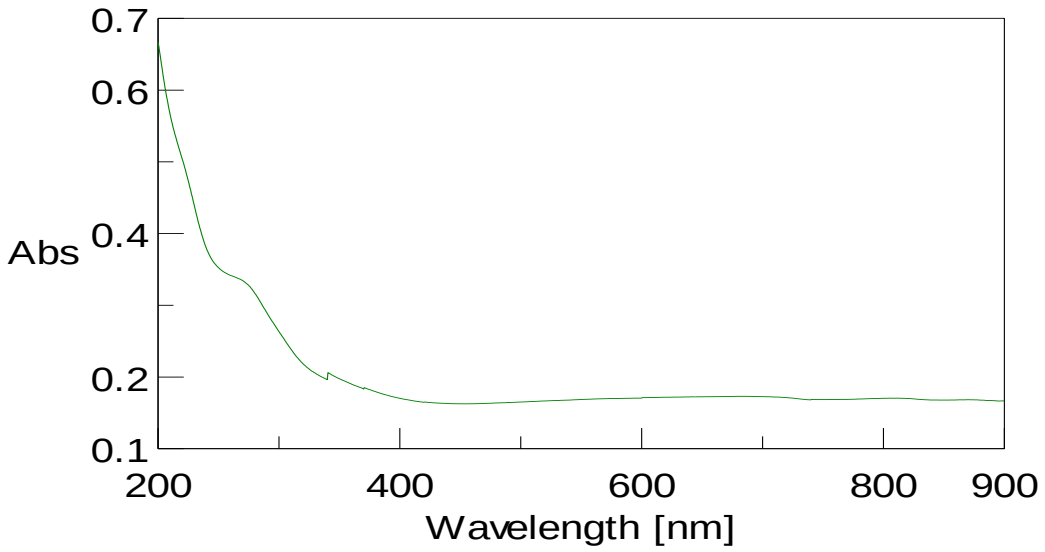


Figure 4: UV-Visible Spectra of Calcium Nanoparticles using *Harpulina lapponica*

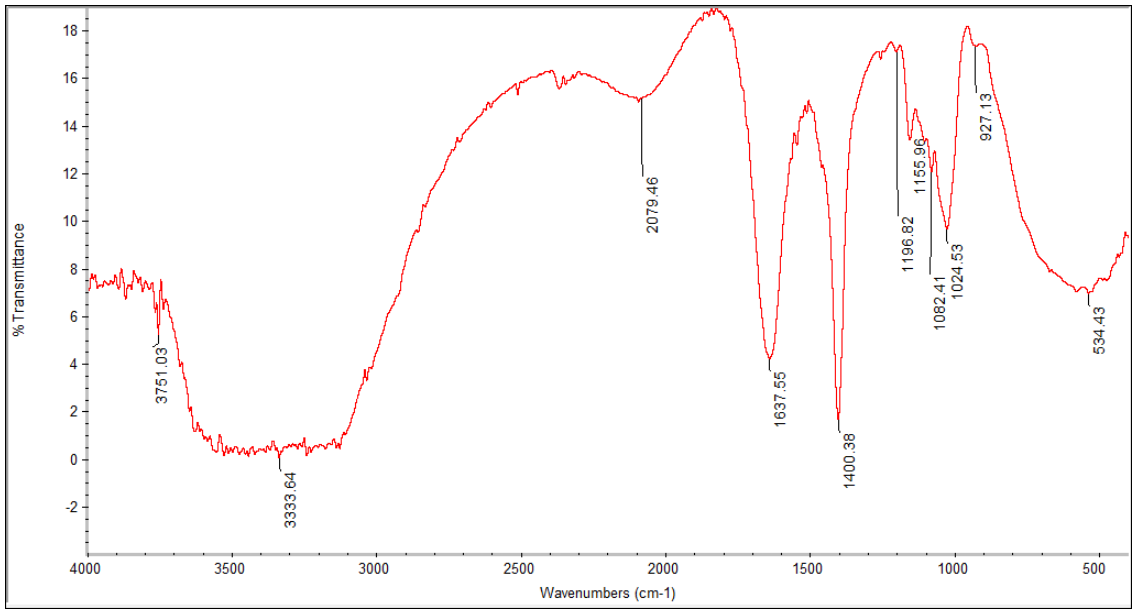


Figure 5: Infra Red Spectra of Calcium Nanoparticles using *Harpulina lapponica*

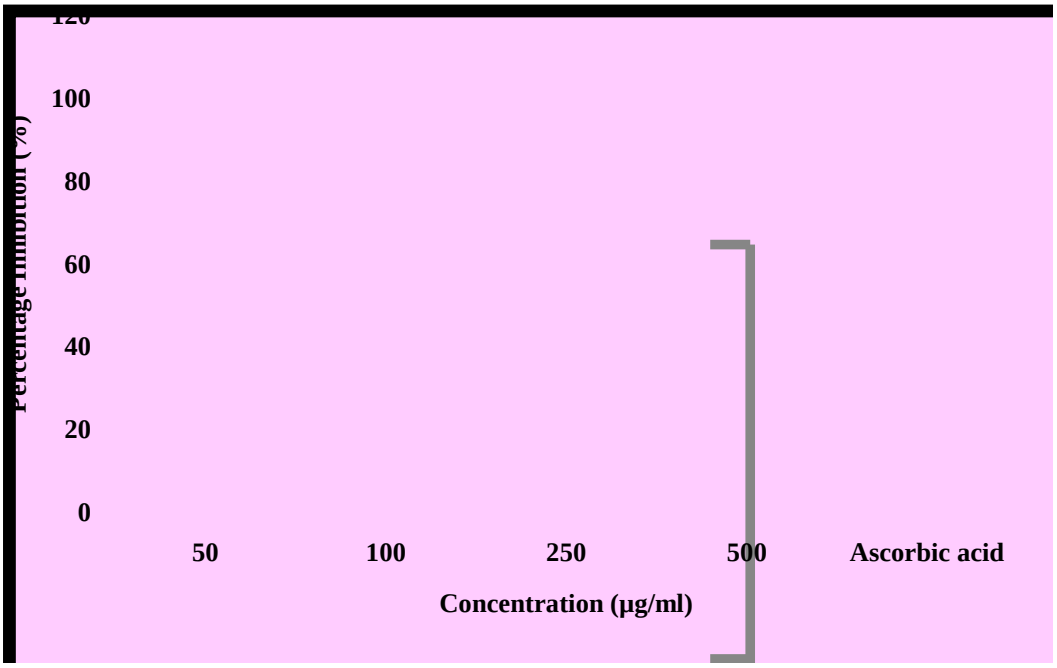


Figure 6: Hydrogen Peroxide Activity of Calcium Nanoparticles using *Harpulina lapponica*

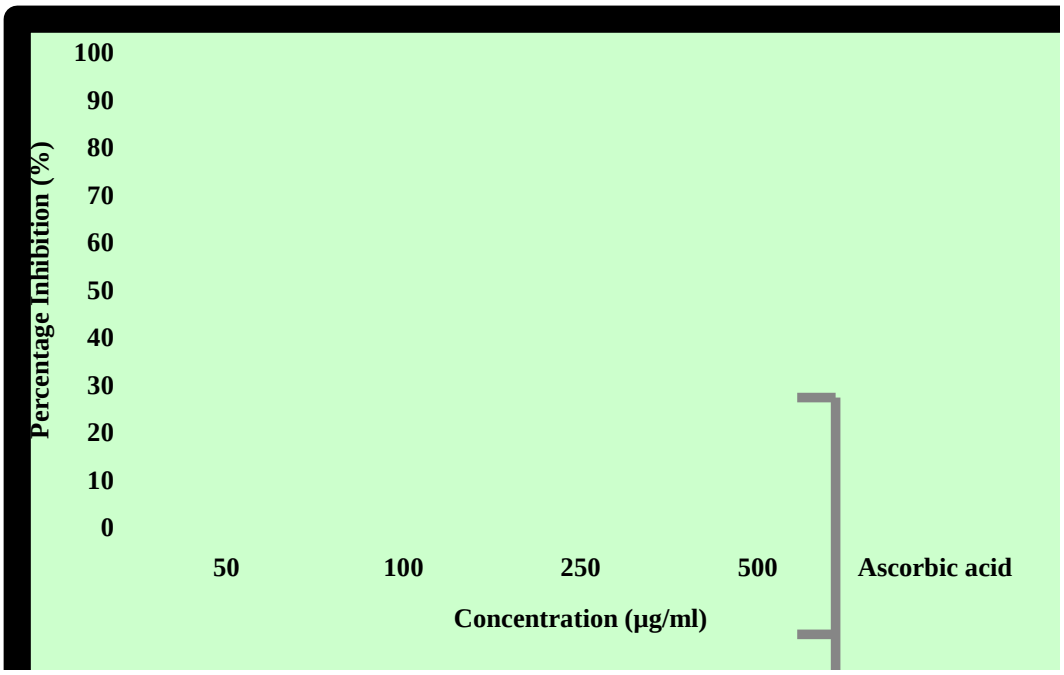


Figure 7: Phosphomolybdenum Assay of Calcium Nanoparticles using *Harpulina lapponica*

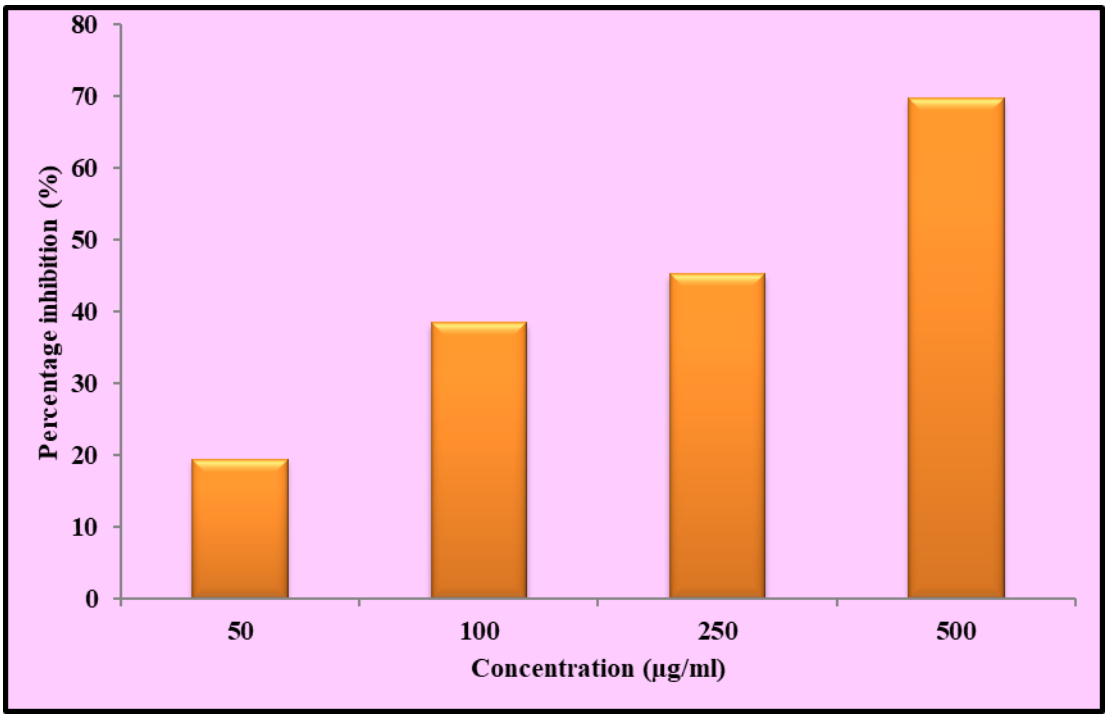


Figure 8: Antidiabetic Activity of Calcium Nanoparticles using *Harpulina lapponica*

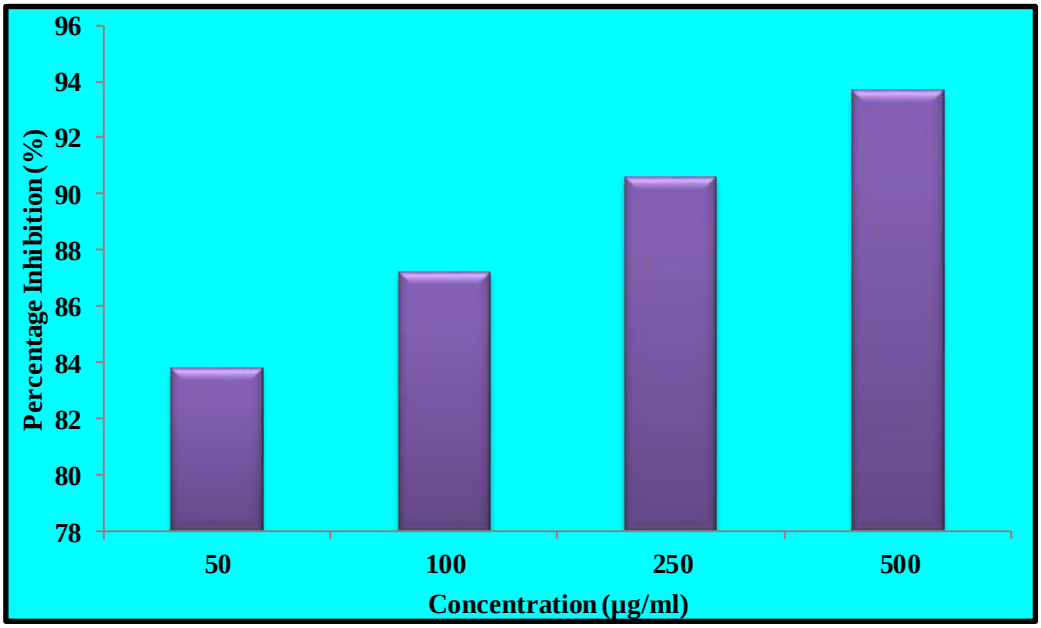


Figure 9: Egg Albumin Denaturation Activity of Calcium Nanoparticles using *Harpulina lapponica*

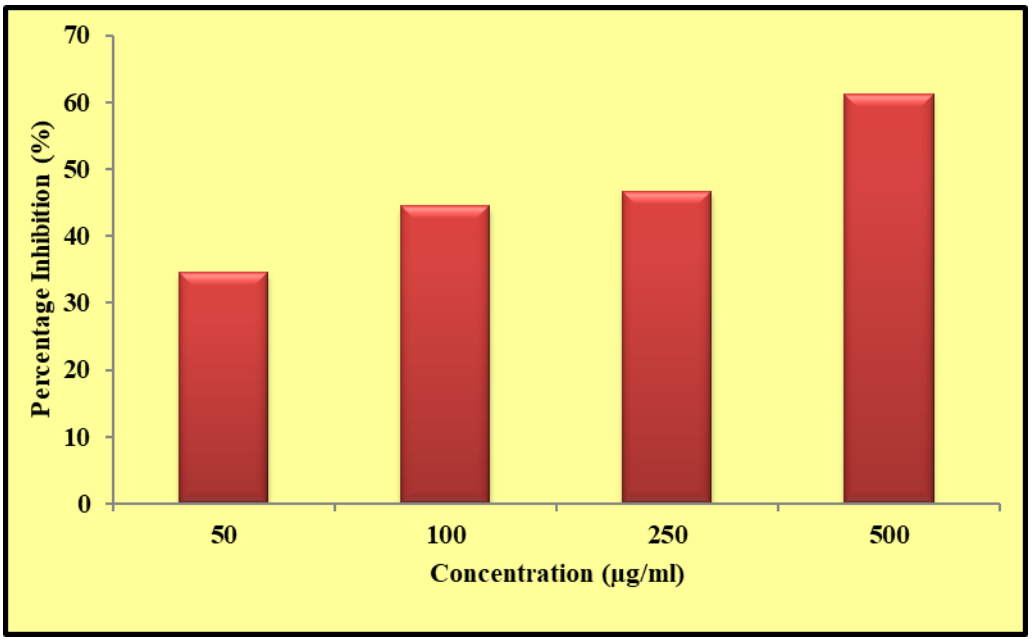


Figure 10: Nitric Oxide Scavenging Assay of Calcium Nanoparticles using *Harpulina lapponica*

7. DISCUSSION

Nanoparticles are referred to as particles with a size of 1-100 nm in at least one dimension (Chen *et al*., 2013). The surface area to volume ratio increases as the size of the nanoparticles decreases and leads to minor changes in their physiochemical and biological properties. In past years, nanoparticles had many biomedical applications such as antimicrobial, antioxidant, anti-inflammatory, antiviral, cytotoxic, anticancer, antidiabetic, anti-HIV, and so on (Abdel-Aziz *et al*., 2014). Presently, several metallic nanomaterials are being synthesized using copper, zinc, titanium, magnesium, gold, alginate, and silver. Nanoparticles are being used for several purposes, from medical treatments, using different branches of industrial production such as solar and oxide fuel batteries for energy storage, to wide incorporation into diverse materials of everyday use such as cosmetics or clothes (Dubchak *et al*., 2010).

Green synthesis of nanoparticles is an eco-friendly methodology which may prepare for scientists over the globe to investigate the capability of various spices so as to synthesis nanoparticles (Savithramma *et al*., 2016). Since, green synthesis is the most ideal alternative to decide on the synthesis of nanoparticles (Anamika *et al*., 2012) and biogenetic production is presently of more enthusiasm because of straight forwardness of the systems and adaptability

(Popescu *et al*., 2010). The synthesis of calcium and calcium oxide nanoparticles by different physical and chemical methods through colloid particles (Sadowski *et al*., 2010), protein cage (Hiroko *et al*., 2011), modified emulsion membranes (Ritika *et al*., 2004), two membrane system (Zeshan *et al*., 2004), saturated carbonate and calcium nitrate aqueous solutions (Romuald *et al*., 2012) and by ethanol assisted synthesis (Shao *et al*., 2013). In recent years, the development of efficient green methods for synthesis of metal nanoparticles has become a major focus. Hence, the present study was an attempt to synthesize Calcium nanoparticles from the shell extract of marine gastropod *Harpulina lapponica*.

UV-Visible spectroscopic analysis confirmed the formation of the biosynthesized calcium nanoparticles using the marine molluscan shell extract *Harpulina lapponica*. The above solutions were subjected to optical measurements by UV-Visible spectrophotometer. In *H. lapponica*, the wavelength obtained around 260 nm suggested the presence of calcium nanoparticles in the solution (Figure 4). This is the specific wavelength which indicates synthesized calcium nanoparticles. The colour changed aqueous solution allowed for UV- visible spectra analysis indicating the generation of calcium nanoparticles, due to the reduction of calcium ions into calcium oxide nanoparticles via the active molecules present in the shell extract of *H. lapponica*. Ahmad *et al*., (2003) analyzed the extracellular synthesis of silver

nanoparticles by *Fusarium oxysporum* fungal extract. The present study agrees well with the above findings.

Fourier Transform Infra Red Spectroscopy (FTIR) is a technique which is used to analyze the chemical composition of many organic chemicals, polymers, paints, coatings, adhesives, lubricants, semiconductor materials, coolants, gases, biological samples, in organics, and minerals. FTIR can be used to analyze a wide range of materials in bulk or thin films, liquids, solids, pastes, powders, fibres, and other forms. FTIR analysis can give not only qualitative (identification) analysis of materials, but, with relevant standards, can be used for quantitative (amount) analysis. FTIR can be used to analyze samples up to ~11 millimetres in diameter and either measure in bulk or the top ~1 micrometer layer. The results of FTIR analysis of this study show different stretches of bonds shown at different peaks ; 3751.03, 3333.64, 2079.46, 1637.55, 1400.38, 1196.82, 1155.96, 1082.41, 1024.53, 927.13, 534.43cm^{-1}. The image shows a strong absorption peak around 3751.03cm^{-1} to 1400.38cm^{-1} which shows the presence of C-H stretching vibration. A peak around 500cm^{-1} to 1100 cm^{-1} shows the presence of C-O stretching frequency. A peak around finger print region confirms the presence of calcium nanoparticles (Figure 5).

Antioxidants from natural sources play a paramount role in helping endogenous antioxidants to neutralize oxidative stress (Sasikumar *et al*., 2009). The generation of reactive oxygen species (ROS) is an unavoidable consequence of life in an aerobic environment. In which, the production of ROS is essential to many organisms for the production of energy to fuel biological processes (Yong-Xu *et al*., 2010). Accumulation of uninhibited H_2O_2 leads to the development of oxygen free radicals (Peroxide and hydroxyl) which causes heavy damage to cell membranes in living systems (Nathan, 2002). Hydrogen peroxide inside a cell at a low dose can accelerate the dissolution of AgNPs and produce much stronger oxidative stress Nathan (2002).

Kharat and Mendhulkar (2016) studied the antioxidant activity of synthesized nanoparticles using DPPH assay and observed the antioxidant potentials of photosynthesized nanoparticles. Priya *et al*. (2016) studied *in vitro* antioxidant activity of biosynthesized nanoparticles from *P. pinnata* extract and found significant free radical scavenging potential. Patra and Baek (2016) demonstrated presence of strong antioxidant activity in terms of DPPH radical scavenging (IC_{50} 385.87 μg/mL). The results strongly recommend the application of AgNPs as useful natural antioxidants for health preservation against different oxidative stress associated with degenerative diseases. In fact,

antioxidant evaluation is essential for Ag NPs before its use *in vivo* models and also human applications.

The hydrogen peroxide radical scavenging activity of marine molluscan shell extract of *H. Lapponica* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 97.31% was observed at 500 µg/ml followed by 79.15% at 250 µg/ml, 70.14% at 100 µg/ml and 31.12% at 50 µg/ml respectively. The percentage inhibition of 87.73% was found for the standard ascorbic acid. The IC_{50} value of 65.1 µg/ml was noted which shows the good antioxidant activity (Figure 6).

The phosphomolybdenum scavenging assay of marine molluscan shell extract of *H.lapponica* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 93.97% was observed at 500 µg/ml followed by 81.87% at 250 µg/ml, 56.41% at 100 µg/ml and 38.90% at 50 µg/ml respectively. The percentage inhibition of 89.95% was found for the standard ascorbic acid. The IC_{50} value of 56.3 µg/ml was noted which shows the good antioxidant activity (Figure 7).

Diabetes mellitus is a group of metabolic diseases in which there are high blood sugar levels over a long period and a therapeutic approach to decrease

hyperglycemia is to inhibit the carbohydrate digestive enzyme. The carbohydrate digestive enzyme α-glucosidase is responsible for the breakdown of carbohydrates into monosaccharides for absorption. Thus natural compounds using traditional medicinal plants that could inhibit the digestive enzyme would be useful for the treatment of non-insulin diabetes.

Comparable results were obtained in the study carried out by Saratale *et al*. (2018) where *Punica granatum* AgNPs potentially inhibited the carbohydrate digestive enzyme α-glucosidase. However, the foregoing result suggests the potential usefulness of synthesized silver nanoparticles using *C. odorata* leaf extract to treat diabetes and could be considered an effective approach for diabetes care. The present study corroborates well with the above findings. The α-amylase activity of marine molluscan shell extract of *H.lapponica* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 69.71% was observed at 500 µg/ml followed by 45.28% at 250 µg/ml, 38.45% at 100 µg/ml and 19.48% at 50 µg/ml respectively. The IC_{50} value of 29.3 µg/ml was noted which shows the good antidiabetic activity (Figure 8).

Protein denaturation is a perfectly documented reason for the inflammation in conditions as rheumatoid arthritis (Viscido *et al*., 2014). The

prevention of protein denaturation is the main mechanism of action of non-steroidal anti-inflammatory drugs (NSAIDs). Adeyemo *et al*. (2022) synthesized the *C. odorata* AgNPs and studied the ability to inhibit protein denaturation (albumin) (35.62%) and its percentage inhibition is low when compared to standard aspirin (53.25%). However, the ability of the AgNPs to inhibit protein (trypsin) action (76.54%) is similar to that of the control (85.29%) which might be a result of the secondary metabolites present in the extract used to synthesize the AgNPs.

The egg albumin denaturation activity of marine molluscan shell extract of *H.lapponica* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 93.68% was observed at 500 µg/ml followed by 90.61% at 250 µg/ml, 87.19% at 100 µg/ml and 83.78% at 50 µg/ml respectively. The IC_{50} value of 21.9 µg/ml was noted which shows the good anti-inflammatory activity. Our results are confirmatory with the reports of Pretsch*et al*. (2014) and Tomer et al. (2019) (Figure 9).

The nitric oxide scavenging assay of marine molluscan shell extract of *H.lapponica* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of

61.29% was observed at 500 µg/ml followed by 46.67% at 250 µg/ml, 44.49% at 100 µg/ml and 34.48% at 50 µg/ml respectively. The IC_{50} value of 28.9 µg/ml was noted which shows the good anti-inflammatory activity (Figure 10).

Synthesis of nanoparticles using a biological agent is eco-friendly and of low cost. The calcium nanoparticles significantly showed antioxidant, anti-diabetic and anti-inflammatory activity. The outcome of the research confirms that the presence of bioactive compounds are responsible for the formation of calcium nanoparticles and also prove to be a good antioxidant, antidiabetic and anti-inflammatory agent.

8. SUMMARY

- In *H. lapponica*, the wavelength obtained around 260 nm suggested the presence of calcium nanoparticles in the solution.
- The results of FTIR analysis of this study show different stretches of bonds shown at different peaks; 3751.03, 3333.64, 2079.46, 1637.55, 1400.38, 1196.82, 1155.96, 1082.41, 1024.53, 927.13, 534.43cm-1.
- The hydrogen peroxide radical scavenging activity of marine molluscan shell extract of *H. lapponica* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml respectively. The highest percentage inhibition of 97.31% was observed at 500 µg/ml followed by 79.15% at 250 µg/ml, 70.14% at 100 µg/ml and 31.12% at 50 µg/ml respectively. The percentage inhibition of 87.73% was found for the standard ascorbic acid. The IC_{50} value of 65.1 µg/ml was noted which shows the good antioxidant activity.
- The phosphomolybdenum scavenging assay of marine molluscan shell extract of *H. lapponica* was observed at various concentrations of 500µg/ml, 250µg/ml, 100µg/ml and 50µg/ml respectively. The highest percentage inhibition of 93.97% was observed at 500 µg/ml followed by 81.87% at 250 µg/ml, 56.41% at 100 µg/ml and 38.90% at 50 µg/ml respectively. The percentage inhibition of 89.95% was found for the

standard ascorbic acid. The IC_{50} value of 56.3 µg/ml was noted which shows the good antioxidant activity.

- The α-amylase activity of marine molluscan shell extract of *H. lapponica* was observed at various concentrations of 500µg/ml, 250µg/ml, 100µg/ml and 50µg/ml respectively. The highest percentage inhibition of 69.71% was observed at 500 µg/ml followed by 45.28% at 250 µg/ml, 38.45% at 100 µg/ml and 19.48% at 50 µg/ml respectively. The IC_{50} value of 29.3µg/ml was noted which shows the good antidiabetic activity. It has noted which shows the good antioxidant activity.
- The egg albumin denaturation activity of marine molluscan shell extract of *H. lapponica* was observed at various concentrations of 500µg/ml, 250µg/ml, 100µg/ml and 50µg/ml respectively. The highest percentage inhibition of 93.68% was observed at 500 µg/ml followed by 90.61% at 250 µg/ml, 87.19% at 100 µg/ml and 83.78% at 50 µg/ml respectively. The IC_{50} value of 21.9 µg/ml was noted which shows the good anti-inflammatory activity.
- The nitric oxide scavenging assay of marine molluscan shell extract of *H. lapponica* was observed at various concentrations of 500µg/ml, 250µg/ml, 100µg/ml and 50µg/ml respectively. The highest percentage inhibition of 61.29% was observed at 500 µg/ml followed by 46.67% at 250 µg/ml,

44.49% at 100 µg/ml and 34.48% at 50 µg/ml respectively. The IC_{50} value of 28.9µg/ml was noted which shows the good anti-inflammatory activity.

9. CONCLUSION AND SUGGESTIONS

The increased interest in nanomedicine and its applicability for a wide range of biological functions demands the search for raw materials to create nanomaterials. Recent trends have focused on the use of green chemistry to synthesize metal and metal – oxide nanoparticles. Marine – derived materials, either whole extracts or pure components, are employed in the synthesis of nanoparticles due to their case of availability, low cost of production, biocompatibility and low cytotoxicity. The marine derived nanomaterials have been employed to treat infectious diseases caused by bacteria, fungi and viruses as well as treat non – infectious diseases such as tumors, cancer, inflammatory response, diabetes and support wound healing.

In the present study calcium oxide nanoparticles from the gastropd *H.lapponica* were synthesized and characterized through UV – Vis and FT – IR. And further its pharmacological activities viz., antioxidant, antidiabetic and anti-inflammatory activities were studied. The results showed good inhibitory activities.

Through significant progress has been made in the production of nanoparticles utilizing extracts from marine sources, relatively little information is known on the synthesis of nanoparticles using pure active compounds. As a

result, future research should prioritize the use of pure active compounds for nanoparticle synthesis.

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