IDENTIFICTION OF BIOACTIVE COMPOUNDS AND ANTIMICROBIAL ACTIVITY OF MARINE ECHINODERMS *PROTOREASTER LINCKII* (BLAINVILLE,1834) FROM THOOTHUKUDI COAST

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THOOTHUKUDI - 628001

April 2023

CERTIFICATE

This is to certify that this dissertation entitled, **"IDENTIFICTION OF BIOACTIVE COMPOUNDS AND ANTIMICROBIAL ACTIVITY OF MARINE ECHINODERMS *PROTOREASTER LINCKII* (BLAINVILLE, 1834) FROM THOOTHUKUDI COAST."** submitted by **S.ISWARYA, Reg No. 21APZO01** to St. Mary's College (Autonomous), Thoothukudi, affiliated to Manonmaniam Sundaranar University, Tirunelveli in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2022 - 2023 under my guidance and supervision. It is further certified that this dissertation or any part of this has not been submitted elsewhere for any other degree.

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DECLARATION

I do hereby declare that this dissertation entitled, "**IDENTIFICTION OF BIOACTIVE COMPOUNDS AND ANTIMICROBIAL ACTIVITY OF MARINE ECHINODERMS *PROTOREASTER LINCKII* (BLAINVILLE, 1834) FROM THOOTHUKUDI COAST**" submitted by me for the award of the degree of Master of Science in Zoology is the result of my original independent research work carried out under the guidance of **Dr.S.R.T. Sherly Cross., M.Sc.,M.Phil., Ph.D., Assistant Professor, Department of Zoology,** St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

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Date: 05. 4. 23 Signature of the Candidate

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INTRODUCTION

1. INTRODUCTION

Marine organisms represent excellent source for bioactive compounds (Bickmeyer et al., 2005). Approximately 7000 marine natural products, 25% of which are from algae, 33% from sponges,18% from coelenterates, 24% from representatives of other invertebrate phyla such as ascidians, opisthobranches, mollusks, echinoderms and bryozoans (Anake, 2004).

The ocean covers three-fourth of the earth's surface and forms an indispensable source of protein for human nutrition, drug discovery and development, however the oceans started to attract interest from pharmaceutical companies and research institutions only approximately 50 years ago with the pioneering work of (Bergman and fenny 1951). About 80% of all life on earth is found under ocean surface and two third of the phyla are exclusively from marine. The rich diversity of marine organisms assumes a great opportunity for the discovery of new bioactive substance. This diversity has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, fine chemicals and agrochemicals (Lohner and Staudegger, 2001).

Since 1950 the drug discovery and development field has blossomed and matured and by the end of 1997 there were 713 papers published on marine natural

products (Marine Lit., 1998). Generally the ocean meets the food requirements of humans, a far better profitability is obtained by the producing human consumables and the highest profitability is currently expected from bioactive compounds (Je. Y *et al.*, 2005; Jeon and Kim, 2002; Kim *at al.*, 2001). Owing to these reasons, this field attracted the attention of not only the chemists but also those of marine biologists, biochemist, pharmacologists, etc. The Indian scientist have screened more than 4000 marine samples (both fauna and flora) for wide spectrum of bioactivity , anticancer, bacterial, fungal, parasitic TB, viral infections as well as against dyplipidemia and diabetes. In recent years, a significant number of novel metabolites with potential pharmacological properties have been discovered from marine organisms (Fenical, 1997).

The biota of marine organism has developed unique metabolic and physiological functions that not only ensure survival in extreme habitat but also offer a potential for the production of novel enzymes and bioactive metabolites for potential exploitation. Various observation indicated that for every dozen organisms examined fewer than six have been shown to have chemical and biological characteristic, life saving drugs. The organisms from marine environment have been found possessing a vast array of new pharmaceutical compounds with novel activities that will provide new drugs leads to combat

microbial pathogens, currently developing resistance to conventional antibiotic therapies (Shanthi, 2011).

Research on bioactive compounds from marine organisms have provided the broad and better support of marine natural products – research throughout the past quarter century (Faulkner, 2005). The number of naturals produced isolated from marine organisms increase rapidly now exceeds with hundreds of new compounds being discovered every year (Faulkner, 2002 Proksh and Muller 2006). From 1960's approximately 300 bioactive marine natural products were filed and patent. Approximately 6,500 bioactive compounds were isolated from marine organisms (Kamboj, 1999). A large proposition of these natural components has been extracted from marine invertebrates, especially sponges, ascidians, and mollusus and some of them are currently in clinical trials (Proksh, *et al.,* 2002).

Antimicrobial peptides are wide spread in the living kingdom, and a large number of these molecules have been isolated from vertebrates and invertebrates (Haung*et al.,* 2002). There is evidence that those molecules are largely found in marine invertebrates especially in tissues such as the gut and respiratory organs (Chisholm and Smith, 1992).

Marine invertebrate's particularly sessile ones are the source of bioactive metabolites (Tincu and Taylor, 2004) which prevent bio-fouling and this can be

considered as kind of autogenic (Bergquist and Bedford, 1978). This mechanism has proved to be timely alternative natural medicine to human beings. Among the invertebrates, the mollusks are very good source for biomedically important products (Shenoy, 1988).

The most interesting phyla with respective pharmacologically active marine compounds include bacterial, fungi, algae, sponges, soft corals and echinoderm (Faulkner, 2000). Natural products obtained from marine sources have provided useful resource having medicinal values. When compared with the natural products obtained from terrestrial sources, marine origin also equally produces mammoth resources of novel compounds with possible pharmaceutical importance. These marine products from the marine invertebrates namely the echinoderms (sea stars) have been evolved over millions of years. The products specifically the secondary metabolites which are produced by them are an integral part of their survival tactic. The secondary metabolites are nothing but the chemical defenses produced by the marine sea star in order to protect them from the predators during attack. These chemical metabolites serve as a sole source of compounds having medicinal value for mankind.

Echinoderms have received great attention as an unexploited source of new bioactive molecules with important antimicrobial, antiviral, antiprotozoal, antifungal, and antihelminthic anticancer activities suggesting their potential

applicability for drug discovery. The peculiarities of these molecules are stability, activity at low temperature, and specificity of action. Often these molecules are part of their innate immune system. As invertebrates lacking adaptive immunity, echinoderms are an excellent model for studying innate immunity. Their defense mechanisms are mediated by cellular and humoral responses (Armirez et al., 2010).

Echinoderms represent an exceptional source of polar steroids of a immense structural diversity, showing a range of biological activities. The steroids are organic compounds which act as an integral part of the cell membrane. The steroidal components namely saponins, asterosaponins, and astropectenol are the major source of compounds abundantly found in sea stars (Malyarenko*et al.,* 2014, Guang *et al.,* 2006). A basic study of these facts reveals the search for "Drugs from the Sea" progresses at the rate of a 10 percent increase in new compounds per year (Faulkner,1995) The isolation and characterization of bioactive compounds from the sea stars in the marine ecosystem is serving a good resource for the human population to fight against the deadly diseases like cancer.

Elucidation of novel compounds from far eastern sea star *Leptasteria sochotensis* illustrated cytotoxic activity towards cancer cell lines RPMI-7951 and T-47D (Malyarenko et *al.,* 2014). Steroidal compounds and asterosaponins were isolated from cold water star fish *Ctenodiscus crispatus* and starfish *Culcitano*

vaeguineae respectively showing cytotoxicity against human carcinoma cell lines HepG2 and U87MG ensuing the apoptosis of the cells hence playing a significant role in the anti tumor chemotherapy (Tran et al.,2014,Xiang*et al.,* 2016). Steroidal compounds were elucidated from one another species of sea star *Astropecten polyacanthus* which showed cytotoxic activity against the Human cancer cell lines HL-60,PC-3 and SNU-C5(Faulkner,1995). The crude extracts of same species starfish *Astropecten polyacanthus* possessing inhibitory effects against the inflammatory components (TNF-α and IL-6) (Nguyen *et al.,* 2013).

Antimicrobial activity is a term coined as an agent that acts against the microbes either making them to be cidal or static against the microbes. The continued existence of the sea stars depends on capable antimicrobial mechanisms to safeguard themselves against microbial infections and fouling. The potent secondary metabolite produced by the sea star shows a rich source of activity against the microbes.

The coelomic cavity is the internal structure of star fish which contains coelomic fluid holding the cells of immunity and the antimicrobial peptides. Antimicrobial peptides form the first line of defenses and hence termed as the host defense peptides. They come under the innate defense response in both unicellular and multi cellular organisms (Maltseva *et al.,* 2007). They have a wide range of activity towards bacteria, fungi, viruses and parasites. Antimicrobial peptides

(AMPs) are vital immune effect of echinoderms, which lack a vertebrate-type adaptive immune system.

Ocean has plenty of organisms which are evolved with potential secondary metabolites being used as medicine (Devi *et al.,* 2011; Rajeev Kumar Jha and Xu Zi-rong, 2004). Sponges, ascidians, bryozoans and mollusks are largely used for the production of novel compounds in a larger proportion (Proksch*et al.,* 2002) but only less than 1% of the isolated compounds examined so far for pharmacological activities from the marine organisms (Fusetani,2000). The multi resistant nature of pathogens to antibiotic is the serious threat and has stimulated.

Search for novel antimicrobial agents from various natural sources (Laila Abu-Bakr *et al.,* 2012). During the last decade, there has been an increase in research on marine crustaceans, mollusks and echinoderms, particularly interest on their secondary metabolites with desirable antimicrobial properties (Casas *et al.,* 2010; Haug*et al.,* 2002). When compared with antibacterial research, little progress has been made in the development of new antifungal agents, which has been justified by the low occurrence of fungal infections. However, the current increase in incidence of fungal infections has led to aggressive research on new antifungal agents as evidenced by the rise in the number of publications since the 1960s (Maertens, 2004; Ngo et al., 2016). Another reason for the slow development of antifungal agents is the fact that fungi are eukaryotic, with a close

evolutionary relationship with human hosts, which complicates the search for antifungal targets. Nonetheless, detailed knowledge regarding the structure, composition and biochemistry of fungal cells, in addition to various facets of fungal infections, has contributed to our understanding about the mechanism of action of many antifungal agents (Borgers, 1980; Kanafani and Perfect, 2008).

Unexpectedly, echinoderms appear as untapped source in the pursuit of the identification of new and useful products (Petzelt,2005). Sea stars are benthic free living echinoderm has evolved with rich sources of bioactive metabolites such as steroidalglycosides, steroids, anthraquinones, alkaloids, glycolipids and phospholipids (De Marino *et al.,* 1997). Especially steroidal glycosides and related compounds are predominant metabolites in sea stars and have a broad variety of biological activities such as cytotoxic, hemolytic, ichthyotoxic, repellent, antineoplastic, antimicrobial, antifungal, antiviral and anti-inflammatory (Andersson *et al.,* 1989);

Sea stars are a group of invertebrates that exist in ecosystems with sea grass, coral, rocky substratum, etc., from shallow areas to the deepest regions in the ocean, at depths of approximately 6000m. There are about 1890 species of sea star. Belonging to 370 genera and 36 families of the class Asteroidea, under the phylum Echinodermata (Cintra-Buenrostro*et al.,* 2005; Mah & Blake, 2012). They are star shaped, oral-aborally flattened, free-living marine deuteron, and some are found to

be distributed ubiquitously throughout the world's oceans. Sea stars have a centrally located disc from which an arm arises and they have the power to regenerate damaged organs. Normally, most sea stars have five arms; and having either more than five arms or less than five arms is considered as abnormal. The reason for such abnormalities might be due to environmental conditions. Sea stars play a vital role in maintaining the benthic community due to their predatory activities and some of them act as key species (Menge, 1982). Hence, they are used as a model organism in areas of community structure and feeding ecology (Menge*et al.*,1999; Ortiz *et al.*,2003). Studies on the biology, or other related aspects, of sea stars are scarce from Indian waters, the sea star *Protoreaster lincki* (Blainville, 1830).

For the past 50 years antibiotics have revolutionized life saving medicine by providing cure for formerly life threatening diseases. However, strains of bacteria and fungi have recently emerged that are virtually and responsive to antibiotics such multidrug resistance arising through antibiotics misuse is now recognized as a global health problem. The situation is exacerbated by the fact no novel chemicals classes of antibiotics have been discovered for 20 years. All though many pre-existing antibiotics have been modified to yield new derivatives, bacterial have the potential to mutate resistance mechanism to combact these derivatives (Hancock, 1998).

In the past 20 years the pharmaceutical industry has been relatively successful in constrain problems due to single resistance determinates, however, the advent of multiple resistance mechanism has severely limited the effective use of major class of drugs (Chopra *et al*.,1996).

Today most infectious diseases can be brought under control with natural or synthetic drug products. We are still in great need of safer, cheaper and effective drugs. Some marine echinoderms have shown pronounced activities, useful in biomedical area. The potential of marine echinoderms as a source of biologically active products is largely and explored in India. Hence a broad waste screening of marine echinoderms for bioactive compounds is necessary. A throughout understanding of chemical structure and biological activity we lead to the formulation of novel drugs with specific action.

At this juncture, the echinoderms received attention not only for their delicacy for seafood next to fishes and crustaceans but also as suggested by (Shenoy, 1988), known for possession of bio active compounds of pharmaceutical interest. Among the different echinoderms, pro branches are proved to be excellent source of bioactive compounds. Considering all the above facts, the presence study has been undertaken to test the marine echinoderms extract against microbs to isolate and characterized the possible antimicrobial compounds from the test Echinoderms.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

In recent years, great attention has been paid to study the bioactivity of natural products, because of their potential pharmacological utilization. Marine organism is expected to serve as source of novel chemicals of pharmacological and other applications. (Riguera *et al.,* 1997), reported the antimicrobial for diseases control and growth promotion animals increases the selective pressure exerted on the natural emergence of bacterial resistance. 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. (Guillaume Mitta *et al.,* 2000), Mytilin B and MGD2, two antimicrobial peptides of marine mussels: gene structure and expression analysis. Finally, the genes encoding two isoforms of these peptides have been cloned and sequenced, revealing that both genes contain four exons and three introns.

(Lohner *et al.,* 2001), studied the development of novel antimicrobial agents: emerging strategies. Result showed the Innate immunity and the development of antimicrobial peptides based on host defences. (Chludil *et al.,* 2002), analysed Cytotoxic and Antifungal Triterpene Glycosides from the Patagonian Sea Cucumber *Hemoiedema spectabilis*. Hemoiedemoside B (2) is a new example of a small number of trisulfated triterpene glycosides from sea cucumbers belonging to the family Cucumariidae. Glycosides 1 and 2 exhibit considerable antifungal

activity against the phytopathogenic fungus *Cladosporium cucumerinum,* while the semisynthetic desulfated derivative 1a is less active.

Kelly M.S (2005), examined the echinoderms: Their Culture and Bioactive Compounds. As this review shows, there have been dramatic advances in the culture methods of sea urchins and sea cucumbers in the last 10-15 years, to the extent that one can conclude that currently the major obstacles to successful cultivation are indeed economic rather than biological. Hence the future of the echinoculture industry is closely linked to that of the fisheries, whose fate will ultimately determine the market forces that will shape this growing industry. (C Li *et al.,* 2010), Antimicrobial peptides in Echinoderms. Future studies on AMPs should be aimed in revealing how echinoderms use these AMPs in the immune response against microbial pathogens.

(Behnam *et al.,* 2013), described that aquatics are a some of bioactive compounds that these compounds have different properties such as antimicrobial activity. In this study, antibacterial activity of methanol chloroform and hexane extracts from body wall, gonads and intestine of sea sea cucumber. (Kolandhasamy Prabhu and Subramanian Bragadeeswaran 2013), Antibacterial activity of starfish *Stellaster equestris* from Southeast Coast of India. The result of the present study indicates that the crude and fractions of starfish *S.equestris* have remarkable antimicrobial activities against human bacterial pathogens. Further

fraction has been characterised by using GC-MS and 1 H and 13NMR spectroscopy analysis.

(Neda Adibpour *et al.*, 2014), Antibacterial and Antifungal Activity of *Holothuria leucospilota* Isolated From Persian Gulf and Oman Sea. The displayed effect was microbiostatic at concentrations of 1000 and 2000 µg/ml rather than microbicidal. The highest activity of hydroalcoholic extracts was exhibited by body wall, cuvierian organs and coelomic fluid against *Escherichia coli, Salmonella typhi, Staphylococcus aureus and Pseudomonas aeruginosa; Aspergillus niger, A. fumigatus, A. flavus and A. brasilensis.* However, none of the methanol, chloroform and n-haxane extracts showed appreciable effects against *Shigella dysenteriae, Proteus vulgaris, Bacillus cereus, S. epidermidis* and *Candida albicans.* Moreover, cuvierian organs did not possess any antifungal potential.

(Chellachurai *et al.*, 2015), studied that starfish are echinoderms that live among coals and occur from the supra – littoral to the hadal zone. Deviation from pentamerrism is a rare phenomenon in starfish and was observed in the red-knobbed starfish *protoreaster linck.* (Ana R. Gomes *et al.*, 2016), showed the echinoderms: A Review of Bioactive Compounds With Potential Health Effects. In this context, this chapter focuses on the phylum Echinodermata and aims at summarizing and highlighting the BCs derived from echinoderms discovered

between 2009 and 2014 with beneficial health effects, clarifying their structure, distribution, biosynthetic origin, biological activity, and when applied, their mode of action.

(Satoshi *et al.*, 2017), reported that marine invertebrates associate with diverse microorganisms even inhabit coelomic fluid CF, namely, the fluid filling the main body cavity of echinoderms. Echinoderms are a renewable resource with an economic valve due to their increasing demand as food and or source bioactive molecules exerting antitumor, antiviral, and anticoagulant, antioxidant, and antimicrobial activities. (Gerardi *et al.*, 2018), resulted the screening of Three Echinoderm Species as New Opportunity for Drug Discovery: Their Bioactivities and Antimicrobial Properties. Therefore, our findings have implications due to the ongoing explosion of antibiotic-resistant infections because of the new opportunistic pathogens and the need to discover antibacterial agents with new modes of action. Also the recorded antioxidant activity taking into account the need to find natural antioxidants useful for human health is intriguing. (Mohammad Reza Shushizadeh *et al.*, 2019), reported that preparation of the Persian Gulf *Echinometra mathaei* Organic Extracts and Investigation of Their Antibacterial Activity. The results clearly showed the high antimicrobial activity of test and spines of the Persian Gulf sea urchin extracts against various Gram-positive and Gram-negative bacteria.

(Hassan A.H. Ibrahim *et al.*, 2020), investigated the antimicrobial activity of the sea star (*Astropecten spinulosus*) collected from the Egyptian Mediterranean Sea, Alexandria. Regarding to investigating the efficacy of some commercial antibiotics (mm), data confirmed that the Gram-negative bacteria were more resistant than Gram-positive bacteria. On the other side, the result of GC-MS/MS of crude extract observed the presence of several bioactive constituents, most of which had antimicrobial activities. (Sukmiwati *et al.*, 2020), reported by antibacterial activity of sea cucumber (*Holothuria astra*) against *Pseudomonas aeruginosa*. The results showed that methanol extract of sea cucumber contained terpenoids, saponins, and phenolic. The diameter of methanol extract inhibition zone at 1 mg concentration of 12.25±0.05 mm and of three types of fractions used hexane fraction had a more dominant antibacterial potency with diameter inhibition zone at 1 mg concentration of 14.61±0.02 mm. Based on the results of research that the methanol extract and hexane fraction of sea cucumber has potential as an antibacterial.

(Sumitha *et al.*, 2022), reported the in vitro Antimicrobial Evaluation of an Isolated Compound from Sea Star *Stellaster equisteris*. The evaluation of antimicrobial susceptibility by tube dilution and well diffusion assay indicated that the isolated purified compound from the sea star *Stellaster equestris* was reported to be evident for all the above-mentioned concentrations by a marked zone of

clearance. A dose-dependent increase was observed in the tube dilution method. Therefore compounds possess antimicrobial activity and can be further subjected for developing the compound as a potent antimicrobial drug.

OBJECTIVES

3. OBJECTIVES

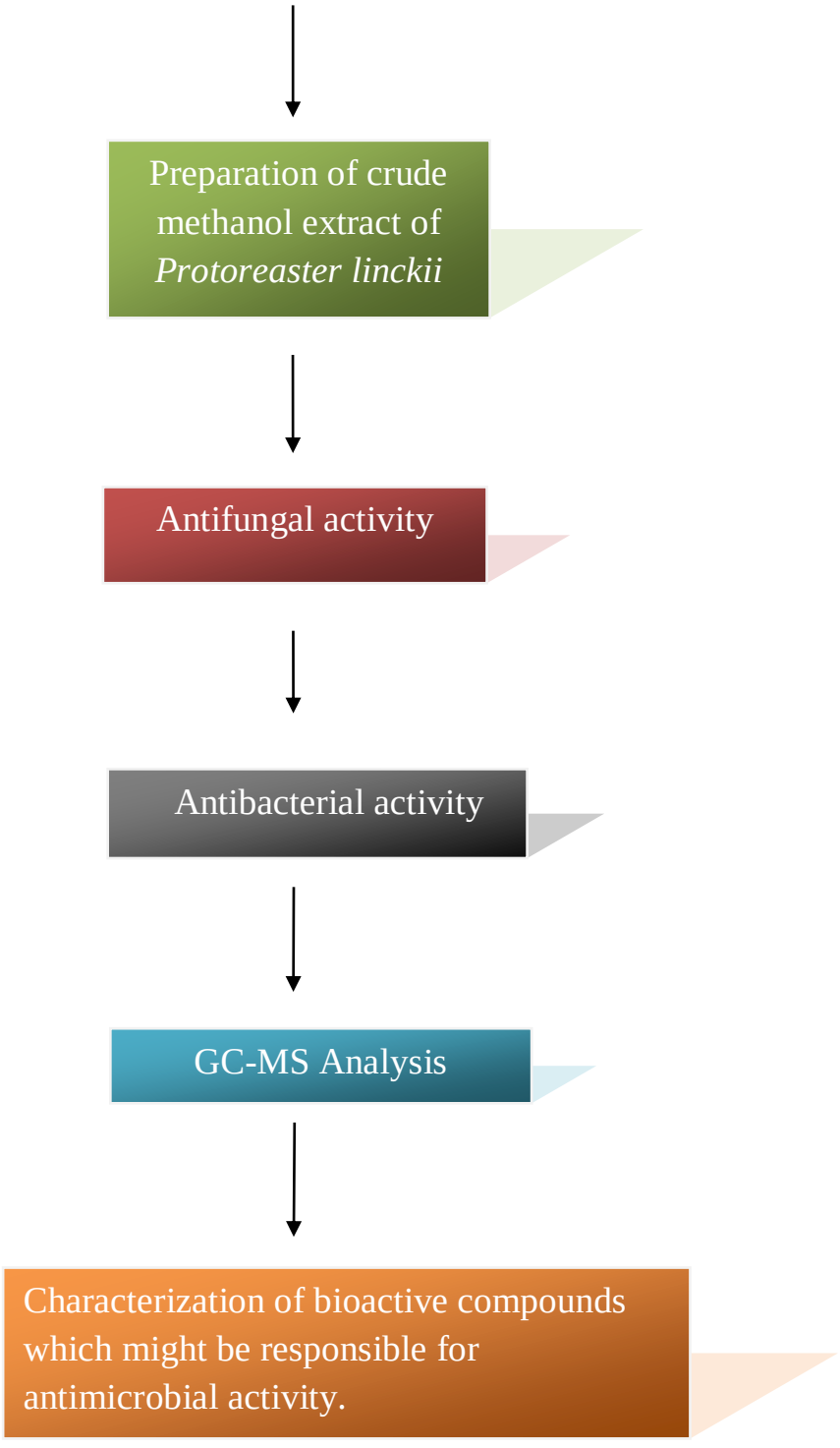
Importance of bioactive substance in the present scenario triggered to carry out this present work with the following objectives.

- To collect the probable bioactive extracts from the body tissue of *Protoreaster linckii*.
- To test the antibacterial activity against pathogenic bacteria.
- To test the antifungal activity against pathogenic fungi.
- To find out the bioactive compounds of the extract by GC-MS analysis.

EXPREMENTAL DESIGNS

4. EXPREMENTAL DESIGNS

Collection of *Protoreaster linckii* from the Gulf of Manner coastal region of Thoothukudi.



MATERIALS AND METHODS

5. MATERIALS AND METHODS

5.1 SYSTEMATIC POSITION OF EXPERIMENTAL ANIMAL

Protoreaster linckii

Phylum : Echinodermata

Class : Asteroidean

Order : Valvatida

Family : Oreasteridea

Genus : *Protoreaster*

Species *: P.linckii*

The red knobbed starfish is a species of starfish best known for its noticeable traffic cone like knobs are very distinguishing. They live in a Indian ocean as well as the eleven armed starfish the red, knobbed star fish can be found in a few other tropical waters, like other starfish, the red knobbed starfish can do major damage to coral reef, but this little, harmful Echinoderm is crucial to the marine food.

EXTERNAL:

Each red knobbed starfish has at least five arms some have less and some have many more. The one truly amazing physical aspect is on its dorsal side. The

red knobbed starfish has multiple red knobs throughout the body. These red knobs are connected to each other by a surfacing. Red knob like wiry materials which make the starfish like a grid. On the ventral side connected to the radial canals, there are thousands of little tube feet which act like a suction cup. These hundreds of tube feet allow the starfish to stick to the side of rocks, and move as it likes throughout the sea.

At the end of each arm, these are a photoreceptor eyespot. This eye spot allows the starfish to see the different between light and dark, and detect movement of the currents of the ocean and possible predators.

INTERNAL:

The internal anatomy of the red knobbed starfish is the same as all other starfish types. All starfish have two stomachs, a cardiac stomach and a pyloric stomach the cardiac stomach is at the very center of the red knobbed starfish and helps with eating mollusks like clams and mussels. Once the red knobbed starfish has a clam in its arms, it pries the mollusks shell open and releases its stomach into the shell. Once the mollusks are dead the stomach sucks up the animal, brings in its stomach and leaves the empty shell on the ocean floor. When he red knobbed starfish digests the mollusks, it goes to the anus to be expelled into the sea.

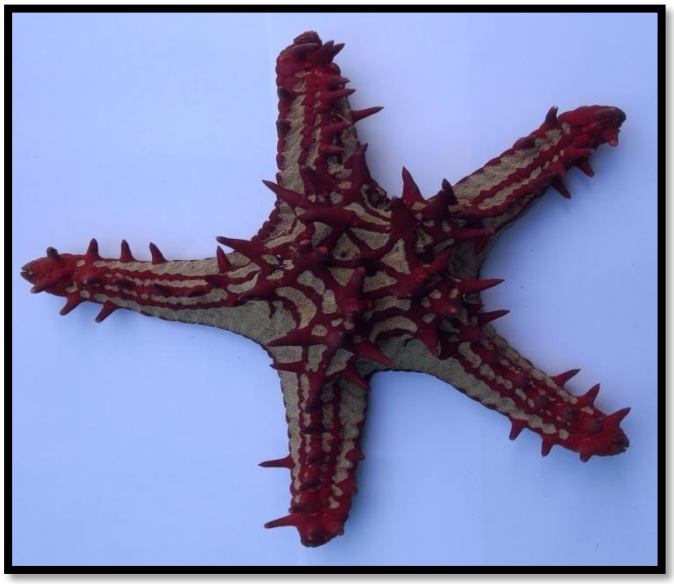
P.linckii grows to a maximum diameter of 12 in (30cm). It has numerous tubercles located along its five arms. These tubercles are bright red and extend upwards from the arms. It has a gray body with red and extends upward from the arms. It has a gray body with red stripes that connect the tubercles. This creates an appearance of a grid made of interconnecting wires.

The skeleton is composed of many calcareous ossicles and spicules. They are located inside the layer of connective tissues. This skeleton supports the larger central disk.

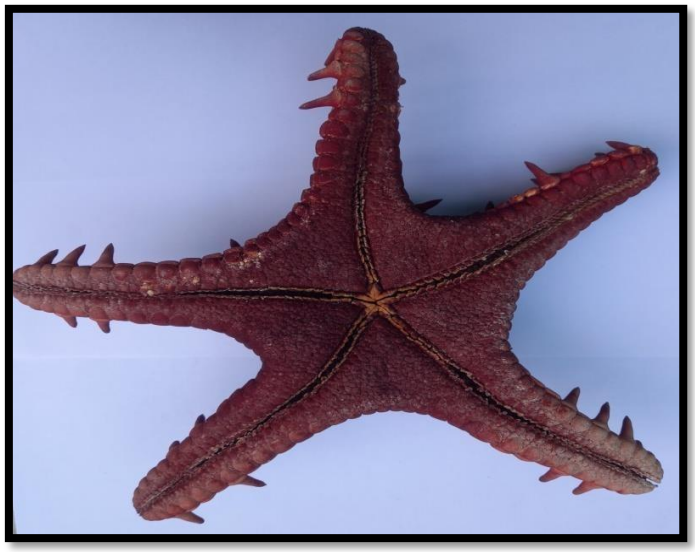
PLATE 1: *PROTOREASTER LINCKII*

STUDY ANIMAL

DORASAL



VENTRAL



5.2 STUDY AREA

Species of *Protoreaster linckii* used in the present study were collected from Gulf of Manner coastal region. Gulf of Manner is situated along the south east coastal of India. This area is remarkable for its richness and variety of fauna and the inshore sea bottom which forms an ideal habitat for the growth of the shell fishes which sustains a good fishery. The Indian part of gulf of manner covers approximately an area of 10,500 km^2 along at $8^0$35 - $9^0$25n long $78^0$08 – $79^0$30E.

It is apart 0f the southward extension of Bay of Bengal, it means in the Indian Ocean. This geographical area runs from Pamban Island including Rameshwaram to cape comarin to a distance of 170 nautical miles. This coastal contains a rich biological diversity of flora largely due to diversified microhabitats such as mangroves, corals, seaweeds, sea grasses, sandy, rocky and muddy shores etc. The faunal diversity is also well pronounced with reference to different echinoderms groups.

Specimens of *Protoreaster linckii* were collected during low tides from the sea in their natural habitat that is intertidal zone and from reefs by divers, brought to the laboratory and maintains under laboratory conditions for further observations.

Figure : 1 MAP SHOWING THOOTHUKUDI SHORE,

THE GULF OF MANNAR

5.3 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.

1. AGAR- WELL DIFFUSION METHOD

a. 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 (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).

5.4 ANTIBACTERIAL ACTIVITY

In the present study whole body tissue extract of *Protoreaster linckii* 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 56^0c for 48hours and used for further studies.

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 10days at room temperature. After filtration with whatmann no1 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 0^0c for further use.

ANTIBACTERIAL ASSAY:

Antibacterial activity of the extract of *Protoreaster linckii* was determined against ten bacterial strains viz, *Pseudomonas, Vibrio cholera, Esherichia coli, Steptococus sps, Salmonella typhi* these pathogens were obtained from the microbiology department of Sri Paramakalyani College, alwarkuruchi.

PREPARATION OF BACTERIAL CULTURE

Nutrient broth medium was prepared and sterilized in an autoclave at 151b pressure for about 30 minutes ten bacterial species were inoculated in the nutrient broth and incubated at 28 2^0c for 24 hours. Nutrient agar medium was also prepared, autoclaved and transfer aseptically into sterile petridishes 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 *Protoreaster linckii.*

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 testing at different concentration viz. The more potent fraction was characterized to know the functional groups through GC-MS study at Indian institute of crop processing technology, Tanjore.

5.5 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 µg/ml composed of 5% diphenyl 95% diphenyl poly giloxane), fopetating 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 0.5s and fragments from 45to450Da.

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 component found in the body tissues of *Protoreaster linckii* 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.

RESULT

6. RESULT

6.1 ANTIFUGAL ACTIVITY OF *PROTOREASTERT LINCKII*

The methanol extracts of *Protoreastert linckii* showed antifungal activity against all the pathogen strain which were concentration dependent. The highest inhibition zone ranging from 9mm to 5mm. The highest activity of 9mm zone was recorded against *Aspergillus niger* at positive control concentration (Amphotericin B), where as a minimum of 7mm inhibition zone was observed against *Aspergillus niger* 500 µg/ml concentration and lowest activity of 5mm was showed at 250 µg/ml concentration There was no activity observed at 100 µg/ml and 50µg/ml concentration. (Table 1) (fig 2) (plate 2.1).

The methanol extracts of *Protoreastert linckii* showed the highest inhibition zone ranging from 8mm to 4mm.. The highest activity of 8mm zone was recorded against *Sporothrix schenckii* at positive control concentration, where as a minimum of 5mm inhibition zone was observed against *Sporothrix schenckii* 500 µg/ml concentration and lowest activity of 4mm was showed at 250 µg/ml concentration. There was no activity observed at 100 µg/ml and 50µg/ml concentration. (Table 1) (fig 3) (plate 2.2).

TABLE 1

ANTIFUNGAL ACTIVITY OF *PROTOREASER LINCKII*

S.NO	Name of the test organisms	Zone of inhibition (mm)				
		500 µg/ml	**250 µg/ml**	**100µg/ml**	**50 µg/ml**	**PC**
1.	*Aspergillus niger*	7mm	5mm	0	0	9mm
2.	*Sporothrix schenckii*	5mm	4mm	0	0	8mm

FIG 2 : ANTIFUNGAL ACTIVITY OF *ASPERGILLUS NIGER* OF *PROTOREASTER LINCKII*

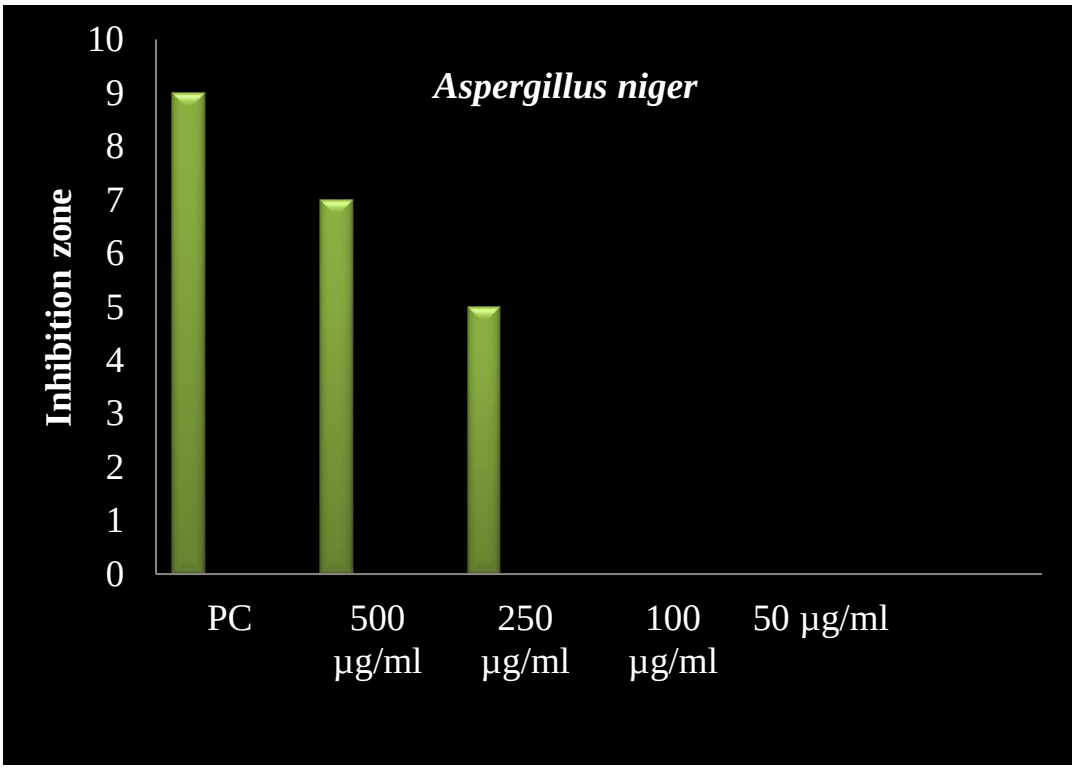


FIG 3 : ANTIFUNGAL ACTIVITY OF *SPOROTHRIX SCHENCKII* OF *PROTOREASTER LINCKII*

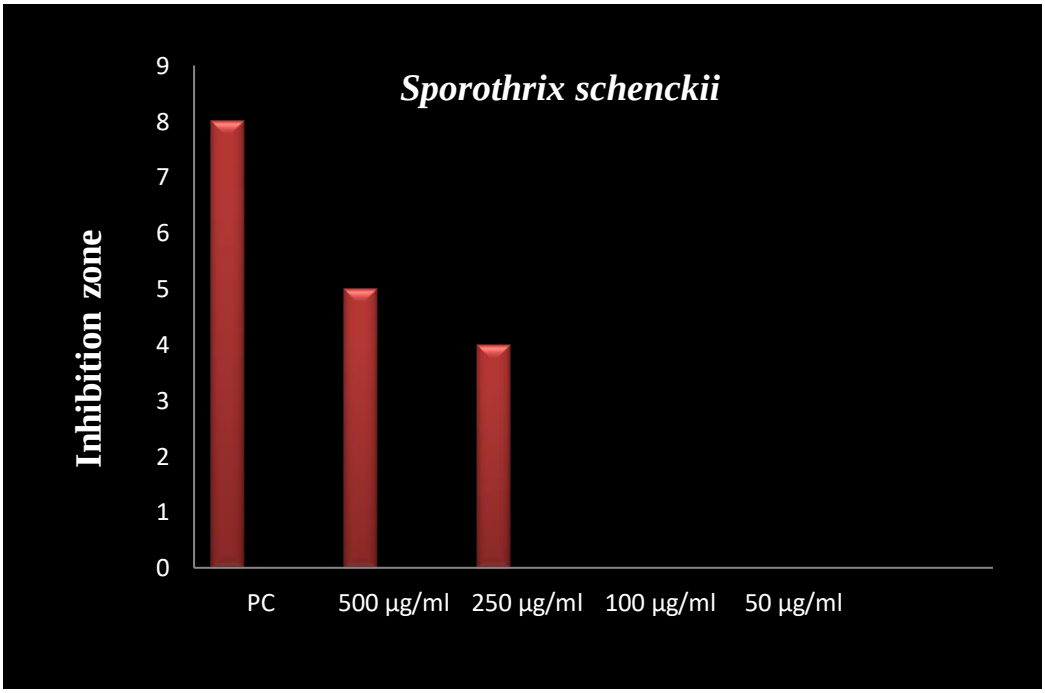
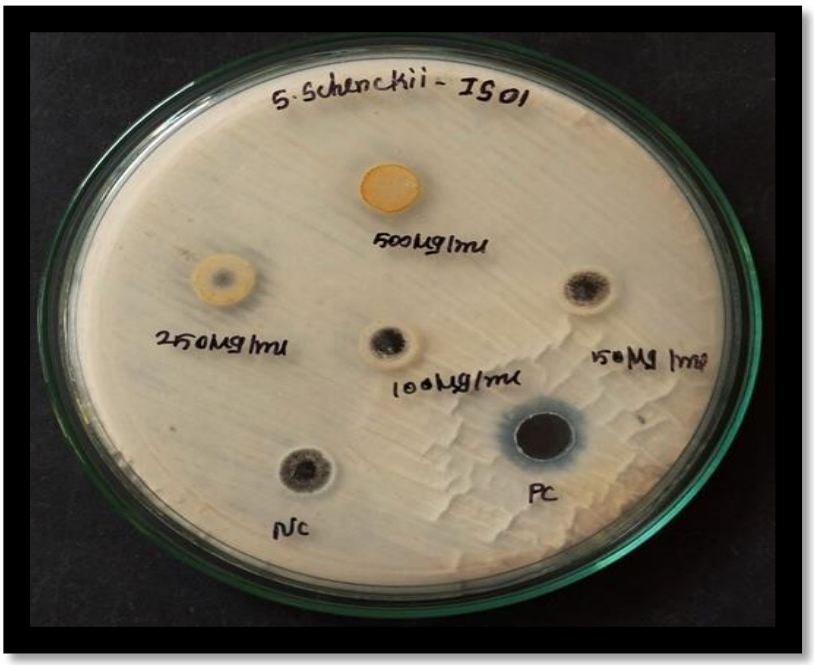


PLATE -2.1. ANTIFUNGAL ACTIVITY OF *ASPERGILLUS NIGER* OF *PROTOREASTER LINCKII*

PLATE -2.2. ANTIFUNGAL ACTIVITY OF *SPOROTHRIX SCHENCKII* OF *PROTOREASTER LINCKII*



6.2 ANTIBACTERIAL ACTIVITY OF PROTOREASTER LINCKII

Antibacterial activity of Methanol, benzene, hexane, chloroform and distilled water (aqueous extract) of the whole body tissue of *Protoreaster linckii was* tested against five bacterial pathogens *Pseudomonas*, *Escherichia coli*, *Salmonella typhi*, *Vibrio cholera*, *Streptococcus sps* in (table 2) (fig 4-8) (plate 3.1-3.5). The level of activity was measured by inhibition zones. The extracts developed different zones of inhibitions at different concentrations.

The methanol extract of *Protoreaster linckii* showed activity with the inhibition zones ranging from 0. 2mm to 0. 5mm. The highest activity of 0.5mm zone was recorded against *Pseudomonas* at 100mg/ml concentration , whereas a minimum of 0.3mm inhibition zone was observed against *Pseudomonas* at 50mg/ml concentration and very negligible activity of 0.2mm was showed at 10 mg/ml concentration.

The benzene extract of the *Protoreaster linckii* showed the activity with the inhibition zones ranging from 0.2mm to 0.5mm. The highest activity of 0.5 mm was observed against *E.coli* at 100mg/ml concentration and minimum activity of 0.3 mm was recorded against *E. coli* at 50 mg/ml

concentration. And lowest activity of 0.2mm was recorded at 10mg/ml concentration of benzene extract.

Hexane extract of *Protoreaster linckii* showed activity by developing the zones of inhibition ranging from 0.1mm to 0.4mm. The highest activity showed 0.4mm at 100mg/ml. The minimum activity of 0.2mm at 50mg/ml concentration and the lowest activity showed in 0.1mm noted against *Salmonella typhi.*

Chloroform extract of *Protoreaster linckii* showed activity with the inhibition zones ranging from 0.2mm to 0.4mm. The highest activity showed 0.4mm at 100mg/ml concentration. And minimum activity of 0.3mm at 50mg/ml concentration recorded against *Vibrio cholera* and very negligible activity was showed in 0.2mm against *vibrio cholera.*

Distilled water (aqueous extract) of *Protoreaster linckii* showed activity with the inhibition zones ranging from 0.1mm to 0.4mm. the highest activity of 0.4mm at 100mg/ml concentration against *Streptococcus sps*. The minimum of 0.3 against *Streptococcus sps* at 50mg/ml concentration respectively and very lowest activity of 0.1mm noted against *Streptococcus* at 10mg/ml concentration. Of the three concentrations of extracts tested in the present study 100mg/ml showed more potent activity

than the 50mg/ml and 10mg/ml. The zone of inhibition increased with increased in concentrations of all the extracts tested in the present study. Methanol and benzene extract showed maximum activities against all the pathogens tested. Hence the methanol extract of *Protoreaster linckii* 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 2

ANTIBACTERIAL ACTIVITY OF *PROTOREASTER LINCKII*

S.NO	EXTRACT	PATHOGENS	10µg/ml	50µg/ml	100µg/ml
1	Methanol	*Pseudomonas*	0.2mm	0.3mm	0.5mm
2	Benzene	*Escherichia coli*	0.2mm	0.4mm	0.5mm
3	Hexane	*Salmonella typhi*	0.1mm	0.2mm	0.4mm
4	Chloroform	*Vibrio cholere*	0.2mm	0.3mm	0.4mm
5	Distilled water	*Streptococcus sps*	0.1mm	0.3mm	0.4mm

FIG 4: ANTIBACTERIAL ACTIVITY OF METHANOL EXTRACT OF *PROTOREASTER LINCKII*

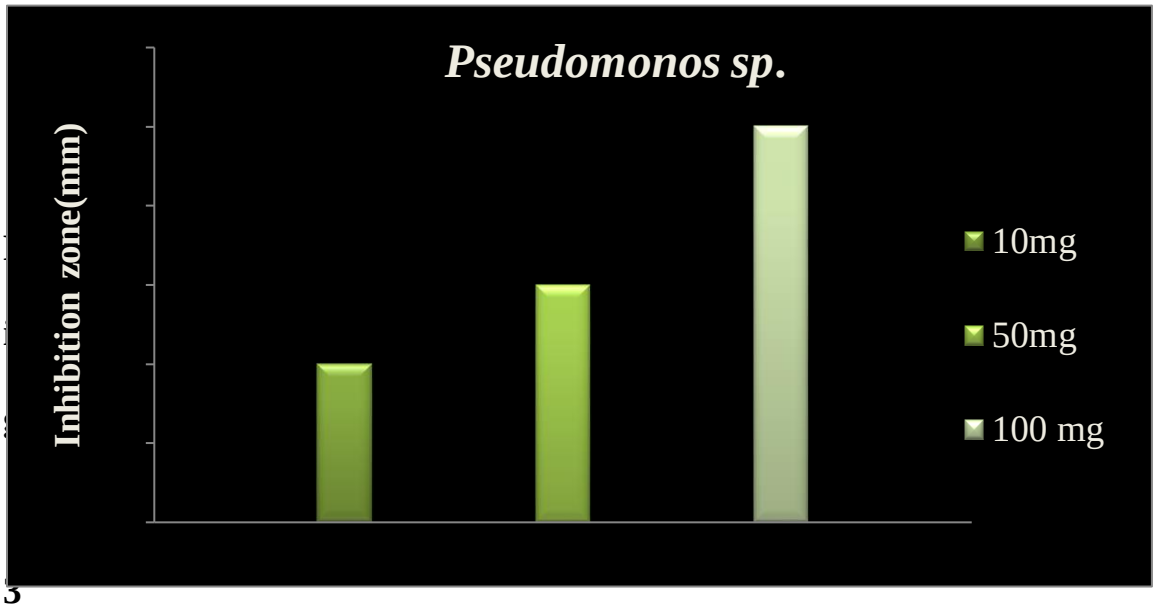


FIG 5: ANTIBACTERIAL ACTIVITY OF BENZENE OF *PROTOREASTER LINCKII*

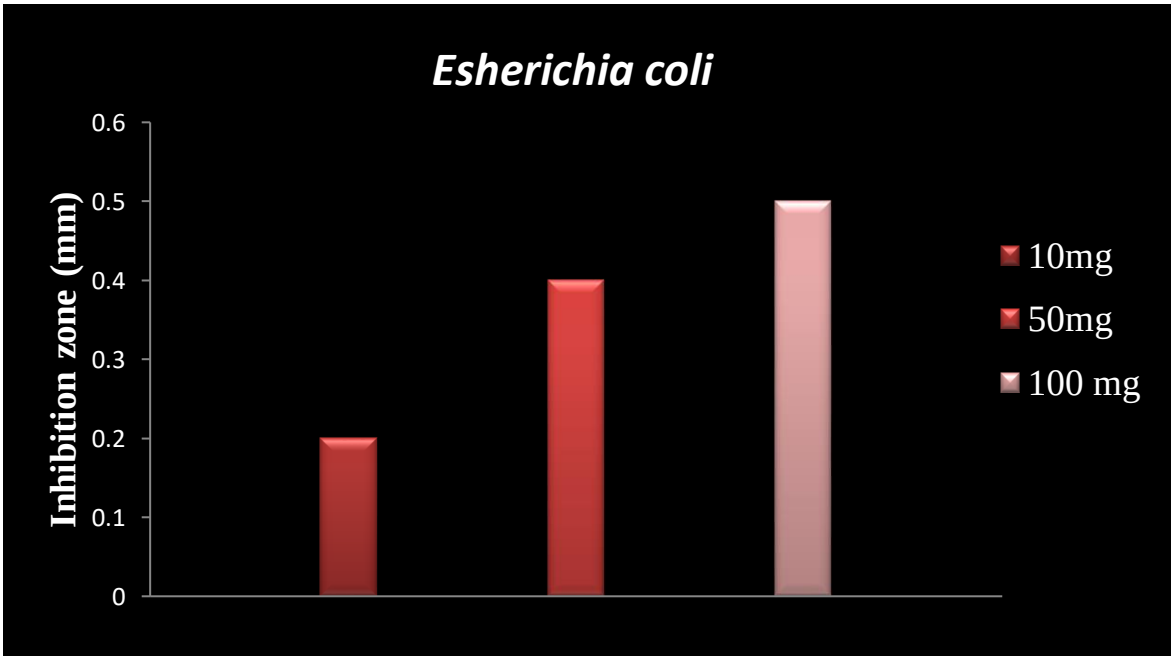


FIG 6: ANTIBACTERIAL ACTIVITY OF HEXANE EXTRACT OF *PROTOREASTER LINCKII*

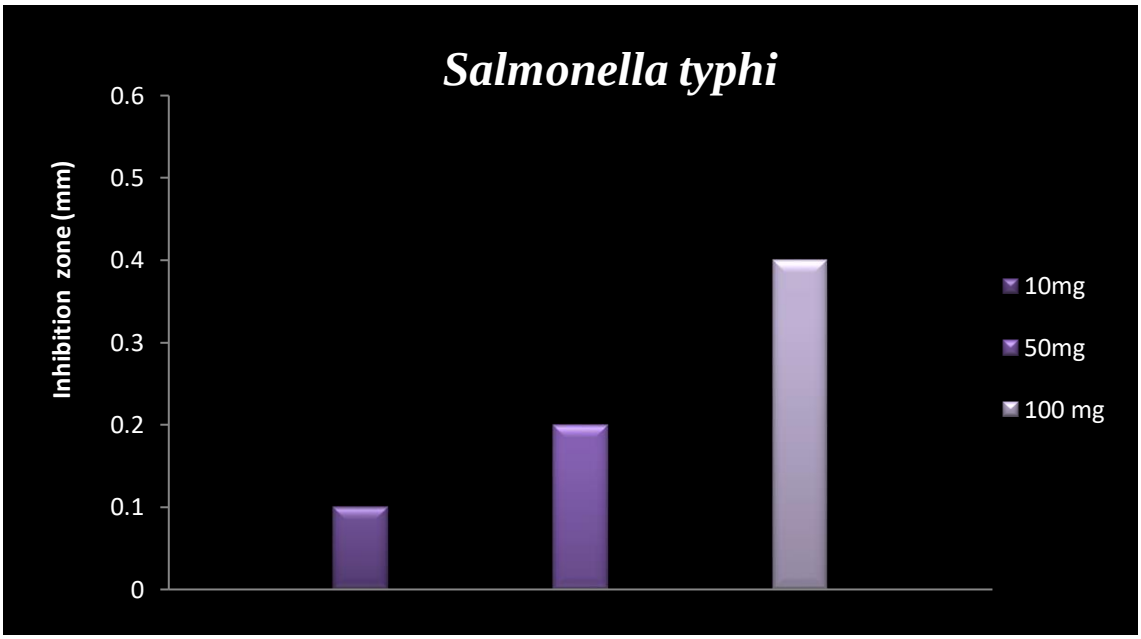


FIG 7: ANTIBACTERIAL ACTIVITY OF CHLOROFORM EXTRACT OF *PROTOREASTER LINCKII*

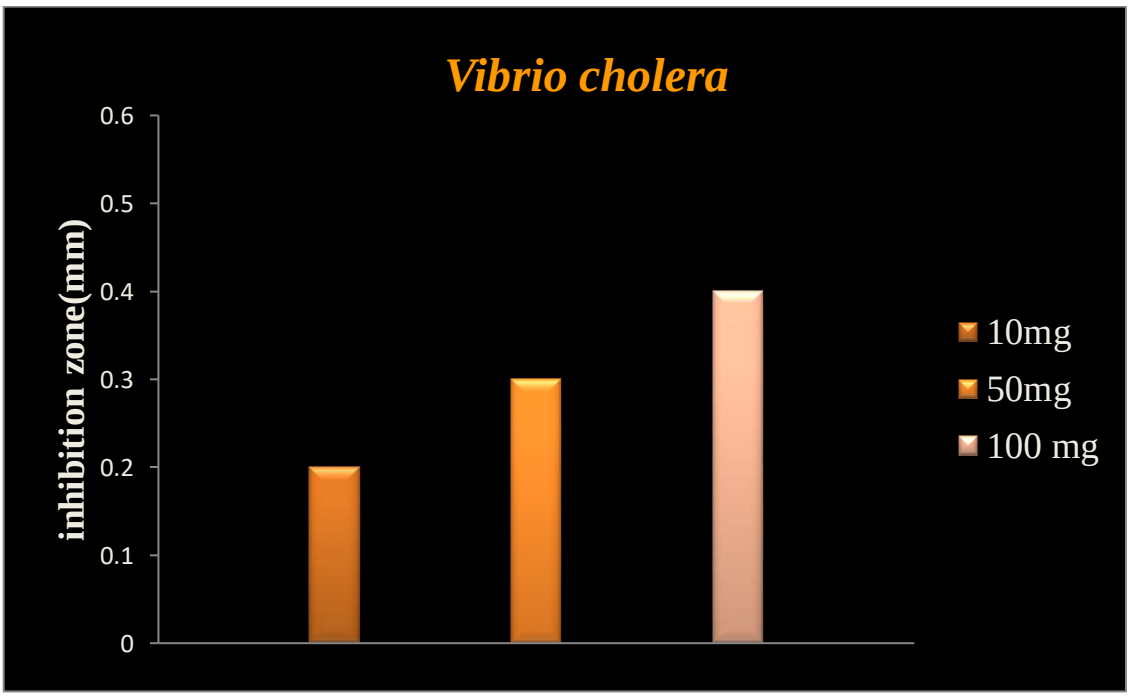


FIG 8: ANTIBACTERIAL ACTIVITY OF DISTILLED WATER EXTRACT OF *PROTOREASTER LINCKII*

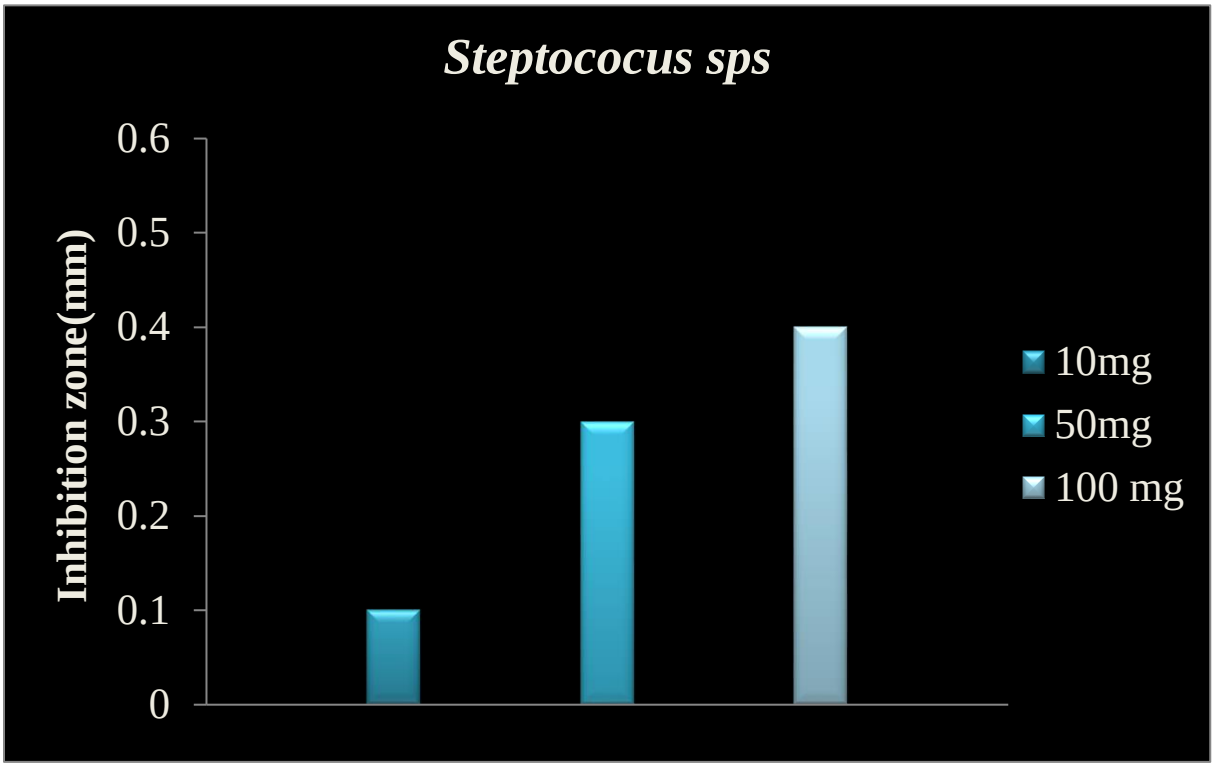


PLATE -3.1. ANTIBACTERIAL ACTIVITY OF CRUDE METHANOL OF *PROTOREASTER LINCKII*

A) Pseudomonas

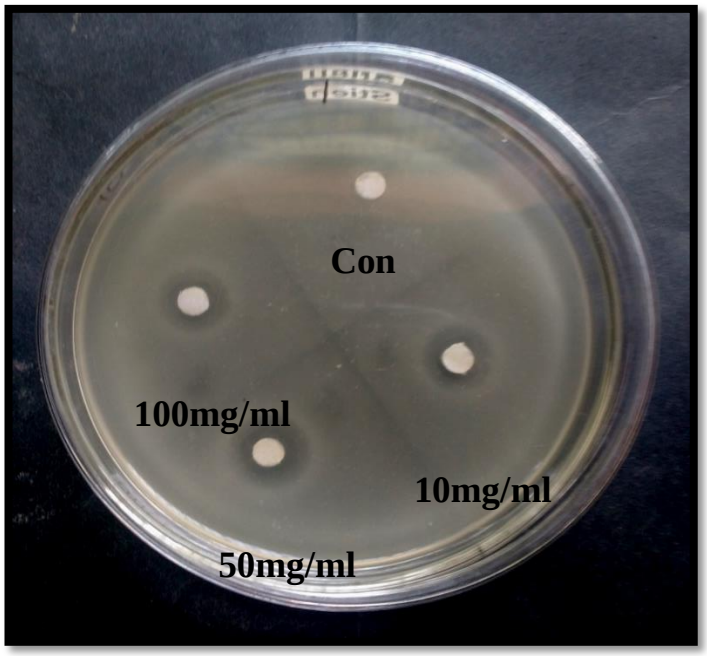


PLATE- 3.2. ANTIBACTERIAL ACTIVITY OF CRUDE BENZENE OF *PROTOREASTER LINCKII*

B) Escherichia coli

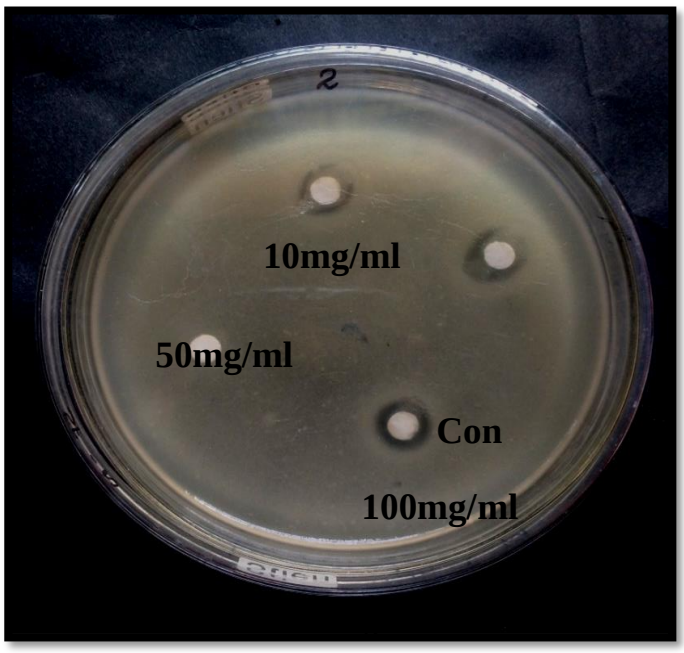


PLATE-3.3.ANTIBACTERIAL ACTIVITY OF CRUDE HEXANE OF *PROTOREASTER LINCKII*

C) Salmonella typhi

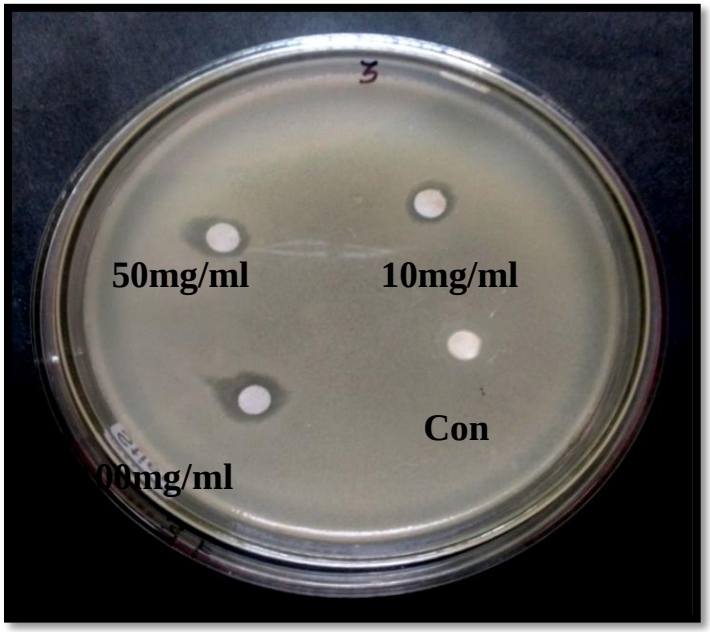


PLATE- 3.4. ANTIBACTERIAL ACTIVITY OF CRUDE CHLOROFORM OF *PROTOREASTER LINCKII*

D) Vibrio cholera

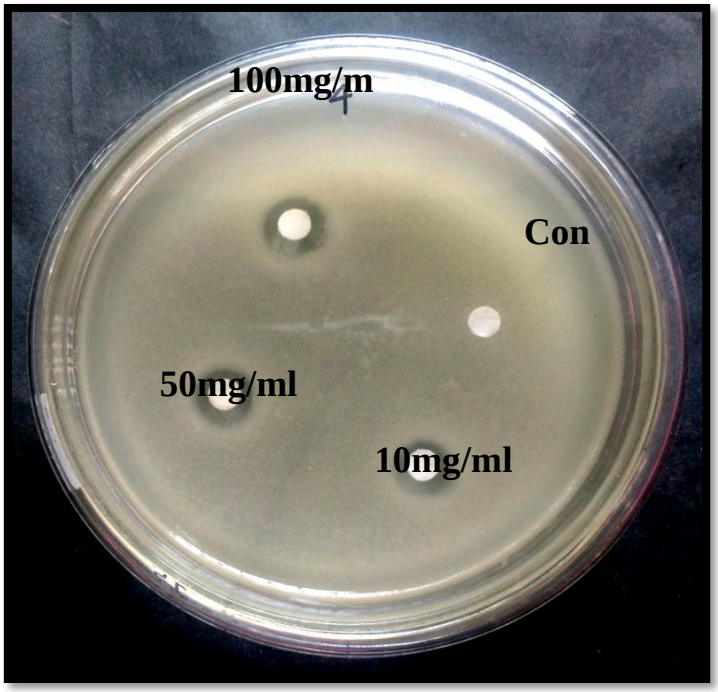
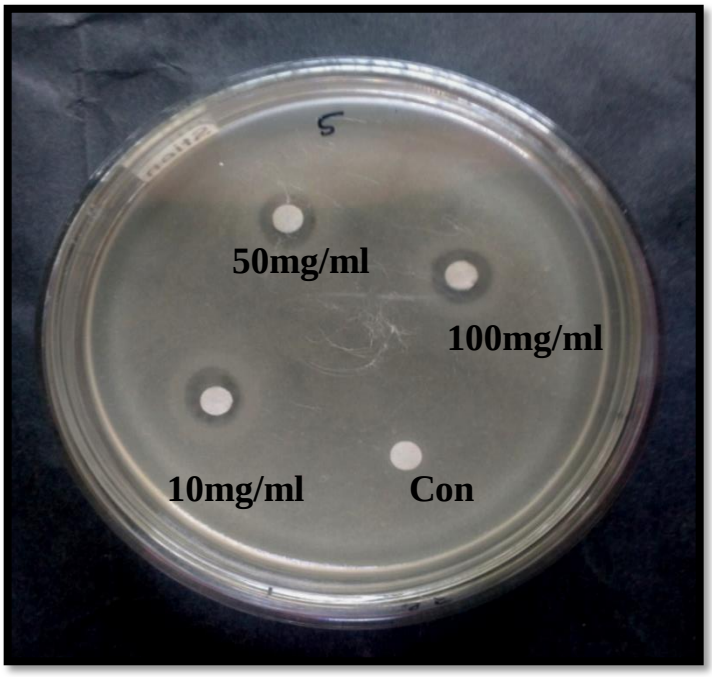


PLATE- 3.5. ANTIBACTERIAL ACTIVITY OF CRUDE DISTILLED WATER OF *PROTOREASTER LINCKII*

E) Streptococcus sps



6.3 GC-MS Analysis:

Methanol extraction the of the whole body tissue of *Protoreaster linckii* showed significant antimicrobial activity. Hence this fraction was subjected to GC-MS analysis to characterise the compounds responsible for antimicrobial activities. (Table 3) (fig 9).

GC-MS analysis of body tissue of *Protoreaster linckii* exhibited 6 peaks, with retention times ranging from 11.692 to 17.818. All the six compounds were characterised as n-Hexadecanoic acid, Octadec-9-enoic acid, Phenylephrine, N-Methyl-1-adamantanea cetamide, 2(Acetoxymethyl 1)-3-(methoxycarbonyl) biophenylene, Benzene 2-(tert-butydimethylsilyl)oxyl)-1-isopropyl-4-methl. (Table 3) (fig 9 to 15).

Among the identified compounds n-Hexadecanoic acid have the role of Antioxidants, Hypocholesterolemic, Nematicide, and Pesticide (fig 10). Octadec-9-enoic acid it act as Cancer preventive, Hypocholesterolemic, Anti-inflammatory(fig 11). Phenylephrine has the part of Antioxidants, Anti cancer (fig 12). N-Methyl-1-adamantanea cetamide have role in major derivative of bael tree ethanol leaf extracts againts dengue mosquito vector and their biosafety on natural predators (fig 13). 2(Acetoxymethyl 1)-3-(methoxycarbonyl)biophenylene function as Antireflective coating polymers can be used reduce out gassing(fig 14).

Benzene 2-(tert-butydimethylsilyl)oxyl)-1-isopropyl-4-methl have role in biological activity and pharmacological activity (fig 15). These compounds constitute a 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 as certain needs bioactivity.

TABLE 3

ACTIVITY OF COMPONENTS IDENTIFIED IN THE METHANOL, EXTRACT OF TISSUE OF *PROTOREASTER LINCKII* BY GC-MS TABLE 3

No	RT	Compound Name	Molecular Formula	MW	Peak area %	Compound Nature	Activity
1	11.692	n-Hexadecanoic acid	C16H32O2	256.42	14.75	Palmitic acid	Antioxidants, Hypocholesterolemic, Nematicide, and Pesticide
2	13.361	Octadec-9-enoic acid	C18H34O2	282.5	32.07	Fatty acid	Cancer preventive Flavor Hypocholesterolemic Anti-inflammatory
3	13.563	Phenylephrine	C9H13NO2	167.20	9.19	Hydrochloride salt	Antioxidants, Anticancer
4	15.041	N-Methyl-1-adamantanea cetamide	C13H21NO	207.31	21.71	Oleic acid	Major derivative of bael tree ethanol leaf extracts againts dengue mosquito vector and their biosafety on natural predators.
5	15.276	2(Acetoxymethyl 1)-3-(methoxycarbonyl)biophenylene	C17H14O4	282.29	13.31	Methy3(cacetyloxy)methyl)-2-biophenylenecarboxylate	Antireflective coating polymers can be used reduce out gassing.
6	17.818	Benzene 2-(tert-butydimethylsilyl)oxyl)-1-isopropyl-4-methl	C16H24O2	248.36	8.97	Thymol	Biological activity, Pharmacological activity.

Fig:9 CHROMATOGRAM OF COLUMN EXTRACT OF *PROTOREASTER LINCKII* BY GC - MS

GC-MS/MS Chromatogram

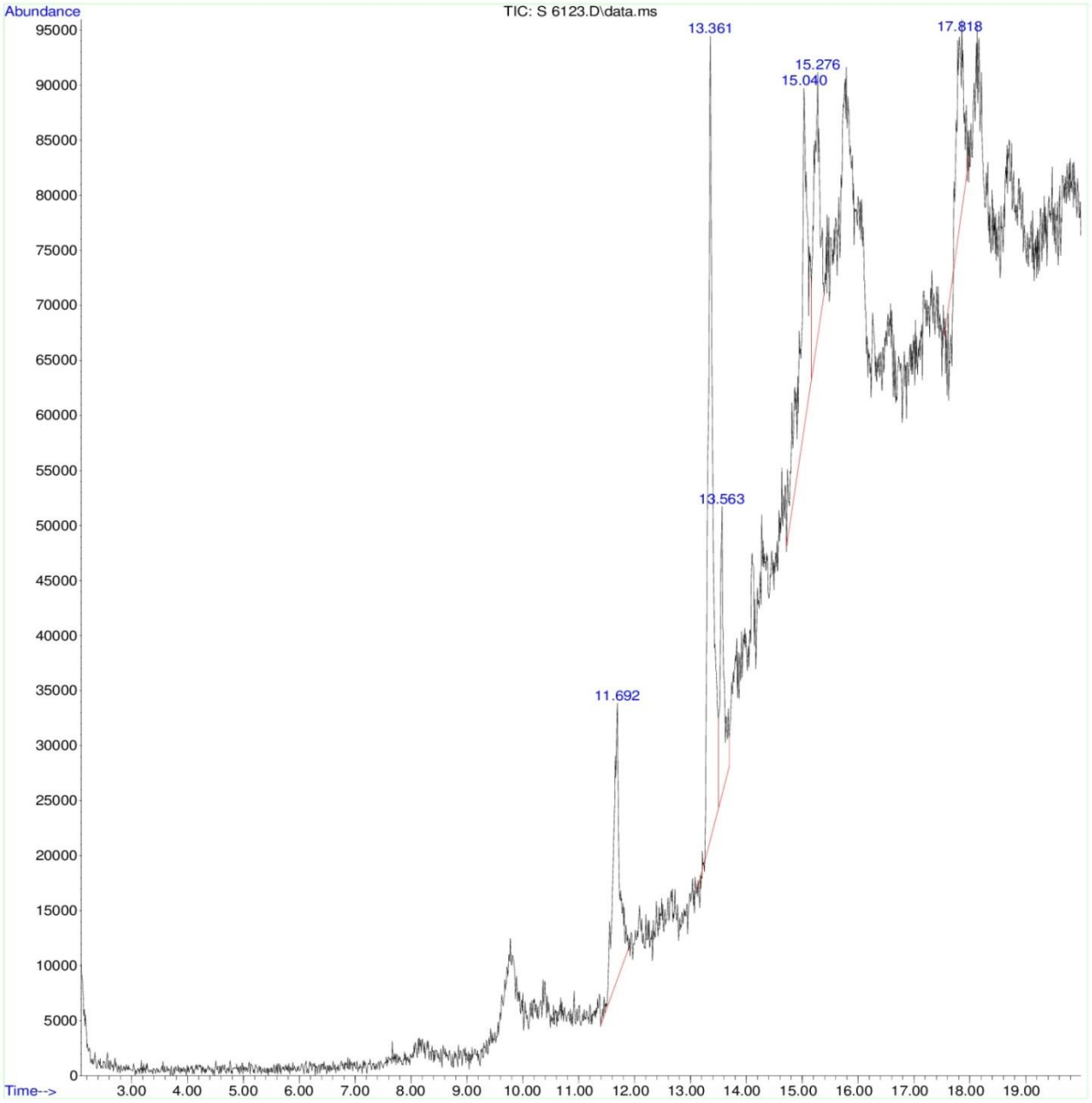


Fig : 10

Name: n-Hexadecanoic acid

Formula : C16H32O2

MW : 256.42

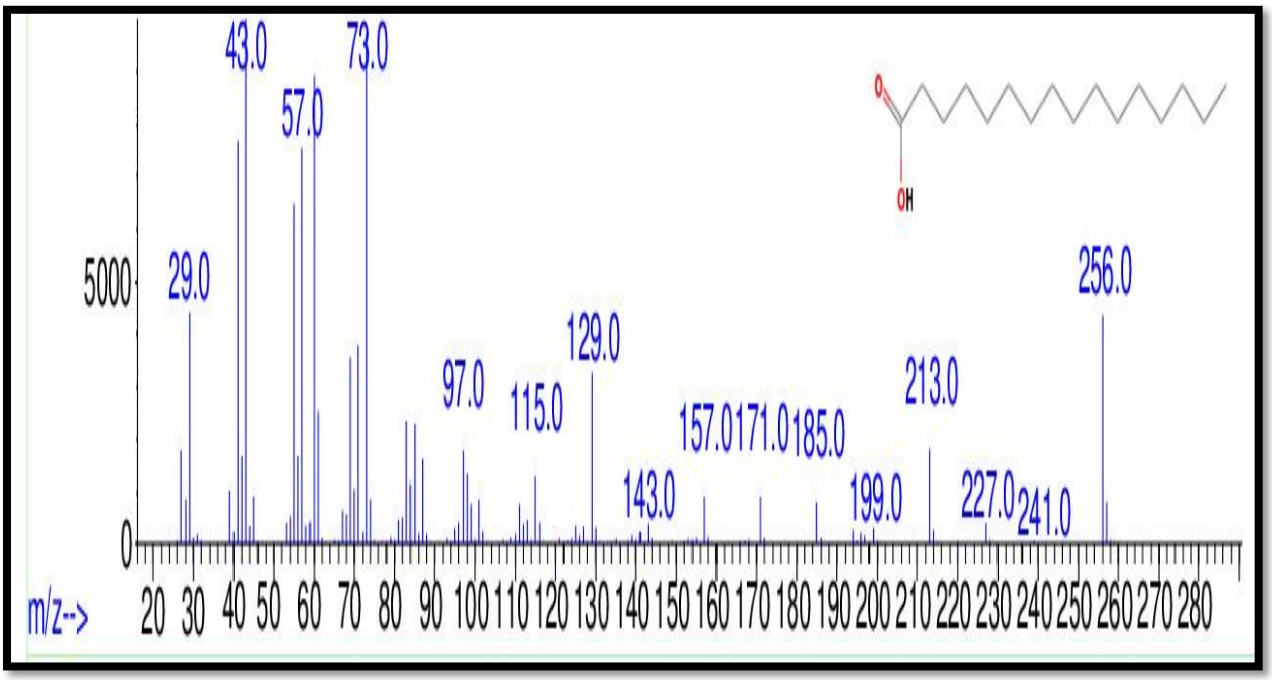


Fig : 11

Name: Octadec-9-enoic acid

Formula : C18H34O2

MW : 282.5

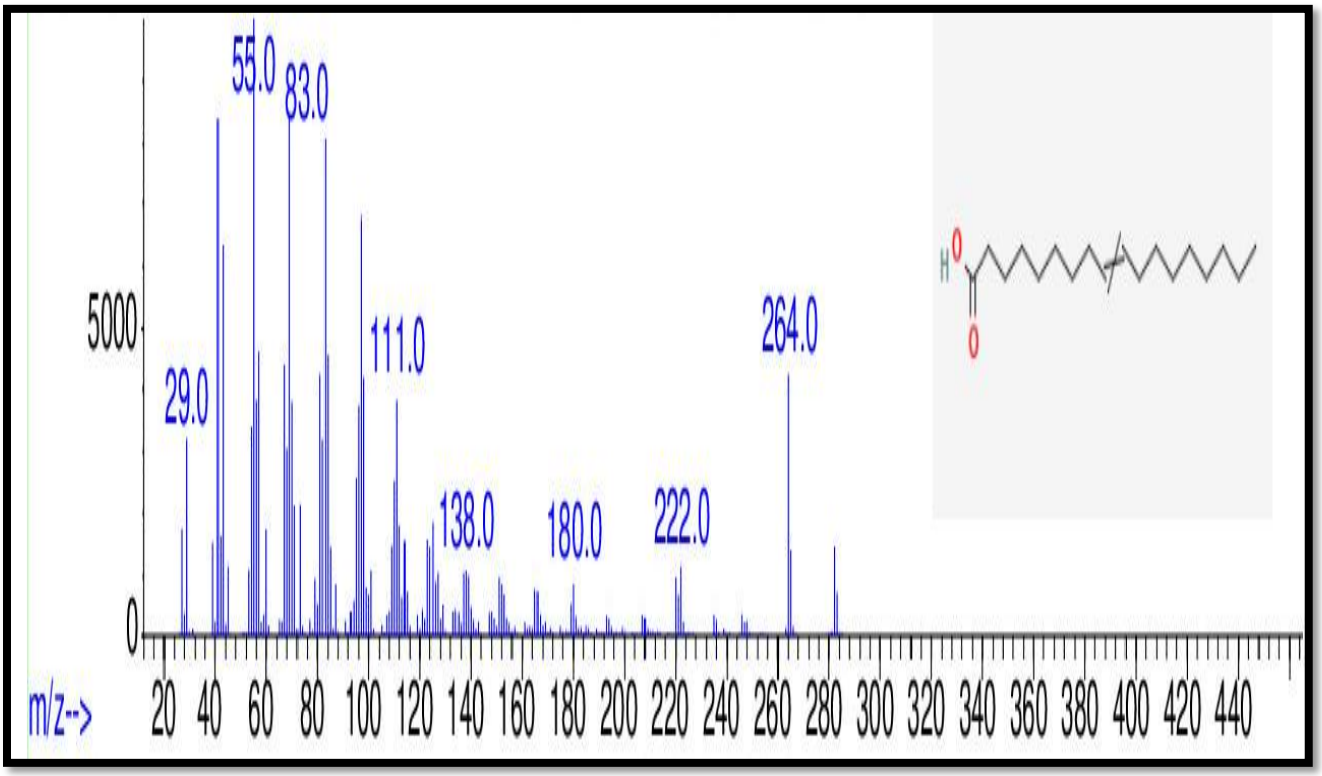


Fig : 12

Name: Phenylephrine

Formula : C9H13NO2

MW : 167.20

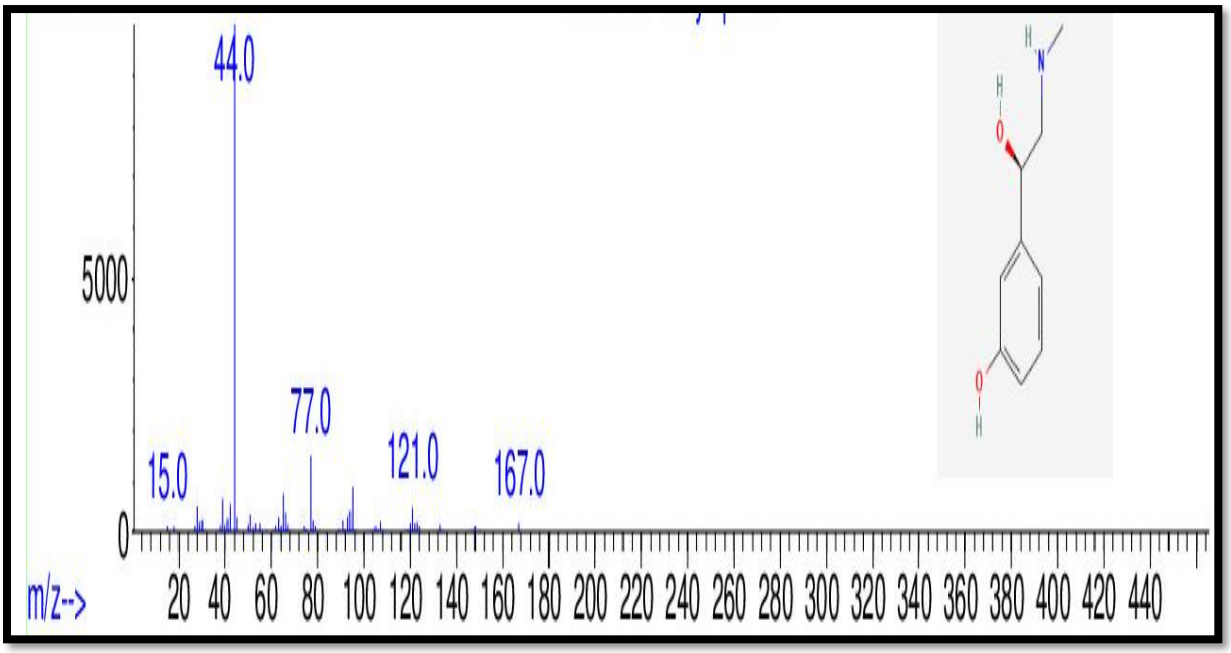


Fig : 13

Name: N-Methyl-1-adamantanea cetamide

Formula : C13H21NO

MW : 207.31

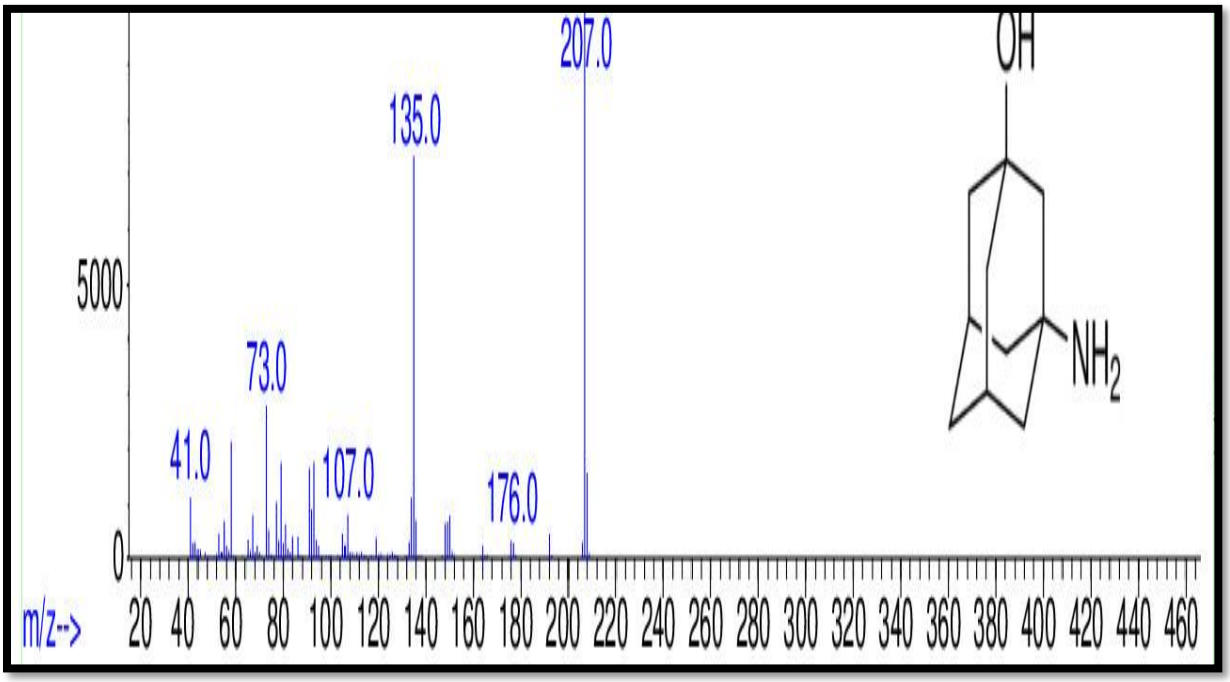


Fig : 14

Name: 2(Acetoxymethyl 1)-3-(methoxycarbonyl)biophenylene

Formula : C17H14O4

MW : 282.29

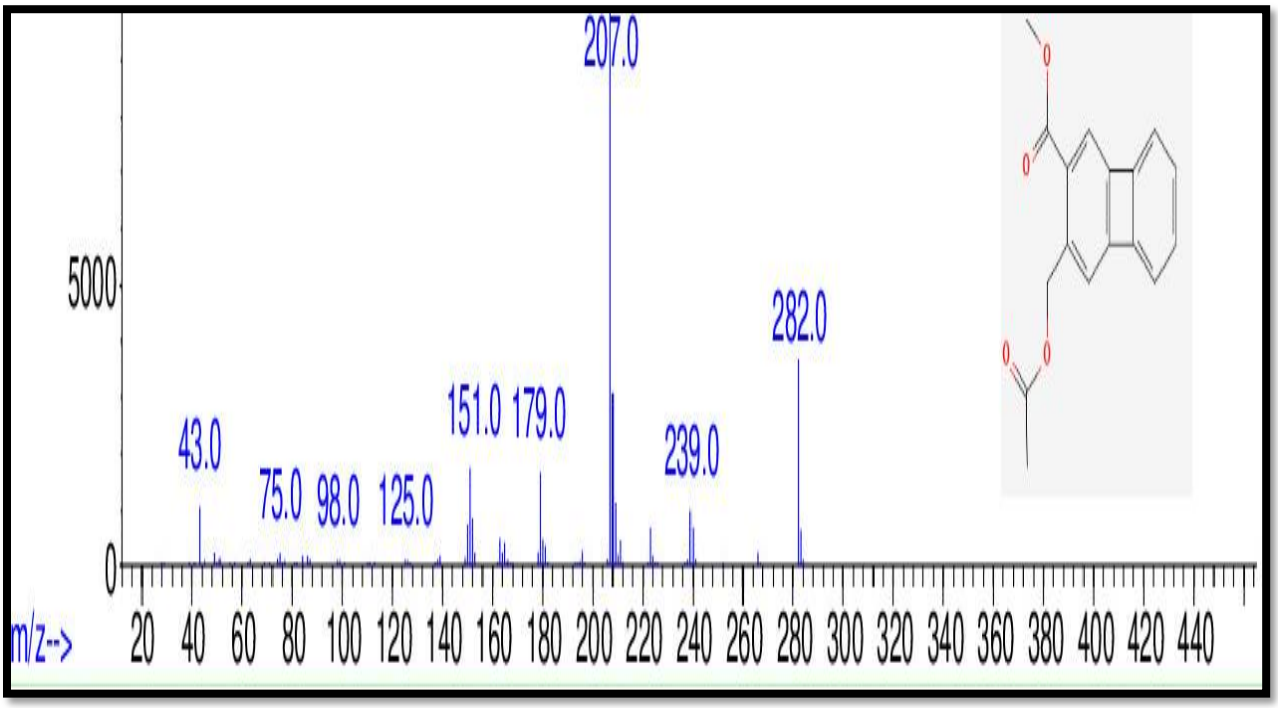
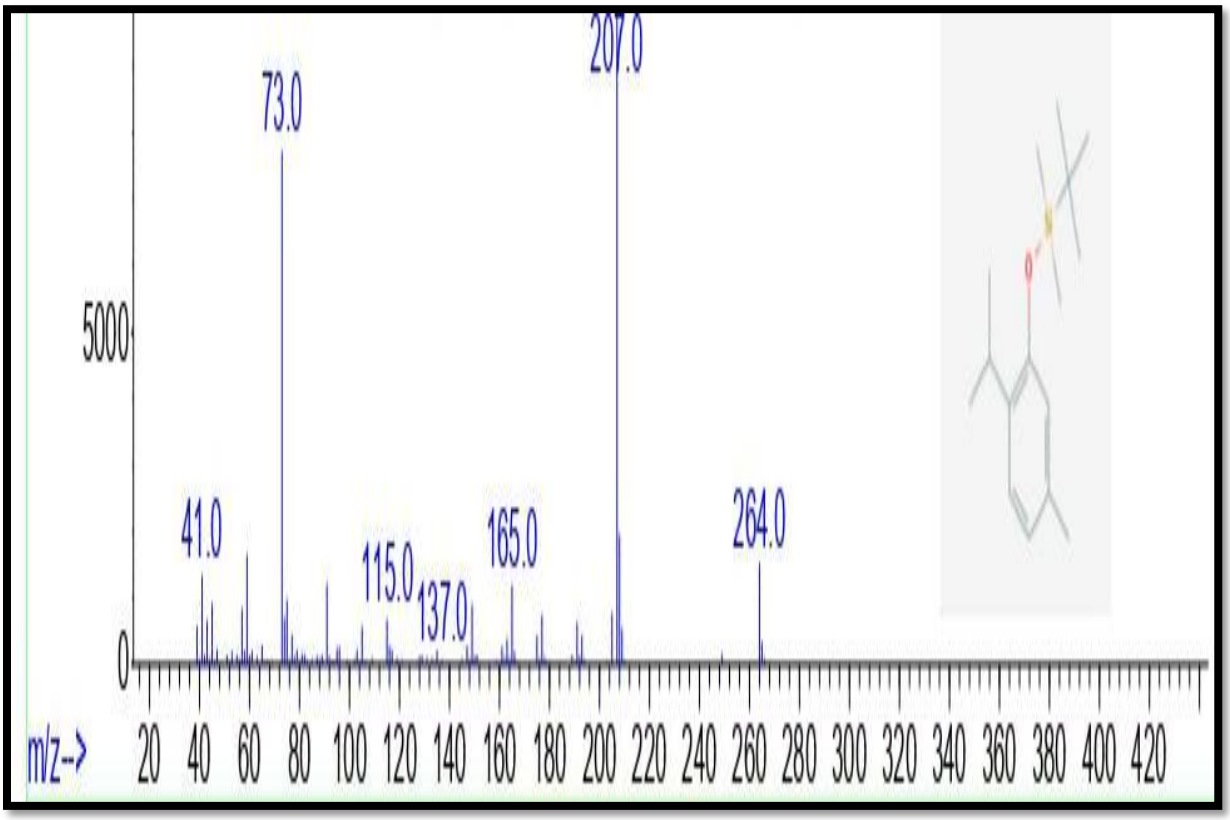


Fig : 15

Name: Benzene 2-(tert-butydimethylsilyl)oxyl)-1-isopropyl-4-methl

Formula : C16H24O2

MW : 248.36



DISCUSSION

7. DISCUSSION

It is globally accepted that natural products play a crucial role in drug discovery. In the last decade, the investigation of marine natural products has resulted in a remarkable number of compounds with promising biological activities. Marine natural products have been shown to display antibacterial, antifungal, anticancer, antiviral, antiparasitic, anti- inflammatory activity and several other pharmacological activities of benefit to humankind (Thilaga, 2005).

In recent years, great attention has been paid to study the bioactivity of natural products due to their potential pharmacological utilization. The rationale of searching for drugs from marine environment stems from the fact that marine plants and animals have adapted to all sorts of habitats in the marine environment and these creatures are constantly under tremendous selection pressure including competition for space, predation, surface fouling and reproduction (Rinehart et al., 1981).

Throughout the ages, natural products have always been the mainstay of disease therapy and are still considered to play an important role in modern medicine. Almost half of the drugs approved since 1994 are based on natural products. It is well known that marine invertebrates are important source where

natural products have derived from (Bruce et al., 2004 and Selva Prabha et al., 2011).

In the last 30 years, the interest in marine bio – prospecting has increased among researchers in the whole world. The marine environment differs from land based ecosystems and offers a great chemical diversity and high biological specificity. Marine organisms are therefore considered as treasures that remain a relatively unexplored source for novel bioactive compounds that could eventually be developed into therapeutics. However as a consequence of the complex molecular structures of natural products, pharmaceutical complains have lately shifted to use synthetic chemical libraries.

Abraham *et al*., (2002), studied the antibacterial activity in alcoholic extracts of holothurians species inhibited *S.typhi.* Vimala (2013) found out that the methylene chloride extract of *P. glaucum* developed maximum inhibition zones against *K.pneumoniae* (25mm) and minimum zone in *E.coli* (6mm). Whereas in the present study (F3) Benzene: Methanol extract of *C. Achatinus* has shown wide spectrum activity by developing inhibition zones of 20mm against *S.aureus*, 18mm in *V.cholerae*, 15mm against *P.vulgaris* and *S.flexneri*, 14mm against *S.pyogenes, S.typhi* and *B.cereus*, 13nn against *E.coli* and *P.aerogenosa* and 12mm against *K.pneumoniae*. Kanagasabapathy *et al*., (2011) observed the antibacterial activity of *Melo melo*. Dhinakaran *et al.*, (2011) observed the antipathogenic activity of

marine gastropod *H.pugilinus* against few pathogens and the ethanol and methanol extracts showed significant activity against proteus mirabilis and *E.coli.* Thilaga (2005) and Jayaprabha (2012) studied the antibacterial activities of *B.spirata* and Turbo brunneus respectively and observed highest activity against *E.coli, K.pneumoniae, P.vulgaris and S.typhi.* Sugesh *et al.,* (2013) noticed highest antibacterial activity with methanol and ethanol extracts of *H.pugilinus* against Human pathogens. All the above findings corroborate with the observations of the present study.

In echinoderms, an antioxidant activity was already evidenced in the viscera of the Atlantic star fish. Moreover, in crude ethanol extract of the star fish *luidia maculate,* a good antioxidant activity was observed by Suguna *et al*., (2014). In all these cases, the activity was assayed in tissue extracts and there are few studies only on sea cucumbers demonstrating the coelomic fluids as a good source of antioxidants.

Jung, 2002 reported that fractions obtained from *Asterias amurensis* and *Asterina pectinifera* showed strong activity against various human bacterial and fungal pathogens and also affirmed that the pre-treated fractions with protease showed stronger results than the positive control used. Had reported many AMPS from echinoderms which exhibited strong antimicrobial activity against bacterial, fungal, and viral pathogens Li *et al.,* 2010. In a coelomocyte extract from the star

fish *Asterias rubens* showed antimicrobial activity and several protein / peptides with molecular mass around 2 kDa were isolated (Maltseva et al., 2004, Maltseva et al., 2007).

The crude whole body methanol extract of *P.persica* showed wide spectrum and bacterial activity against seven pathogens out of ten pathogens tested. Rajaganapathi, (2000) also reported that the methanol extract from the whole body of *Hemifuses pugilinus* exhibited activity against *Bacillus subtailis*, Escherichia coli and *klebsiella pneumonia*. Similarly Santhana Ramasamy and Murugan (2005) experimentally analyzed the methanolic extract of *C. Virgineus* and *C.ramosus* and they also observed the broad spectrum antibacterial activity of body tissue extract. Highest activity was observed against *Klebsiella pneumoni*a and *Staphylococcus* epidermis by the extract of acetone ad against *Salmonella paratyphi* by the extract of chloroform column purified whole body extract of the winged oyster *Peteria chinensis* were reported by Chellaram *et al.*, (2004).

C.virgineus and *P.persica* column fractionated extract at 100mg/ml concentration inhibited *S.typhi, B.cereus, S.flexneri P.aerognosa* and *V.cholerae* classical with more than 10mm inhibitory zone. Gnanambal *et al*., (2005) found that 0.07mg / ml of *Trochus retiatus* inhabitat *Protous mirabilis* and 0.15 mg/ml of extract inhabitat *Serratia marcescens*. Sum of the peptides obtained from Oysters inhabitat *E.coli* and 3.30mg/ml and *Vibrio alginolyticus* at 162mg/ml. (Gueguen *et*

al., 2016; Seo *et al.*, 2005). Antibacterial of various of marine animals and bioactive product at molecular level have been studied by various authors and the molecules responsible for antibacterial activities where identified and characterized (Pawlik,1992; Llijima *et al*.,1995; Silvester *et al*.,1995; San-martin et al.,1996; Pouliquen *et al*.,1997; Chatterji *et al*.,1999; Ogawa *et al*.,1999; Gnanambal *et al*.,2005; Annnamalai *et al*.,2007;). Aplysianin-a isolated from albumin gland of *Aplylsia kurodia* inhibited B*acillus subtilis* (Kamiya et al.,1986), glycoprotein isolated from *Achacina fulica* inhibited *P.floubresens, E.coli, Streptococcus faecalis, Staphylococcus Epidermidis, S.aureus* and *Vibrio angulliarum* (Ogawa *et al*.,1999) and lectin isolated from *Modiolus modiolus* inhibited *Vibrio angulliarian* and *Vibrio salmonicida* (Tunkijianukeriji and Olafsen 1998). In particular, the antimicrobial activity was exerted against *S.auerus, P.aeruginosa, and C.famata.* These microorganisms may become dangerous in particular conditions (body debilitated, etc.) and assume different characteristic of pathogenecity compared to those known. These Nosocomial isolates show increasing resistant towards antibiotics representing a great challenge for the management of hospital accured infections and the modern pharmacology. *Staphylococcus aureus* is indeed notorious for its ability to become resistant antibiotics; *P.aeruginosa* develops antimicrobial resistant rapidly, which complicates medical treatment of infections (Lee ventola 2015).

Brine shrimp lethality assay was used to determine the anti tumour activity of the different solvent extracts of echinoderms. This assay has been established as rapid and simple method for screening starfish extracts for anti tumour activity. The chloroform extract of *Protoreaster linckii* had LC50 of less than 10ppm, the lowest concentration used for all the extracts. It may be considered to contain anti tumour agents since the standard set by the national cancer institute of the US for a bioactive compound to be an effective anti tumour agent is equal or less than 30ppm. Starfishes extract where screened for antibacterial activity. Chloroform, methanol and hexane extract of *Linckiia Laevigata* showed after 8 hours of exposure but immediately ceased at 24 hours. The chloroform extract *Protoreaster linckii* on the other hand showed B bacteriostatic activity (Chludil and Maier 2005, Wang *et al*., 2005).

Chloroform and hexane extract had antibacterial activity more than methanol extract. This could be due to the suitability of chloroform and hexane solvent for dissolving the bioactive compounds presents in the bodies of marine creature. Also, some of the microbes become resistant to antibiotics and drugs. Microorganism use mechanisms that include limiting the antimicrobial agent concentration within cells by reducing penetration are increasing the exit and neutralising of the antimicrobial agent. Antibiotics has necessitated a search for

new antimicrobial substance from other sources including natural sources from any terrestrial or marine sources (Blend et al.,2007).

Prabhadevi *et al*.,(2011) accounted that the body wall extract of sea star pentaceraster affinis showed considerable activity against *Shigella flexineri, acinetobacter sps*., and moderate activity against streptococcus sps they suggested that the compounds inhibited the activity of pathogen may be saponins and saponin like steroid derivative. James (2010) reported that considerable toxicity is found in the echinoderms and the "Crown of thrones" acanthaster planci is highly toxic to human as well as fish predates. Srikumaran et al., (2011) also reported that the Butanol extract of *Protoreaster linckii* showed high activity against *S.Paratyphi* and *K.Pneumonia*. Methanol extract of *P.regulus* showed activity against *K.oxytoca*. Rinehart *et al.,* (1981) as documented as about 43% of 83 unidentified California echinoderms have antimicrobial property. Yeon Jung Jung (2002) reported that the fractions obtained from *Asterias amurensis* and *Asterina pectinifera* showed strong activity against various human bacterial and fungal pathogens.

In the present study the activity of *Protoreaster linckii* 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 difference solvent system extracts of *Protoreaster linckii*

are showing antibacterial properties against the bacterial species tested here. Thus the current studies revealed the presence of potent antimicrobial compounds from Echinoderms *Protoreaster linckii* of tuticorin coast. In the present study GC-MS analysis of *Protoreaster linckii* showed 100 percentage successes in the identification of bioactive compounds responsible for antimicrobial activities. The magnitude of crude extracts of *Protoreaster linckii* possibly reveals the presence of six antimicrobial compounds., n-Hexadecanoic acid, Octadec-9-enoic acid, Phenylephrine, N-Methyl-1-adamantanea cetamide, 2(Acetoxymethyl 1)-3-(methoxycarbonyl) biophenylene, Benzene 2-(tert-butydimethylsilyl)oxyl)-1-isopropyl-4-methl. Identified from GC-MS analysis might be responsible for antimicrobial activity. As the Echinoderms resources are 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 *Protoreaster linckii*.
- Antifugal activity was tested against two fungal pathogens, *Aspergillus niger, Sporothrix schencki.* Antifungal of methanol tissues extract showed the maximum activity in pasitive control (Amphotericin B).
- The antifungal activity of *Protoreaster linckii* showed maximum activity (9 µg/ml) positive control in *Aspergillus niger* than the *Sporothrix schencki* (8 µg/ml) positive control.
- Antibacterial activity was tested against five bacterial pathogens *Pseudomonas, Escherichia coli, Salmonella typhi, Vibriocholere,* and *Streptococcus sps*. The growth of all tested bacterial inhibited by the crude extract of *Protoreaster linckii* and the inhibitory zones varied from 1 mm to 5mm.
- The maximum inhibition zone (0.5mm) was developed against *Pseudomonas* and *Escherichia coli* at 100µg/ml and minimum inhibition zone (0.1mm) was recorded against *salmonella typhi* at 10µg/ml concentration. Benzene and distilled water were 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 *Protoreaster linckii* exhibited six peaks, with the retention

times ranging from 11.692 to 17.818. Among the compounds identified Octadec-9-enoic acid was the most abundant antimicrobial compounds (32.07) present in the methanol tissue extract of *Protoreaster linckii.*

- The result of the present study showed that the hold 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

9. CONCLUSION

- In the present study indicates the whole body extraction of *Protoreaster linckii* would be a good source of antimicrobial agents and would replace the existing inadequate and cost effective antibiotics. It has been widely reported that many bioactive natural products from marine invertebrate have striking similaries to metabolites of their associated microorganisms including bacteria.
- The GC-MS chromatogram of the methanolic extracts of *Protoreaster Linckii* confirmed the presence of the six active compounds that could be identified as n-Hexadecanoic acid, Octadec-9-enoic acid, Phenylephrine, N-Methyl-1-adamantanea cetamide, 2(Acetoxymethyl 1)-3-(methoxycarbonyl)biophenylene, Benzene 2- (tert-butydimethylsilyl) oxyl) -1-isopropyl -4- methl. These compounds which could be responsible for the antibacterial activities in the present study.
- GC-MS analysis has also aided the evaluation of the major and minor compounds present in methanol extract of tissue. 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 strains.

- World oceans could play paramount role in supplying lifesaving drugs in future. 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 distributing the biodiversity of marine organisms. If any animal is found to be suitable candidates species for the exploration of drugs, the animal can be cultured by suitable aquaculture practice and thereby were can conserve the fauna as well as not modifying the diversity for the value of mankind.

SUGGESION

10. SUGGESION

From the result of the present study, it is understood that the rich diversity of the marine biota with unique physiological adaptations exerted or provided by organisms to the harsh marine environment. Affords a fruitful source for the discovery of the lifesaving drugs by using molecular biological approach it is also under investigation to transfer a bacterial gene cluster, responsible for the biosynthesis of desire natural products to a vector suitable for large fermentation.

World oceans could play paramount role in supplying life saving drugs in future. 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 bio diversity of marine organisms. If any animal is found to be suitable candidate species for the exploration of drugs, the can be cultured by suitable aquaculture practice and thereby where can conserve the fauna as well as not modifying the diversity for the sale of mankind. And further research and modification are to be carried out.

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ANTIDIABETIC, ANTIOXIDANT AND GC-MS ANALYSIS OF MARINE PUFFER FISH *DIODON HYSTRIX* FROM THOOTHUKUDI COAST

A dissertation submitted to

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In partial fulfilment for the award of the degree of

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By

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THOOTHUKUDI-628001

April - 2023

CERTIFICATE

This is to certify that this dissertation entitled **"ANTIDIABETIC, ANTIOXIDANT AND GC-MS ANALYSIS OF MARINE PUFFER FISH *DIODON HYSTRIX* FROM THOOTHUKUDI COAST"** submitted by **J.MICHAEL THERES RENISHA, Reg.No. 21APZO02** to St. Mary's College (Autonomous), Thoothukudi in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2022 – 2023 under my guidance and supervision. It is further certified that this dissertation or any part of this has not been submitted elsewhere for any other degree.

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DECLARATION

I do hereby declare that the dissertation entitled,"**ANTIDIABETIC, ANTIOXIDANT AND GC-MS ANALYSIS OF MARINE PUFFER FISH *DIODON HYSTRIX* FROM THOOTHUKUDI COAST**"submitted by me for the award of the degree of Master of Science in Zoology, is the result of my original independent research work carried out under the guidance of **Dr.S. SELVI M.Sc., B.Ed., M.Phil., Ph.D.,** Assistant Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date: 5. 4. 2023

J. Michael Theres Renisha

Signature of the candidate

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CONTENTS

INTRODUCTION

1. INTRODUCTION

The sea is immense almost unexploited source of new potentially useful biologically active substance (Faulkner, 1999). Recently marine microorganisms have been found to produce a variety of chemically interesting and biologically significant secondary metabolites. Some of them being expected to serve a lead compound for drug development or pharmacological tools for basic studies in life science (Kobayashi and Jensen, 1994). The number of natural products isolated from marine organism increase rapidly and now exceeds with hundreds of new compounds being discovered every year (Faulkner, 2002, Proksch and Muller, 2006). Thus the marine environment is an exceptional reservoir for bioactive natural product, many of which exhibit structural features that are not found in terrestrial natural products (Johansson and Soderhall, 1985).

The Gulf of Mannar, situated in the south eastern coast of India extending from Rameshwaram in the north to Tuticorin in the south along with its marine environment has been declared as India's first marine Biosphere Reserve. Marine Biosphere Reserve was established during the year 1989. 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 500,000 species and more than double that of the land (Anand *et al*., 1997). 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 compound of interest in the pharmaceutical industries (Faulkner, 2002; Proksch and Muller, 2006).

Fishes are one of the diverse sources of natural products and bioactive compound with over 40,000 known species. They combat infections caused by viruses, bacteria, fungi and parasites that are similar to those of humans and other vertebrates. Many species of marine fish have been reported as ithyocrinotoxic (Halstead, 1978), releasing into the water toxic secretions. Fish live in intimate contact with an environment containing both saprophytic and pathogenic microbes, capable of digesting and degrading fish tissues (Ellis, 2001; Plouffe *et al*., 2005). Marine organisms not only elaborate pharmaceutically useful compounds but also produce toxic substances and fabricate, some of the most cytotoxic compounds ever discovered but the yields of these compounds are invariably so small that natural sources are unlikely to provide enough material for drug development studies (Mohan Raj *et al.,* 2014). Concisely, fish have evolved a number of innate immune responses to defend themselves against

infection. Tetraodontidae is diverse with species such as puffer fish, Balloon fish, Blow, Bubble fish, Globe fish, Swell fish, Toad fish, Toadies, Honey Toads and Squab (Mills and Passmore, 1988; Ramaiyan and Senthilkumar, 1998; Froese and Pauly, 2007).

Puffer fishes are commonly distributed in the 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). The skin and certain other internal organs of puffer fish are highly toxic to human. Puffer fish poisoning is considered to be the common cause of fish poisoning along the Asian coast (Chew *et al.,* 1983). Puffer will eat all type of food such as shrimp, fish, clams, molluscs and crustaceans etc. It is also important that they consume hard shelled crabs, mussels and shell fish in their diet to wear down their teeth and prevent them from overgrowing. Furthermore toxicity changes with age, sex, season and geographical variations (Homaira *et al.,* 2010). Puffers are able to move their eyes independently and many species can change the colour or intensity of their patterns in response to environmental changes. They are somewhat similar to the terrestrial chameleon. Although most puffers are dark, many have bright colours and distinctive markings and make no attempt to hide from predators (Keiichi *et al.*, 1998).

Puffer fishes are able to produce toxin with associate microbes on the mucus of their body. They are named after their habit of inflating themselves with water or air when threatened making it difficult for a predator to swallow them. This fish is known to carry tetrodotoxin (TTX) (Bilecenoglu *et al.,* 2006; Kasapidis *et al.,* 2007; Sabrah *et al.,* 2006) which is known a non-protein organic compound .The toxin has occasionally been detected in the muscles of these fishes. The toxin is produced by several bacteria species including *Mycobacterium arabinogalatanolyticum, Serratiama scescens, Vibrio alginolyticus* and *Bacillus spp*. (Yu *et al*., 2001; Wu *et al*., 2005).

Tetrodotoxin is a low molecular weight with 319 small molecules with a unique cage structure. The basic molecule for TTX consists of a positively charged Guanidium group. The source of tetrodotoxin is accepted that bacteria in the fish's intestinal tract (Shibamoto and Bjeldanes, 2009). Saxitoxin, the cause of paralytic shell fish poisoning and red tide, can also be found in certain puffers (Lehman, 2006). It is also important to note that puffer fish toxin is 100 times more potent than cyanide (Alipala, 2012). On the other hand, fishes hold the credit of possessing rich protein sources. These marine proteins are not only correlated to the intact proteins, but also to the possibility of generating bioactive peptides (Indumathi *et al.*, 2016).

Diabetes mellitus is a chronic metabolic disorder, is one of the most important problems in public health nowadays (Hashempur *et al*., 2015). It is characterized by high levels of glucose in the blood due to the impaired secretion of insulin insensitivity (Zhang *et al.,* 2014). A globally around 4% population was affecting and could be predictable to increase by 5.4% in 2025. Worldwide the number of adults suffering from diabetes will increase from 194 million in 2003 to nearly 380 million in 2030 (Kaul *et al.,* 2012). Currently, the available therapy for diabetes includes insulin and various oral antidiabetic agents such as sulfonylureas, thiazolidinedione's, and α-glucosidase inhibitors. Hence antidiabetic drug discovery has shifted to focus on natural product and plant sources having minimal side effects. One of the most potent method to induce experimental diabetes mellitus is chemical induction by alloxan and metformin hydrochloride. It is a well-known diabetogenic agent that is used to induce type I and type II diabetes in experimental animals (Viana *et al.,* 2004). Globally 90–95% of people were affected with type-2 diabetes. Obesity-related to high calorie diet and sedentary way of life causes biochemical abnormalities and high glucose concentration in the blood which re-duces β-insulin sensitivity and leads to insufficient secretion of insulin by β-cells of the pancreas (Tfayli *et al.,* 2009; Wu *et al*., 2011).

A global survey stated that the expected incidence of diabetes and projection for the year 2030 is 350 million (Qiao *et al*., 2011). In spite of difficulties in maintaining proper glycemic control with available pharmaceutical approaches (Vuksan *et al.,* 2007). Puffers have a long and rich cooking history in East Asian countries including China, Korea, and Japan that have been locally harvested for food and medicine for centuries. In East Asia and elsewhere, modern technologies have en-abled the demand of puffers supply in global aquaculture trade (Kawata, 2003). The collagen peptides from marine wild fish decrease free fatty acids and regulate nuclear receptor metabolism in type-2 diabetic patients (Zhu *et al.,* 2010).

An antioxidant is something that fights oxidation. Radical peroxidation of lipids is prevented by antioxidants. Because they are willing to surrender their own electrons to free radicals, antioxidants are effective. The chain process of oxidation is stopped when a free radical receives an electron from an antioxidant and no longer needs to attack the cell (Dekkers *et al.,* 1996). Over the past ten years, there has been an increased focus on the function of antioxidants. However, using synthetic antioxidants may be harmful to your health (Park *et al.,* 2001). Antioxidants are found in foods like fruits, vegetables, nuts, and sea food that are consumed on a regular basis (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).

The human body is constantly being exposed to reactive oxygen species, which are characterized by the presence of molecules that carry unpaired electrons that may damage cellular molecules and structures. These reactive oxygen species can trigger the reactions in the cells and result in significant damage to the entire tissue, which is known as oxidative stress (Banach *et al.,* 2001; Lehucher – Michel *et al.,* 2001; Niki, 2000). Anti –oxidative compounds play an important role in various fields such as medical (to treat cancer, cardiovascular disorders and chronic inflammations), cosmetics (Anti - aging process) and others (Ganapathy *et al.*, 2007).

Body processes, such as metabolism, as well as environmental factors, like excess exposure to the sun, cigarette smoke and air pollution, excess alcohol and even x-rays can produce free radicals. The formation of free radicals and other reactive oxygen species is unavoidable during the oxidative metabolic process (Shailaja *et al.,* 2012). These free radicals may oxidise nucleic acids, proteins, lipids or DNA which start chain reactions

that damage cells and it can initiate degenerative disease such as cancer and other diseases like stroke, diabetes, Alzheimer's disease (Devasagayam *et al.,* 2004).

GC/MS is a technique for analysing complex organic and biological mixtures that combines the two analytical methods of gas chromatography and mass spectrometry. The GC is quite good at separating volatile and semi-volatile chemicals, but it is unable to identify them. While MS can accurately identify and quantify the majority of compounds, it cannot easily separate them. MS can offer extensive structural information on most compounds. Gas chromatography (GC) is a type of chromatography in which the stationary phase is a microscopic layer of liquid or polymer on an inert solid support and the mobile phase is typically an inert gas such as helium or an unreactive gas such as nitrogen. A thin capillary fibre is used in gas chromatography as the "column," through which various chemicals pass at various rates depending on various chemical and physical properties. Chemicals are electronically detected and identified as they leave the column at the end. The column's purpose is to concentrate and separate various components in order to increase the detection signal (Veerakumari, 2006).

A mass spectrometer is an analytical tool that can reveal details about the molecular makeup of both organic and inorganic compounds. One of the

few techniques that can be used to qualitatively analyse and characterise various organic substances. It allows for quantitative examination of mixtures (including gases, liquids, and occasionally solids). A mass spectrometer is also used in understanding kinetics and mechanisms of unimolecular decomposition reactions. GC-MS is highly efficient tool widely used to analyse semi volatile and volatile organic personal care products as extremely low levels from environmental samples (Motalab *et al.*, 2015).

In India, studies of puffer fish are very limited. Hence the aim of the present study has been carried out to establish the occurrence of antidiabetic and antioxidant activity and GC - MS analysis of tissue extracts of *Diodon hystrix* collected from Thoothukudi Coast.

OBJECTIVES

The objectives of the present study are

- To investigate the antidiabetic activity of methanol extract of skin and muscle of *D.hystrix.*
- To analyse the antioxidant activity of methanol extract of skin and muscle of *D.hystrix*.
- To evaluate the bioactive compounds present in the crude methanol extract of skin of *Diodon hystrix* using GC - MS analysis.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The marine biota is the largest source for novel discovery of natural product such as pharamacological metabolites and medicines. There has been an extensive research showing that vast bio active substances were identified and characterized from marine organisms, indeed several of them showed promising results to treat human and animal diseases (Sato, 1996; Grabley and Thiericke, 1999).

The oceans stand out as a key component of the biosphere, called to accommodate much of the increasing demands for resources (food, water, and energy) required to meet future projected global human population growth (Duarte *et al.,* (2020). 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 anti–bacterial, antifungal, antioxidant etc (Rajamanikandan *et al.,* (2011).

Marine bioactive agents a short review on new marine antidiabetic compounds was reported by (Barde *et al.,* 2015). The present review focuses on potential marine resources that provide bioactive agents for diabetes treatment.

An antidiabetic property of bioactive components from fish and milk was reported by (Zhou *et al.,* 2001). The recent studies related to the anti-diabetic activities of fish and milk derived bioactive compounds (proteins, peptides, fatty acids) in humans, animals and in vitro, and mechanisms of action of these bioactive compounds in the management of T2DM.

Health benefits of bioactive peptides produced from muscle proteins: Antioxidant, anti-cancer, and anti-diabetic activities was reported by (Islam *et al.,* 2022). This review article aims to summarize the antioxidant, anti-diabetic, and anti-cancer activities of muscle proteins hydrolysates and purified bioactive peptides.

Physicochemical, nutritional and in vitro antidiabetic characterisation of Blue Whiting (Micromesistiuspoutassou) protein hydrolysates was reported by (Rowthwell *et al.,* 2021). The results of insulin secretory activity of the BW-SPHs were 4.5 – 5.4 fold higher than the basal control following SGID. The BW-SPHs generated here in provide potential for anti-diabetic related functional ingredients, whilst also enhancing environmental and commercial sustainability.

Marine organisms with anti-diabetes properties was reported by (Drugs, 2016). This review summarizes recent discoveries in anti-diabetes properties of several marine organisms as well as marine wastes, existing patents and possible future research directions in this field.

Muscle extract of *Arothron immaculatus* regulates the blood glucose level and the antioxidant system in high-fat diet and streptozotocin induced diabetic rats was reported by (Kaleshkumar *et al.,* 2019). This is the first study on the muscle extract of marine puffer fish which is used as antidiabetic agent to treat the diabetes-induced in the animal model.

Marine food derived functional ingredients as potential antioxidants in the food industry (Ngo *et al*.,2011).The reducing power of a sample is an indicater of its antioxidant activity. The reducing power assay is used to evaluate the ebility of an antioxidant to donate an electron or hydrogen (Yildirim *et al.,* 2001). 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). Sampathkumar *et al.,* (2012) reported that purification and identification of antioxidant peptide from the skin protein hydrolysate of two marine fishes, horse mackerel (Magalaspiscordyla) and croaker (Otolithesruber). Studies showed that fish and fishery products have a high radical – scavenging activity comparable to that of some vegetables (Khanum *et al*., 2004).

Antioxidant activity of fermented meat sauce and isolation of an associated antioxidant peptide was reported by (Motoka *et al*., 2016). This study showed an extremely high antioxidant activity against the OH-radical

that was greater than 90%. This substance was anticipated to be the tripeptide Gln-Tyr-Pro.

Effect of cadmium exposure on expression of antioxidant gene transcripts in the river puffer fish, *Takifugu obscurus* (Tetraodontiformes) was reported by (Jin- Hyoung *et al.*, 2010). The results suggested that cadmium exposure modulates the expression of antioxidant genes, and would indicate that the antioxidant genes would be a relevant biomarker of trace metal pollution such as cadmium exposure in *T. obscurus*.

Effect of dietary astaxanthin on the growth performance, non-specific immunity, and antioxidant capacity of puffer fish (*Takifugu obscurus*) under high temperature stress was reported by (Chang – Hong *et al.,* 2018). The results showed that astaxanthin could suppress ROS production induced by high temperature stress and antioxidant defense system and improve resistance against high temperature stress in pufferfish.

Combined effects of low temperature and salinity on the immune response antioxidant capacity and lipid metabolism in the puffer fish (*Takifugu fasciatus)* was reported by (Xin *et al*., 2021). The results indicated that 10 ppt of salinity can alleviate the survival pressure on *T. fasciatus* at 13°C and that at 25°C, 20 ppt salinity stresses the organism. Interactive effects of temperature and salinity on the apoptosis, antioxidant enzymes, and MAPK signaling pathway of juvenile pufferfish (*Takifugu fasciatus*)

was reported by (Chu *et al.,* 2023). The results expressed of p-p38 MAPK and its phosphorylation ratio (p-p38/p38) at low temperatures but significantly affected its downstream transcription factors (ATF2, ElK-1, MEF2, and P53).

The effect of added shiitake mushroom on antioxidative activity of puffer fish stock was reported by (Kim *et al.,* 2017). The antioxidant activities of puffer fish stock increased proportionally with increasing amount of added shiitake, which in turn was due to the increased amount of total polyphenol in the stock.

Quantitative determination of fatty acids from oil using GC - MS method and H-NMR spectroscopy was reported by (Bratu *et al.,* 2013). Estimate using the both analytical methods polyunsaturated fatty acids are important for human health were identified and quantified.

GC - MS determination of organochlorine pesticides (OCPs) in fish form river Cauvery and Veeranam Lake was reported by (Bhuvaneswari and Babu Rajendren, 2012). The study on the risk associated with consumption of fish species that had higher concentration of aldrin, dieldrin and mirex showed significant carcinogenic risk to the human beings.

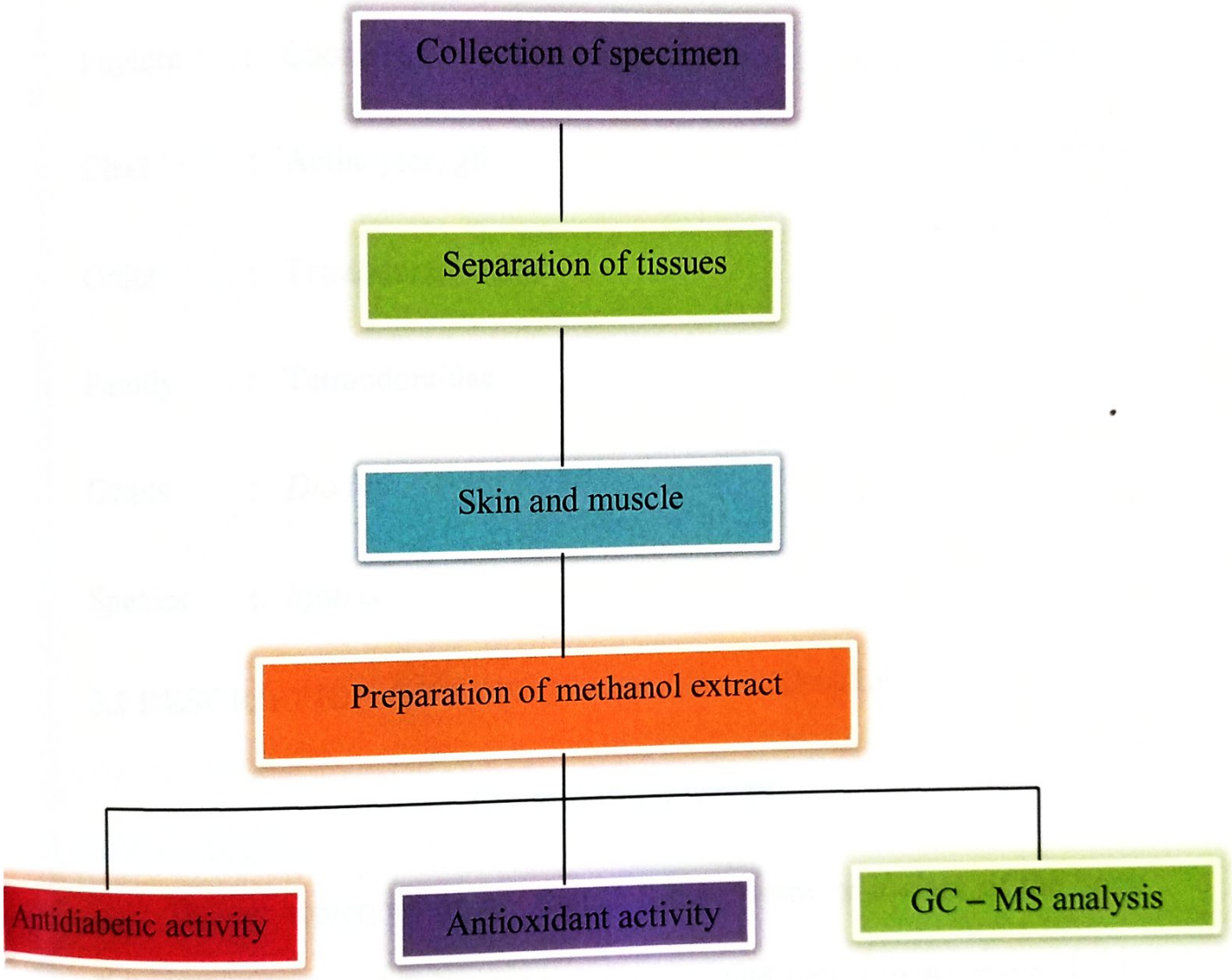
Use of LC - MS and GC - MS methods to measure emerging contaminants pharmaceutical and personal care products (PPCPs) in fish

was reported by (Mottaleb *et al.,* 2015). This review focuses on PPCP emerging contaminants concern with regards to sources, occurrences, analytical methods, fat and biological transformation.

Characterization of odor-active compounds in cooked meat of farmed obscure puffer (*Takifugu obscurus*) using gas chromatography-mass spectrometry-olfactometry was reported by (Ping Tao *et al.,* 2014). analyzed by gas chromatography-mass spectrometry-olfactometry (GC - MS - O). The volatile compounds were extracted by the simultaneous distillation - extraction (SDE) method, then separated and identified by GC - MS.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN



3. MATERIALS AND METHODS

3.1 SYSTEMATIC POSITION OF EXPERIMENTAL ANIMAL:

Kingdom : Animalia

Phylum : Chordata

Class : Actinopterygii

Order : Tetraodontiformes

Family : Tetraodontidae

Genus : *Diodon*

Species : *hystrix*

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 class 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).

***Diodon histrix* (Plate 1).**

This porcupine fish looks similar to its relative, the balloon fish, but its body is uniformly grayish-tan, speckled evenly with black spots, with a white belly. The spines all over its body are modified scales, and when it's threatened, it intakes water, puffing up and making the spikes stand out. It prefers living alone near reefs, caves or ledges, hunting crustaceans and molusks at night. The porcupinefish *D.hystrix* gets its name from the numerous long spines located all over the head and body. There are approximately 20 spines in a row between the snout and dorsal fin. Because it secretes a toxin, it's not considered a food catch, but some are caught for the aquarium trade.

3.3 Collection of Specimen:

Specimens of the puffer fish *D.hystrix* 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^{o}C until use.

Plate 1 ***Diodon hystrix***

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 100A Lack Rotavapor at 30ºC) with reduced pressure to give a dark brown gummy mass. The resultant residue was stored at 4ºC for further analysis.

3.5 Antidiabetic Activity;

The α-amylase activity of *D.hystrix* was measured according to the modified method of (Fei *et al.,* 2014). The α-amylase was dissolved in phosphate-buffer saline (0.02 mol/l, pH 6.8) at a concentration of 0.1 mg/ml. The various concentrations of sample solutions (200, 400, 600, 800 and 1000 µg/ml) were mixed with the α-amylase solution (0.25 ml) and incubated at 37°C for 5 min. Then the reaction was initiated by adding 0.5 ml 1.0% (w/v) starch substrate solution to the incubation medium. After incubation at 37°C for 3 min, the reaction was stopped by adding 0.5 ml reagent (1% dinitrosalicylic acid, 0.05% Na2 SO3, and 1% NaOH solution) to the reaction mixture and boiling at 100°C for 5 min. After cooling to room temperature, the absorbance (Abs) at 540 nm was recorded by a

spectrophotometer. The inhibition percentage was calculated by the following equation:

Inhibition (%) = [(Abs1 - Abs2)/ Abs1] × 100

3.6 Antioxidant Activity:

Total Antioxidant Activity (TAA) by Phosphomolybdenum assay TAA was estimated by phosphomolybdenum assay (Prieto *et al.,* 1999). Sample of concentration 1000 µg/ml were taken in individual test tubes and made up to 1 ml using distilled water and 2 ml of Molybdate reagent solution (0.6 M sulfuric acid, 28 mMsodium Phosphate and 4 mM ammonium molybdate). The test tubes were incubated at 95^0C for 90min. After incubation, the tubes were cooled to room temperature for 20-30 min and the absorbance of the reaction mixture was measured at 695 nm. All experiments were performed in triplcates and the results were expressed as mean ± SD. Ascorbic acid was used as the positive reference standard.

% Antioxidant activity = Abs sample/ Abs Std * 100

3.7 GC-MS Analysis:

GC-MS analysis of methanol extracts of *D.hystrix* 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% phenylArylene-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 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 Antidiabetic activity;

The antidiabetic activity of methanol extract of skin and muscle was assessed by alpha amylase inhibition assay. The five different concentrations were using, namely, 200, 400, 600, 800 and 1000 µg/ml. Among the extract of skin and muscle of *D.hystrix* was evaluated. The skin extract showed antioxidant activity with 20%, 44 %, 56%, 76 % and 76.80% at 200 µg, 400 µg, 600 µg, 800 µg and 1000 µg respectively (Fig 1). The muscle exhibited the strongest antioxidant activity with 36 %, 48 %, 60%, 84% and 84.8% at 200 µg, 400 µg, 600 µg, 800 µg, and 1000 µg respectively (Fig 2).

4.2 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 free radicals by Phosphomolybdenum assay. The free radical scavenging activity of methanol extracts of skin and muscle of *D.hystrix* was evaluated. The skin extract showed antioxidant activity with 8 %, 20 %, 24 %, 28 % and 40% at 200 µg, 400 µg, 600 µg, 800 µg and 1000 µg respectively (Fig 3). The muscle exhibited the strongest antioxidant activity with 64 %, 76 %, 88%, 96% and 98.40% at 200 µg, 400 µg, 600 µg, 800 µg, and 1000 µg respectively (Fig 4).

4.3 GC – MS Analysis:

Fourteen chemical compounds were identified in the skin extract of *D.hystrix* through GC - MS study. They are 2 – Acetylbenzoic acid (RT – 8.658), Phenol, 2-

amino-4 (RT 8.658), Phthalic acid (RT – 8.658), 1- Propene, 3- azido (RT – 12.403), Thiirane (RT – 12.403), Hydrazine, 1-1 - dimethyl(RT – 12.403), Acetamide, 2- fluoro (RT – 14.057), Ethylenimine, N-chloro(RT – 14.057), Silane, (11-fluoroundecyl) oxy (RT- 14.105), 2- Hexenal,(RT -14.105), 1H – Imidazole, 4,5 – dihydro-2,4 (RT – 14.105), 4 – fluorohistamine (RT – 14.294), 1– Hexanamine, 2- ethyl (RT – 14.294) and 1 – Azabicyclo (3.1.0)hexane (RT – 14.294) (Fig 5-19).

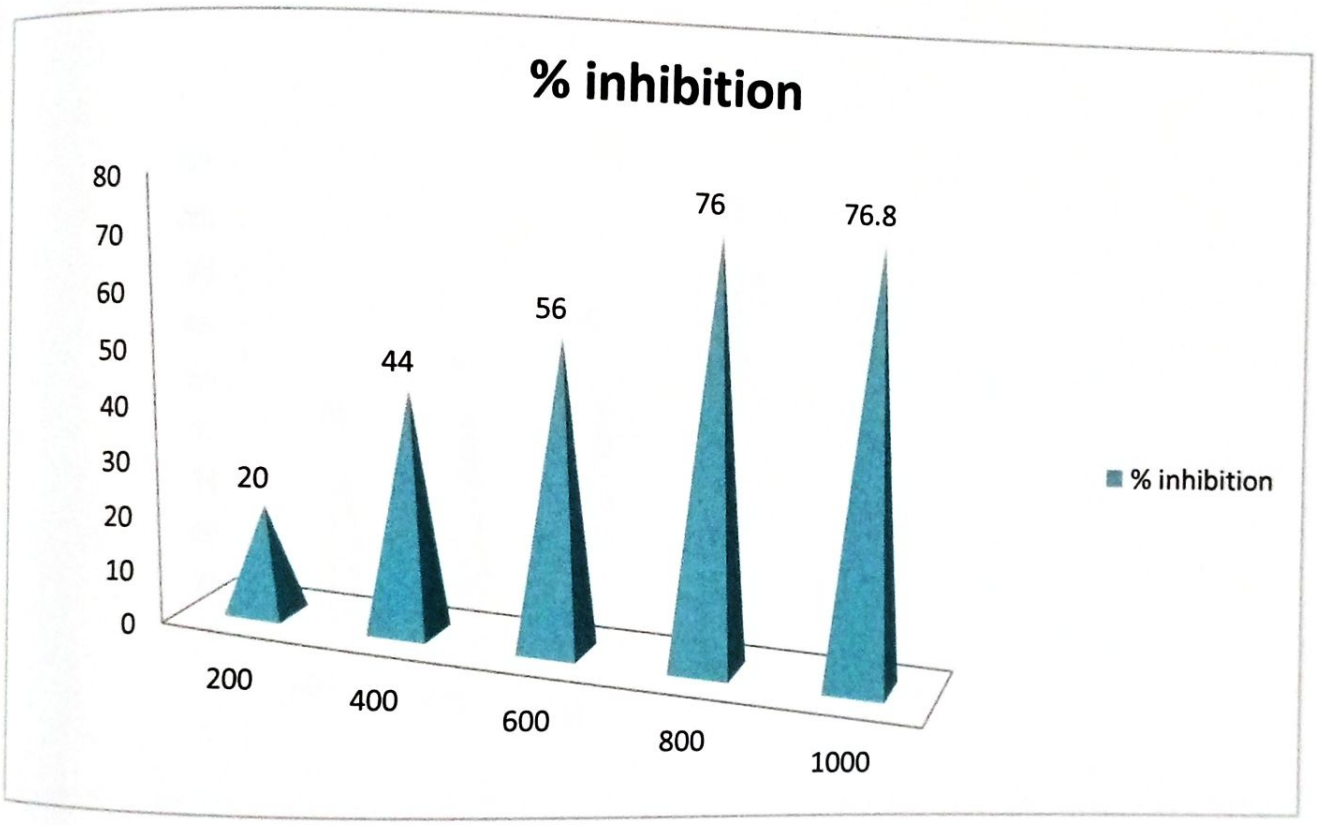


Fig 1 Antidiabetic activity of alpha amylase inhibition assay of skin of *Diodon hystrix*

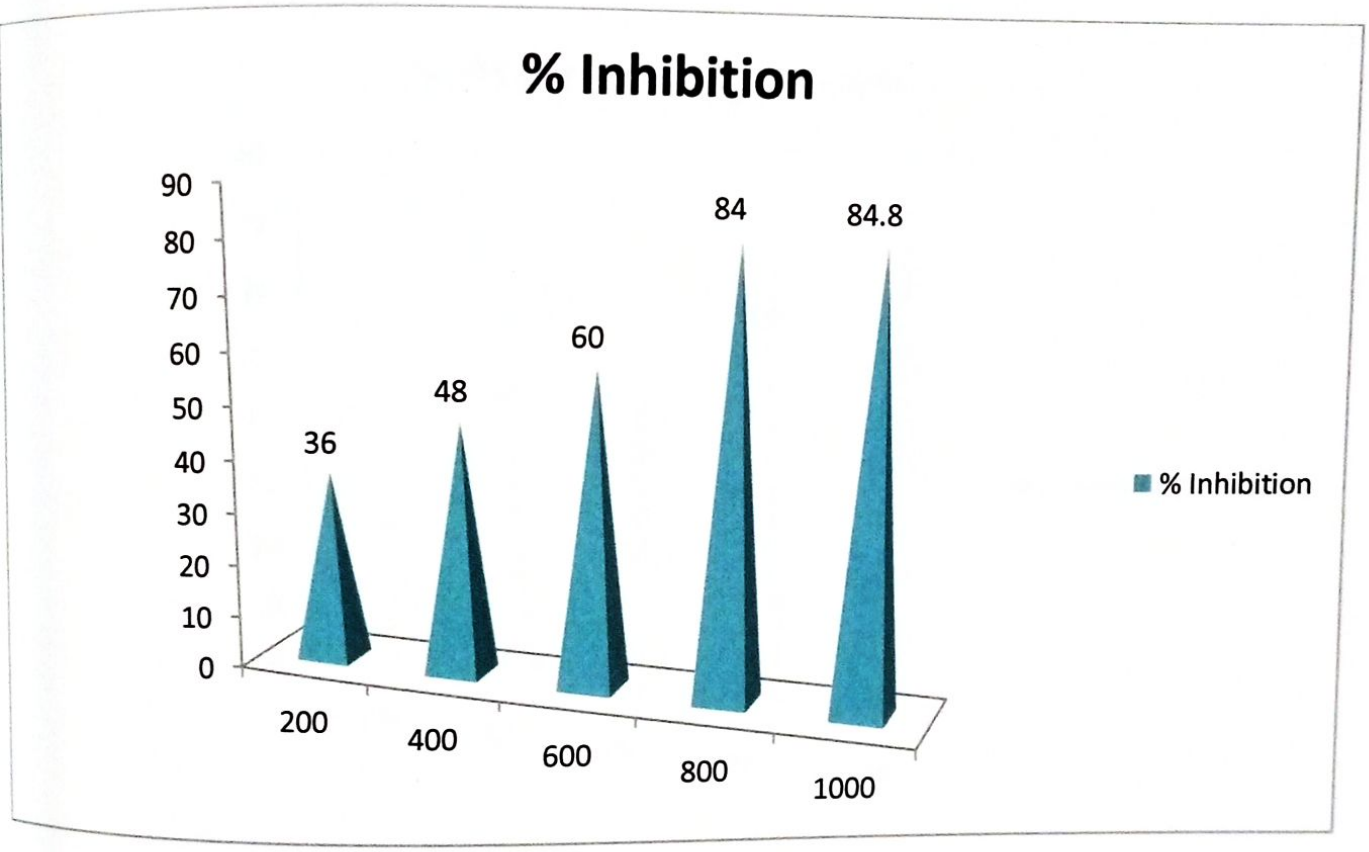


Fig 2 Antidiabetic activity of alpha amylase inhibition assay of muscle of *Diodon hystrix*

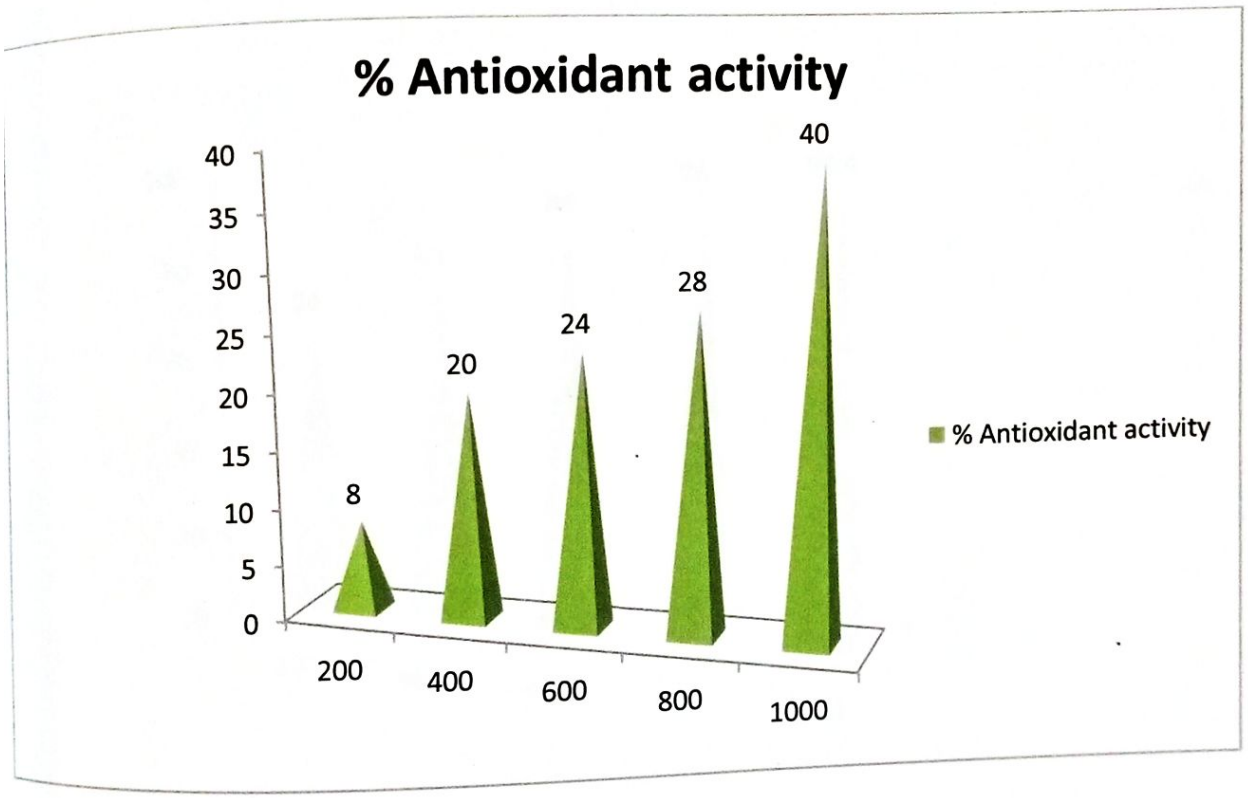


Fig 3 Antioxidant activity of phosphomolymbdenum assay of skin of *Diodon hystrix*

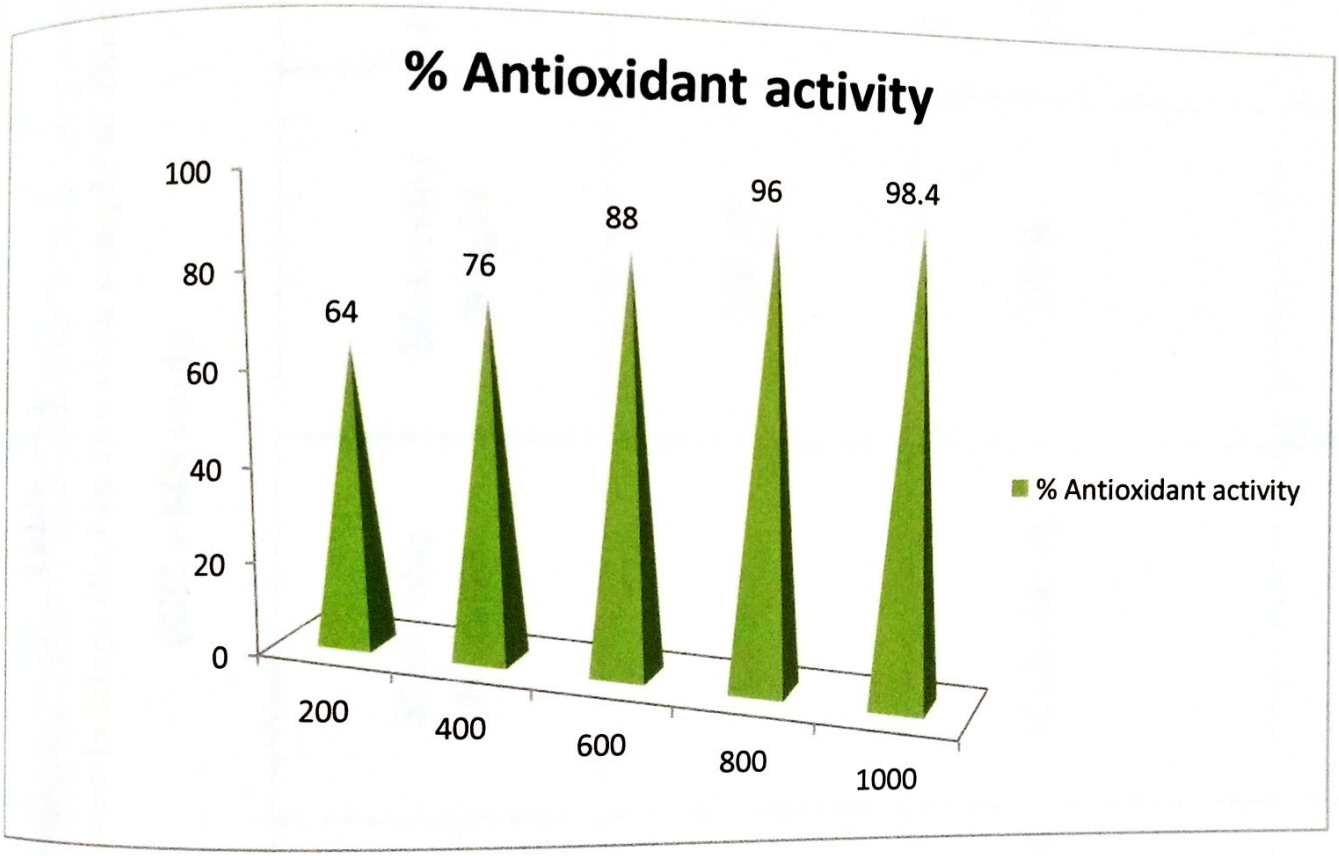


Fig 4 Antioxidant activity of phosphomolybdenum assay of muscle of *Diodon hystrix*

Table – 1

Activity of compounds idencified in the skin sample of *Diodon hystrix*

(GC – MS study

S. NO.	RT	Name of the compound	Molecular formula	Molecular weight	Peak area %	Activity
1.	8.658	2-Acetylbenzoic Acid	$C_9H_8O_3$	164.16	27.78%	Anti- aggregatory Anti-inflammatory
2.	8.658	Phenol, 2-amino-4	$C_{16}H_{15}N_3OS$	297.4	27.78%	Antimicrobial Antioxidant Anti- inflammatory
3.	8.658	Phthalic acid, 2-ethoxyethyl	$C_{14}H_{18}O_5$	266.29	27.78%	Antimicrobial Antifungal Gram positive

4.	12.403	1-Propene, 3-azido-	$C_3H_5N_3$	83.09	6.84%	Anticancer Antibacterial Antifungal
5.	12.403	Thiirane	C_2H_4S	60.12	6.84%	Anti-tumour Anti- metastatic Potential inhibitor
6.	12.403	Hydrazine, 1,1- dimethyl	$C_8H_{20}N_2$	144.26	6.84%	Antioxidant Nitrosamines Anti-nematocides Antimicrobial
7.	14.057	Acetamide 2-Fluoro	C_2H_4FNO	77.06	4.06%	Antioxidant Antimicrobial

8.	14.057	Ethylenimi , N - chloro	C_2H_4ClN	77.51	4.06%	Antimicrobial Antibiotics Anti-bacterial
9.	14.105	Silane, (11-fluoroundecyl)	H_4Si	32.117	45.00%	Antioxidant Antimicrobial
10.	14.105	2-Hexenal	$C_6H_{10}O$	98.14	45.00%	Antifungal Insecticidal activity Anti nematicidal
11.	14.105	1H-Imidazole, 4,5- dihydro-2,4	$C_3H_4N_2$	68.08	45.00%	Antifungal Antibacterial Anticancer Antidepressant Anti-leishmanial

12.	14.294	4-Fluorohistamine	$C_5H_4N_2$	129.14	16.33%	Antioxidant Anti –bacterial
13.	14.294	1-Hexanamine 2-ethyl-	$C_{16}H_{35}N$	241.46	16.33%	Antimicrobial Antitumor
14.	14.294	1-Azabicyclo(3,1,0 hexane	C_5H_9N	83.13	16.33%	Anti-fungi Anti-bacterial

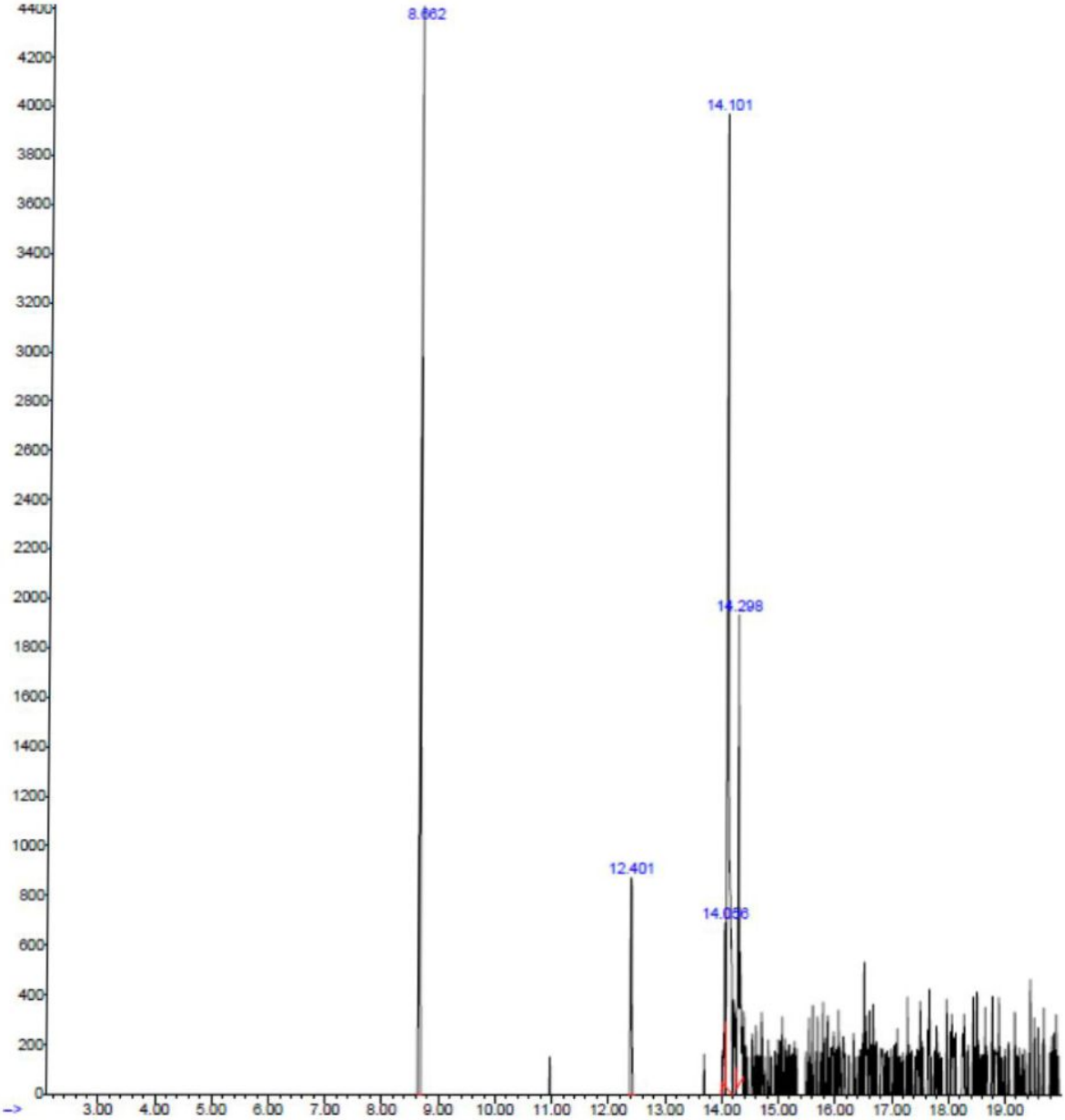


Fig 5 Chromatogram – Methanol extract of skin of *Diodon hystrix*

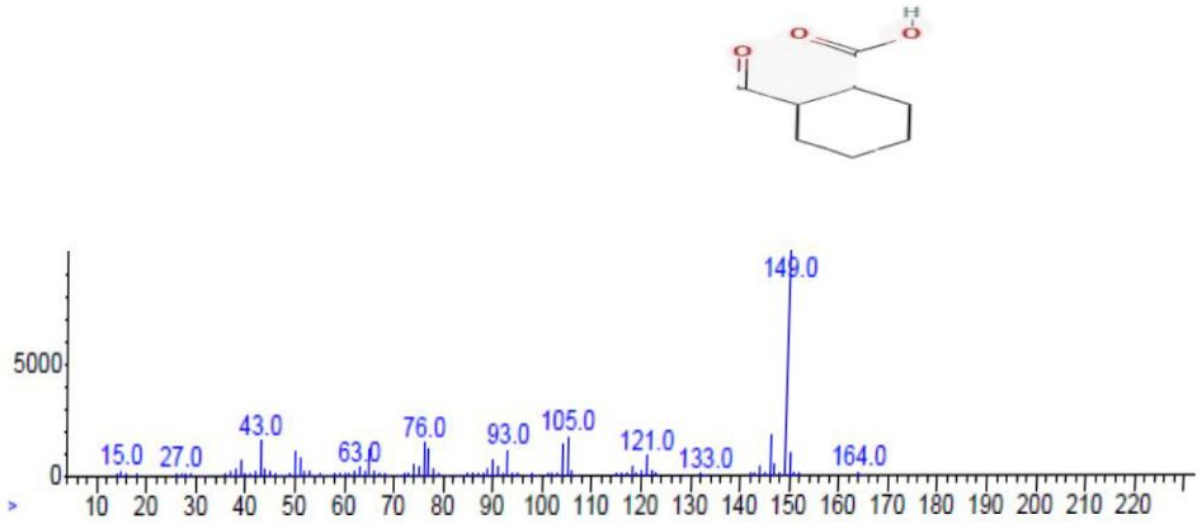


Figure : 6

Molecular Formula : $C_9H_8O_3$

Name : 2- Acetylbenzoic acid

Molecular weight : 164.16

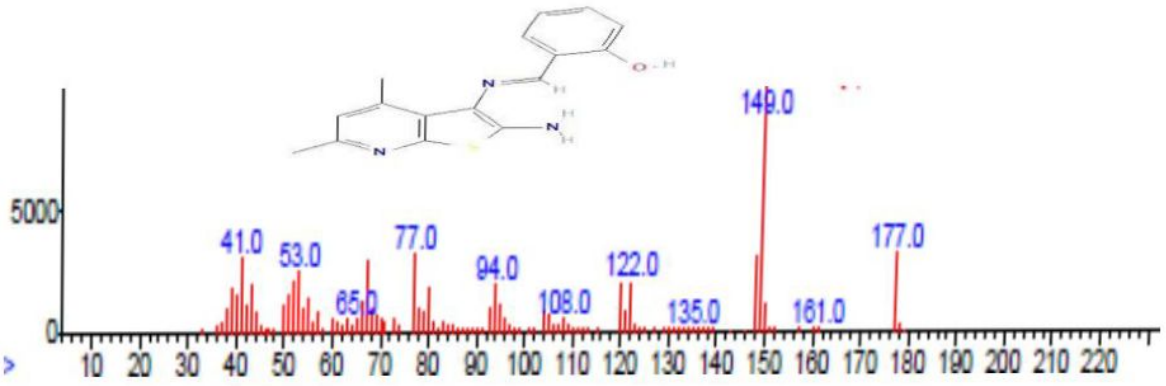


Figure : 7

Molecular Formula : $C_{16}H_{15}N_3OS$

Name : Phenol, 2 amino – 4 -

Molecular weight : 297.4

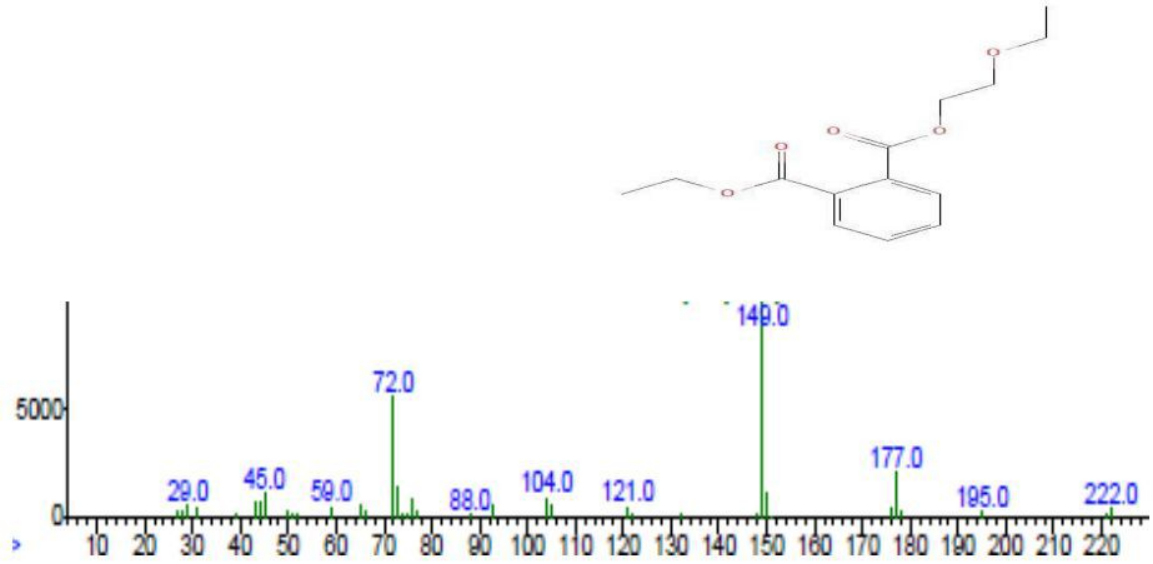


Figure : 8 **Molecular Formula :** $C_{14}H_{18}O_5$

Name : Phthalic acid 2 - ethoxyethyl **Molecular weight :** 266.29

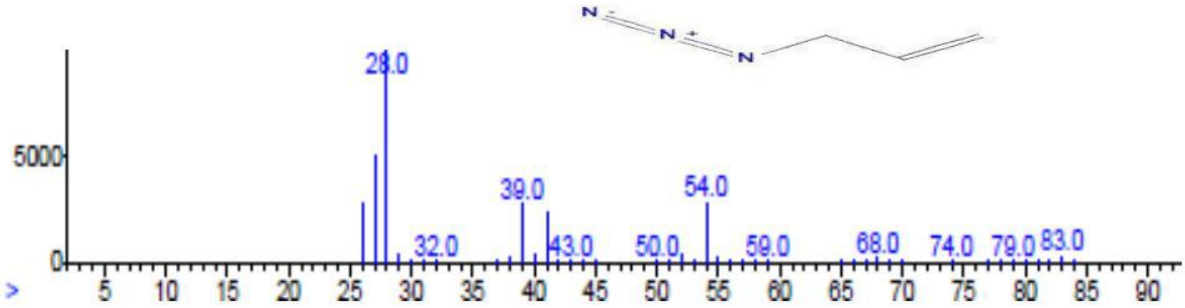


Figure : 9 **Molecular Formula :** $C_3H_5N_3$

Name : 1 – Propene 3 - azido **Molecular weight :** 83.09

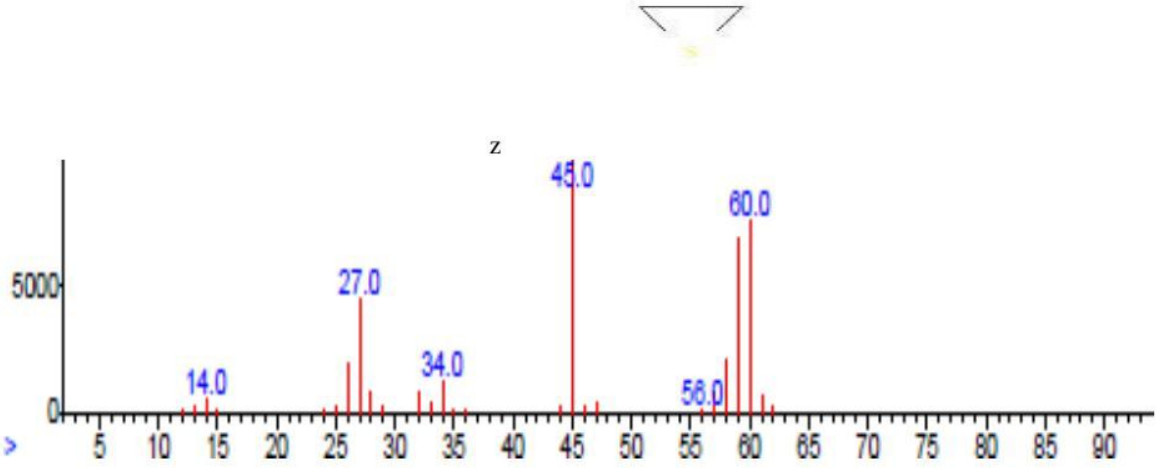


Figure : 10 **Molecular Formula :** C_2H_4S

Name : Thiirane **Molecular weight :** 60.12

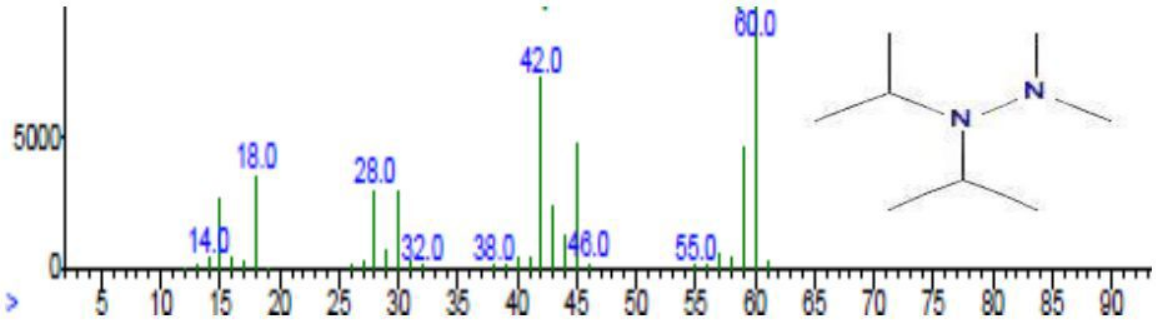


Figure : 11 **Molecular Formula :** $C_8H_{20}N_2$

Name : Hydrazine 1,1 -dimethyl **Molecular weight :** 144.26

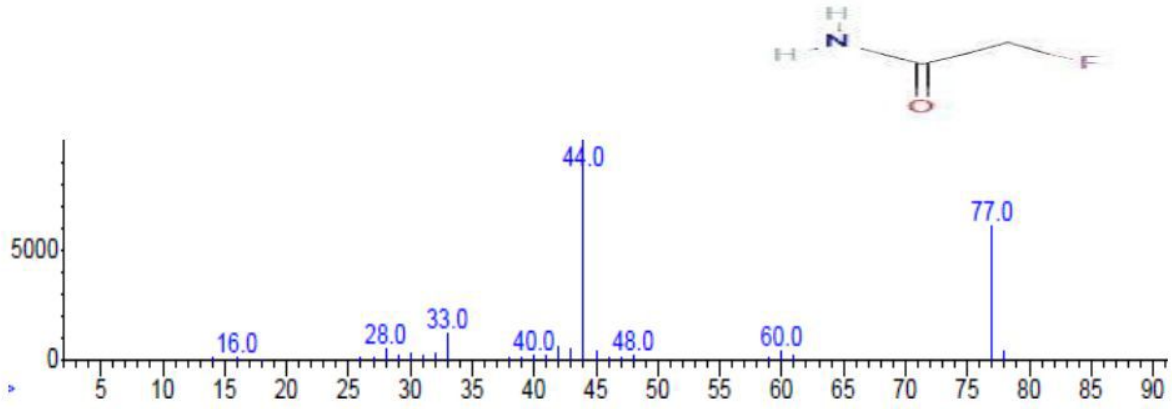


Figure : 12 **Molecular Formula :** C_2H_4FNO

Name : Acetamide 2- fluoro **Molecular weight :** 77.06

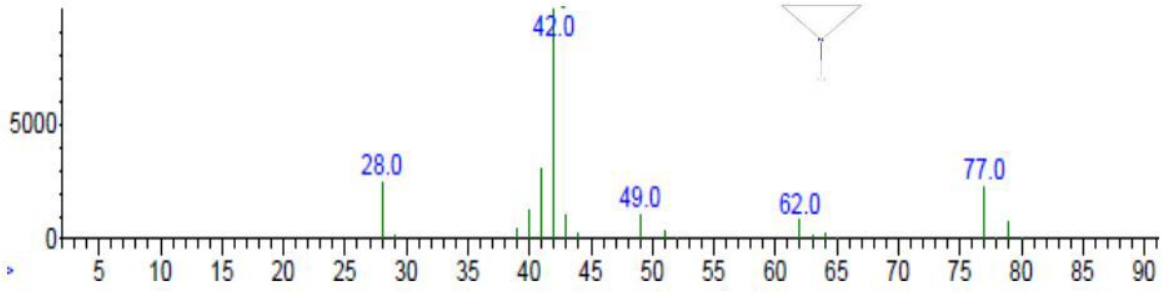


Figure : 13 **Molecular Formula :** C_2H_4CIN

Name : Ethylenimine N-chloro **Molecular weight :** 77.51

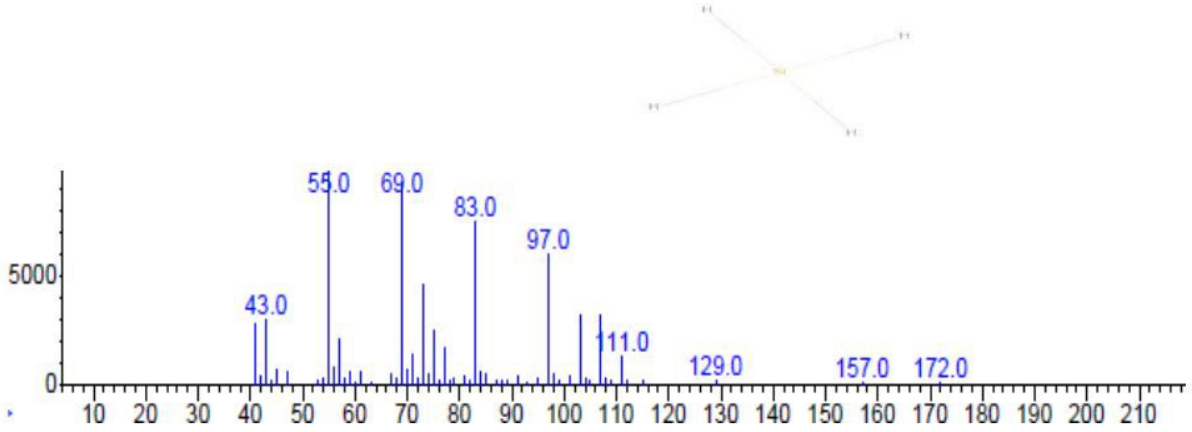


Figure : 14 **Molecular Formula :** H_4Si

Name : Silane (11-fluoroundecyl oxy) **Molecular weight :** 32.117

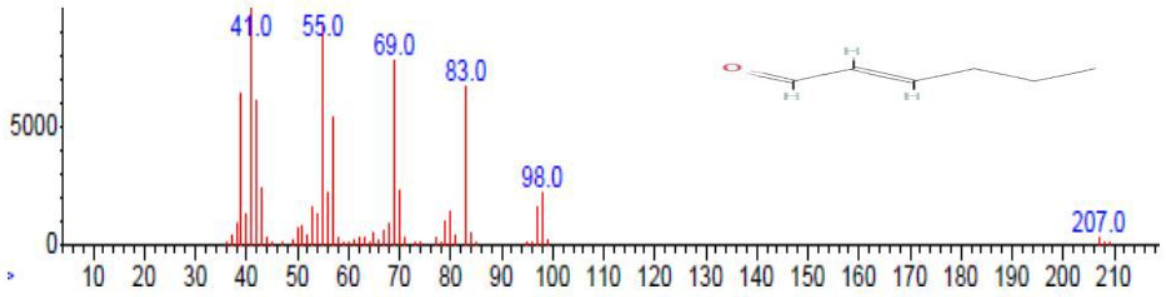


Figure : 15 **Molecular Formula :** $C_6H_{10}O$

Name : 2 - Hexenal **Molecular weight :** 98.14

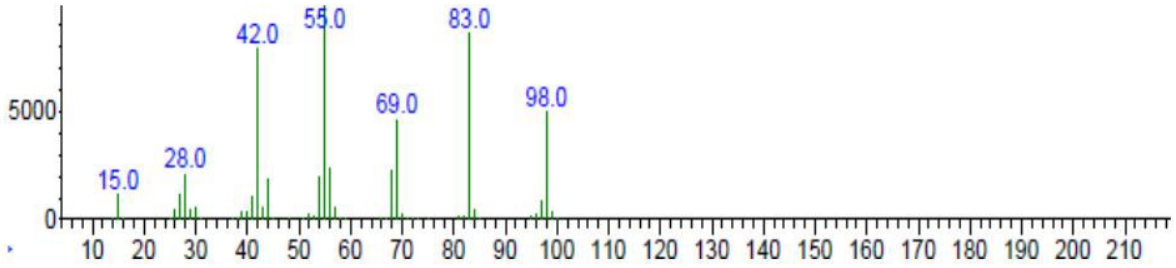


Figure : 16 **Molecular Formula :** $C_3H_4N_2$

Name : 1H – Imidazde, 4,5 - dihydro **Molecular weight :** 68.08

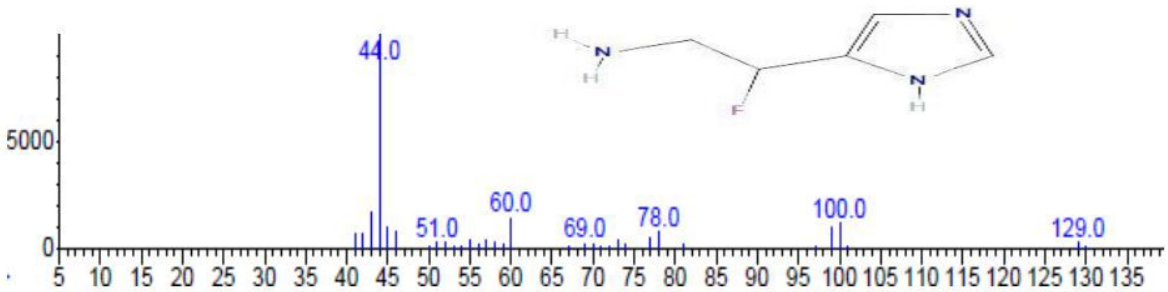


Figure : 17 **Molecular Formula :** $C_5H_4N_2$

Name : 4 - Fluorohistamine **Molecular weight :** 129.14

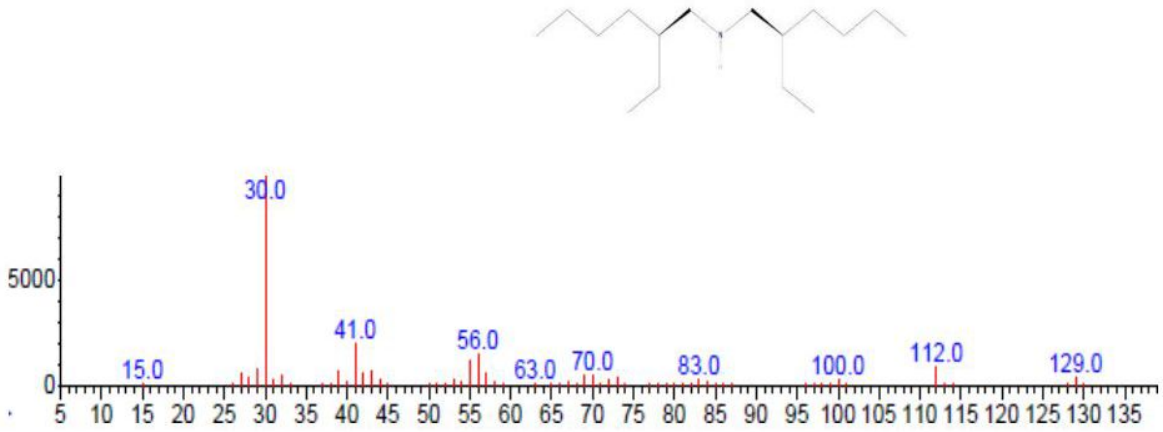


Figure : 18 **Molecular Formula :** $C_{16}H_{35}N$

Name : 1 – Hexanamine 2- ethyl- **Molecular weight :** 241.46

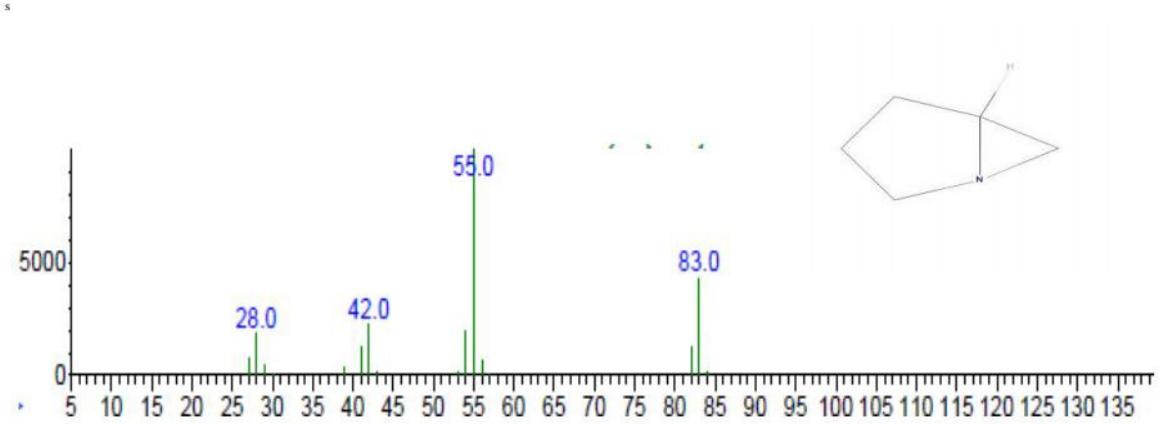


Figure : 19 **Molecular Formula :** C_5H_9N

Name : 1- Azabicycto (3.1.0) hexane **Molecular weight :** 83.13

DISCUSSION

5. DISCUSSION

The bio resources present in the marine ecosystem have potent biomolecules which includes many natural organic compounds. These compound are reported to have biological activities like antitumour, 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 biochemical useful to biotechnology and its researches (Colwell, 2002; Rajeev and Xu 2004; Faulkner and Fenical, 2005).

The aim of our study is to check the antidiabetic potential of the muscle extract of *D.hystrix* in the HFD and STZ-induced diabetic rat models. Our results demonstrated that *D.hystrix* 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. 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. Many drugs are commercially available to overcome this disorder. Now the researchers focus on the marine organisms using their bioactive compounds to treat against the development of the various diseases. Keeping in mind the medicinal value of marine compounds, fish-derived products such as the

peptide obtained from the fish muscle, fish skin have gained a lot of interest to explore the bioactive compounds which can be of medicinal values (Lauritano *et al*., 2016).

α-amylase is a digestive enzyme which has a major role in the conversion of complex starch into smaller oligosaccharides. Inhibitor of α-amylase enzyme helps to delay the hydrolysis of the starch which in turn leads to the reduction of glucose absorption and ultimately leads to lesser postprandial blood glucose level (Etxeberria *et al* .,2012). AIME expressed a better activity in the inhibition of the α-amylase enzyme. Similarly, AIME could also inhibit another important digestive enzyme, α-glucosidase effectively. The enzyme α-glucosidase present in the borders of the small intestine were involved in the reduction of disaccharides into simple sugar. So that it can be easily absorbed into the small intestine. Subsequently, inhibition of these enzymes may aid in controlling the circulating blood glucose (Hemalatha *et al*., 2016). Similar results of inhibition of α-glucosidase enzyme activity were observed in the K. pelamis heart muscle treatment (Ali *et al.,* 2016). Based on the inhibition of the digestive enzymes by AIME, it was attempted to explore the antidiabetic activity of AIME in HFD+STZ induced diabetic rats. Notably, literary reports are available which states that animal administrated with a low dose of streptozotocin leads to the development of diabetes. However, this model does not progress

the insulin resistance typically seen in T2DM. Hence, a combination of HFD with STZ is involved in this study for the development of the disease where HFD leads to the development of insulin resistance and low dose STZ induction will lead to the destruction of pancreatic β cells which mimics the type 2 diabetes pathogenesis (Ghelbi *et al*., 2017). HFD for a period of 14 days followed by a single intraperitoneal injection of STZ at 35 mg/kg concentration forms the best model for the development of type 2 diabetes. Previously, (Wang *et al*., 2011) have reported that HFD induction for 4 weeks and STZ injection at 30 mg/kg forms the best model for the development of diabetes. Increasing the period of HFD for the animals may lead to the death of the animals. Hence in our study, the modified procedure of (Wang *et al*., 2011) has been adopted.

Oxidation stress is closely related to all aspects of cancer, from carcinogenesis to the tumour bearing state and from treatment to prevention (Noda and Wakasugi, 2001). Epideminologic studies have suggested that some antioxidants of dietary constitute exhibit antioxdidant properties may be acting as naturally occurring anticancer agents and may explain some of the difference of being intake (Greenwald *et al.,* 2001). Synthetic antioxidants are suspected of being toxic upon long – term exposure (Kahul and Kappus, 1993). According to Arakawa *et al.,* (2010) many marine puffer fish posses a potent neurotoxin, which is named as tetrodotoxin

(TTX) is originally produced by marine bacteria. It can be found in muscle and skin of puffer fishes can cause death in persons who ingest it (Ellenhorn and Barceloux, 1988).

The methanol extract of puffer fish *D. hystrix* was examined for its antioxidant activity using TAA free radical scavenging by Phosphomolybdenum assay scavenging activity. As TAA picks up one electron in the presence of a free radical by phosphomolybdate assay scavenger, the absorption decreases and the resulting decolouration is stechiometrically related to the number of electrons gained (Silva *et al* ., 2005). Thus the production of such antioxidant compounds and their TAA by phosphomolybdenum scavenging effect expected to be highly variable. Such a large variability in the TAA radical by phosphomolybdenum scavenging activity may possibly due to struggle faced by wild animals for accessing food and acclimatizing to the dynamic conditions. Among the genders, the activity levels were higher in females than in males (Sanaye *et al.,* 2014). Hung, (2008) who found relatively higher scavenging activity in belly portions compared to other body portions. Crude extracts of skin and muscle *D.hystrix* were subjected for the evaluation of antioxidant activity using TAA radical by phosphomolybdenum scavenging activity test. Results of the present study showed that the crude methanol extracts of various tissues of *D.hystrix* exhibited antioxidant activity.

Barwin *et al.,* (2012) studied the inhibitory effect of sulphated chitosan from cuttle bone of Sepia aculata through scavenging activity and observed that the scavenging activity of superoxide radicals was in concentration dependent manner. Higher levels of TAA by phosphomolybdenum activity compared to other body tissues (muscle and skin) in four species of Hippocampus have also been reported by Bhadra *et al.,* (2004). These findings are also supporting the present study. In the present study the crude extracts of puffer fish scavenged TAA radical by phosphomolybdenum assay in a concentration dependent manner. Among the two tissues tested, muscle extract showed strong scavenging activity of TAA radical by phosphomolybdenum assay and clearly suggested that the antioxidant activity of skin extract was related to its ability to scavenge TAA radical by phosphomolybdenum assay.

GC - MS Analysis

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 skin. 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 extracts of puffer fish D. 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.

SUMMARY

6. SUMMARY

The present study has been carried out to establish the occurrence of antidiabetic, antioxidant activity and the characterization of chemical compounds from skin and muscle extract of puffer fish *D.hystrix.*

The current study was designed to evaluate the pharmacological activity of methanol extracts of skin and muscle of *D.hystrix*. Methanol extract of muscle exhibited maximum Alpha amylase inhibition assay- Antidiabetic activity with 36%, 48%, 60%, 84% and 84.8% at 200µg, 400µg, 600µg, 800µg, 1000µg respectively. The skin extract with 20%, 44%, 56%, 76%, 76.80% at 200µg, 400µg, 600µg, 800µg, 1000µg respectively.

Methanol extract of muscle exhibited maximum TAA by Phosphomolybdenum assay – Antioxidant activity with 64%, 76%, 88%, 96% and 98.40% at 200µg, 400µg, 600µg, 800µg, 1000µg respectively. The skin erxtract with 8%, 20%, 24%, 28%, 40% at 200µg, 400µg, 600µg, 800µg, 1000µg respectively.

Chemical compound present in the skin characterized through GC - MS analysis. The result indicated that there are fourteen compounds in the skin with antioxidant, antimicrobial, antidiabetic, antineoplastic agents,

antiepileptics, antidotes, immunomodulators and immunostimulants activities.

CONCLUSION AND SUGGESTIONS

7. CONCLUSION AND SUGGESTION

Bioactive compounds from various marine sources have often been found to be promising pharmaceutical agents. It is worthy, to note that the product from nature source is good for health to devoid of side effects. In conclusion, the emergence of the population with diabetes has been increasing day by day. Hence, the search for a suitable drug with the antidiabetic potential is in great demand. Since diet plays a key role in controlling and the management of the disease by the consumption of the food rich in medicinal value has been increasing. The fish *D.hystrix* showed the extracts of skin and muscle tissues studied, methanolic extract of the presence of many bioactive compounds which has antidiabetic property. This fish can be consumed by people for the control of diabetes when it is carefully handled (TTX) and properly cooked. However, the detailed study is required in order to explore the mechanism in which the bioactive compounds of *D.hystrix* influence to control diabetes.

In the present study, the methanol extract of skin and muscle of puffer fish *D.hystrix* has been examined for their antioxidant activity in particularly the crude methanol extracts showed a broad spectrum of activities. Phosphomolybdenum assay are known to play a definite role in a wide variety of pathological manifestations of pain, inflammation, cancer, diabetes, hepatic damage etc. Antioxidants fight against

Phosphomolybdenum assay and protect us from various diseases. They exert their action either by scavenging the extracts of skin and muscle tissues studied, methanolic extract of scavenging activity on TAA by Phosphomolybdenum assay compounds with the standard antioxidant ascorbic acid.

GC - MS is a useful tool for chemical analysis. Results obtained clearly indicate that, the skin and muscle extracts possess compounds with many biological activities.

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AN EVALUATION OF *PORTUNUS PELAGICUS* SHELL EXTRACT FOR THERAPEUTIC ACTIVITY

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MASTER OF SCIENCE IN ZOOLOGY

by

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

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April – 2023

CERTIFICATE

This is to certify that this dissertation entitled, **"AN EVALUATION OF *PORTUNUS PELAGICUS* SHELL EXTRACT FOR THERAPEUTIC ACTIVITY"** submitted by **B. MUTHULAKSHMI, Reg. No. 21APZO03** to St. Mary's College (Autonomous), Thoothukudi affiliated to Manonmaniam Sundaranar University, Tirunelveli in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2022-2023 under my guidance and supervision. It is further certified that this dissertation or any part of this has not been submitted elsewhere for any other degree.

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DECLARATION

I do hereby declare that this dissertation entitled, **"AN EVALUATION OF *PORTUNUS PELAGICUS* SHELL EXTRACT FOR THERAPEUTIC ACTIVITY"** submitted by me for the award of the degree of Master of Science in Zoology is the result of my original independent research work carried out under the guidance of Dr. Jemma Hermelin Jesy Diaz M.Sc., B.Ed., M.Phil., Ph.D., Assistant Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date: 05.04.2023

B. Muthulakshmi

Signature of the Candidate

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1. INTRODUCTION

The ocean is one of earth's most valuable natural resources. Ocean offers a large biodiversity of fauna and flora which is estimated to be over 5,00,000 species or more than double of the land species (Kamboj,1999). 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 (Johanson *et al.*, 1985). From 1960's approximately 6,500 bioactive compounds have been isolated from the marine organisms (Kamboj,1999).

Marine invertebrates offer a rich source of potential drugs with excellent biological actives. Approximately 7000 marine natural products have been established from marine invertebrates. so far 18% from coelenterates (sea whips, sea fans and sea corals), 33% from sponges, and 24% from representatives ascidians (also called tunicates), Opisthobranch mollusks (nudibranches, sea horse etc.,), bryozoans (mass animals), echinoderms (star fish, sea cucumber etc.,) and crustacean.

Crustaceans are visibly a remarkable group of organisms with a long evolutionary history and prominent adaptability (Chopra and Das, 1937;

Varadharajan, 2012). Living organisms captured from the marine environment are used as food and regarded as seafood or otherwise known as marine-based foods (MBFs) (Hosomi *et al.*, 2012). MBFs may consist of several species of mollusks, fish, echinoderms, seaweed or macroalgae and crustaceans, which are rich sources of fat, protein, vitamins and minerals (Olatunde *et al.*, 2018).

Crabs are decapods crustacean and are the essential part of macro fauna. Crab (brachyura) holds a prosperous diversity and more than 5000 species belonging to 700 genera have been identified worldwide. Crabs are found in all type of environment. They are adapted to various intertidal habitats of rocky, lakes, ponds and freshwater caves. They are also found in deep water of oceans and the abundance decrease with increase in the depth and latitude. They show pelagic, benthic burrowing and terrestrial modes of life. The majority of crab species belongs to the infra order brachyuran, which means “short tails”.

Crabs are one of the extremely diversified and leading groups among crustaceans and considered as healthy food for humans because they contain high quality protein vitamins A and D, minerals, glycogen, free amino acids and less amount of lipids. Crabs are the fundamental parts of the ecosystem; they are supposed to be good for human consumption and taken as food in many countries. Both freshwater and marine crabs are consumed. Crabs are commercially important and fetch high price as there is a rapidly expanding demand for crab

meat both in local and international market. The crab fishery in India is fast developing and there is a vast scope for the crab meat due to its delicacy and nutritional richness. Most of the marine crabs occurring along the Indian coasts are belonging to the family portunida (Soundarapandian *et al.,* 2014).

Dumping of shellfishery waste is a major environmental concern worldwide and a serious threat to the coastal area. By-products are generated during the processing of crustaceans, which can be further transformed to other products with varying bioactivities and increased their value (Gulzar &Benjakul, 2019; Sinthusamran *et al.*, 2018). The shell wastes constitute of many commercially valuable products, such as, chitin, calcium carbonate, proteins, and carotenoids. Processing of shell wastes is a source of wealth. Chitin is one of the constituents of the shell wastes and also the most abundant biopolymer next to the cellulose. Calcium carbonate and proteins are the other valuable constituents of the shell wastes and can serve as a better animal feed supplement.

Crustaceans waste from crab shells is composed of chitin which forms a chitinoproteic complex with proteins. These by-products are composed of valuable components such as proteins (15-50%), minerals (30-50%), chitin (15-30%) (Kurita, 2006) and antioxidant compounds such as seleunium and carotenoids (astataxanthin, astatine & can-thaxanthin) (Cho *et al* .,1998). The

crustacean shell has high potential of bioactive compounds with antimicrobial, antioxidant, and antitumor activities (Shahidi *et al*., 1991).

The microbial associates of marine crustaceans have proven to be a rich source of biologically active substances with antimicrobial, cytotoxic and antineoplastic activities that can be useful for biotechnological and pharmaceutical application. Despite tremendous progress in human medicines today infectious diseases caused by bacteria, viruses, fungi and parasites are still a main threat to public health (Barber 1947 and Thomas 2010). The impact is particularly large in developing countries due to relative unavailability of medicines and the emergence of widespread drug resistance (Roberts *et al.*, 2009, Clarke *et al.*,1952 and Yen *et al.*, 2009). The decreasing therapeutic effect of conventional antimicrobials is associated with the improper use and inappropriate dose of these compounds, which allow the microorganisms to develop the resistance toward them over time (León-Calvijo *et al.*, 2015). During the last two decades, the development of drug resistance as well as the appearance of undesirable side effects of certain antibiotics has led to the search of new antimicrobial agents.

Antibiotics are one of our most important weapons in fishing bacterial infections and have greatly benefited the health related quality of human life since their introduction (Sarkar *et al*., 2003). The problem of antimicrobial (drug)

resistance is emerging and many diseases are increasingly difficult to treat because of the emerging drug- resistant organisms (Sharma *et al*., 2005). It is therefore necessary to search for novel antibacterial compounds with therapeutic potential for which pathogenic may not have resistance (Patil *et al*., 2001; Bansemir *et al*., 2006 and Ilthan *et al*.,2007). The evolution of antibiotic resistant pathogenic bacteria has stimulated the search for alternative antimicrobial agents from natural source. Many antimicrobial peptides show a high specificity for prokaryotes and a low toxicity for eukaryotic cells and their mode of action is considered unlikely to lead to development of resistance. These properties have favored their investigation potential new antibiotics (Bax *et al*., 2000).

Free radicals are harmful to the body. The instability and reactivity of free radicals due to the lone electron in the outer shell can cause them to attack specific biomolecules in the body such as proteins and lipids (Nagmoti *et al*.,2012). Presence of abnormal amount of free radicals in the body leads to oxidative stress. To maintain normalcy, there is a balance between the quantity of free radicals and antioxidants that are produced by the body. Due to increased concentration of free radicals in the body, additional supplements of antioxidants are needed to be consumed.

Antioxidants are compounds capable of either delay or inhibit the oxidation process which occurs under the influence of atmospheric oxygen or reactive

oxygen species. Antioxidants are involved in the defense mechanism of the organism against the pathologies associated to the attack of free radicals. Natural antioxidants can play a more important role in the health of mankind because of having antivirus, anti-inflammatory, anticancer, antitumor and liver protection properties (Atila Gozde *et al.*, 2015; Gupta Pooja *et al.*, 2015; Viahwakarma Pratima *et al.*, 2016 and Bektas Ersan *et al*.,2016). Oxidative stress is involved in the pathology of cancer, arteriosclerosis, malaria and rheumatoid arthritis and could be play a role in neurodegenerative disease and ageing processes (Nakagami, 1995). Current research in the free radicals has confirmed that food items rich in antioxidants play an essential role in the prevention of cardiovascular disease and cancer and neurodegenerative disease, including Parkinson's and Alzheimer diseases, as well as inflammation and problems caused by cell and cutaneous aging (*Li et al.*, 2007).

Antioxidants arises, as environmental supplements or pharmaceutical products, which contains as active principle an antioxidant compound. Among the most important exogenous antioxidants, vitamin E, vitamin C, B- carotenoid, flavonoids, minerals can derive from natural sources (Vitamins, flavonoids, anthocyanins, some minerals compounds) but can also be synthetic compound, like butythyds (Litscu *et al.*, 2011). There is an increasing interest in antioxidants, to prevent the presumed deleterious effects of free radicals in the human body as

well as the deterioration of fats and other constituents of foodstuffs (Molyneux, 2004). Therefore, in recent years, interests have been developed for searching effective natural antioxidants, since they can protect human body from free radical and retard the progress of many chronic diseases. It possible to reduce the chronic disease and prevent disease progression by either enhanching the body's natural antioxidant defenses or by supplement with dietary antioxidants (Stanner *et al*.,2000).

Diabetes mellitus (DM) is a chronic disease caused by inherited or acquired deficiency in insulin secretion and by decreased responsiveness of the organs to secreted insulin. Such a deficiency results in increased blood glucose level, which in turn can damage many of the body's systems, including blood vessels and nerves (Matsui *et al.*, 2007). Diabetes mellitus is currently one of the most costly and burdensome chronic diseases and is a condition that is increasing in epidemic proportions throughout the world (King, Aubert, & Herman, 1998). Based on the World Health Organization report, the number of diabetic patients in the world will rise from 171 million in 2000 to more than 300 million in 2025 (Wild *et al.*, 2004).

Long hyperglycemic periods, through glucose oxidation and protein glycosylation, can lead to production of free radicals, especially reactive oxygen species (ROS). These conditions disrupts the balance between ROS production

and antioxidant defense mechanism in all tissues and results in cell dysfunction and changes in cell function, especially in pancreas (Robertson *et al.,* 2003). Nitric oxide (NO) is significantly involved in pancreatic destruction, oxidative stress and NO pathway are related and seem to modulate each other, leading to ß-cell destruction (Gonzalez *et al.,* 2000). Management of diabetes without any side effects is still a challenge to the medical system (Chakraborty & Rajagopalan, 2002; Kameswararao *et al.,* 2003).

One therapeutic approach for treating diabetes is to decrease the post-prandial hyperglycemia. This is done by retarding the absorption of glucose through the inhibition of the carbohydrate-hydrolyzing enzymes α-glucosidase and α-amylase in the digestive tract. Inhibitors of these enzyme delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise (Rhabasa- Lhoret & Chiasson, 2004). Many natural resources have been investigated with respect to the suppression of glucose production from carbohydrates in the gut or glucose absorption from the intestine (Fernando *et al.,* 1991; Welsh *et al.,* 1989). The wide variety of chemicals classes indicates that a variety of mechanisms of action are likely to be involved in lowering blood glucose levels. Current drugs used for the treatment of diabetes are associated with several side effects such as hypoglycemia, weight

gain, gastrointestinal disorders, peripheral edema and impaired liver function (Mallare *et al.,* 2005), hence, there is a need for effective, safe and better oral hypoglycemic agents.

Hemolysis is widely employed as an important indicator of free radical damage affecting the membrane of erythrocytes (Chansiw *et al.,*2018). Erythrocytes are the most abundant cells in the human body with their own replicative biological and morphological characteristics. The hemoglobins and polyunsaturated fatty acids (PUFA) are redox active oxygen transport molecules and potent promoters of activated oxygen species mainly target the erythrocytes.

Oxidative mutilation to the erythrocyte membrane lipids and proteins may be responsible for hemolysis accompanying with several factors namely hemoglobinopathies, oxidative drugs, excess of transition metals, radiation, and deficiencies in erythrocyte antioxidant coordination (Ebrahimzadeh *et al*., 2009 and Hamidi *et al.,* 2003). The magnitude of hemolysis was appeared to be much more overwhelming, when red blood cells were exposed to any toxicant like hydrogen peroxide (Naim *et al.,*1976). On the other hand, the antioxidant compounds are known to have the ability to scavenge free radicals and protect the cells against hemolysis induced by oxidative stress.

Gas chromatography-Mass Spectra (GC-MS) is a analytical technique that combines the separation properties of gas-liquid chromatography with the

detection feature of mass spectrometry to identify different substances within a test sample. GC-MS can be used to study liquid, gaseous or solid samples. GC is used to separate the volatile and thermally stable substitutes in a sample whereas GC-CM fragments the analyte basis of its mass. The further addition of mass spectrometer in it leads to GC-MS.(Sahil *et al*.,2011 and Jenke, 1996).

GC/MS is also used in analyzing compounds such as alcohol, fatty acids, esters, aldehydes and more in beverages, foods and perfumes. GC-MS is widely used in pharmaceutical industries for analytical research and development, quality assurance, for active pharmaceutical ingredients (API), bulk drugs and formulations. It is an integral part of research associated with medicinal chemistry (synthesis and characterization of compounds), pharmaceutical analysis (stability testing, impurity profiling), pharmacognosy, pharmaceutical process control, pharmaceutical biotechnology etc (CDER, 2000 and CDER, 1994).

Considering the potential of *Portunus pelagicus*, an attempt has been made to study the antibacterial, antioxidant and anti-diabetic, antihaemolytic activities of this crustacean.

2. **REVIEW OF LITERATURE**

Nature has been instrumental as a source of therapeutics as the oceans cover more than 70% of the earth surface and the marine environmental is highly diverse, and it would be a wonderful source of biologically active molecules. Over the past decade, several new therapeutic agents derived from marine source have entered preclinical and clinical trials. This field has expended significantly as a result of improvements in the technology of deep-sea collection, extraction and large-scale production through aquaculture and synthesis. The collection of the marine therapeutics includes molecules with antimicrobial activity.

The fishery processing industry has been producing increasingly large amounts of byproducts, with the globally reported accessible waste of 27.85 million tons per year (Caruso *et al.*, 2020). Among various fishery wastes, marine chitinous wastes (MCWs) including crab shells, squid pens, shrimp shells, and shrimp heads are abundantly available as fishery byproducts. Recently, these materials have been extensively used for the extraction and bioproduction of many active compounds (Wang *et al.*,2019).

The crustacean shell has a rich source of biological polymers, chitin (20%-30%), 30-40% protein, 30- 50% calcium carbonate, 20-30% chitin (Hobel C.F.V,2004, Crini G *et al.*, 2009 and Jo G.H *et al.*,2011), with species and

seasonal variations (Cho Y.I *et al.*,1998), lipids and pigments such as carotenoids and minerals (Yilmaz, 2004). The crustacean shell has high potential compounds with antimicrobial, antioxidant and antidiabetic activities.

Haug *et al.*, (2002) has reported the antibacterial activity in different body-parts of *Pandalus borealis* (northern shrimp), *Pagurus bernhardus* (hermit crab), *Hyas araneus* (spider crab) and *Paralithodes camtschatica* (king crab) against *Escherichia coli, Vibrio anguillarum, Corynebacterium glutamicum* and *Staphylococcus aureus.* Park *et al.*, (2004) observed that 87.8% deacetylated water soluble chitosan from *P. stylifera* shrimp shell wastes showed antibacterial activity against *S.aures* than crude chistosan. This dictated that both chitosan might have the antibacterial activity which could be used in pharmacological research.

Veeruraj *et al.*, (2008) recorded antibacterial activity of crab haemolymph on clinical pathogens and showed the highest zone of inhibition was observed in the haemolymph of *Scylla tranquberica* against *Vibrio cholera* (10mm) and the lowest zone of inhibition was observed in the haemolymph of *Macropthalmus depressus* against *S.paratyphi-B* and *S.typhi* (5mm) and indicates that the haemolymph of crab would be a good source of antimicrobial agents. Ravichandran *et al.*, (2010) investigated antimicrobial activity of crude haemolymph of the crab *Ocypode macrocera* against 11 different bacterial strains

and 5 fungal strains. Ferraro *et al.,* (2010) reported that the haemolymph of crab would be a good source of antimicrobial agents and would replace the existing inadequate and cost effective antibiotics.

Moarul Islam *et al.,* (2011) analyzed the antibacterial activity of crab chitoson against *Staphylococccus aures* and *Escherichia coli*. Arul Prakash *et al.,* (2011) has studied the antimicrobial activity in the haemolymph collected from fresh water crab *Paratelphusa hydrodromous* against five bacterial species and five fungal strains. Trancy and Barry, 2012 studied the antibacterial effect of crab shell on *Klebsiella pneumoniae* and *Pteria mirabilis* isolated from urine and wound specimen.

Varadharajan and Soundarapandian, 2013 carried out the antibacterial activity of crab shell extracts against human pathogenic bacteria through in vitro screening by disc diffusion method against *Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Pseudomonas mirabilis*. Gokilavani *et al.,* (2014) studied the physicochemical characteristics and antibacterial activity of chitoson extract from shell of crab *Parateelphusa hydrodromous.* Packia Lekshmi *et al.,* (2014) investigated antibacterial activity from the isolated peptides from the haemolymph of fresh water crab and snail by SDS – PAGE and **r**eported that the fresh water snail (*Pomacea insularium*) and

crab (*Callinectes sapidus*) having remarkable antimicrobial activity in methanol, di-ethyl ether and water extracts.

Nisha, 2015 evaluated the inhibitory effect of chitosan extracted from crab shell *Portunus sanguinolentus* on pathogenic bacteria *Klebsiella, Solmonella typhi, Staphylococcus aureus, E.coli* and *Salmonella paratyphi B.* Shibana *et al.*, (2017) investigated the antimicrobial activities of the shell of the crab *Portunus pelagicus* from thoothukudi coast and showed the best antibacterial activity against *Streptococcus* (7mm) and ethanolic shell extract showed the maximum antifungal activity against *Aspergillus flavus*. Yoana Krasimirova Kizheva *et al*., (2019) studied reported antibacterial activity of crab *Eriphia verrucosa* haemocyanin against clinical pathogens.

Wei Jiang *et al*., (2018) studied antioxidant and antibacterial activities of modified crab shell bioactive peptides by Maillard reaction. Soundarapandian *et al.*, (2014) has reported the antioxidant activity in hard and soft Shell Crabs of *Charybdis lucifera* and suggest that soft shelled crabs of *Charybdis lucifera* show antioxidant property than hard shell crabs. So the soft shelled crab may be used for the preparation of antioxidant and this will prevent the wastage of useful soft shelled crabs from the landing centers in some extend. Rethna Priya *et al.*, (2015) analyzed the antibacterial and antioxidant properties from the crab *Liagore rubromaculata*.

Antioxidants protect the key cell components by neutralizing the damaging effects of free radicals, which are natural by-products of cell- metabolism (Shenoy and Shirwaikar, 2002). Oxidative stress causes serious cell damage leading to a variety of human disease like Parkinson's disease, Alzheimer disease, Atherosclerosis, Cancer, Arthritis, Immunological incompetence and Neuro degenerative disorders (Devasagayam and lasavan ,2003). Prem Anand *et al.*, (2014) reported the antioxidant properties of natural dietary common sea foods from pulicate coast.

The antioxidant activity of chitosan and its derivatives has been reported by Guo *et al.*, (2005). Yen *et al.*,(2008) reported that crab chitoson showed moderate antioxidant activity of 58.3-70.2% at 1mg/ml and high antioxidant activity of 79.9-85.2% at 10mg/ml. Sudhakar, (2011) observed the total antioxidant activity at varying concentration (0.5 to 10mg/ml) in *P.sanguinolentus* crab shell chitoson sample. Kuppusamy *et al.*, (2013) evaluated the free radical scavenging activity of chitoson. Sawssen Hajji Olfa Ghorbel *et al.*, (2015), extracted chitin from crab shells by *Bacillus* bacteria and biological activities of fermented crab supernatants suggest that crab hydrolysates are good sources of natural antioxidants. Gauhar Rehman *et al.*, (2020) estimated the ethanolic extract of *Allacanthos* crab inhibits cancer cell proliferation, posses anti-inflammatory and antioxidant potentials and showed that *Allacanthos crab* could be a potential source of curing

and preventing cancer with active molecules intended for applications in the pharmaceutical industry.

Oxygen derived free radicals or reactive oxygen species (ROS) formed in the body during energy producing energy producing metabolic process, play an important role in pathophysiology of a number of disease (Cuzzocrea *et al*., 2001). Through a series of oxidant reactions (Pashkow and Campbell., 2008), free radicals in excess can react and cause oxidative damage to cellular components such as proteins, lipids, lipoproteins and deoxyribonucleic acid (Lobo *et al.,* 2010 and Singh *et al.,* 2015) being one the causes of degenerative disease and aging (Valko *et al.,* 2006). However, oxidations of biomolecules can be inhibited by suitable amounts of antioxidants present in balanced daily diet (Bose and Agarwal, 2007 and Thomsan *et al.,* 2007).

Shiny Kachhap *et al*., (2020) investigated the assessment of nitric oxide scavenging activity and total antioxidant activity of chitosan extracted from carapace of freshwater edible crab *Sartoriana spinigera* and found that the percent of scavenging activity of chitosan against nitric oxide anion was found to be 21.95 %, 35.49%, 46.90%, and 66.04% at 0.5 mg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml concentration respectively. Percent of total antioxidant activity of Chitosan was found to be 29.73 %, 33.78%, 50%, and 62.16 % at 50 µg/ ml, 100 µg/ml, 200 µg/ml and 400 µg/ml concentration respectively.

Fatemeh Makalani *et al*.,(2017) Crab shell extract improves serum biochemical markers and histological changes of pancreas in diabetic rats evaluated antioxidant and anti-diabetic effects of crab shell increase total antioxidant capacity of serum and decreased blood glucose, serum nitric oxide and ALT levels. Hana Maalej *et al.*, (2021) analyzed novel digestive α-amylase from blue crab (*Portunus segnis*) viscera. Thi Hanh Nguyen *et al., (*2022) analyzed novel α-amylase inhibitor hemi-pyocyanin produced by microbial conversion of chitinous discards and showed HPC demonstrated potent activity comparable to acarbose, a commercial antidiabetic drug. Fang Yan *et al.,* (2011) analyzed hemolytic properties of hemocyanin from mud crab *Scylla serrata* against vertebrate erythrocytes.

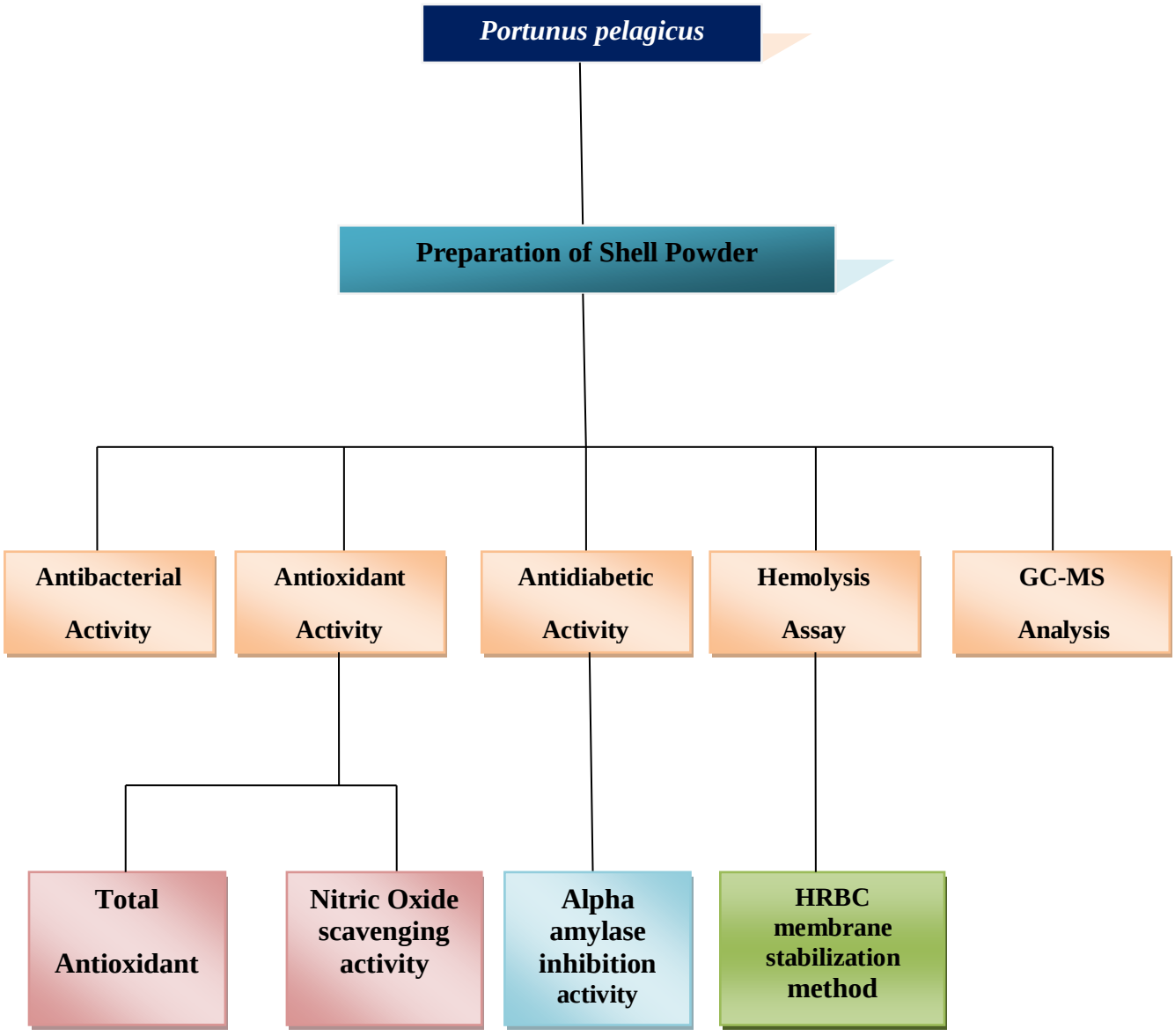
Suganya and Asheeba (2015) **i**solated and characterized astaxanthin from *Portunus sanguinolentus* (three spotted crab), *Callinectes sapidus* (blue crab) and *Paralithodes brevipes* (spiny king crab) by GC-MS. Results revealed that isolated astaxanthin and standard astaxanthin shows the presence of functional groups. Madhubala and Selvamohan (2023) analysed presence of carotenoids pigments in the three spotted crab (*Portunus sanguinolentus)* by GC-MS and reported carotenoid compounds were distributed a varied level in the carapace and muscle.

3. OBJECTIVES

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

- To study the antibacterial activity in the shell extracts of *Portunus pelagicus* against five different pathogens.
- To determine the antioxidant activity in the shell extract of *Portunus pelagicus.*
- To analyze the antidiabetic activity in the shell extract of *Portunus pelagicus.*
- To assess the haemolytic activity of shell extract to prevent oxidative damage to erythrocyte membrane.
- To find out the bioactive compounds in the methanol extract of shell by GC-MS.

4. EXPERIMENTAL DESING

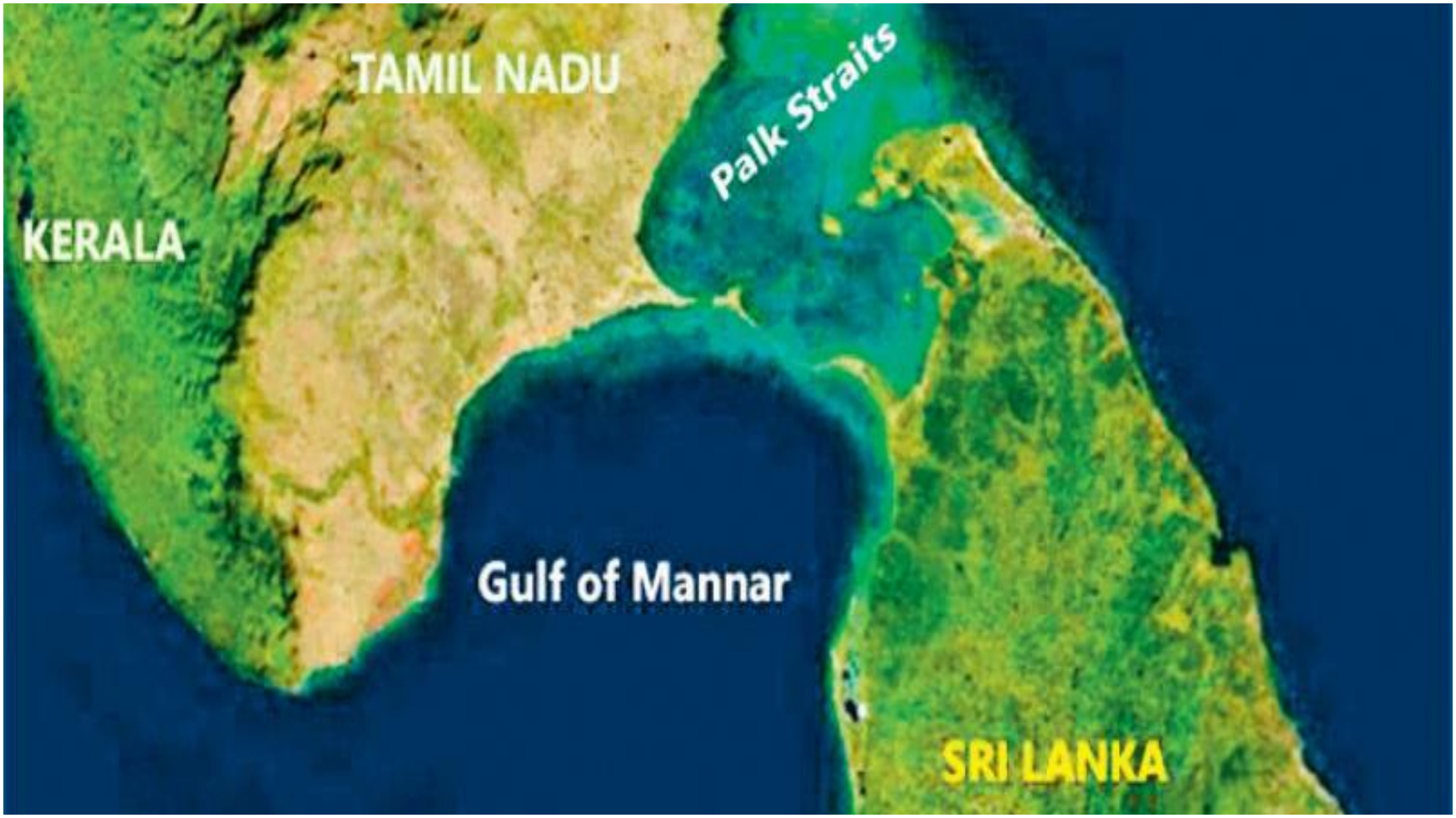


5. MATERIALS AND METHODS

5.1. Description of the study area

Gulf of Manner is placed between India and Sri Lanka (Long. 78º 8” to 79º 30” E and Lat 8º 35” to 9º 25” N). It is a part of the southward extension of the Bay of Bengal and it meets in the Indian Ocean. This geographical area runs from Pamban Island including Rameshwaram to Cape Comarin along the southeast coast of Indian to a distance of about 170 nautical miles. This coast maintains a rich biological diversity of flora and fauna largely due to diversified microhabitats such as mangroves, corals, seaweeds beds, sea grasses, sandy, rocky and muddy shore etc. the fauna diversity is also well pronounced with reference to different crustacean groups. For the present study the animals were collected from the fishing trawlers operated for crab and prawn from the Thoothukudi coastal region.(Fig.-1)

Fig 1: Map showing Tuticorin shore, the Gulf of Manner



5.2Experimental animal: *Portunus pelagicus*(Linnaeus, 1758)

Systematic position of *P.pelagicus*

Kingdom : Animalia

Phylum : Arthropoda

Class : Crustacea

Order : Decapoda

Family : Portunidae

Genus : Portunus

Species : *Portunus pelagicus*

5.2. Description of the study animal

Portunus pelagicus is commonly called as blue swimming crab, one of the best edible crabs. It is also known as flower crab, blue swimmer crab, blue manna crab or sand crab inhabits the sandy mud bottom of sheltered estuaries and inlets throughout the east coast of India. Most of the crab's life is spent just below the sand surface where it waits with only its eyes protruding, watching for fish and invertebrates to venture close enough for a successful attack. This burrowing habit is also for protection, as many species of fish are known to prey on it.

The species is distinguished by the presence of a single spine on the posterior border of merus of the chelipedes. Antero lateral borders are cut into nine teeth including the postorbital spine. The last pair of lateral spines greatly pronounced. Chelipedes have strong spines, its surface scabrous. Carapace covered with irregular granules. The males are pinkish blue with extensive irregular white spots; the tips of the chelae and the distal segments of the legs purple; female sand coloured. The male is larger than the female and its colouration is the source of its common name. The female is smaller and tends to be browner with little of the male's bright blue pigmentation.

Plate 1: Figure showing the experimental animal *Portunuspelagicus*

Dorsal view

Ventral view

5.3. Collection and preparation of the sample

In the present study the animals (*Portunus pelagicus*) were collected from Gulf of Manner. Thoothukudi coastal region by trawl catch, brought to the laboratory, cleaned and washed with fresh sea water to remove all impurities.

5.3.1 Preparation of shell extracts

The shells were broken and the soft tissues were removed and washed thoroughly with distilled water. Shell was dried under the sunlight and powdered with the help of motor and pestle. Approximately 10 grams of shell powder was immersed in methanol and were incubated for 48 hours in a dark place. Then they filtered through filter paper. Samples were centrifuged at 5000 rpm for 15 min in rotary evaporator. The precipitate was collected and it was stored for further use.

5.4 Bacterial cultures

Five bacterial strains namely *Escherichia coli*, *Staphylococcus aures*, *Bacillus subtilis*, *Bacillus cereus*, and *Pseudomonas aeruginosa* were used for antimicrobial activity. (All the bacterial strains were obtained from Government Medical College, Tuticorin).

5.4.1 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 (Mueller hinton) was prepared, and poured into sterile petridish. The 24 hour old bacterial broth cultures were inoculated in the petridishes using a sterile cotton swab.

5.5. Antibacterial activity assay

The antibacterial activity assay was carried out by disc diffusion method (Kirby& Bauer,1966). Mueller hinton agar was prepared and poured in sterile petriplates. Bacterial culture was inoculated on the surface of Mueller hinton 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 concentration of 1000 µg/ml and placed on the surface of inoculated petri plates along with control- ampicillin 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.

5.6. Antioxidant assay

5.6.1 Preparation of extract

The shell (sample) powder (10g) was extracted by stirring with 100ml of 25ºC for 24h and filtering through whatmann No.1 filter paper. The residue then extracted with two additional 100ml portions of the solvent, as described above. The combined extracts were then rotary evaporated at 40ºC to dryness. The combined extracts were kept in an oven at 40ºC for removal of residual moisture. The dried extracts were weighted to determine the percentage yield of the soluble constituents using the formula,

Yield = (Weight of dry extract/ weight taken for extraction) x 100

5.6.2 Total antioxidant activity:

The total antioxidant activity was evaluated by phophomolybdenum method described by Prieto *et al.*, (1990). 1.0 ml of the extract was mixed with 1.0ml of the standard reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95ºC 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 against a reagent blank. The total antioxidant

capacity was expressed as milligram of Ascorbic Acid Equivalence (AAE) per gram of extract.

% Antioxidant activity = Abs sample/ Abs Std x 100

5.6.3Nitric Oxide Scavenging Activity

Sodium nitroprusside (SNP) was used for generation of NO and it was measured by the Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dichloride (NED), and 3% phosphoric acid). SNP spontaneously generates NO in aqueous solution at physiological pH (Marcocci *et al.*, 1994) results in production of nitrite ions by its interaction with oxygen, whose estimation is done by Griess reagent. Scavengers of NO compete with oxygen leading to reduced production of NO. Different concentrations (200–1000 μg/mL) of shell fractions dissolved in ethanol and water was mixed with SNP (10 mM) in phosphate buffer saline (PBS) and incubated at 25°C for 3 h. The samples were then reacted with griess reagent, and absorbance was recorded at 540 nm of chromophore formed as result of diazotization of nitrite with sulphanilamide, and subsequent coupling with NED was done using microplate reader and compared to positive control which in this case was ascorbic acid treated in same way to Griess reagent. The ethanol was used as standard.

Nitric oxide scavenged (%) = (Acontrol−Atest)/Acontrol×100

5.7Antidiabetic Activity:

5.7.1 Alpha amylase inhibition activity:

Alpha amylase inhibition assay was carried out by the method described by Nickavar, Yousefian,2009. 1ml of 1% starch was added to control and test sample tubes along with 0.5ml of amylase enzyme. The test tubes were incubated at 37ºC for 30mins. Add 1ml of DNSA reagent to all the tubes and heat the tubes at 95ºC for 15 minutes. Absorbance was read at 510nm. The percentage of inhibition was calculated by using the following formula,

% Inhibition = Abs control – Abs Test/Abs control x 100

5.7.2Hemolysis assay

Hemolysis assay was evaluated by the method described by Gandhithasan *et al*(1991).3 ml of blood was collected in a heparinized tube. Centrifuge the blood at 2000 rpm for 5 minutes. Discard the supernatant and resuspend the cells in cold PBS. Aliquot 0.1 ml of blood sample was taken in the test tubes. Add different concentration of sample 200, 400, 600, 800, 1000 µg/ml to the test tubes. Make up the tubes to equal volume using PBS. Heat all the tubes to 40ºC for 10 min. Cool the tubes and read absorbance at 540 nm.

% Inhibition = Abs Control- Abs sample/Abs Control x 100

5.8 GC-MS ANALYSIS

The GC-MS spectra analysis was carried out in the methanol extract of the shell.

GC-MS analysis was performed using an Agilent 7820A gas chromatography coupled to an Agilent 5977E mass selective detector in the positive ion electron impact (EI) mode. The separation was achieved using a DB-5 MS fused silica capillary column, 30m x 0.25 mm i.e., 0.25μm film thickness. GC oven temperature was programmed from 100ºC to 270ºC at a rate of 10ºC/min. Helium was used as the carrier gas; inlet pressure was 25kPa; linear velocity: 1ml/min at 210ºC. Injector temperature: 250ºC and injection mode: split 1:50. Ms scan condition: source temperature, 200ºC; interface temperature, 250ºC; energy, 70eV; mass scan range, 40-350 amu.

5.8.1. Identification of compounds

Interpretation on the spectrum was conducted using the data base of National Institute Standard and Technology (NIST), WILEY 8, and FAME having more than 62,000 patterns. The unknown compounds found in the methanol fraction of the shell were matched with the spectrum of the known compounds stored in NIST, WILEY 8, FAME and MS library and predicated

from Dukes ethnobotanical database. The name, molecular weight and structure of the compounds of the test materials were ascertained.

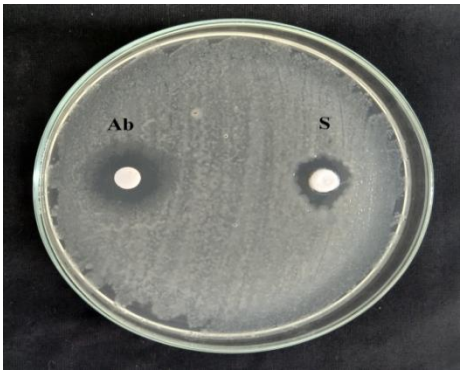
6. RESULTS

6.1Antibacterial activity

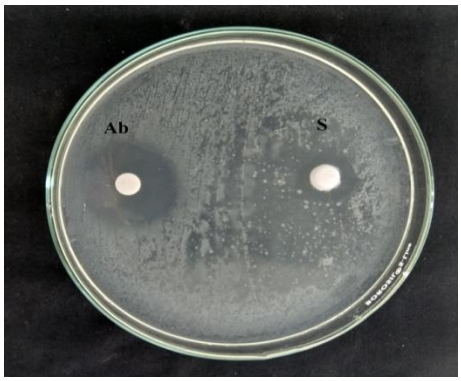
In the present study the antibacterial activity in the methanolic shell extracts of *Portunus pelagicus* was evaluated against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, and *Pseudomonas aeruginosa* .

The methanolic shell extract showed antibacterial activity against all the tested organism. The methanolic shell extract showed activity with the inhibition zones ranging from 9 mm to 12mm (Table-1). The highest activity of 12mm was recorded against *Bacillus subtilis* and lowest activity of 9mm against *Escherichia coli*. The extract also inhibited the growth of *Staphylococcus aureus, Bacillus cereus* and *Pseudomonas aeruginosa* with the inhibition zone of 10mm.(Plate-2). Ampicillin was used as antibiotic control.

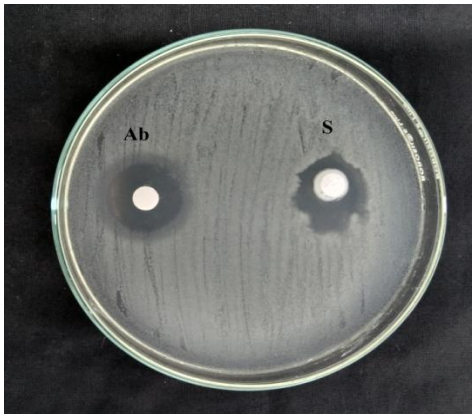
Plate 2: Antibacterial activity in the methanol extracts of shell of *P.pelagicus*



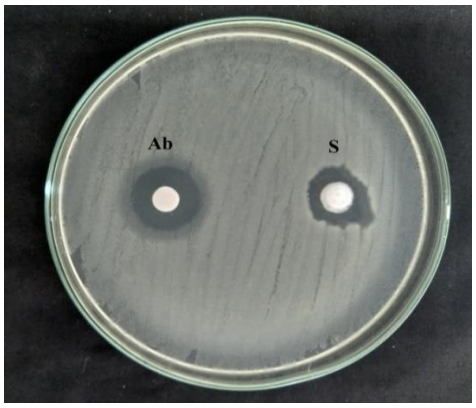
Escherichia coli



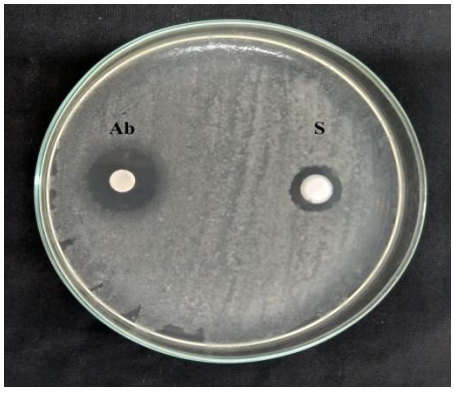
Staphylococcus aureus



Bacillus subtilis



Bacillus cereus

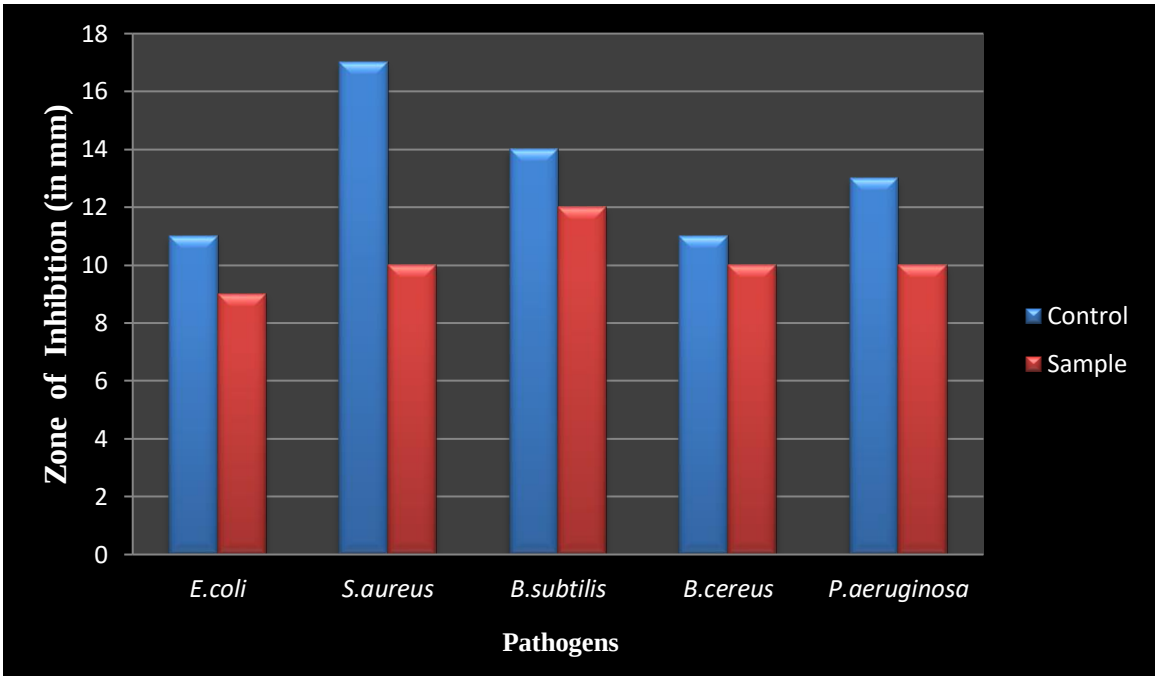


Pseudomonas aeruginosa

Ab- Ampicillin (control)

S-Sample 1000µl

Fig 2: Antibacterial activity in the shell extract of *P.pelagicus*



6.2 Antioxidant activity

The antioxidant activities in the methanolic shell extract has been evaluated by two different methods namely Total antioxidant activity, Nitric oxide scavenging activity at five different concentrations (200µg/ml, 400µg/ml, 600µml, 800µg/ml and 1000µg/ml).

6.2.1 Total antioxidant activity

The total antioxidant activity of the shell extract was measured spectrophotometrically by phosphomolybdenum method which is based on the subsequent formation of Mo (IV) to Mo(V) with a maximum absorption at 695nm. The higher the absorbance, stronger is the antioxidant activity (Table-2).

The total antioxidant activity was found to be higher in the methanolic shell extract of *P.pelagicus* at the concentration of 1000µg/ml (37.69mg/g of extract) and lower at the concentration of 200µg/ml (10mg/g of extract). The activity was found to decrease in the order of the concentration as 1000>800>600> 400>200 (Plate-3 and Fig-3). Antioxidant activity was compared with the standard ascorbic acid.

6.2.2. Nitric Oxide scavenging activity

The percentage of inhibition varied in between 32% to 65%. The percentage of inhibition was increased with increasing concentration. At 200µg/ml concentration the inhibition was 32.3, at 400µg/ml it was 46.54%, at 600µg/ml, 800µg/ml and 1000µg/ml the inhibition was 48.56%, 60% and 64.52% respectively. The results showed that the extract exhibited dose dependent NO scavenging activity (Plate-4 and Fig-4).

Table 1: Total antioxidant activity in the shell extracts of *P.pelagicus*

	Concentration (µg/ml)	**OD @ 695 nm**
Ascorbic acid (Standard)	1000	1.300
Sample	200	0.13
	400	0.21
	600	0.28
	800	0.38
	1000	0.49

Plate 3: Total antioxidant activity in the shell extract of *P.pelagicus*

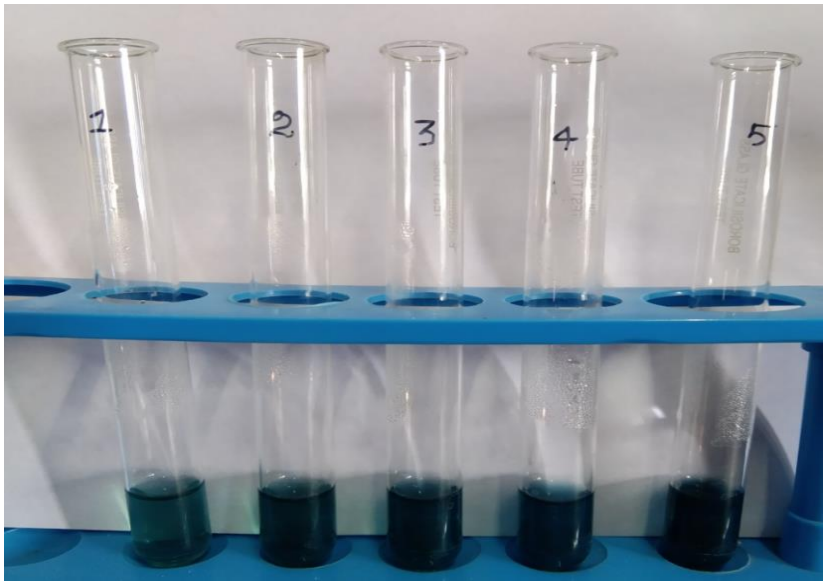


Fig 3: Percentage of inhibition (Total antioxidant activity) in the shell extract of *P.pelagicus*

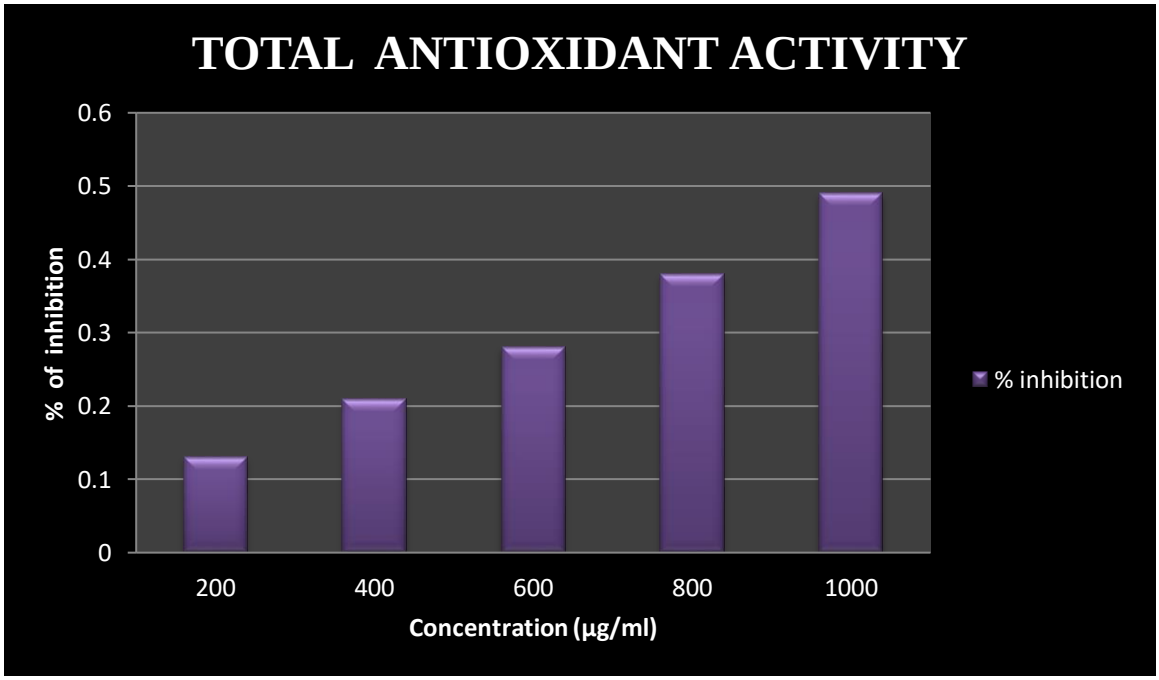


Table 2: Nitric Oxide scavenging activity in the shell extracts of *P.pelagicus*

Sample (µg/ml)	Absorbance at 540nm
Control	1.30
200	0.88
400	0.76
600	0.67
800	0.52
1000	0.46

Plate 4: Nitric Oxide scavenging activity in the shell extract of *P.pelagicus*

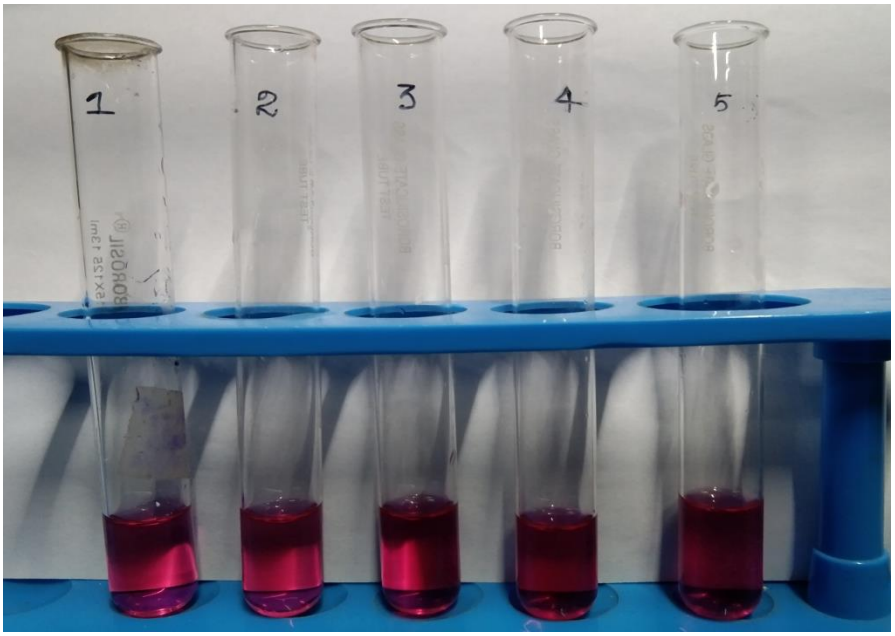
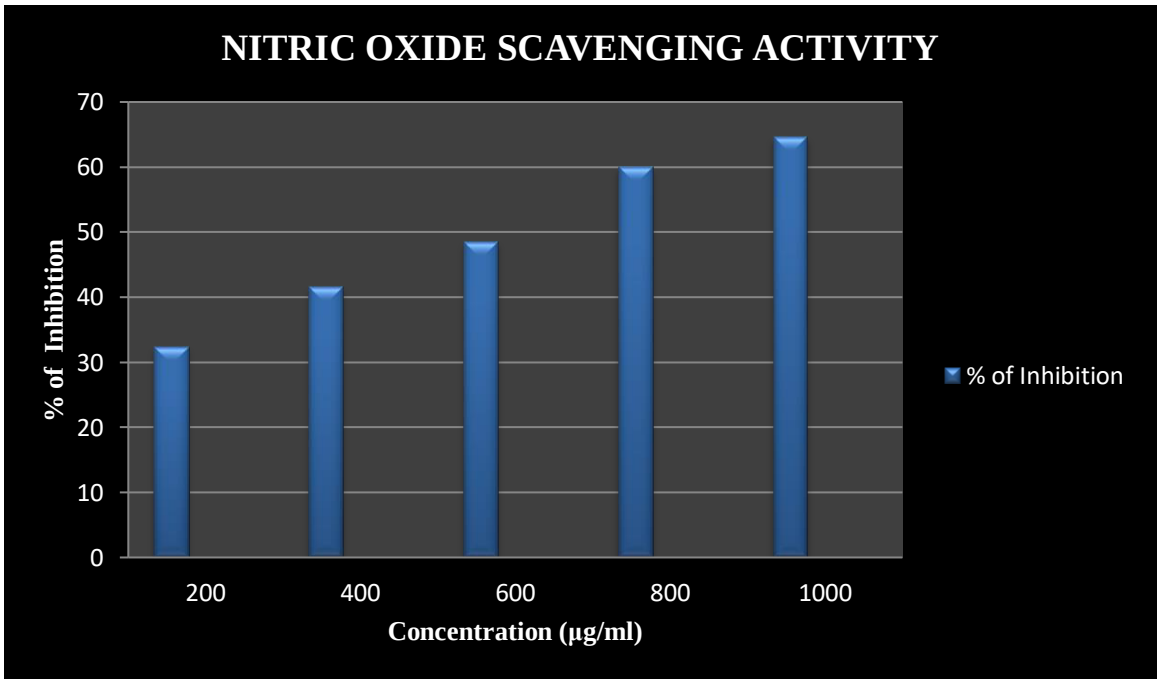


Fig 4: Percentage of inhibition (Nitric Oxide scavenging activity) in the shell extract of *P.pelagicus*



6.3 Antidiabetic activity

6.3.1 Alpha amylase inhibition activity

The methanolic shell etraxct of *P.pelagicus* showed inhibition activity range from 55% to 85% (Table-4).

The methanolic shell extract of *P.pelagicus* exhibits good antidiabetic activity with the percentage of inhibition of 55.83% at 200µg/ml and 75% at 400µg/ml, 78.33% at 600µg/ml, 83.33% at 800µg/ml and 85% at 1000µg/ml concentration. The highest inhibition activity of 85% was observed at 1000µg/ml concentration and the lowest activity of 55.83 was observed at 200µg/ml of the extract concentration. The alpha amylase inhibition activity was increased as of the concentration of extract was increased (Plate-5 and Fig-5). The result of the alpha amylase inhibition activity are given in (Table-4).

6.4. Hemolysis assay

The methanolic extract of *Portunus pelagicus* was tested for haemolysis at different concentrations such as 200, 400, 600, and 1000µg/ml. There is no inhibition activity. The results are shown in Table-5 and Plate-6.

Table 3: Alpha amylase inhibition activity in the shell extract of *P.pelagicus*

Sample (µg/ml)	**Absorbance at 510nm**
Control (Acarbose)	1.20
200	0.53
400	0.30
600	0.26
800	0.20
1000	0.18

Plate 5: Alpha amylase activity in the shell extract of *P.pelagicus*

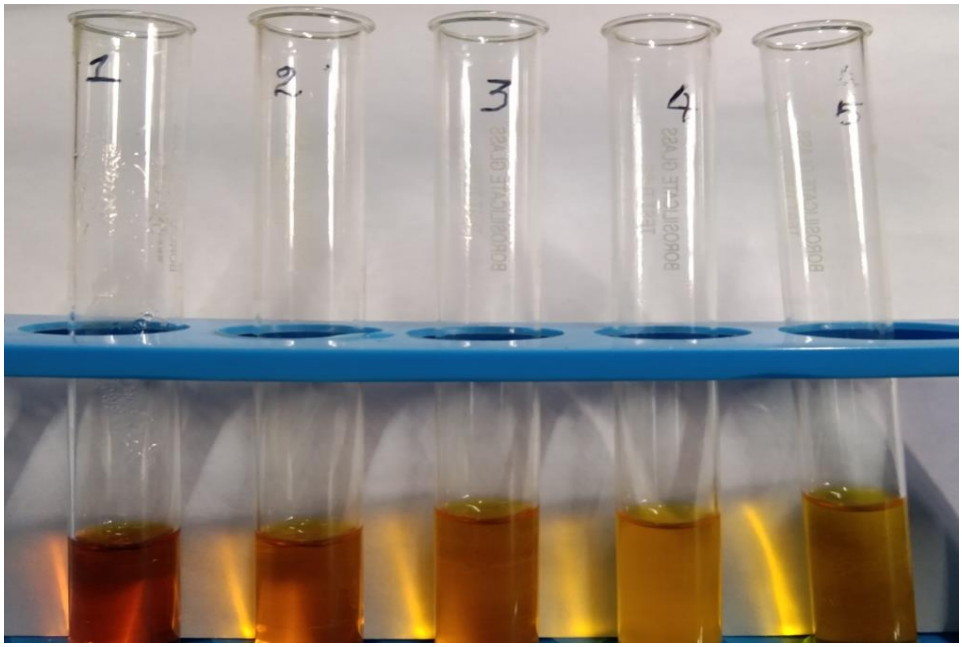


Fig 5: Percentage if inhibition (Alpha amylase activity) in the shell extract of *P.pelagicus*

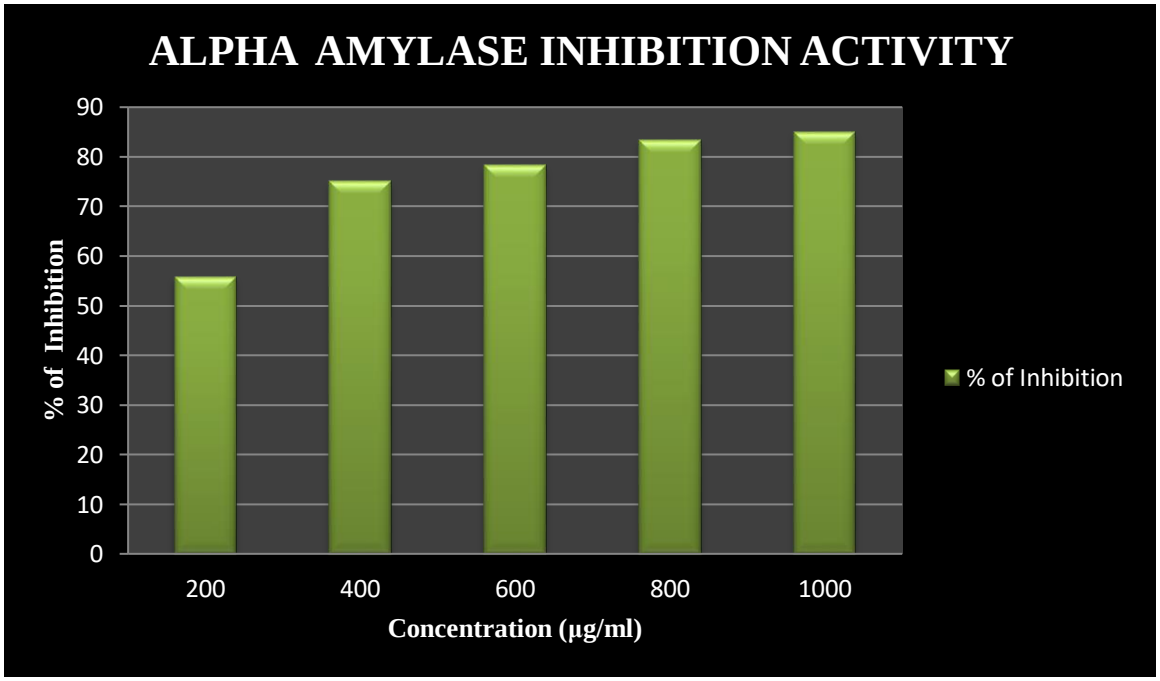
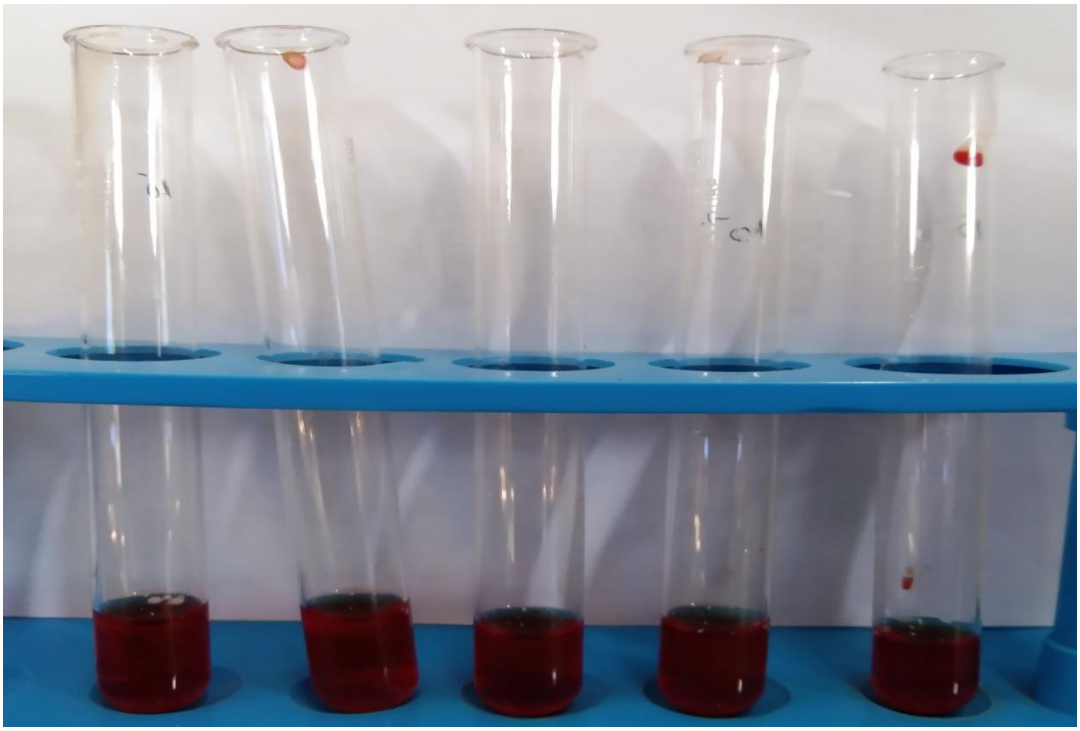


Table 4: Hemolysis assay in the shell extract of *P.pelagicus*

Sample (µg/ml)	Absorbance at 510nm
Control	1.40
200	1.50
400	1.50
600	1.50
800	1.50
1000	1.50

Plate 6: Hemolysis activity in the shell extract of *P.pelagicus*



6.5. GC-MS analysis

Marine environment is a progressive route for attainment of natural novel drugs. Substantially they are of secondary metabolites. This study is about the utilization of marine crab shell waste for the identification of unrevealed bioactive compounds with its pharmacological activity by GC-MS method.

Portunus pelagicus shell waste revealed the presence of various bioactive compounds like Benzene, 1-ethenyl-4-nitro-, Cycloheptene, 4,5-Dimethylthiazole S-oxide, 9-Borabicyclo(3.3.1) nonane, Perhydro-htx-2-one, 2-depentyl-, acetate ester, Heptasiloxane, hexadecamethyl-, Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)]bis[trimethyl-, Pentasiloxane dodecamethyl-, 1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane, Heptasiloxane, hexadecamethyl- and Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-.

The compounds identified by GC-MS analysis was found to have antiviral, antibacterial, anti-inflammatory, nitric oxide scavenger, antineurotic, anticatract, antifungal, insecticide, antiparasitic, anticonvulsant, antihelminths, antidieabetic, antiallergic, alpha amylase inhibitor. glucose oxidase inhibitor, nitric oxide stimulant, antineoplastic (colon cancer), antioxidant, antiprotozoal. These

compounds constitute a promising novel class of pharmaceuticals for the treatment of diseases.

Portunus pelagicus crab shell contains numerous bio compounds with high medicinal values which can be isolated and utilized in rational methods in future for the discovery of innovative drugs.

Table 5: Compounds identified in the methanolic shell extract of *P.pelagicus*

S. No	CAS	Compound name	Rt (in minutes)	Molecular Formula	Molecular weight (g/mol)	Biological activity
1	000100-13-0	Benzene,1ethenyl- 4-nitro-	8.658	$C_8H_5NO_2$	147.1308	Antiviral, Antibacterial, Anti-inflammatory
2	000628-92-2	Cycloheptene	14.105	C_7H_{12}	96.17	Nitric oxide scavenger, Antineurotic,
3	1000112-53-4	4,5-Dimethylthiazole S-oxide	14.303	C_5H_7NOS	129.18	Antiviral, Antibacterial, Anticatract.
4	1000160-35-2	9-Borabicyclo[3.3.1]nonane	15.580	$C_8H_{14}B$	121.01	Antifungal, Insecticide, Antiparasitic.
5	080090-53-5	Perhydro-htx-2-one,2-depentyl-, acetate ester	15.788	$C_{16}H_{27}NO_3$	281.39	Anticonvulsant, Antihelminths, Antidieabetic, Antibacterial, Antiallergic,
6	000541-01-5	Heptasiloxane, hexadecamethyl-	15.844	$C_{16}H_{48}O_6Si_7$	533.1472	Antiviral, Anti-inflammatory, Alpha amylase inhibitor.
7	056114-62-6	Silane,[[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2 phenylene]bis(oxy)]is[trimethyl-	15.930	$C_{20}H_{42}O_4Si_4$	458.9	Glucose oxidase inhibitor, Nitric oxide stimulant.

8	000141-63-9	Pentasiloxane, dodecamethyl-	15.977	$C_{12}H_{36}O_4Si_5$	384.8393	Antineoplastic, Anti-inflammatory
9	038147-00-1	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	16.052	$C_{13}H_{39}O_5Si_6$	443.96	Antioxidant, Antibacterial, Antifungal
10	000541-01-5	Benzoicacid,4-[(trimethylsilyl)amino]-, trimethylsilyl ester	16.090	$C_{13}H_{23}NO_2Si_2$	281.50	Antibacterial, Antiviral, Antihelminthic, Anti-inflammatory,
11	000541-01-5	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	16.128	$C_{12}H_{38}O_5Si_6$	430.94	Antifungal, Antibacterial, Antiprotozoal.

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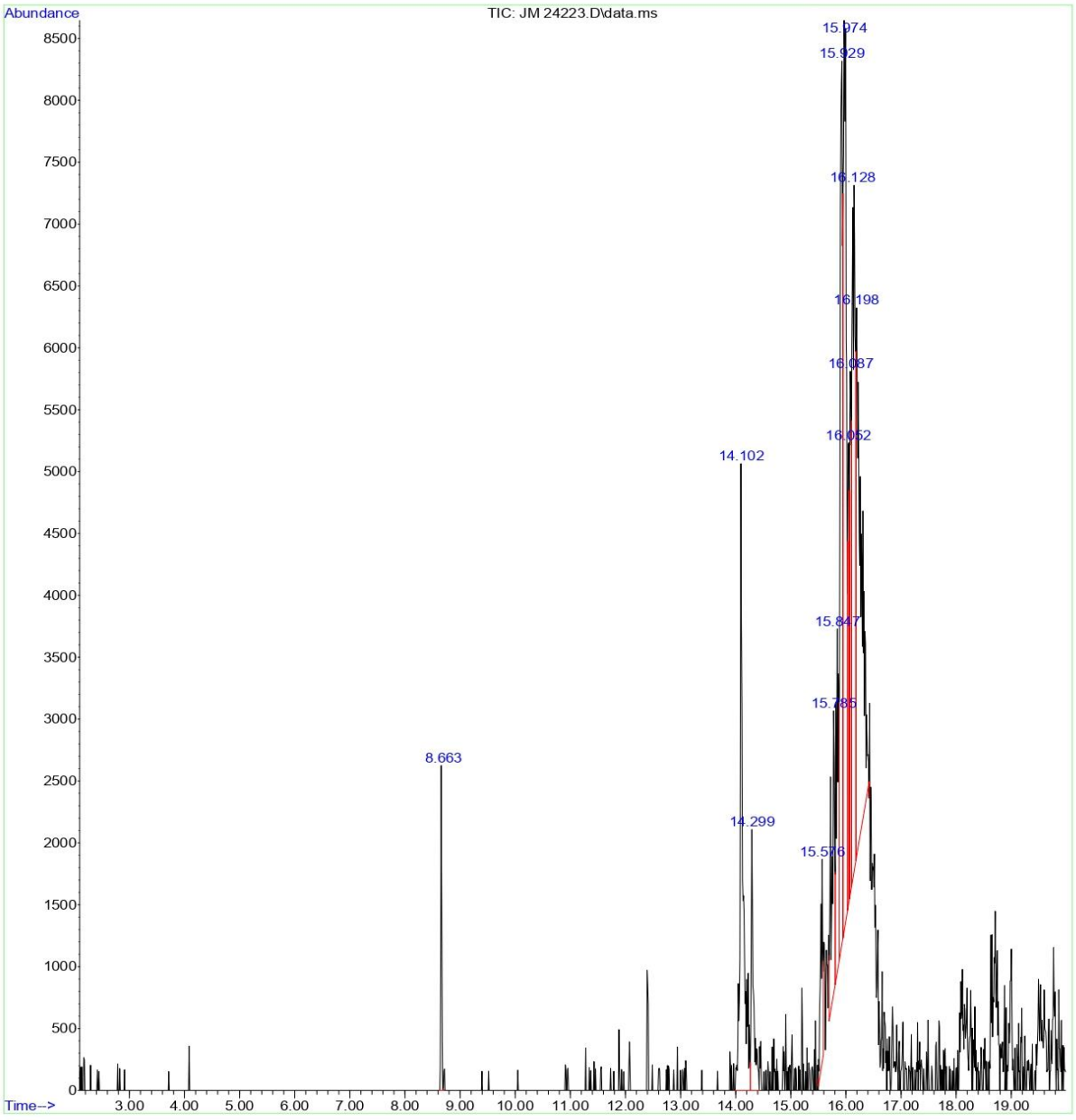

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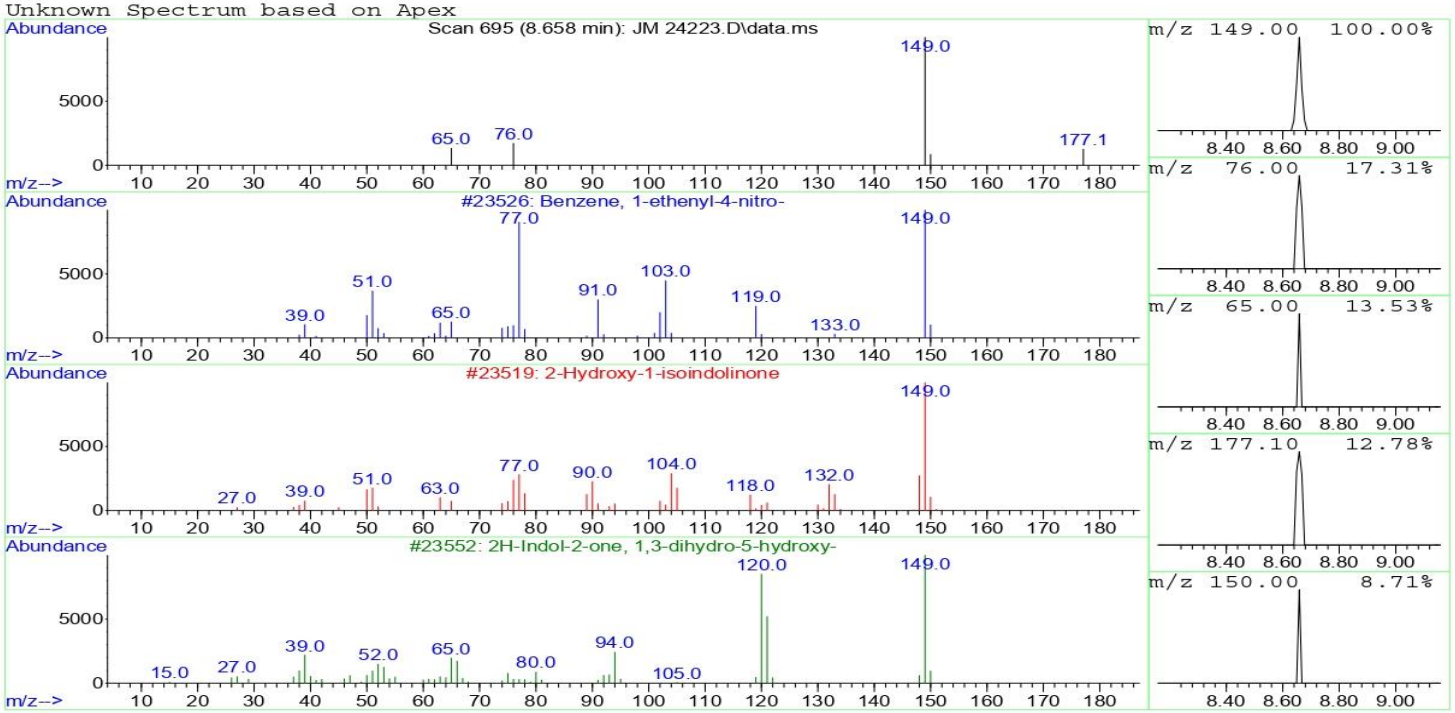

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2 2-Hydroxy-1-isoindolinone	23519	1000289-12-0	9
3 2H-Indol-2-one, 1,3-dihydro-5-hy...	23552	003416-18-0	9

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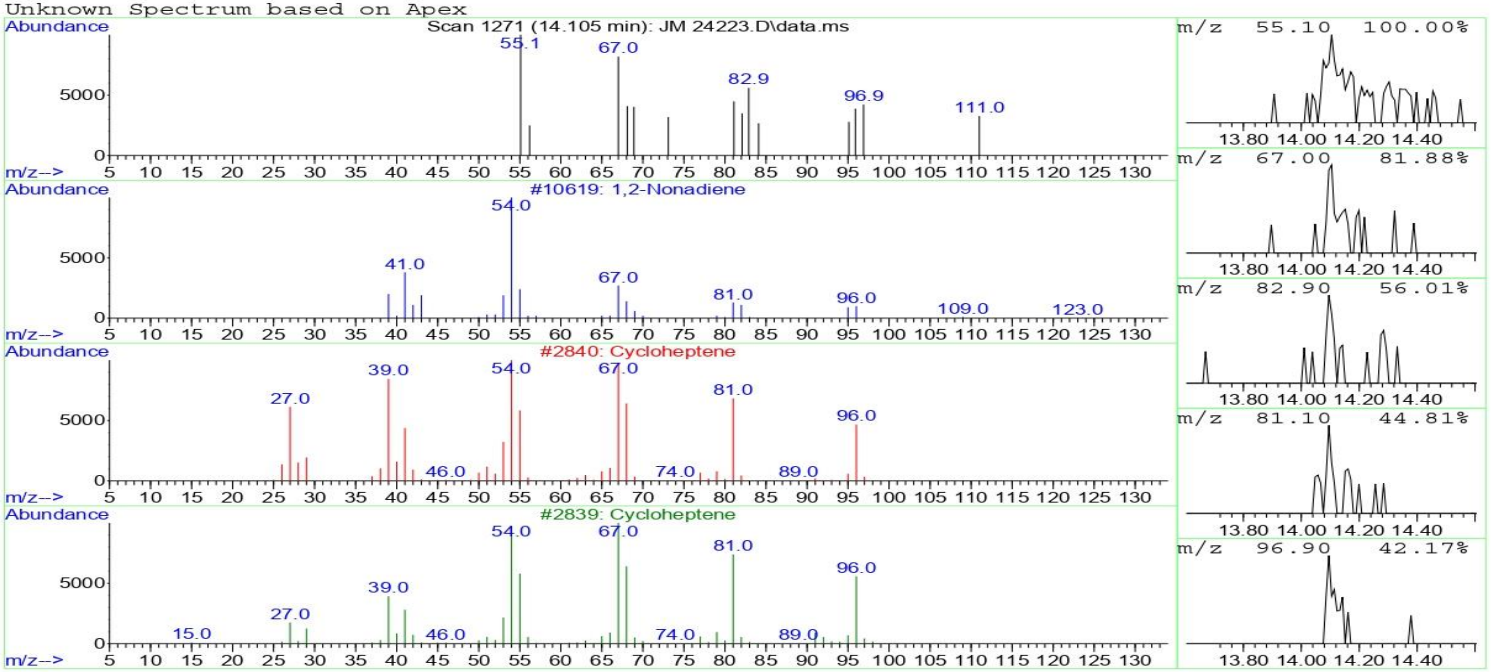

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1 1,2-Nonadiene	10619	022433-33-6	43
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3 Cycloheptene	2839	000628-92-2	38

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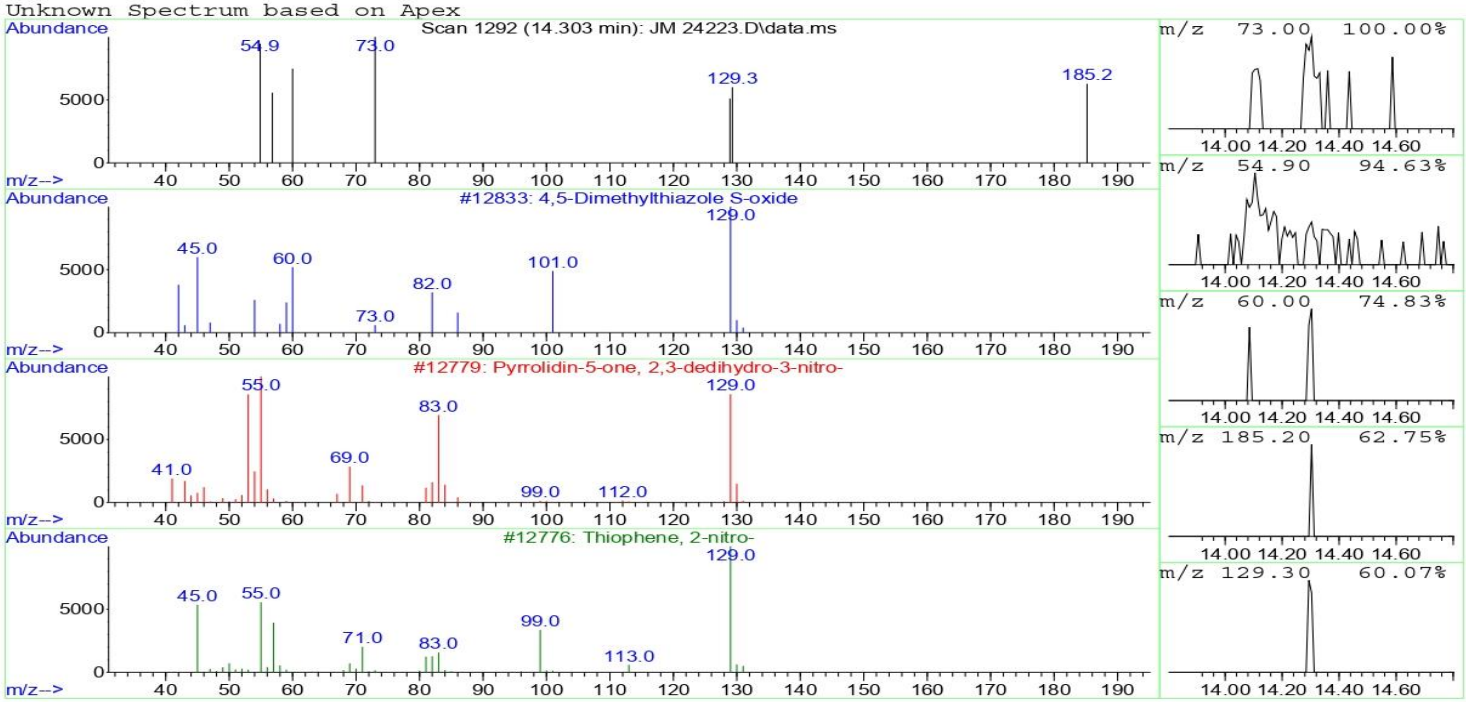

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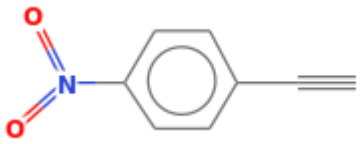
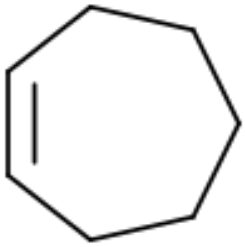
1 4,5-Dimethylthiazole S-oxide	12833	1000112-53-4	5
2 Pyrrolidin-5-one, 2,3-dedihydro-...	12779	1000124-44-1	4
3 Thiophene, 2-nitro-	12776	000609-40-5	4

Structure of Benzene, 1-ethenyl-4-nitro-, Cycloheptene and 4,5-Dimethylthiazole S-oxide

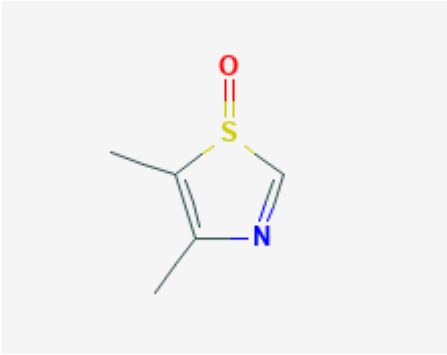
1. Benzene, 1-ethenyl-4-nitro-



2. Cycloheptene



3. 4,5-Dimethylthiazole S-oxide



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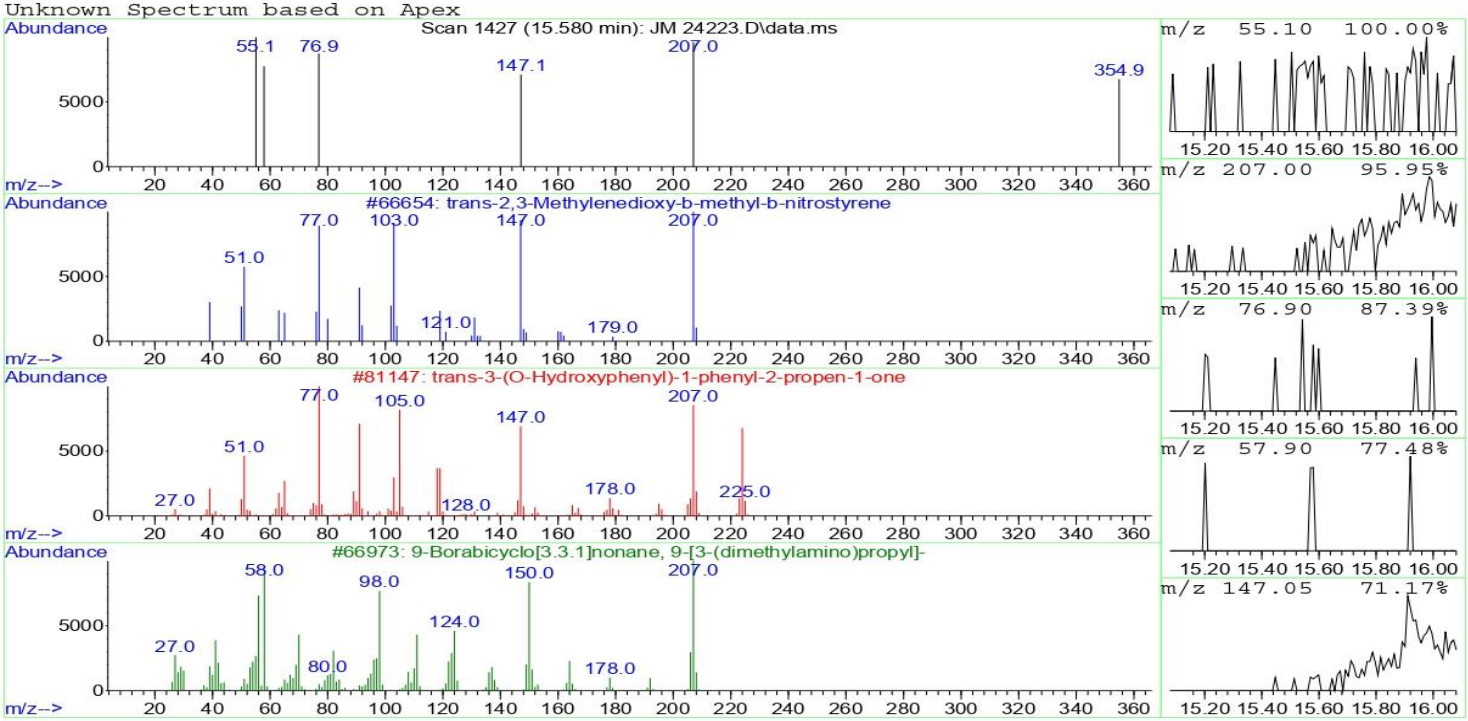

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2 trans-3-(O-Hydroxyphenyl)-1-phen...	81147	042224-53-3	9
3 9-Borabicyclo[3.3.1]nonane, 9-[3...	66973	1000160-35-2	5

Library Search Report - ChemStation Integrator

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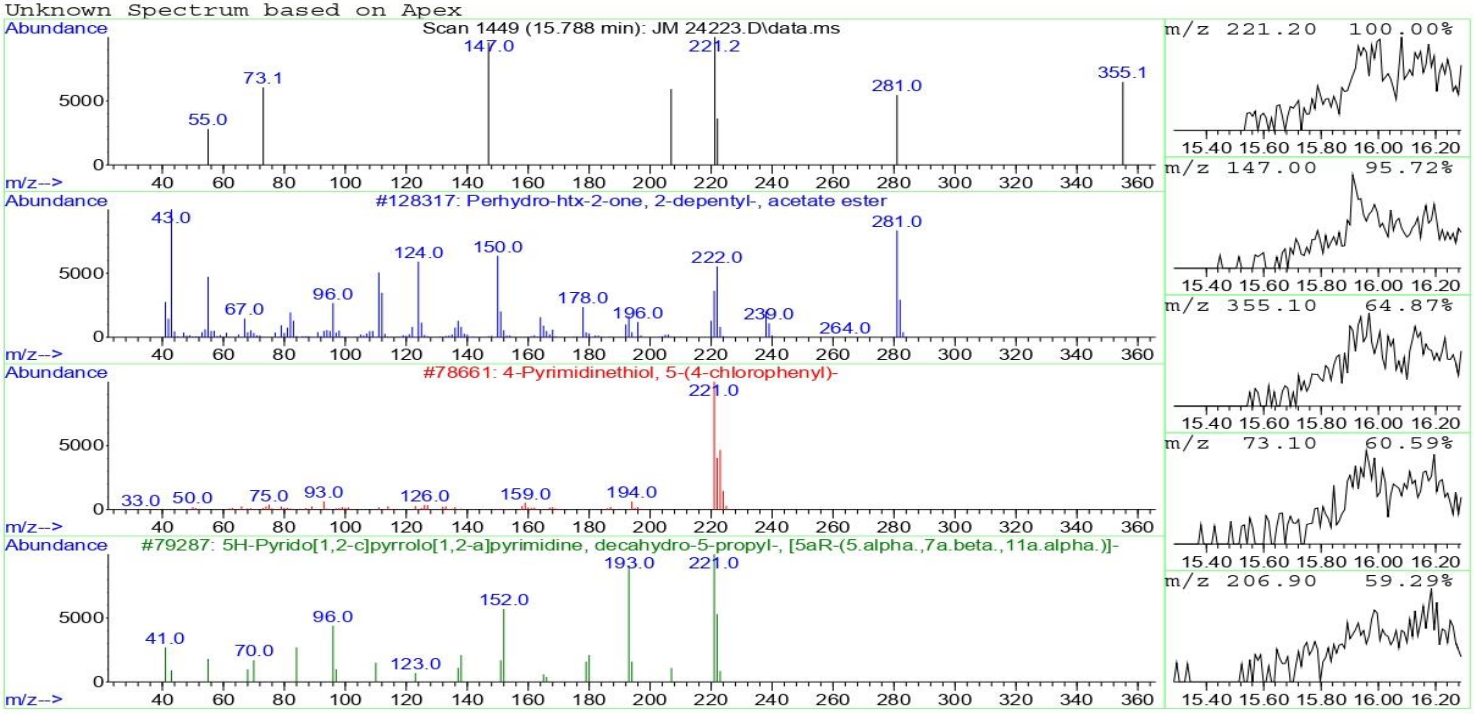

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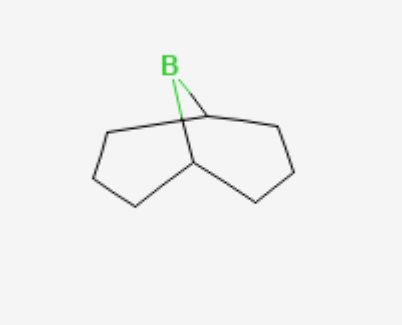
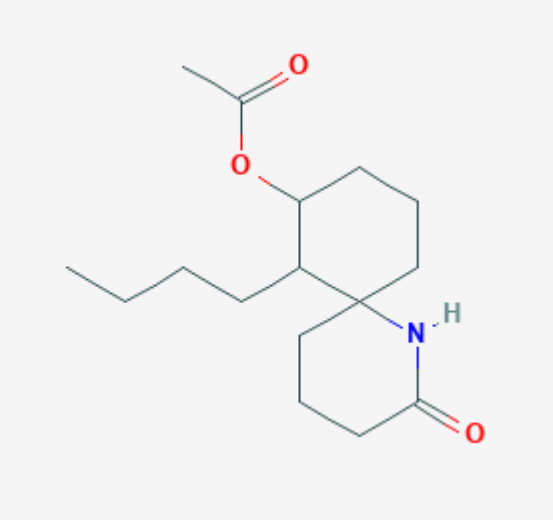
2 4-Pyrimidinethiol, 5-(4-chloroph...	78661	1000319-28-4	5
3 5H-Pyrido[1,2-c]pyrrolo[1,2-a]py...	79287	1000115-02-7	5

Structure of 9-Borabicyclo[3.3.1]nonane and Perhydro-htx-2-one, 2-depentyl-, acetate ester

4. 9-Borabicyclo[3.3.1]nonane



5. Perhydro-htx-2-one, 2-depentyl-, acetate ester



Library Search Report - ChemStation Integrator

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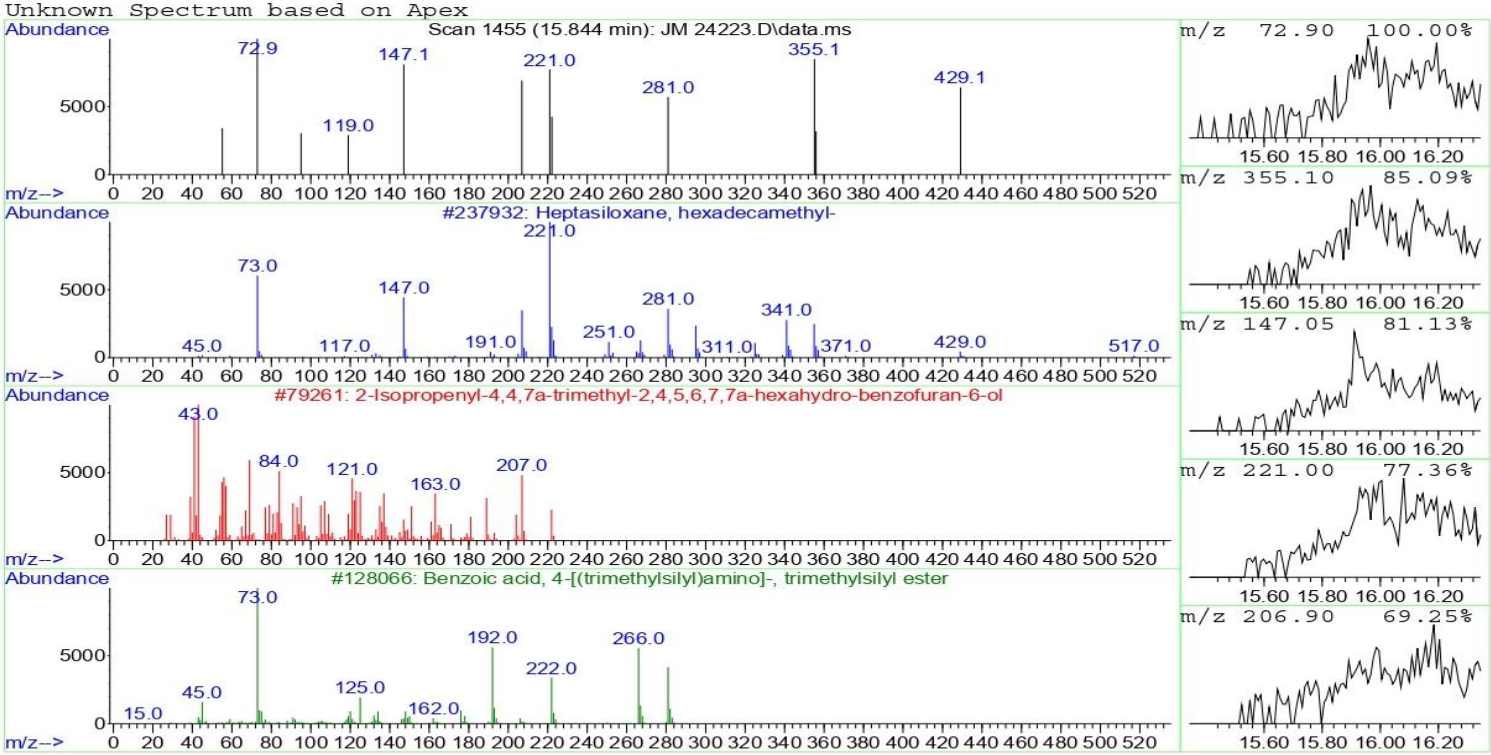

Data File: D:\Data\anjac\2015\ANJAC\st.marry's college\JM 24223.D
Sample : JM 24223

Peak Number: 6 at 15.844 min Area: 80311 Area % 4.89

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1 Heptasiloxane, hexadecamethyl-	237932	000541-01-5	25
2 2-Isopropenyl-4,4,7a-trimethyl-2...	79261	1000189-13-5	9
3 Benzoic acid, 4-[(trimethylsilyl...	128066	018406-05-8	9

Library Search Report - ChemStation Integrator

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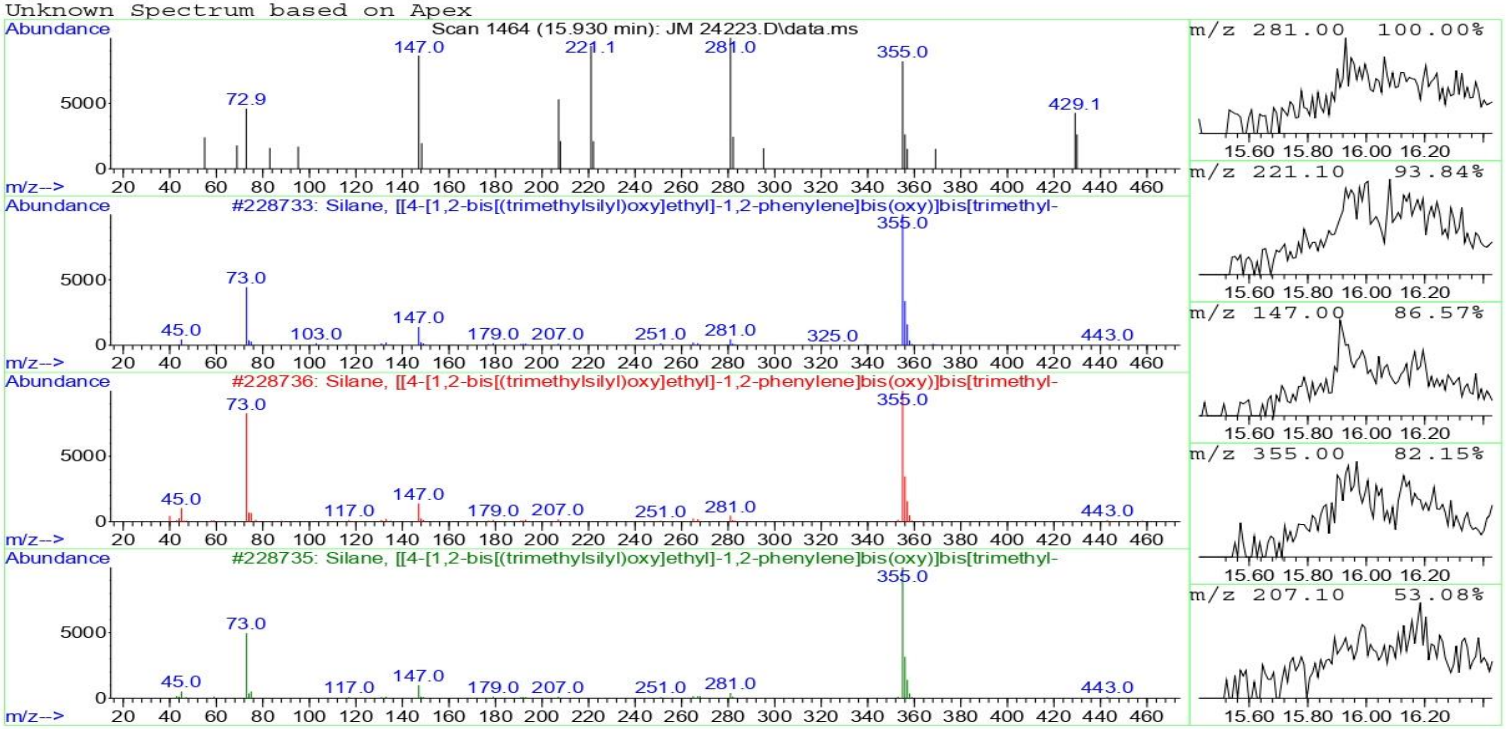

Data File: D:\Data\anjac\2015\ANJAC\st.marry's college\JM 24223.D
Sample : JM 24223

Peak Number: 7 at 15.930 min Area: 221298 Area % 13.47

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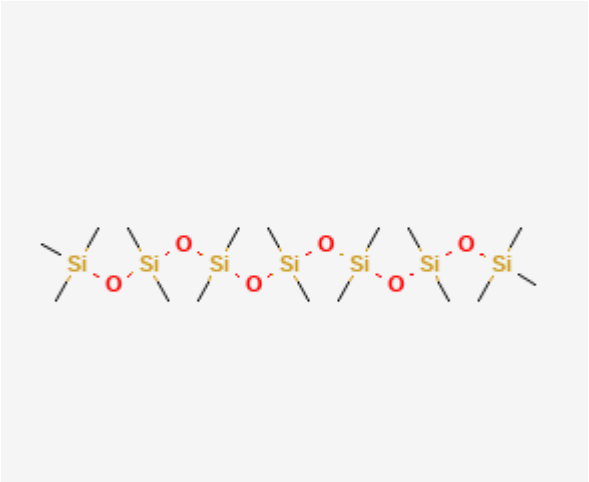
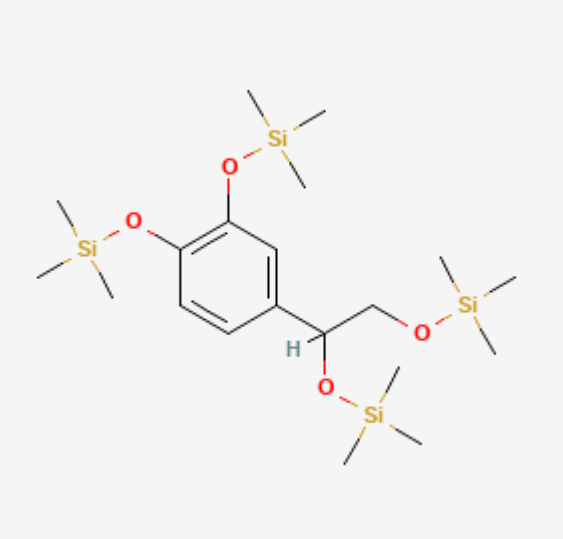
1 Silane, [[4-[1,2-bis[(trimethyls...	228733	056114-62-6	35
2 Silane, [[4-[1,2-bis[(trimethyls...	228736	056114-62-6	35
3 Silane, [[4-[1,2-bis[(trimethyls...	228735	056114-62-6	35

Structure of Heptasiloxane, hexadecamethyl- and Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)]bis[trimethyl-

6. Heptasiloxane, hexadecamethyl-

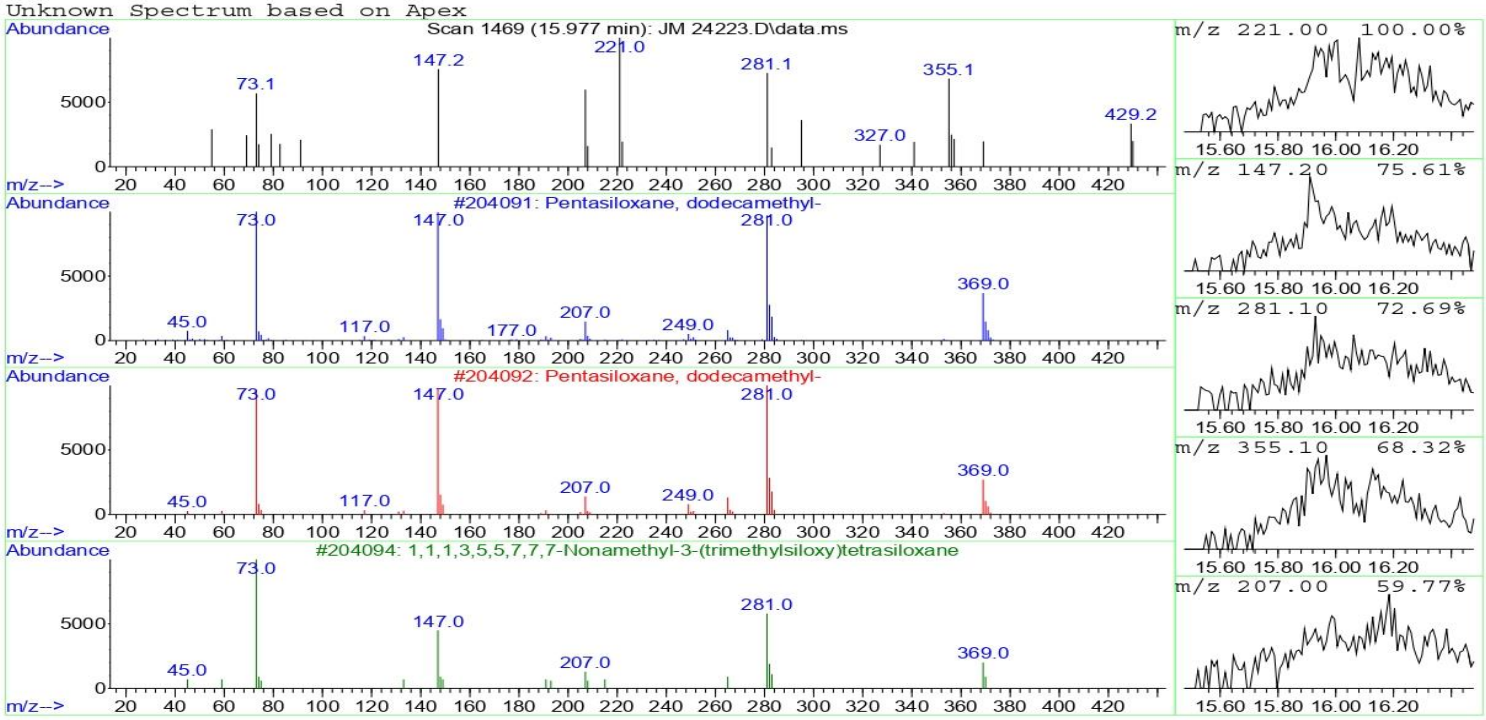


7. Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)]bis[trimethyl-



Library Search Report - ChemStation Integrator

Unknown Spectrum based on Apex



Data File: D:\Data\anjac\2015\ANJAC\st.marry's college\JM 24223.D
Sample : JM 24223

Peak Number: 8 at 15.977 min Area: 292226 Area % 17.79

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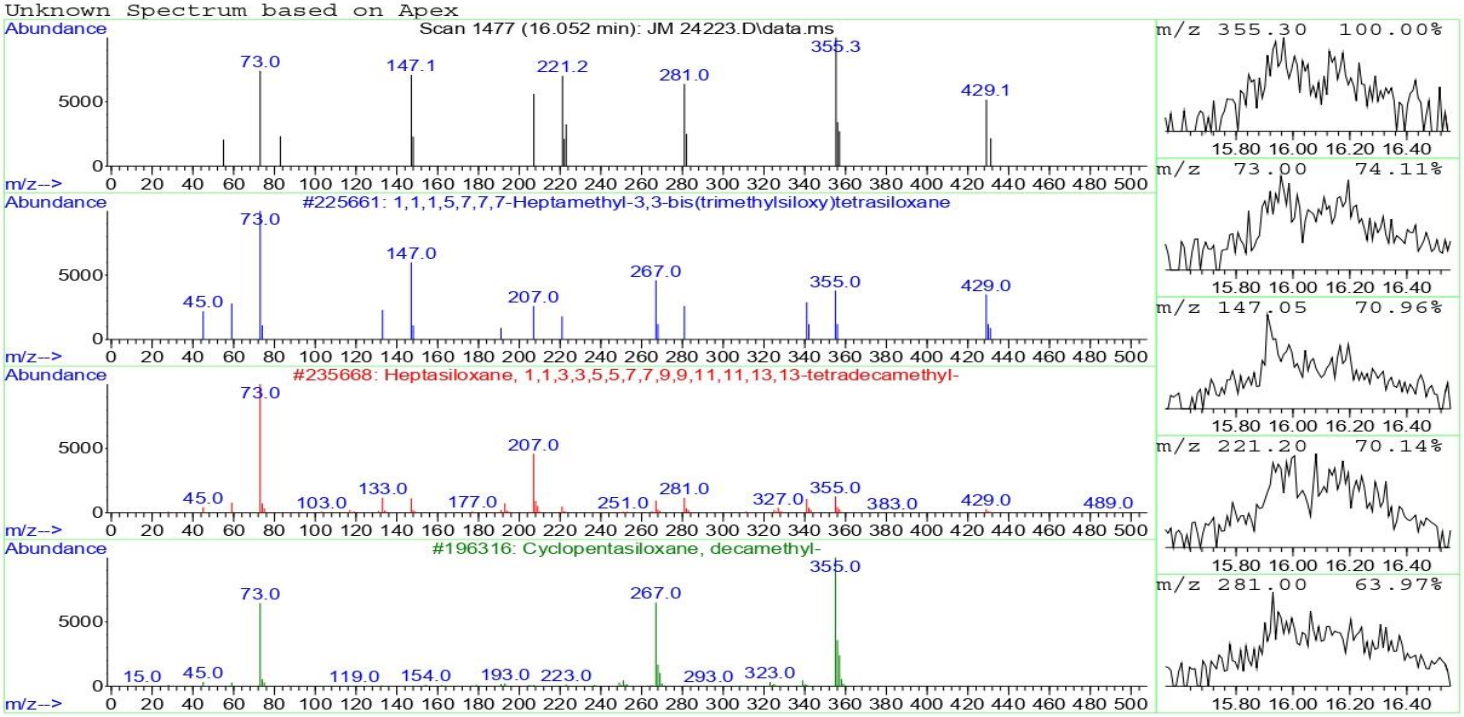
2 Pentasiloxane, dodecamethyl-	204092	000141-63-9	22
3 1,1,1,3,5,5,7,7,7-Nonamethyl-3-(...	204094	038146-99-5	22

Library Search Report - ChemStation Integrator

Unknown Spectrum based on Apex



Data File: D:\Data\anjac\2015\ANJAC\st.marry's college\JM 24223.D
Sample : JM 24223

Peak Number: 9 at 16.052 min Area: 71643 Area % 4.36

The 3 best hits from each library.	Ref\#	CAS\#	Qual
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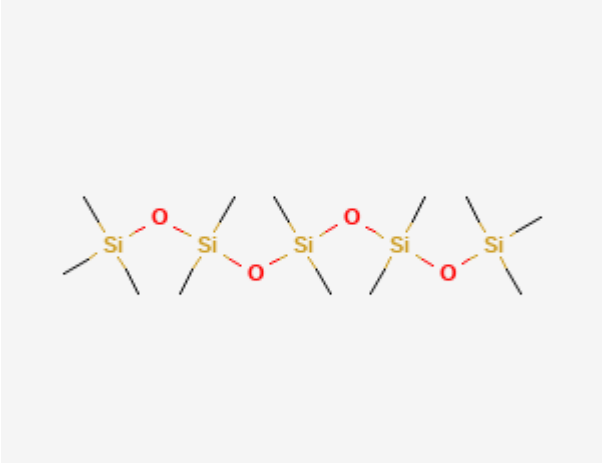
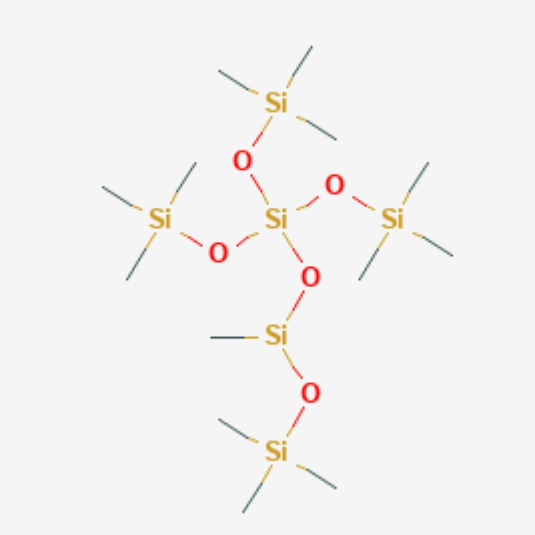
1 1,1,1,5,7,7,7-Heptamethyl-3,3-bi...	225661	038147-00-1	33
2 Heptasiloxane, 1,1,3,3,5,5,7,7,9...	235668	019095-23-9	28
3 Cyclopentasiloxane, decamethyl-	196316	000541-02-6	10

Structure of Pentasiloxane, dodecamethyl- and 1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane

8. Pentasiloxane, dodecamethyl-

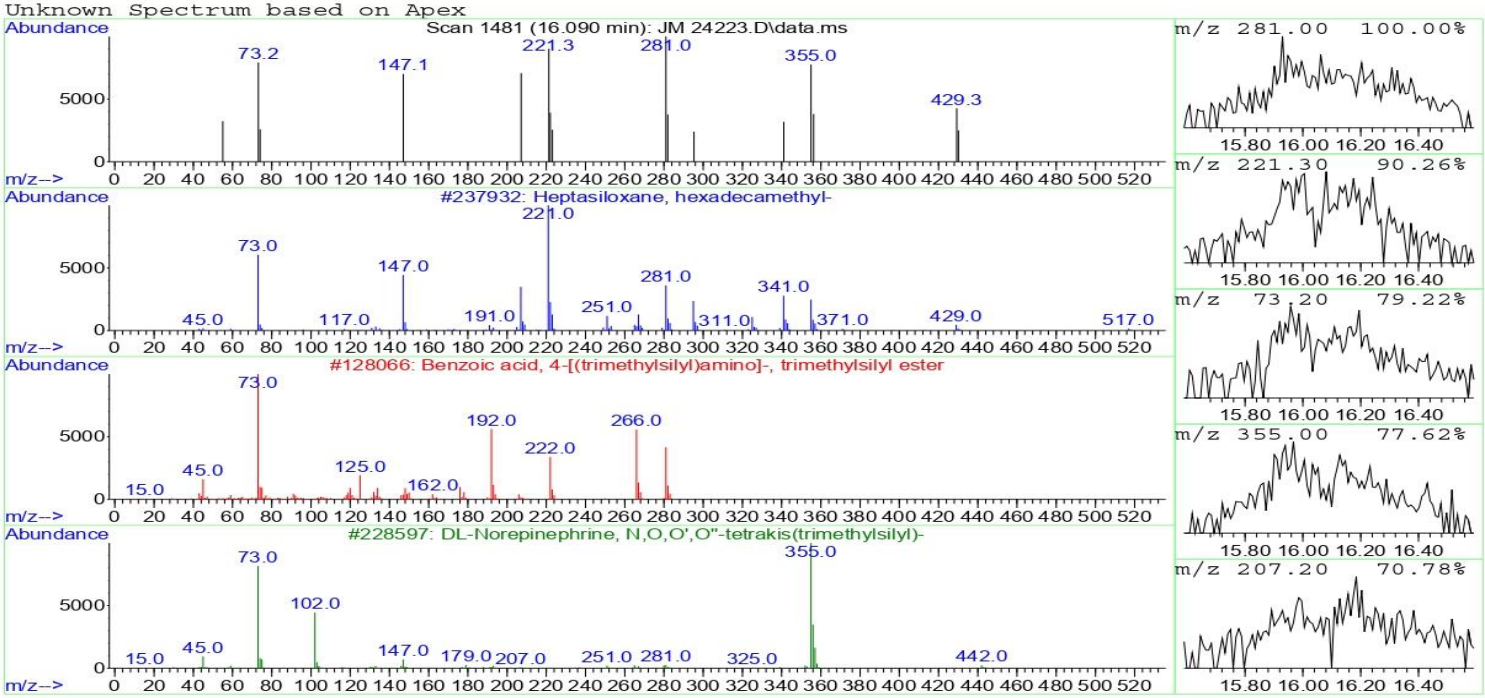


9. 1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane



Library Search Report - ChemStation Integrator

Unknown Spectrum based on Apex



Data File: D:\Data\anjac\2015\ANJAC\st.marry's college\JM 24223.D
Sample : JM 24223

Peak Number: 10 at 16.090 min Area: 83920 Area % 5.11

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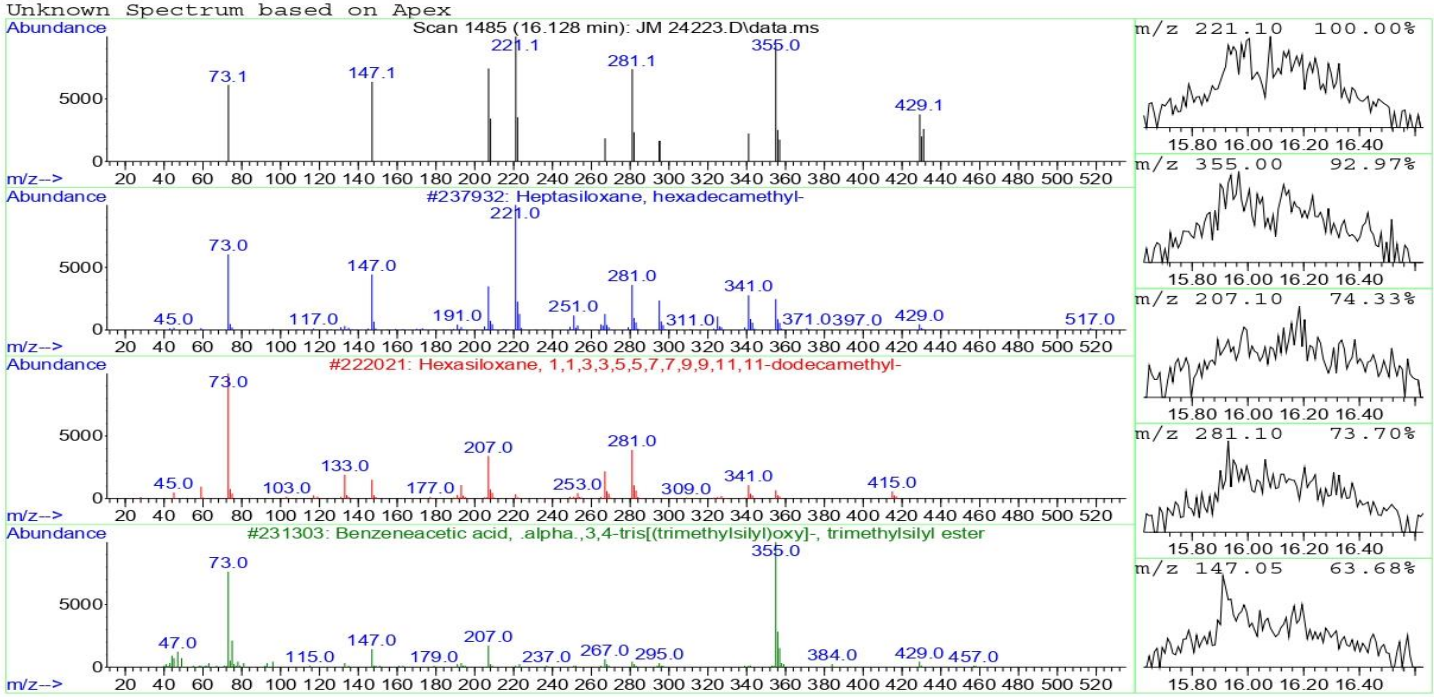
1 Heptasiloxane, hexadecamethyl-	237932	000541-01-5	25
2 Benzoic acid, 4-[(trimethylsilyl...	128066	018406-05-8	9
3 DL-Norepinephrine, N,O,O',O''-te...	228597	1000332-93-5	9

Library Search Report - ChemStation Integrator

Unknown Spectrum based on Apex



Data File: D:\Data\anjac\2015\ANJAC\st.marry's college\JM 24223.D
Sample : JM 24223

Peak Number: 11 at 16.128 min Area: 223454 Area % 13.61

The 3 best hits from each library.	Ref\#	CAS\#	Qual
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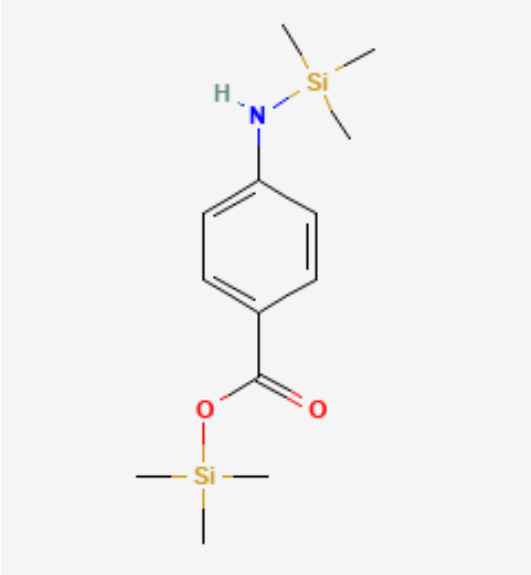
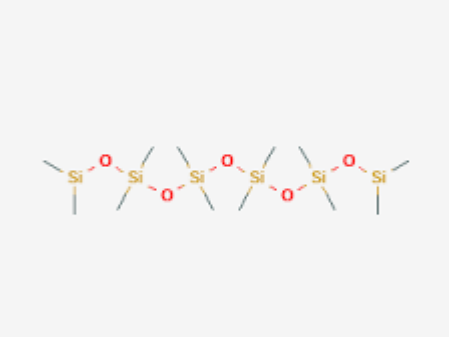
2 Hexasiloxane, 1,1,3,3,5,5,7,7,9,...	222021	000995-82-4	25
3 Benzeneacetic acid, .alpha.,3,4-...	231303	037148-65-5	16

Structure of Benzoic acid, 4-[(trimethylsilyl)amino]-, trimethylsilyl ester and Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-

10. Benzoic acid, 4-[(trimethylsilyl)amino]-, trimethylsilyl ester



11. Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-



7. DISCUSSION

In recent years great attention has been paid to study the bioactivity of natural products and their potential pharmacological utilization. Crustaceans are native of aquatic ecosystem and inhabiting the harmful effect of microbial growth. A microbial infection has been the major concern of aqua culturist worldwide. The extracts shell of crab shows good activity against microbial strains.

In the present study the antibacterial activity in the methanolic shell extracts of *Portunus pelagicus* was evaluated against *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Bacillus cereus,* and *Pseudomonas aeruginosa*. The highest activity of 12mm was recorded against *Bacillus subtilis* and lowest activity of 9mm against *Escherichia coli*. The extract also inhibited the growth of *Staphylococcus aureus, Bacillus cereus* and *Pseudomonas aeruginosa* with the inhibition zone of 10mm. Similar to the present study Shibana *et al.,* (2017) investigated the antimicrobial activities in the five solvent shell extract of *P.pelagicus* against five different pathogenic bacterial strains. Benzene, and Ethanol showed the best activity against *Streptococcus* and *Bacillus sps* (7mm) and the lowest activity was found in *E.coli* and *Enterobacter sps* (3mm). Different extract of six brachyuran crabs were evaluated against five different

pathogenic bacterial and four fungal strains. Maximum antibacterial effect of crude tissue is shown by *Dromia aprolhensis* against *E.coli* and the minimum against the *Scylla serrata* crab against *K.oxytoca*. (Rameshkumar, 1798)

Ravichandran *et al*., 2018 demonstrated that the hemolymph of *D. dehaani* exhibit broad spectrum activity against pathogenic bacteria. The intensity of the antimicrobial action varied depending upon the microorganism, where the activity of *D. dehaani* displayed highest antimicrobial activity, the fractions collected through sephadex G25 was tested against the selected clinical isolates also showed potent antimicrobial activity. Among the twelve fractions tested, fraction 5 was found to show maximum inhibition against the growth of bacterial pathogens and the most potent extracts were obtained from the crab *D. dehaani* which displayed activity against *Vibrio cholerae* at a concentration of 7.5 μg/mL and 15 μg/mL respectively.

Veeruraj *et al*., 2008 studied the antibacterial activity in the haemolymph of six different species of crab such as *Scylla tranquebarica, S.serreta, Nanosesarma minutum, Neoepisesarma tetragonum, Metapograpsus maculatus* and *Macropthalmus depressus* against ten different bacterial strains. The higest zone of inhibition was observed in the haemolymph of *S.tranquebarica* against V.cholerae (10mm) and lowest zone of inhibition was observed in the haemolymph of *M.depressus* against *S.paratyphi-B* and *S.typhi* (5mm).

Sakthivel *et al.*, 2015 revealed the presence of antibacterial activity in the mangrove crab, *Sesarmaplicatum*, with the inhibition zone of 7 mm and 1 mm against *Micrococcus sp* and *V. parahaemolyticus* bacterial strain, respectively. The hemolymph extract of freshwater crab, *Oziotelphusa senex senex* showed an inhibition zone of 14 ± 1 mm, 10 ± 1 mm, 11 ± 1 mm, 8 ± 1 mm and 8 ± 1 mm against *E.coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pyogenes* and *Bacillus subtilis*, respectively (Sumalatha *et al.*, 2016).

The antibacterial activity of chitosan extracted from the shells of fresh water crab, *Paratelphusa hydrodromous*, against *Yersinia ruckeri* at different concentrations of 0.75%, 0.50% and 0.25%. Showed an inhibition zone of 85.61 ± 12.85%, 72.31 ± 10.59% and 44.20 ± 10.8%, respectively, suggesting that there is an increase in antimicrobial activity of crab extracts with increasing chitosan concentration (Gokilavani *et al.*, 2014). Packia Lekshmi*et al.*, 2015 checked the antimicrobial activity in the methanol fraction of *Callinocts sapidus* against bacterial strains, the maximum zone of inhibition was recorded in *E. coli* sp. (32.16±0.28 mm) and minimum zone of inhibition was observed in *Aeromonas* sp. and *Proteus* sp. (18.0±0.5 mm).

The prawn shell (*Macrobrachium nipponense*) extracts showed activity against pathogenic bacteria. The highest antibacterial activities were measured in *B. subtilis, S. aureus*, and *V. cholerae* with the zone of inhibition being 12.12 ±

0.32 mm, 12.51 ± 0.14 mm, and 12.35 ± 0.27 mm, respectively. Among all the strains, *S. aureus* exhibits a significant zone of inhibition against all extracts ($P < 0.05$). (Karimzadeh and Pormehr 2017). The antibacterial activity test of the chitosan from freshwater lobster shells (*Cheraxquadri carinatus*) formulated in the form of a hand sanitizer gel against *Escherichia coli* and *Staphylococcus aureus* showed that it can inhibit *E. coli* bacteria with the best inhibition zone of 8mm at a concentration of 4.5% and *S. aureus* with the best inhibition zone at 10 mm. (Zulmai Ranil *et al.*,2023).

The ethanolic extracts of freshwater crab *Scylla Serrata* were tested for their antimicrobial efficiency against pathogenic bacteria such as *Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli* and *Enterobacter aerogenes* The ethanol extract of crab shell showed maximum zone of inhibition (13 mm) against *P.aeruginosa* and moderate zone of inhibition of 11 mm against*K.pneumoniae*. The soft muscle extract showed the maximum activity of 9mm against *K.pneumoniae* and minimum zone of inhibition (7 mm) against *P.aeruginosa*. It indicated that the shell of crabs would be a good source of antimicrobial agents and would replace the existing inadequate and cost effective antibiotics.(Leena Grace Beslin and Geni G, 2021). The antibacterial activity of methanol extract of *Portunus pelagicus, Scylla tranquebarica* tissue against *Vibrio alginolyticus*, *Klebsiella pneumoniae*, *Streptococcus agalactiae*,

and *Escherichia coli* using disc diffusion method. The results indicate that both types of crab extract are bactericidal at a high concentration and bacteriostatic at low concentration.(Laith *et al*.,2017).

Antioxidant activity is fundamental property and much important for life. Many of the biological functions such as anti-mutagenicity, anti-carcinogenicity and anti-aging, among others originate from this property (Cook and Samman 1996). Therefore, in recent years, interests have been developed for searching effective natural antioxidants, since they can protect human body from free radical and retard the progress of many chronic diseases. In the present investigation total antioxidant activity of the shell extract was measured spectrophotometrically by phosphomolybdenum method. The total antioxidant activity was found to be higher in the methanolic shell extract of *P.pelagicus* at the concentration of 1000µg/ml (37.69mg/g of extract) and lower at the concentration of 200µg/ml(10mg/g of extract). This study is in corroborated with the findings of Soundarapandian *et al.*,2014. He reported that the total antioxidant activity of soft shelled crab, *Charybdis lucifera* exhibited maximum antioxidant potential of 49% and minimum effect of 32% was recorded in hard shelled crab. Sudhakar (2011) recorded the total antioxidant activity ranged from 28.52% to 80.26% at varying concentrations (0.5 to 10 mg/ ml) in *P. sanguinolentus* crab shell chitosan sample. Likewise Shiny Kachhap (2019) reported that the chitosan

extracted from carapace of *Sartoriana spinigera* showed total antioxidant activity. The percentage of inhibition was found to be 29.73 %, 33.78%, 50%, and 62.16 % at 50 μg/ml, 100 μg/ml, 200 μg/ml and 400 μg/ml concentrations respectively.

The results of the present study shows that the methanolic shell extract of *P.pelagicus* exhibit good NO antioxidant activity. The percentage of inhibition varied between 32% to 65%. The percentage of inhibition was increased with increased concentration of 400μg/ml to 1000μg/ml of concentration. The results showed that the extract exhibited dose dependent NO scavenging activity. Similar study was carried by Shiny Kachhap (2019) in the chitosan extracted from carapace of freshwater edible crab *Sartoriana spinigera.* Percentage of scavenging activity of chitosan against nitric oxide anion was found to be 21.95 %, 35.49%, 46.90%, and 66.04% at 0.5 mg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ ml concentration respectively. Chitosan concentration showed positive correlation with its percent scavenging activity, indicating that as concentration of chitosan increased, its scavenging activity against nitric oxide free radical also increased.

But in contrast to the present study Wan Roslina Wan Yusof *et al.*,2017 studied the antioxidant activity by DPPH method in the muscle extract of mud crab of *S. serrata. The extract* had a maximum antioxidant activity with 49% inhibition. The antioxidant activity of DPPH resulting from the hydrolysis of *Portunus pelagicus* chitooligosaccharides ranging from 2.27 to 5.21μmolTE/g

(SiskaAmellia and Dedin Finatsiyatull Rosida 2023). The present study show the antioxidant capacity of shell extract is highly efficient in scavenging the free radicals. Thus the waste shell extract can be encouraged to be used as a natural antioxidant in pharmaceutical industry.

The treatment goal of diabetes patients is to maintain normal levels of glycemic control, in both the fasting and post-prandial states. Many natural resources have been investigated with respect to suppression of glucose production from carbohydrates in the gut or glucose absorption from the intestine (Matsui *et al*., 2007). α-Amylase catalyses the hydrolysis of α -1,4-glucosidic linkages of starch, glycogen and various oligosaccharides and α-glucosidase further breaks down the disaccharides into simpler sugars, readily available for the intestinal absorption. The inhibition of their activity, in the digestive tract of humans, is considered to be effective to control diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes (Hara & Honda, 1990). Therefore, effective and nontoxic inhibitors of α -amylase and α -glucosidase are required. In the present study the anti-diabetic potential of the crab shell extract of *P.pelagicus* was tested by alpha amylase inhibition assay.

The methanolic shell extract of *P.pelagicus* exhibits good antidiabetic activity with the percentage of inhibition of 55.83% at 200µg/ml and 75% at 400µg/ml, 78.33% at 600µg/ml, 83.33% at 800µg/ml and 85% at 1000µg/ml

inhibition at concentration. The highest inhibition activity (85%) was observed at 1000µg/ml concentration and the lowest activity (55.83) was observed at 200µg/ml of the concentration. The alpha amylase inhibition activity was increased as of the concentration of extract was increased.

Alpha amylase inhibitors have been proved efficient for the management of type 2 diabetes. Thi Hanh Nguyen *et al.,* 2022 suggested that hemi-pyocyanin a cost-effective bioproduct of novel potential α-amylase inhibitor from the discard shrimp head and it is proved to be a good candidate for the development of antidiabetic drug. The results from this study give support to the use of crab shell extract for the treatment of diabetes, and show for the first time, the potential role of α-amylase inhibition in its activity. Due to its inhibitory effect, the extract was subjected to Hemolytic assay. Hemolytic assays were performed because compounds possessing potent biological activity may not be useful in pharmacological preparations if they possess hemolytic effect.

In the present study methanolic shell extract of various concentration was tested against human erythrocytes and the results shows that there is no haemolytic effect on human erythrocytes and the extract significantly protected the membrane of the red blood cells from hemolysis. These results clearly demonstrated that the extracts were able to scavenge free radicals and thereby protecting erythrocyte membrane. The finding clearly demonstrated the important

stabilizing effect of shell extract on the erythrocyte membrane. But in contrast to the present study Fang Yan *et al.*,(2011) tested the hemolytic activity of hemocyanin from mud crab *S.serrata*, Haemocyanin was incubated with erythrocytes of human, mouse, rabbit, or chicken at 37^0C for 1hour. Hemolysis was observed with all types of erythrocytes tested, with hemolytic activities ranging from 69.7 ± 2.4%–99.5 ± 0.8%.

The present study indicates that the methanolic shell extract of *P.pelagicus* extracts possess considerable antibacterial, antioxidant, antidiabetic and antihemolytic capacities. So GC-MS analysis was carried out to identify the compounds present in the shell extract. Shell extract showed the presence of 11 compounds with various activities. Similar study was carried out by Madhubala and Selvamohan (2023) in the carotenoids extracted from the three spotted crab (*Portunus sanguinolentus*) and reported forty one compounds in the extract. Shuang *et al*., 2021 isolated more than 18 fatty acids, including 6 saturated fatty acids (SFAs), 4 monounsaturated fatty acids (MUFAs), and 8 polyunsaturated fatty acids (PUFAs), by GC-MS in the lipids extracted from carp bones and crustacean shells. Therefore, taking into account the biological properties of the identified metabolites, the consumption of this crab could have a positive impact on the human health.(Andreia *et al.*,2019).

8. SUMMARY

The production of crab waste from crab processing industries has undergone a dramatic increase in recent years. Continued production of this biomaterial without corresponding development of utilizing technology has resulted in waste collection, disposal, and pollution problems. An ecofriendly crab shell waste management strategy for preventing environmental pollution and converting crab waste into a valuable product in the need of the hour. The crab waste contains several bioactive compounds such as chitin, pigments, amino acids, and fatty acids. These bioactive compounds have a wide range of applications including medical, therapies, cosmetics, paper, pulp and textile industries, biotechnology, and food applications.

Development of resistance by the microorganisms to chemotherapeutic agents appears to be a continuous process since the discovery of antibiotics. Marine natural products serve as an alternate source of combating infections in human beings which may also be of lower cost and lesser toxicity. In view of the above applications, the shell extract of the blue swimming crab, *Portunus pelagicus* were screened for various pharmacological analysis.

In the present study the antibacterial activity of the shell extract of *P.pelagicus* (methanol) was evaluated against five human pathogens. The

methanolic shell extract showed antibacterial activity against all the tested organism. The highest activity of 12mm was recorded against *Bacillus subtilis* and lowest activity of 9mm against *Escherichia coli.*

The role of free radical in disease pathology is well established and is known to be involved in many acute and chronic disorders in human beings, such as diabetes, atherosclerosis, aging, immunosuppression and neurodegeneration. An imbalance between ROS and the inherent antioxidant capacity of the body, directed the use of marine products which have indicated the presence of antioxidant such as phenolics, flavonoids, tannis, and proanthocyanidins.

Hence, the antioxidant activities in the methanolic shell extract of *P.pelagicus* has been evaluated two different methods namely Total antioxidant activity and Nitric oxide scavenging activity at five different concentration (200µg/ml, 400µg/ml, 600 µg/ml, 800µg/ml and 1000µg/ml).The total antioxidant activity was found to be higher in the methanolic extract of *P.pelagicus* at the concentration of 1000µg/ml (37.69mg/g of extract) and lower at the concentration of 200µg/ml (10mg/g of extract). The percentage of inhibition varied in between 32% to 65%.The percentage of inhibition was increased with increasing concentration.

The positive results on antibacterial and antioxidant activities of the shell extract lead to the analysis of antidiabetic and hemolysis assay. The alpha amylase activity in the methanolic shell extract of *P.pelagicus* showed inhibition activity range from 55% to 85%. The highest inhibition activity of 85% was observed at 1000µg/ml concentration and the lowest activity of 55.83 was observed at 200µg/ml of the extract concentration. There is no haemolytic activity in the different concentration of the extract.

Portunus pelagicus shell waste revealed the presence of various bioactive compounds like Benzene, 1-ethenyl-4-nitro-, Cycloheptene, 4,5-Dimethylthiazole S-oxide, 9-Borabicyclo(3.3.1) nonane, Perhydro-htx-2-one, 2-depentyl-, acetate ester, Heptasiloxane, hexadecamethyl-, Silane, [[4-[1,2 bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)]bis[trimethyl-, Pentasiloxane dodecamethyl-, 1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane, Heptasiloxane, hexadecamethyl- and Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-.

The total utilization of crab waste for recovery of bioactive compounds that would serve as therapeutic drugs might be useful to replace the pollution caused by marine crab wastes along with an alternative technology. This study will serve as a baseline data for further studies and a thorough understanding of chemical

structure and biological activity will lead to the formulation of novel drugs with specific actions.

9. CONCLUSION AND SUGGESTION

Crabs are commercially important and fetch high price as there is a rapidly expanding demand for crab meat both in local and international market. Crabs are found inall type of environment. The evolution of antibiotic resistant pathogenic bacteria has stimulated the search for alternative antimicrobial agents from natural source. 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 disease. A further big concern is the development of resistance to the antibiotics in current clinical use.

The industrial processing of marine product creates large amount of bio waste. These bio wastes mainly consist of crab shell which may cause environmental pollution. Marine wastes can be recycled in an appropriate way and the components extracted were found to have nutritional value and other pharmacological applications. Recently much attention has been paid to natural nutrients due to their non-toxicity. Crab muscle alone does not act as a good source of protein but crab shell also has enormous amount of protein. The crab shell is found to contain high amount of protein and other important minerals which are essential to human body.

Thus the effective utilization of crab shell waste enhances biomedical research for the development of natural drug for many chronic diseases without side effects and at the same time can reduce environment pollution. Studies should be conducted to ensure better use of the product as a functional food, which assists in the primary prevention of disease generated by oxidative stress. Hence the present investigation is said to have a specific influence on health as a low-coast natural substitute to overpriced drugs.

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ANTIACNE AND NEMATICIDAL ACTIVITY OF *EUDISTOMA VIRIDE* TOKIOKA, 1955

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affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

Master of Science in Zoology

by

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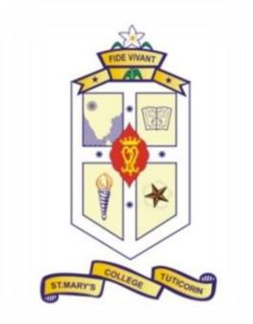

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CERTIFICATE

This is to certify that this dissertation entitled, "**ANTIACNE AND NEMATICIDAL ACTIVITY OF *EUDISTOMA VIRIDE* TOKIOKA, 1955**" submitted by **C. PRATHEEPA, Reg. No. 21APZO04** to St. Mary's College (Autonomous), Thoothukudi, affiliated to Manonmaniam Sundaranar University, Tirunelveli in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2022 - 2023 under my guidance and supervision. It is further certified that this dissertation or any part of this has not been submitted elsewhere for any other degree.

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DECLARATION

I do hereby declare that this dissertation entitled, "**ANTIACNE AND NEMATICIDAL ACTIVITY OF *EUDISTOMA VIRIDE* TOKIOKA, 1955**" submitted by me for the award of the degree of Master of Science in Zoology is the result of my original independent research work carried out under the guidance of Dr. M. Paripooranaselvi M.Sc., M.Phil., B.Ed., Ph.D., SET., Assistant Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

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CONTENTS

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INTRODUCTION

1. INTRODUCTION

Beauty has the power to spawn aspiration and passion, thus becoming the impetus to achieve our dreams. Beauty will always have the power to inspire us. It is that enigmatic, unknowable muse that keeps you striving to be better, to do better, to push harder. The concept of beauty is a permanent obsession that permeates cultures around the world. A person's definition of beauty is an abstract, complicated and highly personal ideal that becomes a guiding light throughout life. Skin is a marker of health and beauty. Skin is the organ that comes in contact with the environment.

The most common skin condition worldwide is acne. Most people experience acne at some point in their lives. Acne can happen in adults, although it is more frequent in teenagers and young adults going through hormonal changes. Acne lesions can appear anywhere on the body, but the most common sites include the face, chest, shoulders and back. According to the lesion type, acne can be classified into non-inflammatory acne and inflammatory acne.

Non-inflammatory acne includes blackheads and whiteheads. These normally don't cause swelling. They also respond relatively well to over-the counter treatments. Blackheads occur when a pore is clogged by a combination of sebum and dead skin cells. The top of the pore stays open, despite the rest of it being clogged. This results in the characteristic black color seen on the surface. Whiteheads can also form when a pore gets clogged by sebum and dead skin

cells. But unlike with blackheads, the top of the pore closes up. It looks like a small bump protruding from the skin. Whiteheads are more difficult to treat because the pores are already closed.

Inflammatory acne includes mild papular, scarring papular and nodular. Pimples that are red and swollen are referred to as inflammatory acne. Although sebum and dead skin cells contribute to inflammatory acne, bacteria can also play a role in clogging up pores and cause an infection deep beneath the skin's surface. This may result in painful acne spots.

Grading upon the severity of acne, it can be categorized into,

1. **Mild acne**: Blackheads and whiteheads are the most common breakouts in mild acne. Some papules and pustules could be present. Typically, there are fewer than 30 lesions overall.
2. **Moderate acne**: A person with moderate acne may develop more papules and pustules, as well as more blackheads and whiteheads. There are between 30 and 125 total lesions.
3. **Severe acne**: A person with severe acne will develop a great deal of uncomfortable, big papules, pustules, nodules or cysts. Scars from acne are another possibility. 125 lesions are generally present overall.

Acne – Types:

Acne rosacea:

It is a skin disease of adults often affected by women in which blood vessels of the face enlarge indicating a flushed appearance. Rosacea is a

common, chronic, incurable, adult acne-like skin condition that is easily controllable and curable medically. Rosacea usually acts upon the central third of the face, especially the nose with periodic aggravation and relief. The symptoms may come and go and the skin may be clear for weeks, months, or years and then may emerge time and again. Rosacea incline to develop in certain stages and causes to create inflammation of the skin of the face, especially the forehead, cheeks, nose as well as chin. Symptoms and signs of rosacea are redness of the face, tiny red pimples, fine red lines on the facial skin, an enlarged bulbous red nose, eye problems like swollen, red eyelids and conjunctivitis.

Acne vulgaris:

It is the most common form of acne. It usually affects people from puberty to young adult hood. Acne is a small inflamed elevation of the skin. A pustule or papules are common symptoms in acne. Unlike common acne, rosacea is not primarily a disease of teenagers but occurs most often in adults (ages 30-50), especially in those with fair skin. Different than acne, there are usually no blackheads or whiteheads in rosacea. Certain people get one or two spots off and on while others get frequent eruption of spots with lots of pus-filled pimples indicates acne which is a chronic or prolonged condition that affects many teens and adults. More or less all human beings in the world gets pimples at some point of time sooner the body enter into puberty stage at the age of 12, there commence to release hormones and start to function in the bodies of man or

woman irrespectively and at this juncture food or pollution, ought to upset hormonal balance thereafter.

Types of pimples:

Pimples or spots come out when the skin produces much more oil, causes breeding bacteria which clog the existing pores creating swelling and redness on the skin. Pimples are not at all contagious.

Whiteheads – Remain under the surface of skin and are very small.

Blackheads – Vividly look black and rise to the surface of the skin but are not formed due to dirt. Black heads are because of dirt, they are black in color. Generally, air oxidizes the protein called keratin.

Papules – They are small tender pink bumps which are clearly seen on the skin.

Pustules – Pustules (pimples or zits) are red at the bottom level consisting of pus at its top and can be looked on the surface of the skin.

Nodules – Obviously visible on the surface of the skin. They are painful, large, solid pimples existing deeply in the skin and can be seen on the skin surface.

Cysts – They are clearly visible on the surface of the skin. They are deeply rooted, painful and pus filled and easily prone to form scars.

Causes of acne:

Hormones: Common acne in teenagers starts with an increase in hormone production. During puberty, both boys and girls produce high levels of androgens, the male sex hormones that include testosterone. Testosterone

signals the body to produce more sebum, the oil produced by oil glands of the skin.

Bacteria: The bacteria that cause acne are *Propionibacterium acnes* and *Cutibacterium acnes*. Excess sebum clogs the openings of hair follicles on the face, neck, chest and back allowing the bacteria to grow in these clogged follicles. This causes blackheads or whiteheads also known as "comedones," to form on the surface of the skin. Sometimes, this clogging causes the follicle wall to break under the pressure of this buildup causing the sebum to leak into nearby tissues and forms a pustule or a papule which is inflammatory acne.

Diet: Consumption of food having high glycemic index, dairy products, spicy and oily food products exaggerate sebaceous glands activity leading to acne. Smoking and alcohol consumption also leads to acne.

Cosmetics: The pores on your skin can become clogged by makeup, moisturising creams, lotions and hair treatments that include pore-clogging sulphates, mineral oil, coconut and cocoa butter and silicones.

Stress and Anxiety: Cortisol and adrenaline levels are strongly impacted by psychological and emotional stress, which worsens acne. These stress chemicals cause the creation of testosterone by oil glands, which increases oil production and clogs pores.

Pimple popping: It is simple to transmit the bacterial infection beneath the skin when we attempt to pop a pimple. The acne will only spread because of the

additional blockage, swelling and redness this creates. Scarring is also more probable because of this.

Too much sun: The skin dries out after getting a sunburn, which causes extra oil to be produced to make up for it. More acne is brought on by too much oil.

Drugs: Prolonged use of some drugs burn the skin of that area causing scar formation. The oral contraceptives, injectable contraceptives, intrauterine birth control devices, steroids taken by bodybuilders and athletes may also cause acne.

Genetics: Acne is influenced by genetics and as a result, the skin condition has a propensity to run in families.

Psychological issues:

People with acne are up to three times as likely to experience depression than people without acne. For certain acne sufferers, suicide can be a problem in addition to sadness. Some acne sufferers can develop agoraphobia. Acne patients typically struggle with social anxiety and avoid engaging in any activity that will draw attention to their condition. Severe acne is associated with increased depression, anxiety, poor self-image and poor self-esteem.

Treatment of acne:

Treatment of acne depends on its condition and degree of severity which may vary from a mild non inflammatory comedons to an inflammatory papule or pustule. This usually signifies the presence of *Propionibacterium acnes*. Topical as well as systemic therapy is available for the treatment of acne. While traditional treatments in the inflammatory phase are topical and systemic

antibiotics acting as both antimicrobial and anti-inflammatory agents, modern acne therapy has been designed to interrupt the pathogenic pathway at one or more points. The excessive use of antibiotics for long periods has led to the increased resistance in acne causing bacteria i.e., *Staphylococcus epidermis*, *Propionibacterium acnes* against a number of antibiotics used to treat acne. WHO noted that majority of the world's population depends on traditional medicine for primary health care.

Retinoids and retinoid-like drugs: Moderate acne is frequently treated with medications that contain retinoic acids or tretinoin. Creams, gels and lotions are available. Examples: Tazortene, adapalene and tretinoin.

Antibiotics: They play a major role in eliminating surplus skin germs and minimising irritation and redness. Examples: Clindamycin with benzoyl peroxide and Erythromycin with benzoyl peroxide.

Azelaic acid: Azelaic acid is a naturally occurring acid produced by a yeast. It is antibacterial in nature. When applied twice daily, a 20% azelaic acid cream or gel appears to be just as effective as several common acne treatments. It can also be applied to treat some types of acne-related discolouration.

Dapsone: Dapsone (Aczone) 5% gel twice day is advised for inflammatory acne.

Anti-androgen agents: If oral antibiotics are ineffective, women and adolescent girls may want to explore the medication spironolactone (Aldactone). It

functions by preventing androgen hormones from having an impact on the oil glands.

Isotretinoin: Isotretinoin (Amnesteem, Claravis) is a vitamin A derivative. If other treatments haven't worked for someone's moderate or severe acne, it might be given.

Light therapy: Blue light therapy is most frequently used to treat acne outbreaks**.**

Chemical peel: A chemical solution, such as salicylic acid, glycolic acid or retinoic acid is applied repeatedly to treat minor acne.

Drainage and extraction: Cysts and comedones may be gently removed by the doctor using specialised tools.

Steroid injection: A steroid medication can be injected directly into nodular and cystic lesions to treat them.

Side effects of the treatment:

The sun sensitivity of skin is increased with topical retinoids. Particularly in those with brown or black skin, they can also cause dry skin and redness. Depression, severe birth abnormalities and inflammatory bowel disease are among the possible side effects of oral isotretinoin. Possible side effects of anti-androgen agents include breast tenderness and painful periods. Skin thinning and discolouration in the treated region are possible side effects of steroid injection.

Conventional acne treatments, like salicylic acid, niacinamide or benzoyl peroxide, are proven to be the most effective acne solutions, but they can be expensive and have undesirable side effects, such as dryness, redness and irritation.

Natural products have received a lot of attention for preparing novel drugs due to their relative safety and abundancy in nature. Pharmaceutical companies are now exploring for alternatives due to the rise in microorganism resistance to the already prescribed antibiotics and the expensive expense of producing synthetic substances. Due to their generally low risk of side effects, lower cost and ability to combat a variety of antibiotic-resistant pathogens, ascidians may be used as substitute. Natural products may be helpful in treating acne because there are adverse effects from conventional treatments. Antiacne effects of marine resources have not been well reported.

To overcome the side effects, ascidians have been studied as alternative treatments for acne.

Nematicidal activity:

Nematodes:

The nematodes that have been fossilised lived about 25 million years ago. Nematodes live at both high and low elevations, in polar and tropical climates, in fresh water, seawater and on land. They have adapted to nearly every ecosystem on the world. Nematodes are thread-like worms, mostly about 1 mm

in length and the most ubiquitous organisms on Earth. One hundred grams of soil typically houses about 3000 individuals (Gaugler *et al*., 2004). Plant-parasitic nematodes display a wide variety of interactions with their hosts. All have hollow, protrusible stylets or mouth spears used to penetrate cells to allow feeding and for endoparasitic forms, entry into the host. Some nematodes are migratory ectoparasites that never enter the host, but simply migrate through the soil, using roots as an ephemeral food source as they encounter them. Migratory endoparasites enter the host and migrate through host tissues causing extensive damage. Semi-endoparasitic nematodes may have migratory stages, but also partially penetrate the host plant in order to feed at one stage of the life cycle. Plant-parasitic nematodes are also called as Root Knot Nematodes (RKN).

They attack plants and cause roughly US $70 billion of crop losses annually in fruit and vegetable production (Jones *et al*., 2013). The majority of dangerous nematodes that affect soil-based agriculture are of the gall, lance or sting varieties. The nematodes are often tiny soil-borne pathogens that can eat all plant components including roots, stems, leaves, flowers and seeds. The majority of species eat roots only. Any agricultural crop, including staples like cereals, legumes, potatoes, sugar cane, sugar beets, bananas, coconuts and sweet potatoes can be negatively impacted by the activity of some species of phytoparasitic nematodes.

Meloidogyne spp. being the most common and widespread group of Root Knot Nematodes in the world (Mai *et al*.,1985), increase the severity of soil

borne diseases such as Fusarium wilt in watermelon (Sumner and Johnson, 1973). Plant growth impairment caused by *Meloidogyne* spp. to vegetable crops is influenced by nematode species and physiological race as well as the initial nematode population density in the soil at sowing or transplanting (Sasanelli, 1994).

The life cycle of root knot nematode has six stages (Plate: 1). The egg state, J1 or first stage larva, J2 or second stage larva, J3 or third stage larva, J4 or fourth stage larva and the adult stage. The first four stages are the immature stages and are known as juvenile stages. Inside the host tissues, *Meloidogyne* spp. pass through an embryonic stage, four juvenile stages (J1−J4). Juvenile *Meloidogyne* species hatch from eggs as J2s, while the first molt occurs within the egg. Newly hatched juveniles live for a short period of time in the rhizosphere of the host plants without feeding. Then J2s invade host root in the root elongation region and migrate until they find a place to settle and feed. In that area, parenchyma cells near the head of the J2s become multinucleate giant cells, from which the J2s and later the adults feed. After further feeding, the J2s undergo morphological changes and then without further feeding, they molt three times. After development to J3 and J4, adults are produced. In females, the reproductive system develops and they can produce hundreds of eggs, while male adults leave the root and do not harm the host. The length of the life cycle is temperature-dependent (Ntalli *et al*., 2012). It has long been recognized that chemotaxis is the primary means by which nematodes locate host plants.

Chemotaxis is a movement in the direction of higher concentrations of semiochemicals such as plant chemical signals. Nematodes J2 are attracted to plant roots via soluble and gaseous attractants produced by the root itself or by attendant rhizosphere microorganisms (Bird 1959 and Young *et al*., 1996).

Root-knot nematodes often occur on roots of banana which is known as 'Apple of paradise'. Bananas are among the most produced, traded and consumed fruits globally. More than 1000 varieties of bananas exist in the world, which provide vital nutrients to all age group of people. The most traded variety is the Cavendish banana, which accounts for just under half of global production at an estimated annual production volume of 50 million tonnes. Bananas are particularly significant in some of the least developed and low-income, food-deficit countries, where they can contribute not only to household food security as a staple but also to income generation as a cash crop. Nematodes from the genera *Helicotylenchus, Pratylenchus, Rotylenchulus* and *Meloidogyne* are widespread. They cause severe problems in banana plantations as toppling, decreasing the production and fruit quality (Pestana & Cravo 1999). The damage caused by nematode species is more visible (root necrosis) and more destructive (toppling of plants).

Classification of nematodes

Based on their feeding environments, the nematodes are classified into

1. **Ectoparasitic:** Some nematodes are migratory ectoparasites, which means they move across the soil while feeding intermittently on roots when they come across them.
2. **Endoparasitic:** Endoparasites that move through host tissues after entering the host cause significant damage.
3. **Semi-endoparasitic:** Nematodes may go through migratory stages, but they can also partially enter the host plant and feed there.

Based on the types of plant affected, the nematodes are classified into

Root-knot nematodes: They induce conspicuous “knots” or gall-like swellings on roots. They can attack any of more than 2,000 different higher plant species. Losses are frequently significant, particularly in warm areas with lengthy growth seasons. However, some species, like the northern root-knot nematode - *Meloidogyne hapla*, may survive in soil that can freeze up to a metre below the surface. Attacks on orchard trees, cotton, strawberries and vegetables are frequent. Through nursery stock, ornamentals and garden plants commonly get infected.

Root-lesion nematodes: *Pratylenchus* species are endoparasites that seriously harm hundreds of different crop and ornamental plants by piercing the roots and feeding on the tissues, which results in cell death. Nematodes enter the soil in search of healthy roots. Root rot frequently happens as fungus and bacteria invade damaged tissues and cause lesions to grow in the root. While perennial plants and orchard trees may not experience a drop for a number of years, annual

crops could fall early in the season. *Pratylenchus goodeyi* is a slender nematode with roughly 0.5 mm 6 long and a diameter of 20 µm, an annulated lip region, a strong stylet with large basal knobs and an oesophagus that overlaps the intestine ventrally. Females of this species have a posterior vulva position at 73-75% and the males with paired slender spicules and usually with the bursa enveloping the tail. The tail is conoid with a small irregular peg which is a distinguishing feature of this species (Machon & Hunt 1985; Loof 1991). Nonetheless, the identification of *Pratylenchus goodeyi* based on morphological characters is complex because they are difficult to detect and also due to a high intraspecific variability. Furthermore, biochemical and molecular characters are not well established to this species (De Waele & Elsen 2002) and the identification is still being done on the basis of morphological characters. *Pratylenchus goodeyi* can be found in roots, rhizomes, tubers and in the host pseudostem. After penetrating the roots, they multiply rapidly reaching 1000 to 3500 specimens per gram of root.

All life stages are considered to be infective, capable to enter and leave the root tissues. The life cycle is completed within the root in 24-30 days at 24-25 ºC, thus several generations may develop during one growing season (Gowen & Quénérhervé 1990). *Pratylenchus goodeyi* are also found in the rhizosphere where they can survive for some time and search for new roots to infect (Machon & Hunt 1985). Females produce eggs and the first moult occurs in the egg as J1. After hatching as J2, these nematodes will try to localize roots from a susceptible

host and following root penetration they complete the life cycle moulting to J3, J4 and adults (female or male). The feeding of migratory endoparasitic nematodes in the cortical tissue destroys the roots 7 and their functions become severely impaired. Roots infected by *Pratylenchus goodeyi* have small brownish-red elongated lesions that tend to enlarge and coalesce, causing an extensive root necrosis (Gowen & Quénérhervé 1990; Loof 1991).

Potato cyst nematode: The nematodes are tiny, less than 1 mm in length and they generate pinhead-sized spherical cysts on roots. Nematode eggs found in cysts number in the hundreds and are so hardy that they can live in soil for up to 20 years. Cysts are easily spread by wind, rain and water, as well as by infected soil that sticks to seeds, animals, tools, clothing and plants. Potatoes are harmed by nematodes, which also cause plant dwarfism, root cysts, diminished roots, leaf withering and discoloration and less yields. If potato cyst nematode is not controlled, a crop can completely perish.

Sugar beet nematode: The causative agent is *Heterodera schachtii*. The nematode survives in the soil as cysts that contain eggs and juveniles. Cysts can be spread by machinery, animals and water from harvested beets. In the soil profile, cysts can be found from the surface to 24 inches deep, but the highest numbers are found in the root zone. The infestation initially appears as circular to oval areas of stunted plants. Infested plants tend to become pale yellow and wilt, especially in the afternoons of warm and sunny days.

Citrus nematode: Wherever citrus is planted, the citrus nematode - *Tylenchulus semipenetrans* can be found and it severely reduces fruit quality and yield. In many groves older than 15 years, steady decline, yellowing and decaying leaves and dieback of twigs and branches are typical indications. The nematode has been widely dispersed by infected nursery stock.

Burrowing nematode: The burrowing nematode - *Radopholus similis* is a dangerous endoparasite that affects over 200 valuable crops, trees and ornamentals including abaca, banana, avocado, tomato, black pepper and citrus.

Other nematodes: Dagger nematodes – *Xiphinema*; stubby-root nematodes - *Trichodorus*; spiral nematodes – *Rotylenchus*, *Helicotylenchus*; sting nematodes - *Belonolaimus* and pin nematodes are just a few significant ectoparasites that feed on plant roots. Vegetable and ornamental bulb crops, clovers, alfalfa, strawberry, sweet potato, orchids, chrysanthemums, begonias and ferns are all severely harmed by leaf nematodes - *Aphelenchoides* species and stem worm - *Ditylenchus dipsaci*.

Effect of nematodes on plants:

Many plant-parasitic nematodes eat plant roots. The root system of plants is harmed during the feeding process, which also limits the plant's capacity to absorb nutrients and water. Root mass decrease, root structure distortion and root expansion are common signs of nematode damage. The plant is further weakened by nematode damage to its roots since it opens up a pathway for other plant infections to enter and spread throughout the root. Depending on the

nematode species, shoot-feeding nematodes can cause diminished vigour, distorted plant parts or even the death of infected tissues.

Control of nematodes:

Cultural practices: A number of cultural practices can be applied. By removing their food source, interrupting their mating grounds and altering their habitat, these methods seek to lower the nematode population. Fallowing, flooding, hot-water treatment, adoption of resistant and antagonistic crops, quarantine and soil supplements such oil seed cakes, seed powder and botanicals are examples of other cultural techniques.

Crop rotation: Nematode populations in the soil can be brought down to levels that will be less harmful to subsequent plants grown in the same area by rotating the most susceptible crops, like tomato, bean, capsicum, carrot and egg plant with less susceptible crops like Jerusalem artichoke, asparagus, sweet corn, broccoli, Brussels sprouts and mustard. Avoid planting the same family of plants and root crops in the same year.

Early-season cropping: Brassicas, lettuce, onions, radishes, leafy greens, green peas and radish are a few examples of vegetables that can be planted early without suffering severe nematode damage. Cooler temperatures promote the growth of these plants while lowering nematode activity and reproduction. Also, these plants are picked before major nematode damage occurs. Late-season plantings are especially susceptible to nematode damage.

Root destruction: It is wise to kill and remove crop roots as soon as the plants are harvested since nematodes continue to feed on and breed in root fragments in the soil. Prior to planting the following crop, this should reduce the population of nematodes.

Organic matter: Plants can withstand nematode assault with more water and nutrients. Organic matter, such as compost or soil improver, helps the soil to retain moisture and increases the amount of plant nutrients that are readily available and are given to plants through microbial action. The rise in soil bacteria favours the development of organisms that eat other soil microbes, such as nematodes. Green manure crops like legumes, clover, vetch, or rye can also build up organic matter in the soil. These crops are planted in autumn and tilled into the soil in early spring, giving enough time for decomposition before the next crop is planted. Some evidence suggests that the incorporation of a green manure crop produces compounds that are toxic to nematodes. This is especially true for green, leafy brassicas. The soil disturbance occurring with this incorporation can also reduce the nematode levels.

Bio-pesticides: Bio-pesticides are the products made from plants or compounds produced from plants. To lessen the infestation of plant parasitic nematodes, they are employed in a variety of methods, including by using their chopped plant parts, directly combining with soil, using their extracts, residues and oil cakes. It has been shown to be quite helpful in many ways to use these plant compounds against phytonematodes.

Soil solarisation: Nematodes and some other plant diseases that survive in the soil can be managed with the aid of high temperatures. Rake out any plant residue from the treated garden bed sections, smooth and moisten the soil surface, then lay and firmly peg down a piece of thin clear plastic. Midway through the summer, keep this in place for at least a month. The heat of the sun will destroy several undesirable species by penetrating deeply into the soil. The soil temperature needs to be between 37 and 52°C for several months in order to be effective.

Bio-fumigation: Cover crops of marigolds and mustard have been shown to reduce the numbers of root knot nematodes in soil. Sow mustard seeds densely as a green manure crop and dig it into the soil at flowering. Keep the soil moist and as the mustard decomposes it will release chemicals that fumigate the soil and reduce nematode numbers. Plant marigold seeds densely at least two months, preferably three or four months, before sowing vegetable crops. Do not let weeds become established as they can become nematode hosts. The roots of the living plants produce a chemical that inhibits the hatching of nematode eggs. There is no further benefit by digging the plants into the soil. French marigolds - *Tagetes patula* are thought to be effective against a larger range of root knot nematode species than African marigolds - *Tagetes erecta*.

Chemical control: The nematicides ethylene dibromide and 1,2-Dibromo-3-Chloropropane and carbamate are widely used. The mixture of 1,2-dichloropropane and 1,3-dichloropropene is widely used as a successful

nematicide. Initially, applying chemical nematicides to contaminated soil can significantly reduce the damage caused by plant parasitic nematodes, but many of the regularly used nematicides are expensive.

Side effects of the nematicides:

- The nematicides ethylene dibromide and 1,2-Dibromo-3-Chloropropane may cause cancer in humans.
- The nematicidal carbamate is carbofuran, which is also offered in granular and liquid forms and it was linked to deaths of birds.
- Groundwater contamination is one of the more serious environmental issues sometimes connected to the use of nematicides.
- Toxins that affect the nervous system can also effectively control nematodes.

Marine environment has been recognized to be a rich source of bioactive metabolites with varied biological and pharmacological activities. Ascidians rank second with most promising source of drugs. The ecologically friendly ascidian is helpful for treating the nematodes because using chemicals to control plant nematodes has numerous negative impacts. Ascidians are marine invertebrates which ranks second with promising the source of drugs (Azumi *et al*., 1990). Many different secondary metabolites have been produced by ascidians, some of which have physiological purposes, mostly for protection against their natural predators (Pisut and Pawlik, 2002). In India, studies on antiacne and nematicidal property of ascidians especially in *Eudistoma*

viride are lacking. As ascidians are available along the Tuticorin coast an attempt has been made to assess antiacne and nematicidal property of ascidians. The thesis consists of 2 parts

Part I: Deals with antiacne activity of ethanolic extract of *Eudistoma viride*

Part II: Deals with nematicidal activity of ethanolic extract of *Eudistoma viride*

OBJECTIVES

2. OBJECTIVES

The objectives of the present study were to

- collect and identify the colonial ascidian *Eudistoma viride* Tokioka, 1955.
- prepare crude extract by soxhlet extraction and rotary evaporator.
- evaluate the antiacne activity of *Eudistoma viride* Tokioka, 1955 against *Propionibacterium acnes* using agar well diffusion method.
- evaluate the MIC, MBC and IC_{50} values of *Eudistoma viride* Tokioka, 1955.
- estimate the nematicidal activity of *Eudistoma viride* Tokioka, 1955.

REVIEW

OF

LITERATURE

3.1 Antiacne activity

Kumar *et al*., (2007) reported about the antimicrobial effects of Indian medicinal plants against acne inducing bacteria and found that the extract of *Coscinium fenestratum* produced strong inhibition zones against *Propionibacterium acnes*. Phytochemical screening of *Coscinium fenestratum* revealed the presence of alkaloid which could be responsible for activity. The extract of *Selaginella involvens* is a safe non-antibiotic antiacne source in the therapeutic application of the treatment of acne as analysed by Joo *et al*., (2008). According to Tsai *et al*., (2010) Minimum Inhibitory Concentration (MIC) values of 2000 g/mL, 500 g/mL and 1000 g/mL respectively of *Rosa damascena* Mill (Rosaceae), *Eucommia ulmoides* Oliv. (Eucommiaceae) and *Ilex paraguariensis* A. St.-Hil. (Aquifoliaceae) suppressed the development of *Propionibacterium acnes*.

According to Nand *et al*., (2012) the dichloromethane and methanolic extracts of *Azadirachta indica* showed very little activity against *S. aureus* and *S. epidermidis*, but did not show any antimicrobial activity against *Propionibacterium acnes*. Muddathir and Mitsunaga (2013) evaluated the antiacne activity of selected 29 Sudanese medicinal plants and found that the methanol and 50 % ethanol extracts of *Terminalia laxiflora* Engl and Diels wood exhibited good antibacterial activity with MIC 0.13 mg/ml

against *Propionibacterium acnes*. According to Sharma and Lall (2014) the ethanol bark extract of *A. galpinii* demonstrated the best activity against *Propionibacterium acnes* with the MIC value of 62.5 μg/mL.

The ethanol extract of the *Garcinia mangostana* has been found to be anti-acne and the compound a-mangostin, was responsible for the anti-bacterial activity as stated by Khumsupan and Gritsanapan (2014). Nelson *et al*., (2016) evaluated the anti-acne activity of Italian medicinal plants used for skin infection and found that the species used in traditional medicine for the skin exhibited significantly greater ($p < 0.05$) growth inhibitory and biofilm eradication activity than random species, supporting the validity of an ethnobotanical approach to identifying new therapeutics. Acetone extract displayed a potent antibacterial activity in the dose-dependent manner. MIC of acetone extract of *P. indica* indicating that these plants could be a good source for the anti-acne medicine.

According to Kaur and Prasad (2016) the acetone extract displayed a potent antibacterial activity in the dose-dependent manner and MIC of acetone extract of *P. indica* indicating that these plants could be a good source for the antiacne medicine. Kok *et al*., (2016) suggested that the extracts of *S. polycystum* serve as a promising source that could be developed for topical application against acne vulgaris. In another study conducted by Budiman *et al*., (2017) *Morus nigra* showed 2.5% MIC against *Propionibacterium acnes* and the MBC values was 5%. Vora *et al*., (2018) analysed the antibacterial and

antioxidant strategies for acne treatment through plant extracts and found that the plant extracts of *R. officinalis, M. chamomilla* and *A. nilotica* showed significant activity against *P. acnes* with diameter of 8 mm, 6 mm and 4 mm inhibition zone respectively.

Satpute and Kalyankar (2018) stated the basil oil, ethanolic extract of neem can be used for the treatment of *acne vulgaris*. Taleb *et al*., (2018) stated that the oregano oil nanoemulsion is a potential natural and effective alternative for treating acne and overcoming the emerging antibiotic resistance. According to Jin and Lee (2018) *Kaempferia parviflora* extract could be developed as a potential natural antiacne agent. Febriyani *et al*., (2018) evaluated the antiacne activity from extracts and fractions of surian (*Toona sinensis*) leaves planted in Sumedang, West Java, Indonesia and the results showed that ethyl acetate extract demonstrated the highest inhibition against *Propionibacterium acne* (49.93%).

Waranuch *et al*., (2019) stated the antiacne hydrogel containing a combination of mangosteen rinds, aloe vera gel and green tea leaf extracts was superior to 1% clindamycin gel in antiacne and antiblotch activities when measured by Total Acne Lesions and erythema and melanin values. Nakyai *et al*., (2021) reported that the *M. ferrea* flower extract may serve as the alternative natural antiacne formulations.

The mangosteen peel extracts alpha-mangostin possessess a strong antimicrobial against *Cutibacterium acnes* as analysed by Rizaldy *et al*., (2021).

Alnabati *et al*., (2021) suggested that *Rhazya stricta, Azadirachta indica*, *Camellia sinensis* and *Ocimum basilicum* could be used in skin care product to prevent acne as they exhibited good antibacterial activity with MIC values of 50 mg/mL, 25 mg/mL, 100 mg/mL and 100 mg/mL. Ardiansyah *et al*., (2021) found that the sea cucumber *Holothuria impatiens, Holothuria scabra, Pseudocolochirus sp., Stichopus vastus* and *Holothuria atra* have antiacne activity.

Zhang *et al*., (2022) evaluated the *in-vitro* antiacne activities *of Ocimum basilicum* L. water extract and found that the inhibition zone of *Ocimum basilicum* L. water extract to *Cutibacterium acnes* was 18.71 mm diameter. Krzemińska *et al*., (2022) found the *Cotoneaster* plants species are the most promising antiacne agent.

The gel preparation of 10% aloe vera ethanol extract concentration has the best inhibition against *Propionibacterium acnes* as analysed by Bilal *et al*., (2023). *Aloe barbadensis* pulp was collected and mixed with the extract of *Vigna radiata* and formulated into a gel using carbopol 940, triethanolamine and propylene glycol and the formulation showed a promising effect on acne as reported by Chellathurai *et al*., (2023). Muhsinin *et* al., (2023) stated the fermented turmeric kombucha can be formulated as an antiacne facial toner because it inhibits *Propionibacterium acnes* bacteria. Sani *et al*., (2023) found that the high concentration of clove extract used in antiacne cream has high potential to treat acne.

3.2. Nematicidal activity

The root-knot nematodes were significantly inhibited by *A. viridis*, but plant growth was negatively impacted (Costa *et al*., 2003). Few essential oils from several Eucalyptus species are harmful to nematodes, weeds, insects, fungi and bacteria (Batish *et al*., 2008). The root exudates *E. hirta* demonstrates nematicidal behaviour towards *Meloidogyne incognita* juveniles (Kumar *et al*., 2010). Oka *et al*., (2012) analysed the nematicidal activity of the leaf powder and extracts of *Myrtus communis* against the root-knot nematode *Meloidogyne javanica* and found that the nematicidal activity may be due to carvacrol, *t*-anethole, thymol and carvone. Caboni *et al*., (2013) found that the EO from *Mentha spicata* showed a nematicidal activity with an EC_{50} of 358 mg/L.

According to Dourado *et al*., (2013) the use of neem oil *in vitro* was effective only in the immobility of *Meloidogyne incognita* at concentrations of 1% and the neem oil reduces the number of galls of *Meloidogyne incognita* when in foliar application and soil at concentrations of 1%. According to Liu *et al*., (2014), *Triadica sebifera, Leptopus chinensis, Glochidion eriocarpum, Croton tiglium, Phyllanthus urinaria, Ricinus communis* and *Euphorbia* spp., at the concentration of 1 mg/ml after exposure of 72 h, inhibited the second stage of *M. incognita* juveniles. Rahul *et al*., (2014) evaluated the nematicidal activity of microbial pigment from *Serratia marcescens* and found that the test pigment was found effective against juvenile stages of *Radopholus similis* and *Meloidogyne javanica* at low concentrations (LC_{50} values, 83 and 79 μg/mL,

respectively). Characterisation of extracted pigment with TLC, FTIR, HPLC, HPTLC and spectroscopic analysis confirmed the presence of prodigiosin as a bioactive metabolite and the use of microbial secondary metabolites can be effective for nematode control rather than using whole organism.

Machado *et al*., (2015) found that the dichloromethane and ethyl acetate fractions showed to be the most active among the hydroalcoholic leaf extracts and the percentages of *C. elegans* larvae immobility were 98.13 and 89.66%, respectively, at a concentration of 1000 $\mu g.ml^{-1}$. Aissani *et al*., (2015) found that the *Eruca sativa* can be considered as a promising companion plant in intercropping strategies for tomato growers to control root-knot nematodes.

The soil treatments with the *Artemisia herba-alba, Citrus sinensis, Rosmarinus officinalis* and *Thymus satureioides* EOs generally resulted in a significant reduction of root-knot nematode infestation on tomato as analysed by Avato *et al*., (2017). Nandakumar *et al*., (2017) studied the nematicidal activity of aqueous leaf extracts of *Datura metel, Datura innoxia* and *Brugmansia suaveolens* found that the aqueous leaf extract of *Brugmansia suaveolens* possessed maximum mortality on second stage juveniles of *Meloidogyne incognita* when compared with *Datura metel* and *Datura innoxia* and found that the nematicidal activity may be due to the presence of phytocompounds in leaf.

Eloh *et al*., (2019) reported the EOs of *Ocimum sanctum* L., *Cymbopogon schoenanthus* (L.) Spreng and *Cinnamomum zeylanicum* Blume were found to

be effective. Rajasekharan *et al*., (2019) studied about the nematicidal activity of 5-iodoindole against root-knot nematodes and found 5-Iodoindole effectively killed juveniles and freshly hatched juveniles by inducing multiple vacuole formation and microscopic analysis confirmed that the rapid death was due to the generation of reactive oxygen species.

Nguyen *et al*., (2020) evaluated the nematicidal activity of cinnamon bark extracts and chitosan against *Meloidogyne incognita* and *Pratylenchus coffeae* and the results suggest that cinnamon mixed with chitosan may be used as an effective eco-friendly pesticide against plant-parasitic nematodes. Laquale *et al*., (2020) found that the *E. angustifolia*, can be used for sustainable nematode management against root-knot nematode *Meloidogyne incognita*.

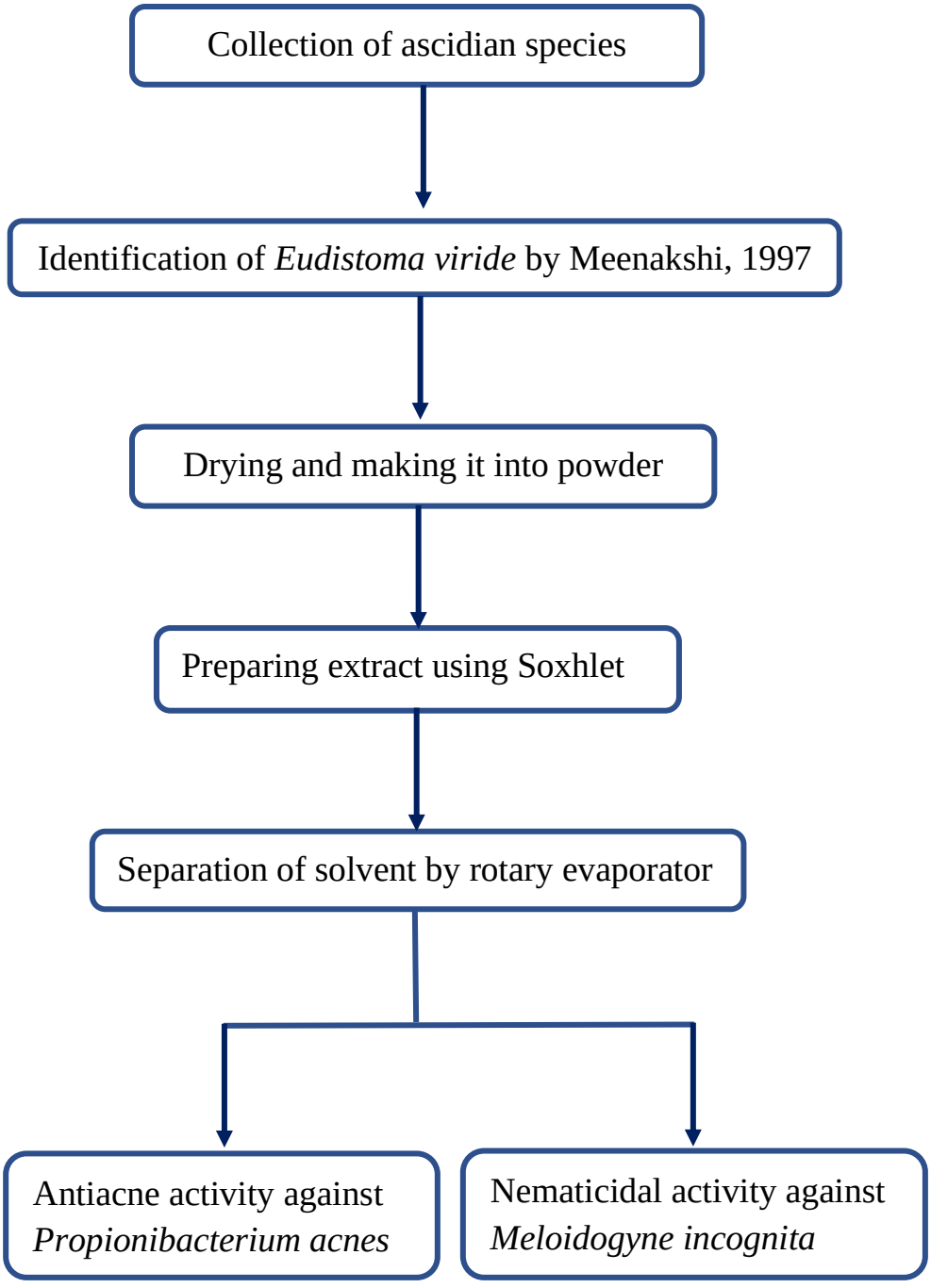
Afzal *et al*., (2021) analysed the nematicidal activity of different plants extracts such as *Amaranthus viridis*, *Chenopodium album*, *Solanum nigrum*, *Carica papaya* and *Euphorbia hirta* against root knot nematodes and found that these plant species can be utilized for biocontrol of root knot nematodes and this method of management is cheap, environmentally friendly and free from any hazards. Arshad *et al*., (2021) evaluated the nematicidal activity of plant extracts for management of *Meloidogyne incognita* in local cultivars of eggplant (*Solanum melongena* L.) in Pakistan and the results revealed that the Marigold exhibited promising results to manage *M. incognita* egg masses. Moreover, Neem brought about significant results after Marigold in controlling *M. incognita* egg masses.

The extracts of sweet annie *Artimisia annua* and garden cress *Lepidium sativum* represent promising nematicide alternatives and have potential use in crop management as reported by Mohammad *et al*., (2022). The ethyl acetate leaf extract of *M. oleifera* Lam. shows great potential for combating agricultural nematodes as suggested by León *et al*., (2022).

D'Addabbo *et al*., (2023) reported that the garlic-based nematicides could be an effective tool for *Xiphinema index* management in organic and integrated vineyards. Shi *et al*., (2023) stated that the EO from *Seseli mairei* H. Wolff roots and their isolates may be developed as a promising natural nematicide. Adande *et al*., (2023) found that the *Conyza bonariensis* and its acetylenic constituents could be considered as potent botanical and insecticidal and nematicidal agents.

MATERIALS AND METHODS

4.1. EXPERIMENTAL DESIGN



4.2. Collection of Animal Material

Samples of *Eudistoma viride* Tokioka, 1955 were collected during the low tide from the intertidal rocky area of Hare Island (Plate: 2).

The samples were washed with sea water to remove sand, mud and overgrowing organisms at the site collection and then transported to laboratory. Identification upto the species level was carried out based on the key to identification of Indian ascidians by Meenakshi, 1997.

4.2.1. Systematic Position

Eudistoma viride belongs to

Phylum	:	Chordata
Subphylum	:	Urochordata
Class	:	Ascidiacea
Order	:	Enterogona
Family	:	Polycitoridae
Genus	:	*Eudistoma*
Species	:	*Viride*

4.2.2. Animal Material

Ascidians commonly called ‘sea squirts’ are an interesting group of marine, sedentary organisms found to occur abundance in Tuticorin coast. It is sessile and filter feeding. It lives on plankton that it filters from seawater with a mucous net.

Plate: 3 depicts *Eudistoma viride*. *Eudistoma* genus 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.

4.2.3. Preparation of Powder

The specimens were dried under shade. The dried animals were homogenized to get a coarse powder. The dried powder of *Eudistoma viride* was used.

4.2.4. Preparation of extract

Soxhlet extraction is a method used for the extraction of valuable bioactive compounds from various natural sources (Plate: 4). It is used to extract the compounds 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 *Eudistoma viride* 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 (Plate: 5). 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.

4.3. Antiacne activity

4.3.1. Microbial strains used

Antibacterial activity was determined against gram positive bacteria *Propionibacterium acnes*.

4.3.2. Preparation of agar medium and broth

Nutrient Agar Medium

The medium was prepared by dissolving 2.8 g of the commercially available Nutrient Agar Medium (HiMedia) in 100 ml 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 100 mm petriplates (25-30ml/plate) while still molten.

Nutrient broth

Nutrient broth was prepared by dissolving 2.8 g of commercially available nutrient medium (HiMedia) in 100 ml 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.

4.3.3. Antimicrobial assay

The antimicrobial assay was measured by agar well diffusion method. 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, (*Propionibacterium acnes*-1951) Wells were cut and ethanolic etract of *Eudistoma viride* was added at various concentration (500, 250, 100 and 50 µg/ml). 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).

4.3.4. Determination of minimum inhibitory concentration (MIC)

The MIC is the lowest concentration of an antimicrobial agent that inhibits a bacterium from growing visibly within a specified amount of time. The MBC was obtained after determining the MICs. The MBC is the least amount of drug required to kill 99.9% of bacteria. Half-maximal inhibitory concentration (IC_{50}) is the most widely used and useful indicator of a drug's

effectiveness. It provides a measure of an antagonist drug's potency in pharmacological research by indicating how much medication is required to block a biological process by a factor of two. The broth microdilution assay method was used to determine the MICs for the susceptibility of *Propionibacterium acnes* (MTCC. No. 1951) in sterile disposable flat-bottomed 96-well microtiter plates. Briefly, the 0.5 McFarland inoculum suspensions were further diluted 1:100 in nutrient broth before inoculation, 50 µL of the bacterial suspension was seeded into a 96-well plate containing 50 µL of test sample with serial concentrations. The final inoculum of the bacteria was approximately 5×10^5 CFU/mL. The final concentrations of *Eudistoma viride* (500, 250, 125, 62.5 and 31.25 µg/mL). The broad-spectrum antibiotic Ciprofloxacin was chosen for testing as a control and the concentration of antibiotic in the well was 100 µg/mL. The plates were then incubated at 37 °C for 18–24 h. The results were read at 600 nm using a microplate reader. Changes of color were observed and recorded. All the tests were done in triplicates.

$$\%\ \text{inhibition} = \frac{Absorbance\ of\ Control - Absorbance\ of\ Reaction\ Mixture}{Absorbance\ of\ Control} X\ 100$$

4.4. Nematicidal activity

4.4.1. Isolation of *Meloidogyne incognita*

Meloidogyne incognita was isolated from the root of the Banana. Egg masses of *M. incognita* were collected using sterile forceps from the heavily infected roots. The egg masses were washed three times with sterile distilled

water and then placed in Petri dishes containing just enough sterile water to keep the eggs wet. The hatched juveniles were harvested from the Petri dishes every 24 h to be used for inoculation and fresh water was added to the dishes to prevent the eggs from drying.

4.4.2. Nematicidal activity of *Eudistoma viride* against *Meloidogyne incognita*

The nematicidal activity of *Eudistoma viride* against *M. incognita* was carried out by maintaining the nematode numbers as constant, but with various concentrations of bio pesticide. Nematodes were divided into six groups of five animals (n=5) each.

Group I - Normal control without any ascidian extract

Group II - 10 µg/ml of ethanolic extract of *Eudistoma viride*

Group III - 50 µg/ml of ethanolic extract of *Eudistoma viride*

Group IV - 100 µg/ml of ethanolic extract of *Eudistoma viride*

Group V - 250 µg/ml of ethanolic extract of *Eudistoma viride*

Group VI - 500 µg/ml of ethanolic extract of *Eudistoma viride*

One set of six petri plates were arranged on a table. Using a filler, nematodes of *Meloidogyne incognita* were transferred to petri plates. The sets of plates were labelled as a, b, c, d, e, and f. The concentrations 500 µg/ml, 250 µg/ml, 100 µg/ml, 50 µg/ml and 10 µg/ml of ascidian extract were taken in each petri plates respectively. The plate labelled ‘f’ acted as the control without any ascidian extract. The plates with each concentration were maintained at 28^{o} C

for 10 days. The death rate of nematodes readings was noted with 2 days intervals. The experiment was conducted in triplicate and the mean was taken.

RESULTS

5.1. Antiacne activity

In the present investigation, ethanolic extract of *Eudistoma viride* were tested against gram positive, anaerobic bacteria *Propionibacterium acnes*. Antibiotic - Gentamicin was used as positive control. Table: 1; Figure: 1 and Plate: 6 depict the antiacne activity of *Eudistoma viride*. The zone of inhibition noted was 4.5±0.7mm at the highest concentration of 500 µg/ml of ethanolic extract of *Eudistoma viride*. Zone of inhibition was absent in 250, 100 and 50 µg/ml of ethanolic extract of *Eudistoma viride*. In control, the zone of inhibition was 14.5±0.7mm. Statistical analysis of the antimicrobial activities showed that the p value was less than 0.05, which indicates that there was a significant difference in the antimicrobial activity of ethanolic extract of *Eudistoma viride*.

The ethanolic extract of *Eudistoma viride* has significant antibacterial activity against *Propionibacterium acnes*. The efficacy of antibacterial activity was quantitatively evaluated with reference to the MIC as well as IC_{50} values attained in mg/ml by the technique of 96 microtitre well plate method. The MIC and MBC of the extract were evaluated to determine their bacteriostatic and bactericidal properties. Table: 2 and 3; Figure: 2 and 3; and Plate: 7 depict the minimum inhibitory concentration of *Eudistoma viride*.

IC_{50} value of ethanolic extract of *Eudistoma viride* against *Propionibacterium acnes* was found to be 156.5 µg/ml. The MBC value of

ethanolic extract of *Eudistoma viride* against *Propionibacterium acnes* was found to be 62.5 µg/ml - 8.503401.

5.2. Nematicidal activity

In the present investigation, ethanolic extract of *Eudistoma viride* were tested against root-knot nematode *Meloidogyne incognita*. Table: 4 and 5; Figure: 4 and Plate: 8 and 9 depict the nematicidal activity of *Eudistoma viride*. Mortality percentage exhibited was 30, 50 and 70 at the concentrations of 100, 250 and 500 µg/ml ethanolic extract of *Eudistoma viride*. The maximum juvenile morality was recorded as 70% at the highest concentration. The minimum juvenile mortality was noted as 30% in 100 µg/ml concentration. No juvenile mortality was noticed in control, 50 µg/ml and 10 µg/ml of the extract. The juvenile mortality was increased in a dose dependent manner.

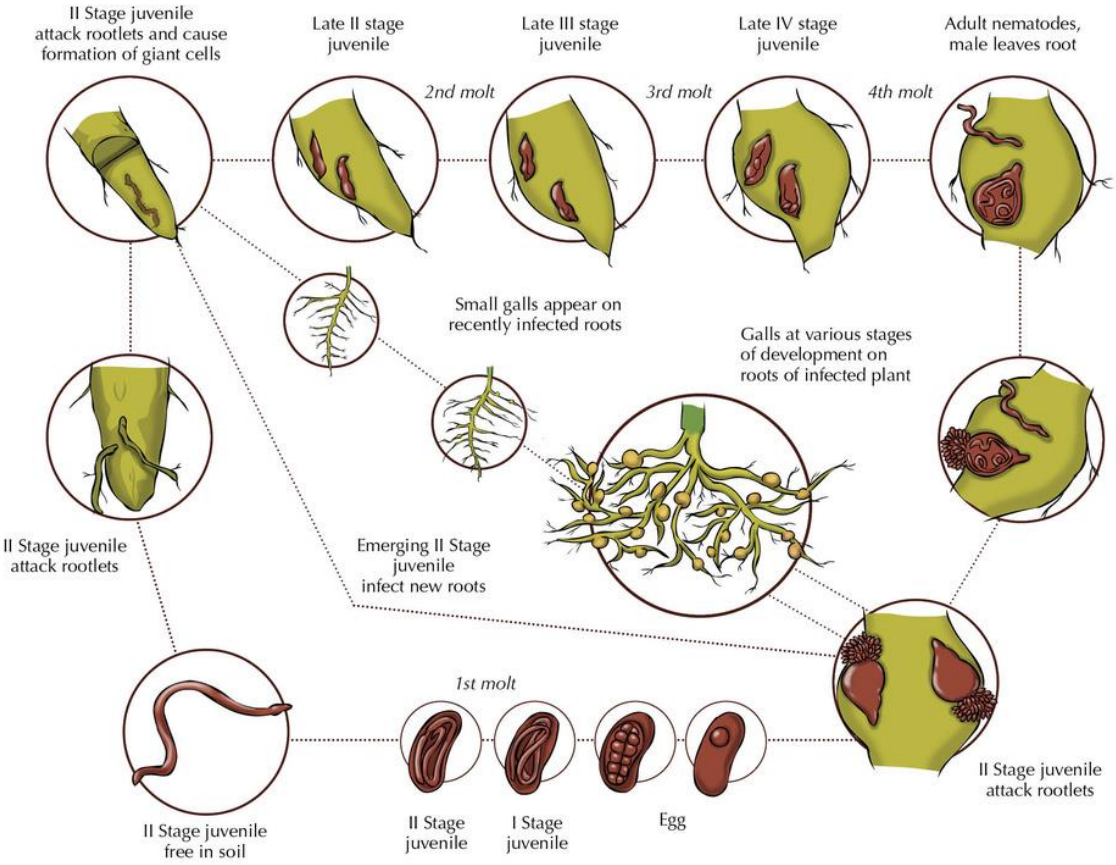


Plate: 1 Life cycle of root knot nematode

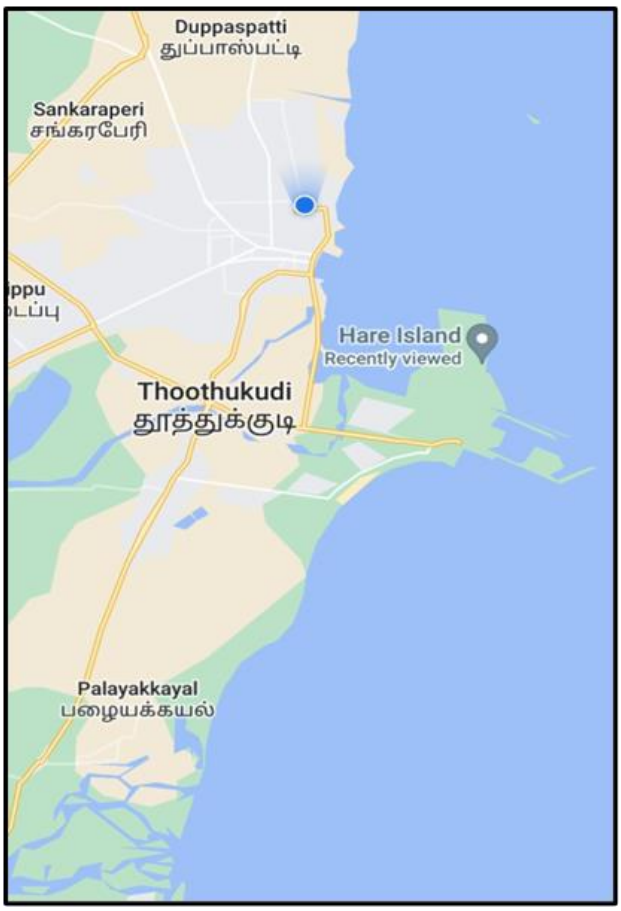


Plate: 2 Study area – Hare Island

Plate: 3 *Eudistoma viride*

Plate: 4 Soxhlet apparatus

Plate: 5 Rotary evaporator

Plate: 6 Antiacne activity of ethanolic extract of *Eudistoma viride* against *Propionibacterium acnes*

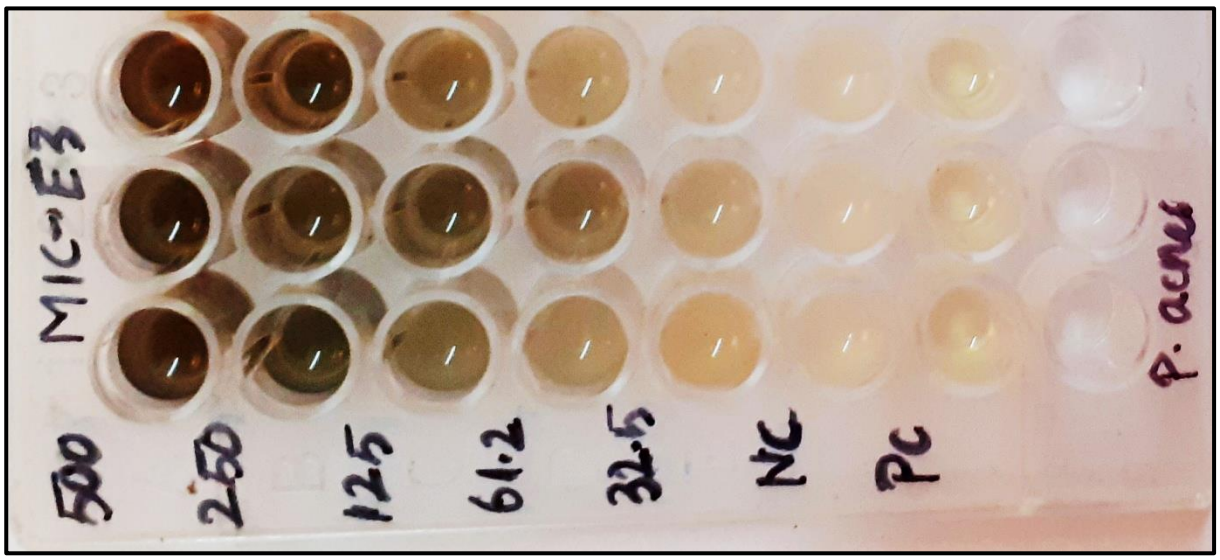


Plate: 7 96 Well Microtiter Plate

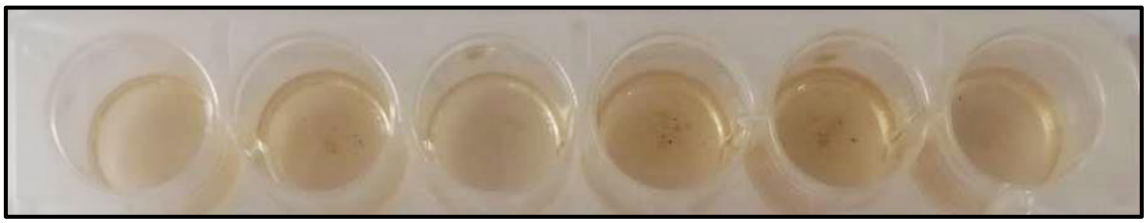
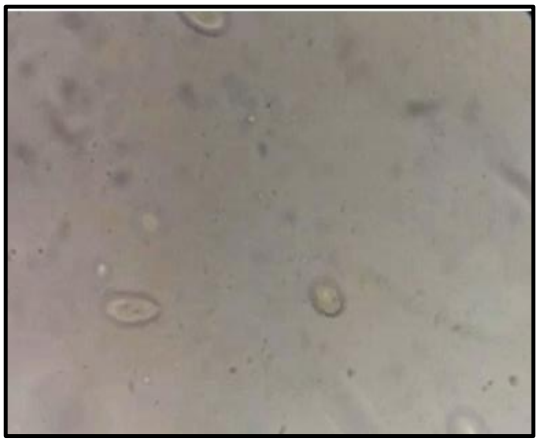
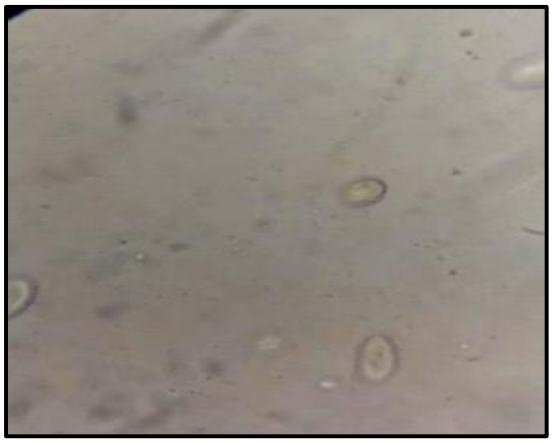


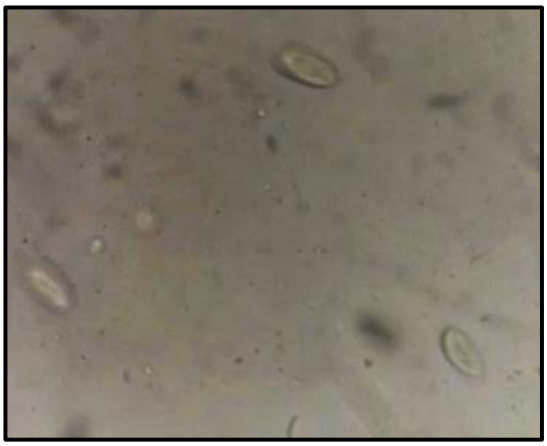
Plate: 8 Nematicidal activity of ethanolic extract of *Eudistoma viride* against *Meloidogyne incognita*



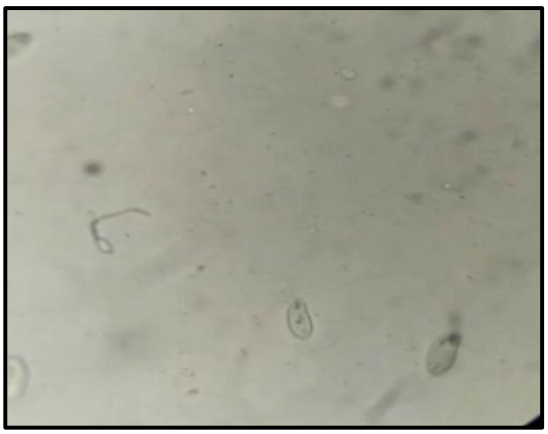
500 µg/ml ethanolic extract of *E. viride*



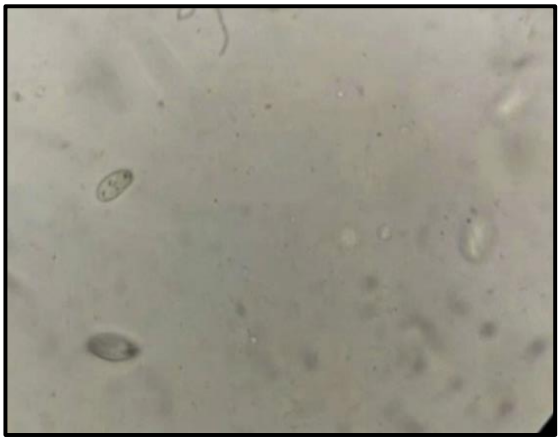
250 µg/ml ethanolic extract of *E. viride*



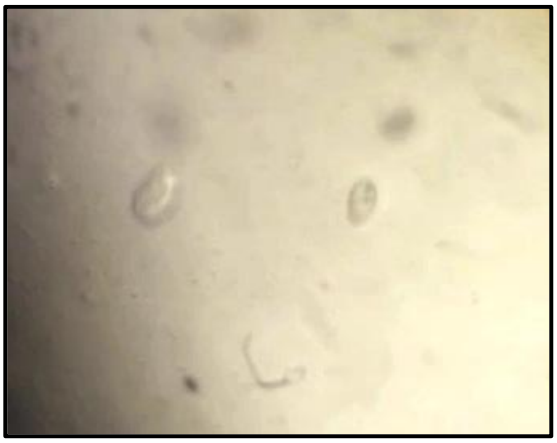
100 µg/ml ethanolic extract of *E. viride*



50 µg/ml ethanolic extract of *E. viride*



10 µg/ml ethanolic extract of *E. viride*



Control

Plate: 9 Nematicidal activity of ethanolic extract of *Eudistoma viride* against *Meloidogyne incognita*

Table: 1 Antiacne activity of ethanolic extract of *Eudistoma viride* against *Propionibacterium acnes*

Zone of inhibition (mm)				
500 µg/ml	**250 µg/ml**	**100 µg/ml**	**50 µg/ml**	**Gentamicin**
4.5±0.7	0	0	0	14.5±0.7

Significance - $p < 0.05$

Table: 2 OD Value at 600 nm

S. No	**Tested sample concentration (µg/ml)**	**OD Value at 600 nm (in triplicates)**			**Mean value**
1.	Control Ciprofloxacin	2.335	2.336	2.386	2.352
2.	Gentamicin	0.526	0.502	0.566	0.531
3.	500 µg/ml	1.489	1.731	1.733	1.651
4.	250 µg/ml	1.765	1.793	1.972	1.843
5.	125 µg/ml	1.999	2.025	2.079	2.034
6.	62.5 µg/ml	2.199	2.079	2.178	2.152
7.	31.25 µg/ml	2.211	2.290	2.287	2.262

Table: 3 Percentage of inhibition

S. No	Tested sample concentration (µg/ml)	Percentage of inhibition (in triplicates)			Mean value (%)
1.	Gentamicin	77.47	78.57	76.28	77.42
2.	500 µg/ml	36.23	25.90	27.37	29.83
3.	250 µg/ml	24.41	23.24	17.35	21.67
4.	125 µg/ml	14.39	13.31	12.87	13.52
5.	62.5 µg/ml	5.82	11.00	8.72	8.51
6.	31.25 µg/ml	5.31	1.97	4.15	3.81

Table: 4 Nematicidal activity of ethanolic extract of *Eudistoma viride* against *Meloidogyne incognita*

Name of the sample	Groups											
	I - Control		II - 10 µg/ml		III - 50 µg/ml		IV - 100 µg/ml		V - 250 µg/ml		VI - 500 µg/ml	
	D	L	D	L	D	L	D	L	D	L	D	L
Eudistoma viride	0	5	0	5	1	4	2	3	3	2	4	1
	0	5	0	5	1	4	1	4	2	3	3	2

D – Dead nematode; L – Live nematode

Table: 5 Mortality percentage of ethanolic extract of *Eudistoma viride* against *Meloidogyne incognita*

Name of the sample	**Groups**					
	I - Control	II - 10 µg/ml	III - 50 µg/ml	IV - 100 µg/ml	V - 250 µg/ml	VI - 500 µg/ml
Eudistoma viride	0	0	0	40	60	80
	0	0	0	20	40	60
Mean value	0	0	0	30	50	70

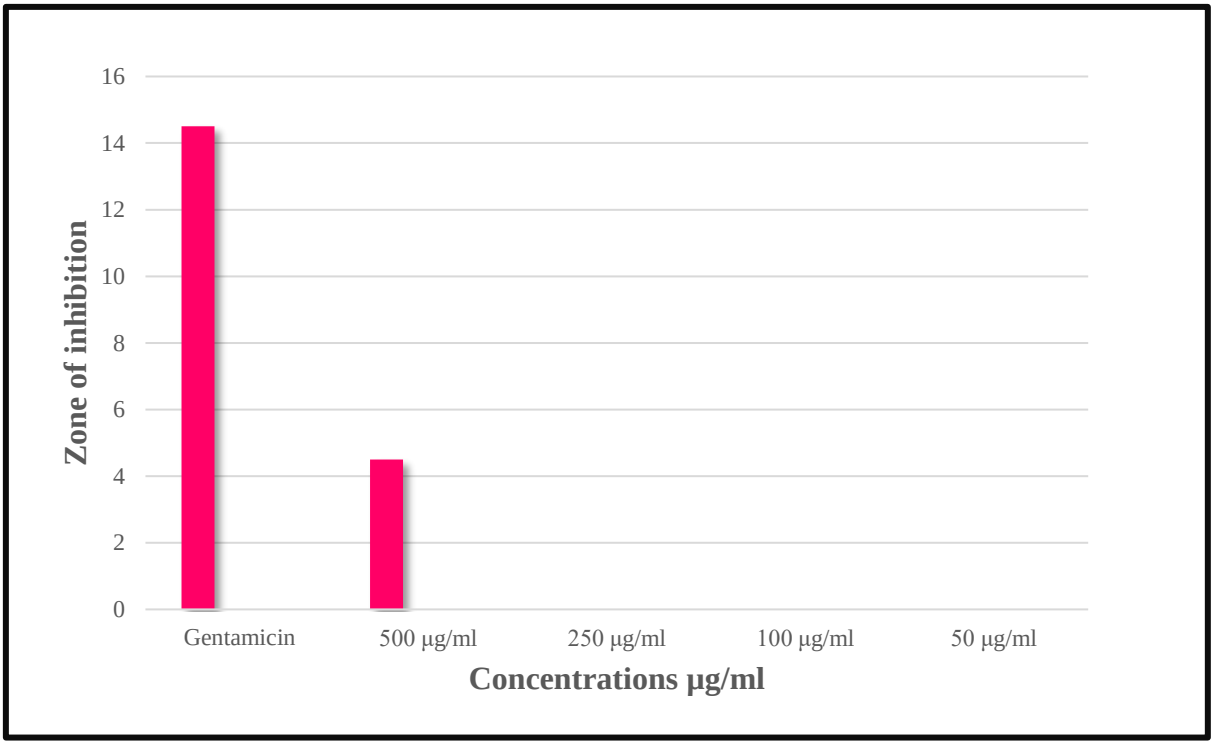


Figure: 1 Antiacne activity of ethanolic extract of *Eudistoma viride* against *Propionibacterium acnes*

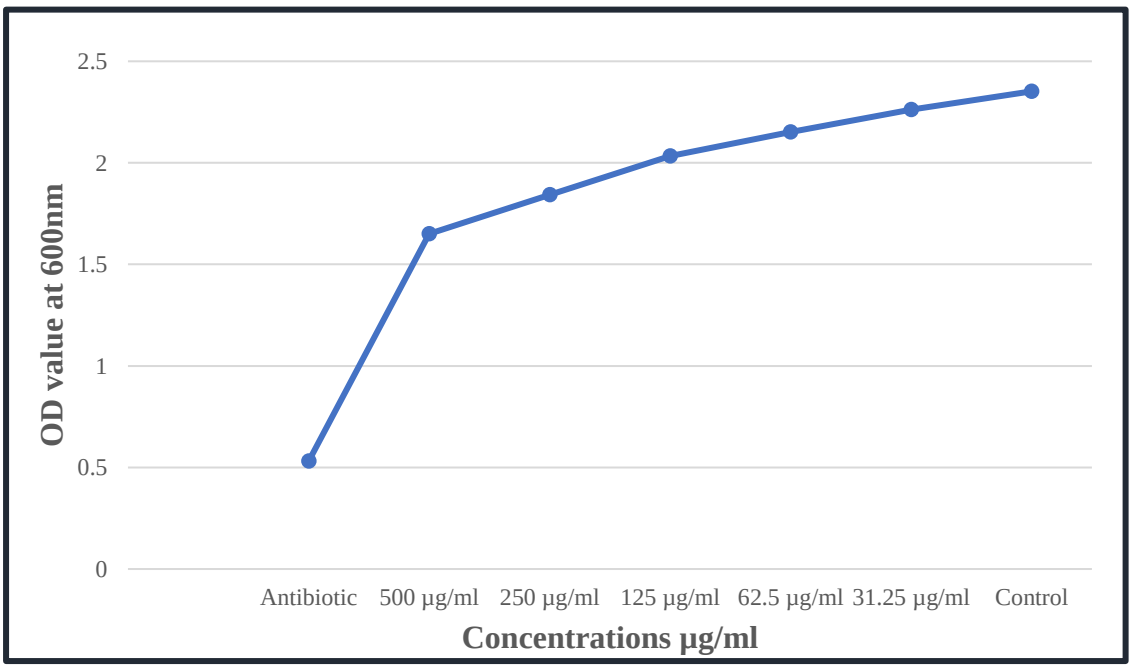


Figure: 2 OD Value at 6oo nm

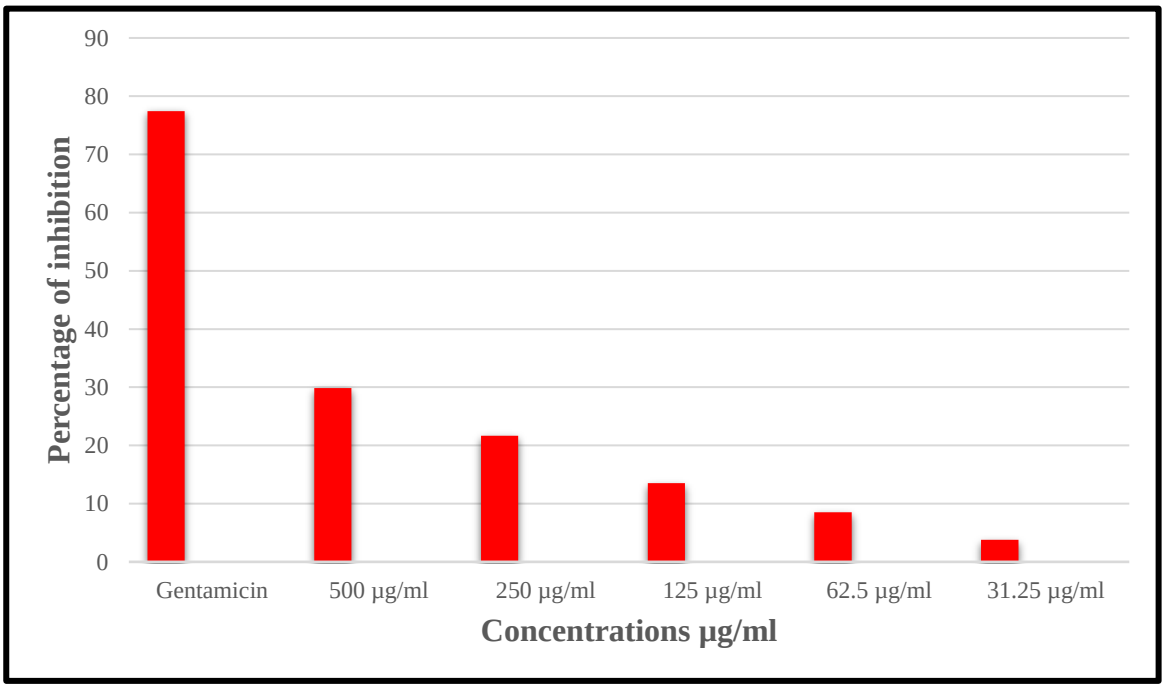


Figure: 3 Percentage of Inhibition

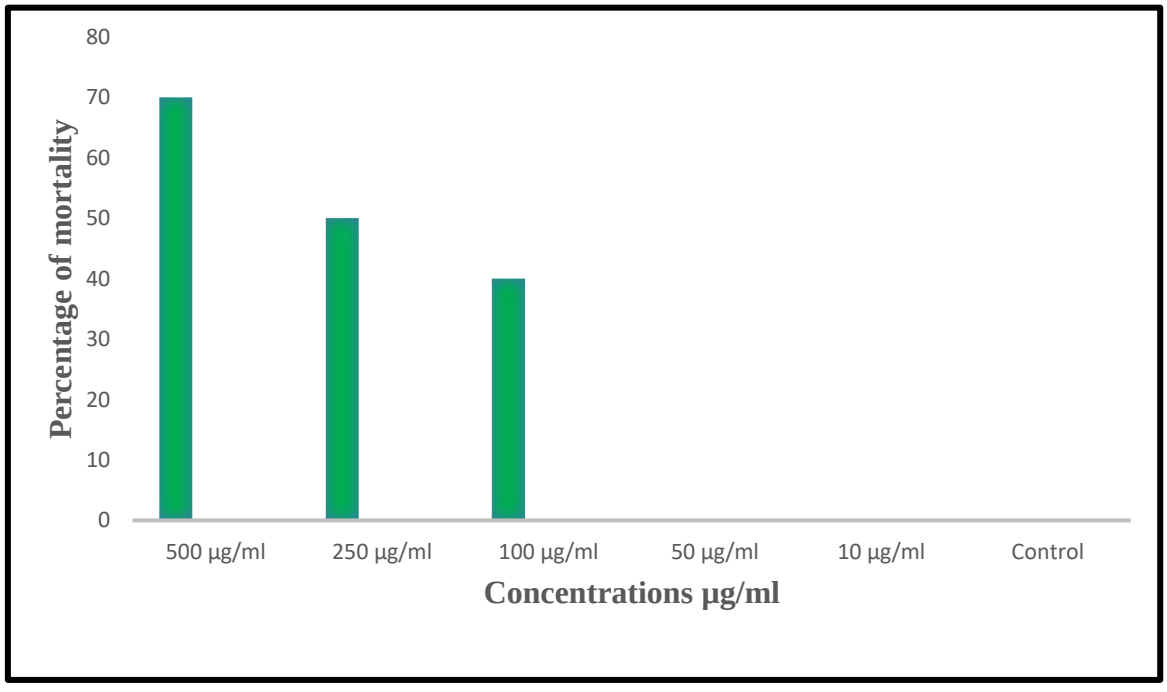


Figure: 4 Mortality percentage of ethanolic extract of *Eudistoma viride* against *Meloidogyne incognita*

DISCUSSION

The ethanolic extract of *Eudistoma viride* showed antiacne effect against *Propionibacterium acnes* with 29.83 as percentage of inhibition. IC_{50} value was found to be 156.5 µg/ml. The total minimum bacterial concentration was found to be 62.5 µg/ml-8.503401. Qidwai *et* al., (2016) reported the oil of *Citrus limetta* (MIC: 2.99, IC_{50}: 1.889) was found to be against *P. acnes* and the antibacterial activity may be due to flavonoids, phenols, tannins and L-ascorbic acid present in the *Citrus limetta*. The *Apis cerana* honey from Banyuwangi has IC_{50}, MIC and MBC values of 59.85 mg/L, 125 mg/mL, and 500 mg/mL respectively against *P. acnes* and it may be due to phenolics and flavonoids present in it (Djakaria *et al*., 2020). The presence of antiacne activity possessed by sea cucumbers may be due to saponin compounds and terpenic compounds in sea cucumbers (Ardiansyah *et al*., 2021). Flavonoids are well known antioxidant, with antibacterial and antimicrobial properties (Qian and Nihorimbere, 2004). Flavonoids are reported to synthesize in plant by the stimulation of microbial infection, as effective antimicrobial substance. Therefore, it is considered as antimicrobial active against wide range of microorganisms, possibly due to its capability of forming complex with bacterial cell walls by interacting with extracellular and soluble proteins. Further microbial membrane may also disrupt by lipophilic flavonoids (Tsuchiya, 1996). Priya *et al*., 2016 stated that the *Eudistoma viride* contains phenols and

flavonoids. The antiacne activity of *Eudistoma viride* may be due to flavonoids and phenols present in it.

The purpose of this research is also to evaluate ascidian as a viable substitute for dangerous and harmful chemical nematicides. In the present study, ethanolic extract of *Eudistoma viride* were tested against root-knot nematode *Meloidogyne incognita*. The maximum juvenile morality was recorded at the highest concentration (500 µg/ml). The juvenile mortality was increased consequently with an increase in the concentration of the extract in a dose dependent manner. In earlier studies, phenolic substances such caffeic acid, benzoic acid and p-cumaric acid caused the death of juvenile *Meloidogyne javanica* (Shaukat and Siddiqui, 2001). Nimin is regarded as a neem-derived chemical that can considerably lower the population of soil and root knot nematode *Meloidogyne incognita* and enhance plant growth (Mojumder *et al*., 2004). Essential oils of *Carum carvi, Foeniculum vulgare, Mentha rotundifolia* and *Mentha spicata* has carvacrol, *t*-anethole, thymol and carvone that immobilized the juveniles of root knot nematode (Oka *et al*., 2007). The nematicidal activity of *Serratia marcescens* was found effective against juvenile stages and it may be due to the presence of prodigiosin (Rahul *et al*., 2014). The nematicidal activity of aqueous leaf extract of *Brugmansia suaveolens* possessed maximum mortality on second stage juveniles of *Meloidogyne incognita* and it may be due to the presence of phytocompounds in leaf (Nandakumar *et al*., 2017). The nematicidal activity of 5-iodoindole was effective to killed juveniles

by inducing multiple vacuole formation and the rapid death was due to the generation of reactive oxygen species (Rajasekharan *et al*., 2019). Priya *et al*., 2016 stated that the *Eudistoma viride* contains phenols and flavonoids. The nematicidal activity of *Eudistoma viride* may be due to phenols present in it.

SUMMARY

7. SUMMARY

- Samples of *Eudistoma viride* Tokioka, 1955 were collected during the low tide from the intertidal rocky area of Hare Island.
- The samples were dried and made into powder. Then the crude extract was prepared using Soxhlet extraction method and the solvent was separated by rotary evaporator.
- The antiacne assay was measured by agar well diffusion method.
- The antiacne activity was assessed by measuring the diameter of the inhibition zone formed around the wells.
- The zone of inhibition noted was 4.5±0.7 mm at the highest concentration of 500 µg/ml of ethanolic extract of *Eudistoma viride*.
- The MIC was measured by broth microdilution assay method.
- The MBC and IC_{50} values were also found.
- The maximum percentage of inhibition was noted as 29.83% at the highest concentration of 500 µg/ml.
- IC_{50} value was found to be 156.5 µg/ml.
- The total MBC was found to be 62.5 µg/ml-8.503401.
- The result from the study showed that the ethanolic extract of *Eudistoma viride* had antiacne activity, hence it can be used in skin-care products for the treatment of acne.

- The ethanolic extract of *Eudistoma viride* were tested against root-knot nematode *Meloidogyne incognita*.
- The nematicidal activity of *Eudistoma viride* against *Meloidogyne incognita* was carried out by maintaining the nematode numbers as constant, but with various concentrations of bio pesticide.
- The maximum juvenile morality was recorded as 70% at the highest concentration of 500µg/ml. The juvenile mortality was increased consequently with an increase in concentrations in a dose dependent manner.
- The ethanolic extract of *Eudistoma viride* exhibited nematicidal activity, hence it can be used as ecofriendly nematicide which will increase the banana yield by controlling the growth of root knot nematode.

CONCLUSION

8. 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 (Martinez, 2021). YONDELIS (trabectedin) derived from Caribbean tunicate *Ecteinascidia turbinata* indicated for the treatment of metastatic liposarcoma (Carter and Keam, 2007). 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 *Eudistoma viride* has confirmed a promising inhibitory effect in acne. The ethanolic extract of *Eudistoma viride* has great potential to be used in skin-care products for the treatment of acne. The ethanolic extract of *Eudistoma viride* exhibited nematicidal activity against root knot nematode *Meloidogyne incognita*. The present study indicated that the ethanolic extract of *Eudistoma viride* can be utilized for biocontrol of root knot nematode and this method of management is cheap, environmentally friendly and free from any hazards.

SUGGESTIONS

- As the extract of *Eudistoma viride* showed antiacne activity, other acne causing bacteria strain such as *Cutibacterium acnes* can also be tried.
- Similarly, antiacne activity of other species of ascidians can also be done.
- Nematicidal activity of other nematodes such as *Heterodera schachtii, Tylenchulus semipenetrans, Meloidogyne hapla etc*, can also be done.
- Similarly, nematicidal activity of other species of ascidians can also be tried.
- A further study on isolation, purification, structure determination and subsequent recognition of the novel mechanism of action of the clinically effective agent is suggested.

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IMPACT OF HONEY SUPPLEMENTATION ON THE LARVAL CHARACTERS AND ECONOMIC PARAMETERS OF THE SILKWORM *BOMBYX MORI L.*

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in partial fulfilment for the award of the degree of

Master of Science in Zoology

by

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THOOTHUKUDI-628 001

April – 2023

CERTIFICATE

This is to certify that this dissertation entitled, **"IMPACT OF HONEY SUPPLEMENTATION ON THE LARVAL CHARACTERS AND ECONOMIC PARAMETERS OF THE SILKWORM *BOMBYX MORI L*"** submitted by **M. PUSHPA JENIFER, Reg. No. 21APZO05** to St. Mary's College (Autonomous), Thoothukudi affiliated to Manonmaniam Sundaranar University, Tirunelveli in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2022-2023 under my guidance and supervision. It is further certified that this dissertation or any part of this has not been submitted elsewhere for any other degree.

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DECLARATION

I do hereby declare that this dissertation entitled, **"IMPACT OF HONEY SUPPLEMENTATION ON THE LARVAL CHARACTERS AND ECONOMIC PARAMETERS OF THE SILKWORM *BOMBYX MORI L"*** submitted by me for the award of the degree of Master of Science in Zoology is the result of my original independent research work carried out under the guidance of **Dr. S. MARY BAPTISTA JANET M.Sc., M.Phil., B.ED., Ph.D.,** Associate Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

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Date: 05.04.2023

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Signature of the Candidate

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CONTENT

INTRODUCTION

Silk worm *Bombyx mori* L the source of fabulous silk has been on the domestication for the past 5000 years . india has a long tradition of producding and using silk ,the queen of fabrics. Sericulture is an art of raising monophagous silkworm, *Bombyx mori* for silk production. Since sericulture is one of the high employment potential land based practice in India, capable of significant contribution towards easing the problems of unemployment and improving the socioeconomic status of the rural families.

Silk worm, *Bombx mori L* is a very important economic insect that contributes to the national economy of India. Sericulture has indeed become business oriented and helps in uplifting millions of small and poor sericulturists and others involved in silk industry. India has unique distinction of being the country producing all the five kind of silk mulberry, eri, muga, tropical tasar. But in Tamil Nadu, mostly mulberry silk in produced. India is the second largest producer of silk in the world with annual production of around 16,500m.

The present global scenario clearly indicates the enormous opportunities for the Indian silk industry. The need of the hour is to produce more bivoltine silk with reduced cost of production to meet the growing demands of quality silk

India has made tremendous progress in the production of mulberry silk for which there is an increasing international demand. In recent years, sericulture has achieved enormous progress in evolving suitable mulberry varieties and techniques. Central silk board and its research and training institution have now made possible big take off towards unprecedented growth in sericulture.

Mulberry silkworm being monophagous solely depends on mulberry leaf for its nutritional requirement for its growth ,development and ultimately high quality cocoon production .Nutrition is the major factor that influences the growth and development of *Bombyx mori* (kanafi *et al.*,2007). The growth and development of larva and subsequent cocoon production are greatly influenced by nutritional quality of mulberry leaves. Silkworm nutritionists have always been searching for better food supplements that can be manipulated to the benefits of the rearers (Nair and kumar 2004). A series of studies has been established the better food supplementing the mulberry leaves with different nutrients such as protein, vitamins , amino acid , mineral , hormones and antibiotics etc for better performance and to get high yield with quality cocoons.(Kanafi *et al.*,2007 Sheeba *et al.*, 2006 venkataramana p and Srivastava Rao 2003)

The improvement in the quality of raw silk mainly depends upon the quality of mulberry leaves and favorable environmental factor. The influence of mulberry varieties on silk yield has been investigated by Nataraju *et al*., (1980) and Venugopalapillai *et al., (*1980). Technical improvement in the conditions has also been attempted with the view to increase the silk yield by giving optimum environmental conditions for the silkworm. (Venugopalapillai and Krishnawami,1978).

Enriching the silkworm diet (i.e mulberry leaves) with exogenous nutrients such as protein , carbohydrates , amino acid , vitamins, minerals ,hormones , antibiotics and assessing their impact on larval growth, metabolism and silk production has become the order of traditional research in sericulture (chakrabarty and kaliwal , 2011)

Larva of *Bombyx mori L* were reared on various kinds of dietary(protein soybean, mushroom , corn flour and mixture of them)using semi artificial diet .larvae fed on semi -artificial diet containing corn flour throughout the 5^{th} instar larvae gave the highest records on the larval duration ,weight of larvae, silk gland , pupa , cocoon and cocoon shell , as well number of deposited eggs. The same diet gave the lowest mortality percentages.(mona Mahmoud,2013)

Shayamala and gowda, 1981 have shown that supplementation of phenyl alanine to Mysore – 5 and local varieties increase and weight and shell radio of *Bombyx mori*. Supplementation of glycine, alanine, serine (1:1:1) to MR2 variety of mulberry on the commercial and biochemical character of Bombyx mori have shown that these amino acids are very useful to increase the silk yield (Saminathan 1985). This amino acid is the major compound of fibroin and sericin.

Mulberry leaves supplementation with spirulina, as a feed to *Bombyx mori*, orally found to be effective in enhancing the larvae and cocoon character. (venkataramana, 2003). Various researches have been carried out on the dietary supplementation included vitamin such as ascorbic acid, thiamine, and niacin, folic acid and multivitamins (Etebari *et a*l., 2005 and Hiware,2006). For the enhancement of silk production various methods were tired and among them supplementation of vitamins C gives wide range of scope for improving the economical parameters of the silkworm

Silkworm nutritionists have always been searching for better food supplements that can be manipulated to the benefits of the rearers (Nair and Kumar,2004). A series of studies has been established the better food supplementing the mulberry leaves with different nutrients such as protein, vitamins, amino acid, mineral, and antibiotics etc for better performance and to get high yield with quality

cocoons.(Kanafi *et al*.,2007 , Sheeba *et al.*,2006,Venkatesh and Srivastava 2003) or using extracts of plants

In recent years, many attempts have made to improve the quantity and quality of silk (Hiware,2006), through enhancing leaves with nutrients. Spraying with antibiotic, JH-mimic principles or using extracts of plants Recently, much research has been done on the diet supplementation of mulberry leaves fed to silk worm. This supplementation includes vitamins such as ascorbic acid, thiamin, niacin, folic acid multivitamins and silver nano particles (Etebari *et al*., 2004; Ganesh prabhu *et al*; 2012).

Nutritional study of silkworm is an essential perquisite for its proper commercial exploitation. Nutrition of silkworm is sole factor which almost individually augment quality of silk(Lakskar and Datta,2000.)

On earth, plants are the richest source of organic chemicals, Enrichment of mulberry leaves with phytochemical supplementation enhanced the silk productivity .Extracts of various medicinal plant such as Aloe vera, Moringa oleifera, ocimum sanctum etc have been elicited various response on silk worm and were shown to have influence on economical characters such as body weight, cocoon weight, shell weight, shell radio and thread length in *Bombyx mori.* (Prabhakar Reddy, and Mohana Lakshmi,2014)

The present study is an attempt to evaluate the effect of supplementation of honey on the biological and economic performance of the mulberry silkworm *bombyx mori* .Honey has been considered one of the most illustrious and most valued natural products. It exhibits great impact on human nutrition and health. It has been used since ancient times in various forms to sweeten and flavor different types of foods.(Al-Ghamdi, A.A.; Ansari, *et al*.,2021) M.J. Biological and Therapeutic Roles of Saudi Arabian Honey.

Furthermore, honey has been used for therapeutic purposes in order to treat or to prevent several clinical conditions, such as dermatitis, diabetes or cancer. It possesses a wide range of curative properties due to the presence of more than 200 bioactive compounds. By using flower nectar, honeybees perform considerable work in order to obtain this appreciable natural product. The nectar source dictates the physical properties of honey, such as flavor, odor, and color. Furthermore, the geographical origins also exhibit an impact on honey's features. It has also been shown that the floral source and the geographical origin regulate the most important therapeutic effect of honey, specifically the antimicrobial activity. Moreover, honey represents an important alternative agent against antibiotic resistant bacteria. Hydrogen peroxide is the main compound that is responsible for the antibacterial activity. (Wang, X. Peng, X. Liu S. et al., 2019) Genetic Manipulation of MicroRNAs in the Silk Gland of Silkworm.

It is a well-known fact that Bombyx mori's biological parameters are determined by the nutritional value of the mulberry leaves. It has been shown that the use of honey increases the nutritional value of mulberry leaves, and therefore, honey exhibits a great advantage for the economic features of Bombyx mori. Furthermore, honey plays a key role in the biomaterials field. It is currently used as an additive in silk-fibroin -based biomaterials due to its hygroscopic and antibacterial properties. There have been described a wide range of biomaterials that consist of silk fibroin and honey.(Chen, S. Liu, M. Huang, et al.,2019 Mechanical Properties of Bombyx Mori Silkworm Silk Fiber and Its Corresponding Silk Fibroin Filament)

This study highlights the effect of honey on the growth, food consumption, weight of silk gland, cocoon characters and silk quality of the silk worm *Bombyx mori*.

OBJECTIVES

The present study is under taken to analyse the effect of supplementation of honey on the following factor of the silk worm *Bombyx mori*

- Larval growth
- Food consumption
- weight of silk gland
- Cocoon characters
- Silk quality

REVIEW OF LITERATURE

A survey of literature adds knowledge regarding the enormous amount of research works that have been carried out on food supplementation in *bombyx mori*. Studies on the nutritional parameter of sericigenous insect is considered to be an important field of work for better management and development of the sericulture industry apart from its physiological importance . As a phytophagous insect , *Bombyx mori* depends exclusively on mulberry for its growth and survival. Perusal of literature showed that supplemented nutrients have much effect in increasing the cocoon quality which in turn depends on the utilization of food by the silk worm larvae.

Regarding the supplementation and assessment of feeding capacity , the only available reference is the observation of (Mathavan *et al*., 1984) on food utilization single cell protein used by him as a supplementary diet exhibited an enhanced consumption in *Bombyx mori* larvae.

Larval nutrition is of great importance, which influences growth , development and silk gland function in *Bombyx mori* (Ito 1980; Akkai,1982).Segupta *et al*.,(1982) Showed that *Bombyx mori* requires specific essential sugar , amino acid , protein and vitamins for its normal growth , survival

and also for the gland development . Akhtal Asghal (1972) found that vitamins and mineral salts played an important role in the nutrition of silk worm . The effect of vitamin supplementation on the growth of *Bombyx mori* have been investigated by many researches (Etebari *et al.,* 2004 ;Rajabi *et al.,)*

Zah *et al*.,(2011) reported that linseed oil and hemp oil influence the larval cocoon parameters of *bombyx mori* . Aloe vera acts as a physiological carrier for many active biological agents .

Venkataramana (2003) supplemented spirulina , a blue green algae which contains 18 amino acid ,Viz ., glutamine , glycine , histidine, proline, tryptophan, asparagines , pyruvic acid , and vital vitamins as a feed to *Bombyx mori* orally and found be effective in enhancing the larval and cocoon characters.

Improvement in larval character of *Bombyx mori* is quite essential as they are directly related to economic characters and reproductive capacity of adult .Increased growth rate and the larval duration was observed by Bhasker *et al.,(*1983), when prolactin and single cell protein were supplementing . The effect of supplementing the amino acid , glycine , on the larval characters was also studied by Sridhar and Radha (1987) in different larval stages of *Bombyx mori*

Saha and khan (1996) described the extensive effects of multivitamins incercompounds as diet factors on growth , interruption and the decrease of

commercial characteristics of cocoon. It is showed that multivitamin and mineral compounds could increase the food intake , growth and conversion efficiency of silk worm (Muniandy *et al* .,2001)

Mazo (2004) reported that spirulina contains high level of antioxidants such as vitamin B1 and B2 , carotenoids and phycobilins. The efficacy of spirulina supplementation to silkworm was reported by venkataramana (2003) . Venkatesh kumar (2009) found significant improvement in all the qualitative cocoon characters except in silk percentage.

Jang, (1986) has reported that the elimination of niacin (nicotinic acid) from the diet of Ceratitis capitata caused the increased morality of larvae and decrease in the proportion of pupal to adult emergence.

Chang and Li(2004) reported that nutritional interaction exist between vitamin B3 and other groups of vitamin B. various extracts of medicinal plants have been tested by supplementation in the silkworm in the silkworm Bombyx mori and were seen to influence the body weight , silk weight , silk gland weight , silk gland weight and the silk gland weight and the silk thread length in Bombyx mori

Studies of Rajabi *et al*., (2007) have determined that generally vitamins present in the mulberry leaves satisfy minimum needs of silkworm but the amount of vitamin present in mulberry leaves varies on the basis of environmental condition, usage of

fertilizers in field and mulberry varieties and other field practices. Sengupta *et al.*, (1972) have showed that *Bombyx mori* requires specific essential sugar, amino acids, proteins and vitamins for its normal growth, survival and also for the growth of silk gland. Akhtar and Asghar *et al.*, (1972) have found that vitamins and mineral salts played an important role in the nutrition of silk worm.

Karaksy,(1990) showed that ascorbic acid increase silk yield of the mulberry silk worm. Mulberry leaf is a rich source of ascorbic acid and contains about 1.8mg. Per grams of leaves (Legay, 1958)

Ito (1961) has recorded the relationship of ascorbic acid supplementation and growth of silk worm. The absence of ascorbic acid in the diet of first and second instar larvae postponed the growth and development of silk worm. There is enough vitamin C in mulberry leaves and ascorbic acid content of growing larvae is dependent on amount of this vitamin in diet. In supplementation of mulberry leaves more than any other vitamin ascorbic acid has been used (Etebari *et al.*, 2004)

Several research demonstrated phagostimulatory effect of ascorbic acid in insects. (Ito, 1978; Balasundaram *et al.*, 2008)

Sengupta et al., (1972) have reported that silk production increased with 1% ascorbic acid in the diet of silk worm. Etabari *et al.*, (2005) have demonstrated that

feeding on mulberry leaves enriched with ascorbic acid at 3% concentration decreased larval weight due to hypervitaminosis.

Etabari *et al.,* (2004) have reported the yield decrease, when ascorbic acid concentration is enhanced in silk worm diet. Saha and khan, (1996) have reported the same effects from multi-vitamins. Vitamins B3 (niacin) comes in two forms, nicotinic acid and nicotinamide.

The healthy growth of silkworm and ultimately the economic traits such as larval characters cocoon and grainage parameters are influenced largely by the nutritional status of the leaves fed to the worm (Krishnaswami *et al.*, 1971).

Nutritional background of the larval stage is significant in influencing the status of the resulting larva, pupae, adult and silk fiber (Fukuda *et al*., 1963; Takno and Arai, 1978; Aftab Ahmed *et al.*, 1999 Rahmathulla *et al.*, 2006).

Supplementation of vitamin c to mulberry leaves improved economic trait in the silk worm as studied by Babu *et al*., 1992, Chauhan and singh, 1992 and Prassad, 2004.

Venkataramana *et al* (2003)., has found out the effect of spirulina on the larval and cocoon characters of silk worm , *bombyx mori* .

Ascorbic acid has many important functions in the animal body. It is a powerful antioxidant, protecting against oxidative damage to DNA, membrane lipids and

proteins. Antioxidant activity of ascorbic acid decrease reactive oxygen and oxidative pressure and as a result, the absorption of nutritious substance in the miggut would increase (Felton and summers, 1993)

Balasundaram *et al*.,(2008) demonstrated the phagostimultatory effect of ascorbic acid in insect. Rouhollah (2010) reported the effect of artificial diet on the growth and development of silkworm.

Naga Jothi *et al*., (2010) have analysed the effect of ultrasound on biochemical parameters of protein metabolism in the silk gland of silkworm, *bombyx mori L.*

Ganesh prabu *et al.*, (2011) have studied the comparative feed efficiency *bombyx mori L,* fed with silver nanoparticles and spirulina treated MR2 mulberry leaves in relation to growth and development.

Mahmound (2013) has tested the impact of dietary protein viz., soyabean, black gram, mushroom and mixture of them on the weight of larvae, silk gland, pupae, cocoon, cocoon shell and larval duration.

AparupaBorgohain (2015) has studied the effect of nutritional supplementation of mulberry silkworm *bombyx mori L*.

Balasundaram *et al.*, (2013) showed the Studies on the Nutritional Supplementation of Vitamin C Treated MR2 Mulberry Leaves Fed by V Instar Larvae of Silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae) in Relation to

Feed Efficacy and Growth Rate and feed efficacy and growth rate of silkworm larvae (V instar), enhanced by 0.2% Vitamin C treated group than control and other Vitamin C treated groups (0.1%, 0.4% and 0.8%). This study has been indicated that the Vitamin C exhibits the presence of certain growth stimulant activity and can be used to increase the feed efficacy in commercial silkworm

s. karnjanaprapratum et al ., (2022) The observed variability of the composition data reported could be due to differences in diet, growth ,strains, pretreatments, and origin of the silkworm analyzed. However, all these variables were not always available and should be reported in future studies to simplify the data comparison.

Gm Baci *et al.*, (2021) analysed Applicability of Honey on Silkworms *(Bombyx mori)* and Quality Improvement of Its Biomaterials by discussing the applicability of honey on *Bombyx mori* and beyond, the importance of honey for life sciences and related fields is spotlighted.

Y .Hori *et al*., estimated Nutrition of the silkworm, *Bombyx mori*—X. Vitamin requirements and the effects of several analogues No competitive effect of this compound was observed, when 1·5 mg were added together with 0·15 mg of the vitamin

Luca Tassoni *et al.,(* 2022) Nutritional Composition of *Bombyx mori* Pupae have determined that The observed variability of the composition data reported could be

due to differences in diet, strains, pretreatments, and origin of the silkworm analyzed. However, all these variables were not always available and should be reported in future studies to simplify the data comparison. Elizabeth *et al*., (2021)

Ramesh *et al* .,(2018) demonstrated the Nutritional supplementation of amino acid L-Serine on the silkworm *bombyx mori L* larvae in relation to growth rate and silk production .

Baci et al., (2021) analysed the applicability of honey on silkworm (*Bombyx mori*) and quality improvement of its biomaterials.

Elizabeth *et al* ., (2021) studied the impact of amino acid supplementation on the growth and yield of silkworm , *Bombyx mori. L* and reported its positive effect.

Neha pande and sharma (2023) have investigated the impact of spirulina supplemented mulberry leaves on the cocoon parameters of silkworm *bombyx mori*

EXPERIMENTAL ANIMAL

BOMBYX MORI

SYSTEMATIC POSITION

KINGDOM : Animalia

PHYLUM : Arthropods

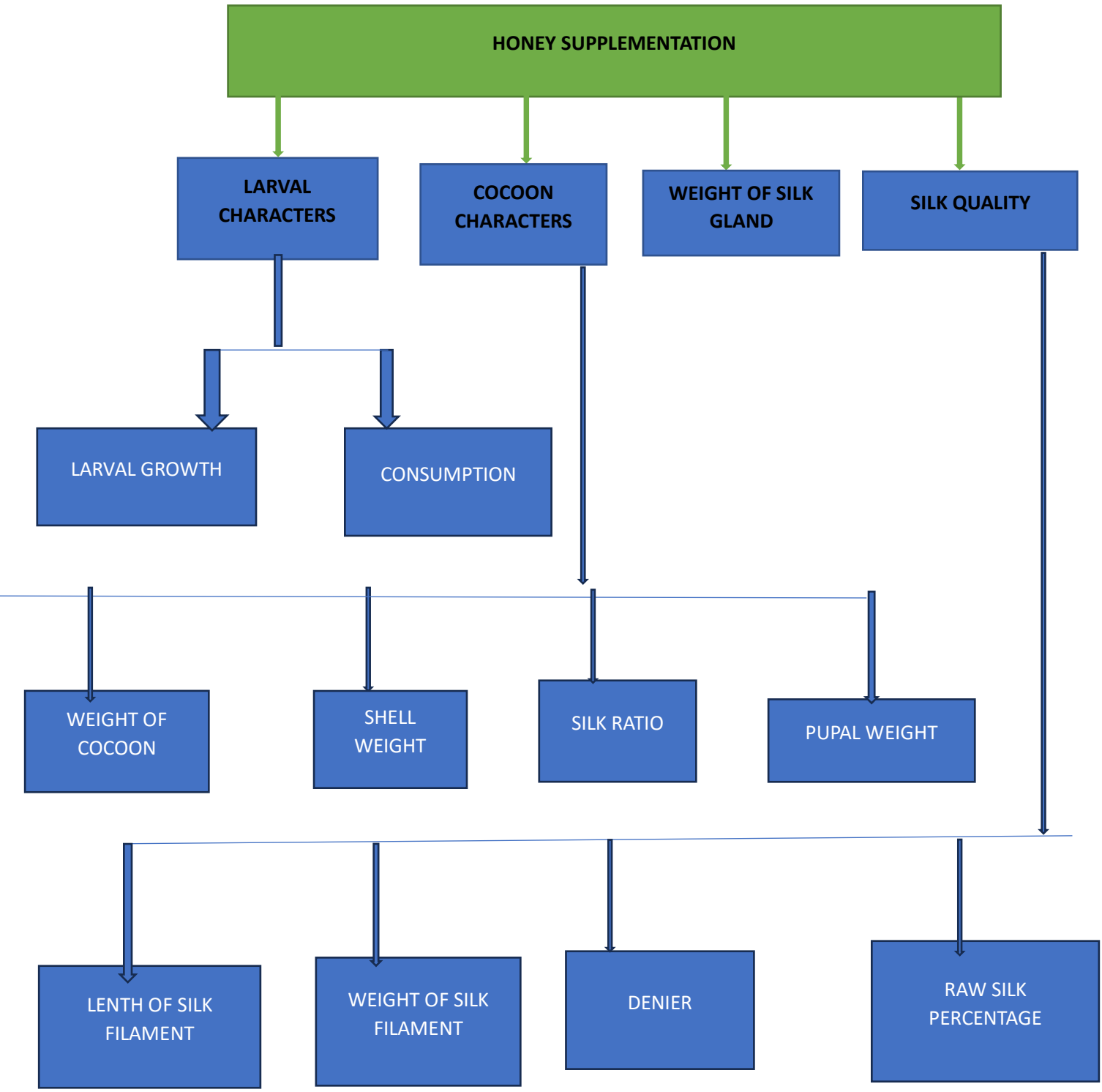
CLASS : Insecta

ORDER : lepidoptera

FAMILY : Bombycidae

GENUS : Bombyx

SPECIES : mori


HONEY SUPPLEMENTATION
LARVAL CHARACTERS
COCOON CHARACTERS
WEIGHT OF SILK GLAND
SILK QUALITY
LARVAL GROWTH
CONSUMPTION
WEIGHT OF COCOON
SHELL WEIGHT
SILK RATIO
PUPAL WEIGHT
LENTH OF SILK FILAMENT
WEIGHT OF SILK FILAMENT
DENIER
RAW SILK PERCENTAGE

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The experimental animal selected for the present study is the mulberry silkworm , *bombyx mori* L. belonging to the order *lepidoptera*, family *Bombycidae*, of the class Insecta. The eggs of the silkworm race L **x** NB4D2 were obtained from sericulture extension and training center V.M. chatram, Tirunelveli. It is a hybrid variety between the mysore local variety and a Japanese bivoltine variety, introduced by center sericulture research and training (CSRTI),Mysore.

DISINFECTION

All the rearing equipments rearing chamber and rearing room were sprayed with 2% formalin solution two days prior to rearing . mild detol solution was used to wash hand before and after handling the worm during rearing.

REARING

The larvae were reared inside the rearing chamber (60 x 89 x 6.5 cm)

By box rearing method. The larvae were reared in wooden tray (29 x 24 x 6.6 cm) with 35 larvae in each tray . The trays were placed in such position to get sufficient

ventilation get sufficient ventilation. The test individual were reared at constant laboratory condition of 30±1 c, 70±10 hr and (12:12) (L:D) of photoregime .

FEEDING:

Fresh mulberry leaves were used in the present investigation . they were collected during cool hours of the day and stored in wet gunny bags. They were chopped to the required size prior to feeding. The larva were fed five times a day .that is around 6 am , 10 pm, 2 pm , 6 pm , and 9 pm respectively. The beds were cleaned every day prior to feeding at 6 am.

ACCULIMATIZATION:

The larvae were allowed to grow up to 111 instar by feeding them with untreated mulberry leaves following the rearing method of Krishnaswami (1978). The freshly moulted 1v instar larvae were used for the present investigation.

EXPERIMENTAL REARING:

About 175 freshly moulted 1v instar larvae obtained from the same moth were selected and used for the experiment . they were reared in the experimental tray in 5 sets , 4 for experimental purpose and one as control each containing 35 larvae selected for the tender coconut water were chopped and provided to each of the respective set of larvae in required quantities . the leaves provided to the larvae in required quantities . the leaves provided to the larvae each time for feeding were

weighed accurately (20mgs) and the quantity of leaf give to set were noted down. The larvae were fed on uniform quantities each time . In all set the left out leaves and the fecal pellets were collected separately from each trial at the time , of cleaning the bed each day . the initial weight of the larvae was measured. All weightings were was made in digital balance . the procedure was followed till the larvae reached the spinning stage . the matured larvae were isolated and mounted on separated chandrike. Few cocoons from each treatment were allowed to hatch out. When the moth emerges out they were allowed to mates and introduced in the black box and allowed to lay the eggs.

GROWTH:

Growth in weight was estimated by subtracting the initial weight of the larva from the final weight .

Growth = Final weight of the larvae – Initial Weight of the larvae

CONSUMPTION :

Standard gravimetric method (Waldbares,1968) was used to estimate the food consumption.

Consumption = Amount of food provided – Amount of food left out

RATE OF CONSUMPTION :

While waldbanes 's (1968) consumption index (C1) related to the feed consumption with mean body weight of the larvae per instar , the term feeding rate refers to the amount of food consumption per unit live body weight of larvae per unit time (mg/live body weight / day).

$$\text{Feeding rate} = \frac{\text{Total food consumed}}{\text{Mid body weight x instar duration}} \times 1000$$

$$\text{Growth index} = \frac{\text{Final weight of the larvae (g) -Initial weight of the larvae(g)}}{\text{Initial weight of the larvae (g)}}$$

ESTIMATION OF SILK PROTEINS IN SILK GLAND

The silk gland was dissected from the larvae (v instar) of maximum maturity (7 days) and washed with 0.9% NACL. The intraglandular fibroin was extracted separated from the middle and posterior part of the silk gland according to the procedure of (Tashiro et al., 1968). After complete extraction of fibroin the extracts from both part of the silk were pooled and weighed.

ANALYSIS OF COCOON CHARACTERS

15 cocoons from each group were taken after harvesting from the silk worm treated with different concentration of tender coconut water for analysis of cocoon characters. Important character like cocoon weight, shell weight, silk

ratio, filament length, weight of reeled silk, denier, cocoon percentage, raw silk percentage , cocoon shell weight, pupae weight, fecundity ERR by number and ERR weight were determined by standard procedures (sywalker, 1992).

SILK RATIO

The ratio between shell weight and cocoon weight is considered as cocoon-shell ratio. The ratio for randomly selected 10 cocoons was calculated by the formula.

$$\text{Silk ratio} = \frac{\text{weight of the cocoon shell (g)}}{\text{Weight of the green cocoon (g)}} \times 100$$

COCOON PERCENTAGE

The number of cocoon formed against available matured larvae is indicated as cocoon percentage and it is calculated by the following formula

$$\text{Cocoon percentage} = \frac{\text{Total number of cocoon}}{\text{Total number of mature larvae}} \times 100$$

COCOON WEIGHT

Fifteen cocoons were randomly taken out from the chandrike (mountages) of different treatment and weighed accurately in an electronic balance. The mean weight of the cocoon were found out and expressed in gram.

COCOON SHELL WEIGHT

Randomly selected ten cocoons from different concentration were cut opened with the help of a sharp stainless steel blade and the shell weight was taken accurately after removing the pupae from them. The mean weight of the shell was calculated and expressed in gram.

PUPAL WEIGHT

Randomly selected 10 pupae were taken out by cutting the cocoon with a new stainless steel blade and the pupae were separated and weighed accurately in an electronic balance. The mean weight of the pupae was calculated and expressed in gram.

ECONOMIC CHARACTERS

EFFECTIVE REARING RATE (ERR)

One of the most important economic parameter in silk worm rearing is the effective rate of rearing (ERR) which indicate the yield per 10,000 silk worm larvae brushed, by both number and weight. The parameter denotes the viability of the larvae and is calculated after harvesting and counting of cocoon in different experimental set up. It is calculated by the following formula.

ERR by number = $\frac{\text{Total number of good cocoon harvested}}{} \times 10000$

Total number of larvae retained after 4^{th} moult

ERR by weight = weight of good cocoon harvested in kg x 10000

Total number of larvae retained 4^{th} moult

TOTAL FILAMENT LENGTH

The total length of the reelable silk bave in the cocoon is considered as filament length and is expressed in meter.

FILAMENT WEIGHT

The weight of the silk thread reeled from a single cocoon is considered as filament weight and expressed as gram/cocoon.

DENIER

Denier is the wt in gram of 9000 m of yarn per filament, which is indicative of the quality of the silk. It is computed by the following formula

DENIER = weight of the filament in gram x 9000

Length of the filament in meter

RAW SILK PERCENTAGE

The total silk extracted from a single cocoon is called raw silk. Its percentage is computed as follows

Raw silk percentage = $\frac{\text{silk weight}}{\text{Green cocoon weight}} \times 100$

Statistical analysis

The statistical analysis of the data was preformed as per the method described by snedcor and Cochran (1967)

1.Mean

The average ($\bar{x}$) is calculated as follows

$$\bar{x} = (\Sigma x) / n$$

where

x = data obtained

$\sum \times$= sum of value of sample

n = total number of samples

2. Standard deviation(SD)

$$SD = \sqrt{(\sum \mathbf{d^2} / \mathbf{n - 1}}$$

d = deviation of each score from mean

n = total number of sam

PLATE : 1 EXPERIMENTAL SETUP

RESULTS

The present study on honey supplementation indicated and enhanced effect on biological economic characters of silkworm *Bombyx mori* in higher concentration .

GROWTH OF IV INSTAR LARVAE

Honey supplementation had sightly elevated the growth of IV instar larvae only in higher concentration (75%) .The growth in weight of the larvae increased from 9.5 gm growth in weight of the larvae increased from to 11.7 gm and 12.7 gm for 10 larvae respectively in 25%, 50% and 75% growth in control was 12.2 gm /10 larvae.(Table I,Fig 2).

GROWTH OF V INSTAR LARVAE

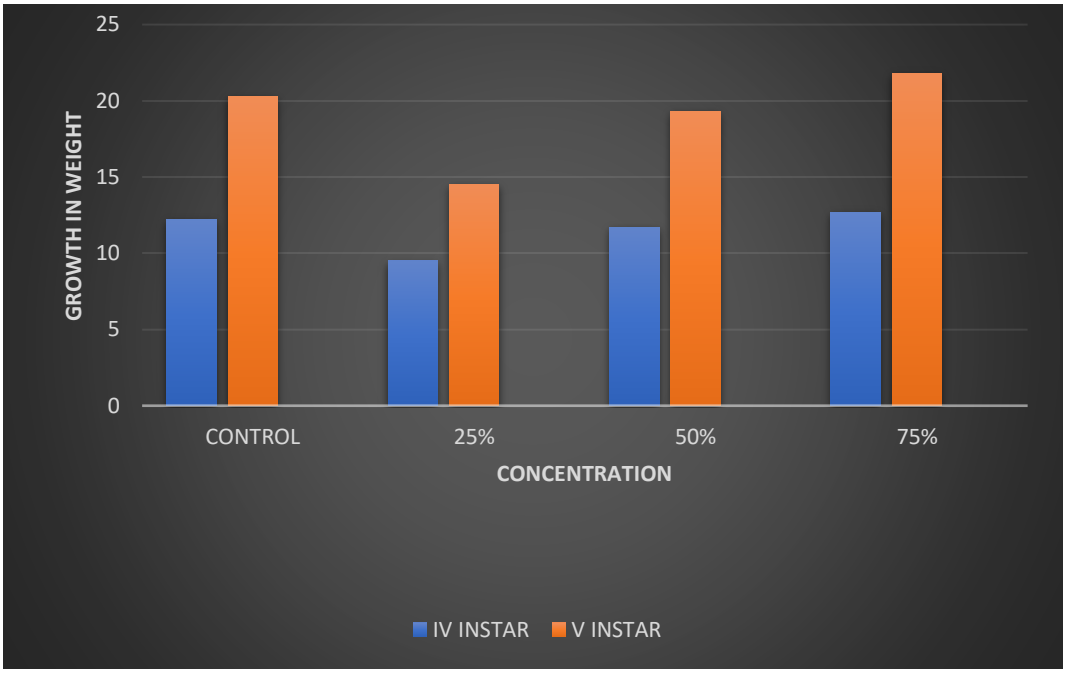
Honey supplementation was observed to have a positive effect on the growth of V instar larvae in higher concentration but it was low in lower concentration of honey and had a repative effect . The growth in weight of V instar larvae was 20.3 gm .but it was reduced to 14.5 gm in 25 % 19.3 gm in 50% and elevated to 21.8 gm in 75%concentration (Table I,Fig 2).

Table I : Growth and growth index of IV and V instar larvae of Bombyx mori fed on honey supplemented mulberry leaves

(values are expressed in gram /10 larvae)

INSTAR	CONCENTRATION	GROWTH IN WEIGHT (gm)	GROWTH INDEX
IV	Control	12.2	0.54
	25	9.5	0.20
	50	11.7	0.48
	75	12.7	0.60
V	Control	20.3	0.66
	25	14.5	0.52
	50	19.3	0.64
	75	21.8	0.71

Fig 1:Growth of IV and V instar larvae of *Bombyx mori* fed on honey supplementation



FOOD CONSUMPTION

Study of food consumption in honey supplemented *Bombyx mori* during IV instar showed that honey has a negative impact on the food consumption in lower concentration . The amount of food consumed was 96.6 gm in control. But in 25% honey supplemented larvae it was reduced to 82:2 gram and 90.8 gram in 50% honey and it was high (104.6 gm) in 75% honey supplementation

In V instar larvae also the same effect was observed amount of food consumed was 242.5 in control. But it was reduced to 229.9 gm in 25% . 251.2 in 50% and 273.1gm in 75% honey supplemented larvae.(Table II,Fig 2).

RATE OF CONSUMPTION:

The rate of consumption was observed to be 2402.98 in control. But it was reduced to 2362.06 and 2316.32 in 25% and 50% honey supplementation respectively in the higher concentration of honey (75%) the rate of consumption was more (2538.83) than the control.

In case of V instar larvae honey supplementated had an elevated effect on the rate of food consumption .(Table II,Fig 2)

Table II Effect of honey on the consumption and rate of consumption of consumption of IV and V instars larvae of *Bombyx mori*

(Values are expressed in gram /10 larvae)

INSTAR	CONCENTRATION	CONSUMPTION (gm)	RATE OF CONSUMPTION
IV	Control	96.6	2402.98
	25	82.2	2362.06
	50	90.8	2316.32
	75	104.6	2538.83
V	Control	252.5	2220.7
	25	205.5	2137.5
	50	237.6	2189.8
	75	269.7	2232.6

Fig : 2 Effect of honey on consumption of IV and V instar of *Bombyx mori L*

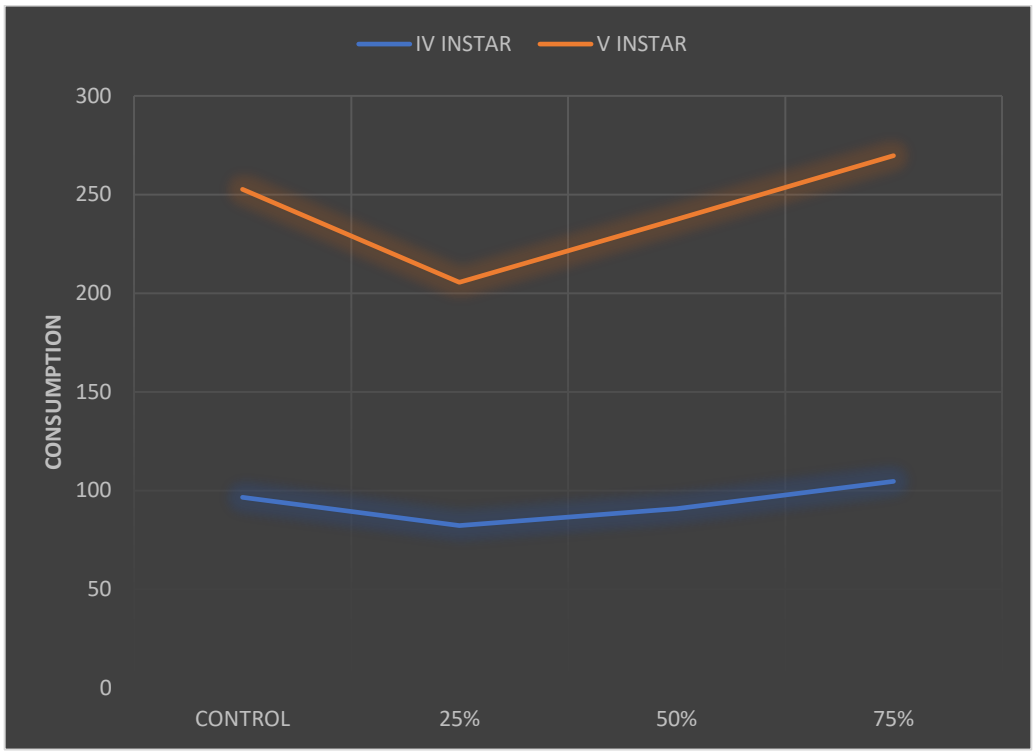
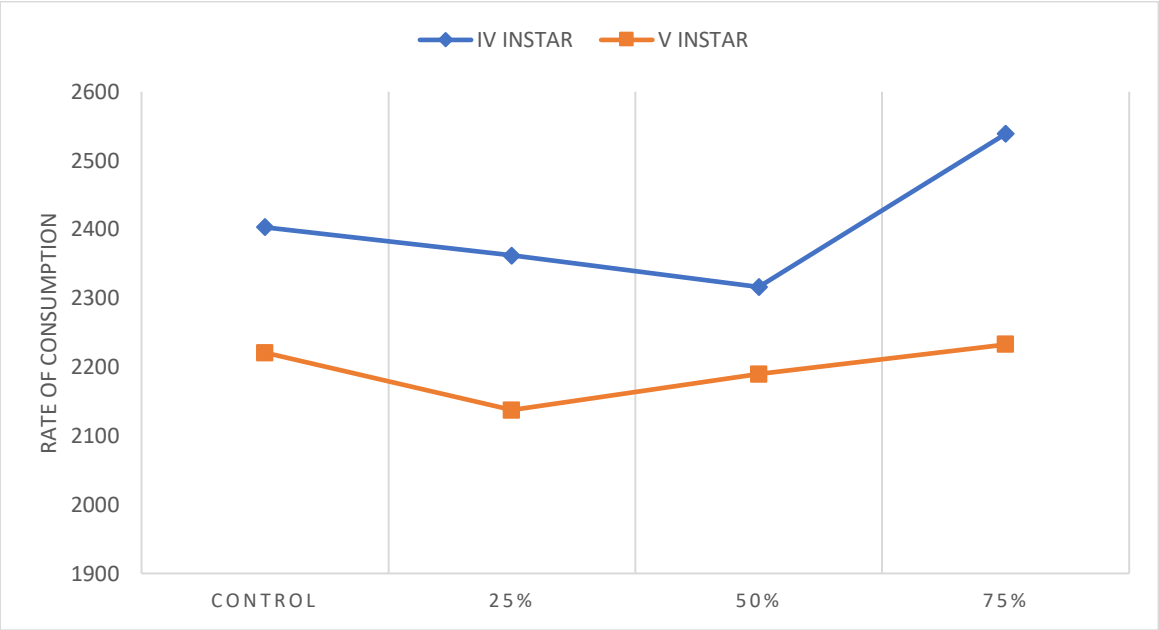


Fig 2.2 :Effect of honey on the rate of consumption of IV and V instar larvae of *Bombyx mori L*



Weight of silk gland

The effect of honey on the weight of silk gland is negative in the minimum concentration (25%) . but it had an happening effect in the higher concentration of honey and the weight was greater than the control.It was very and weighed only 0.55 gm in 25% but the weight of the gland gradually increased as the concentration of the honey increased . it was 0.74 gm and 0.87 gm respectively in 50% and 75% of honey .but the weight of silk gland is control was 0.76gm (Table III, Fig 3)

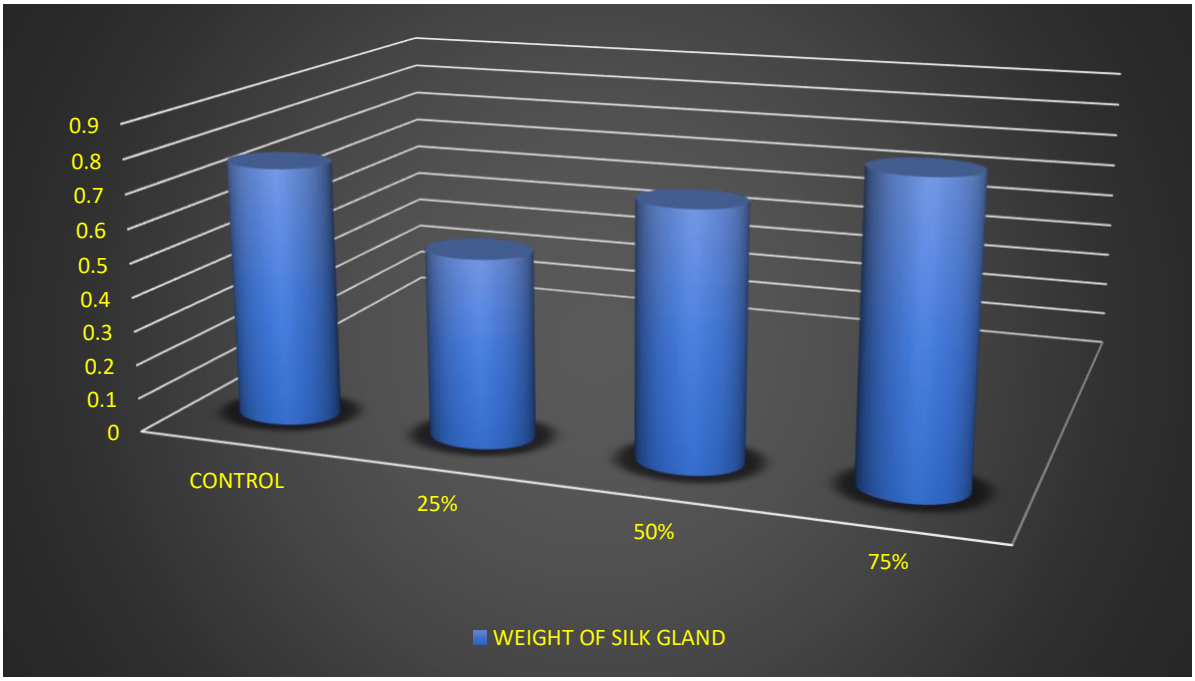
PLATE 2 : SILK GLANDS OBTAINED FROM THE LARVAE FED ON HONEY SUPPLEMENTED MULBERRY LEAVES

Table .III Effect of honey on the weight of silk gland of *Bombyx mori* .

(Values are expressed in gram/gland)

CONCENTRATION	WEIGHT OF SILK GLAND
CONTROL	0.76
25%	0.55
50%	0.74
75%	0.87

Fig 3 : Effect of honey on the weight of silk gland



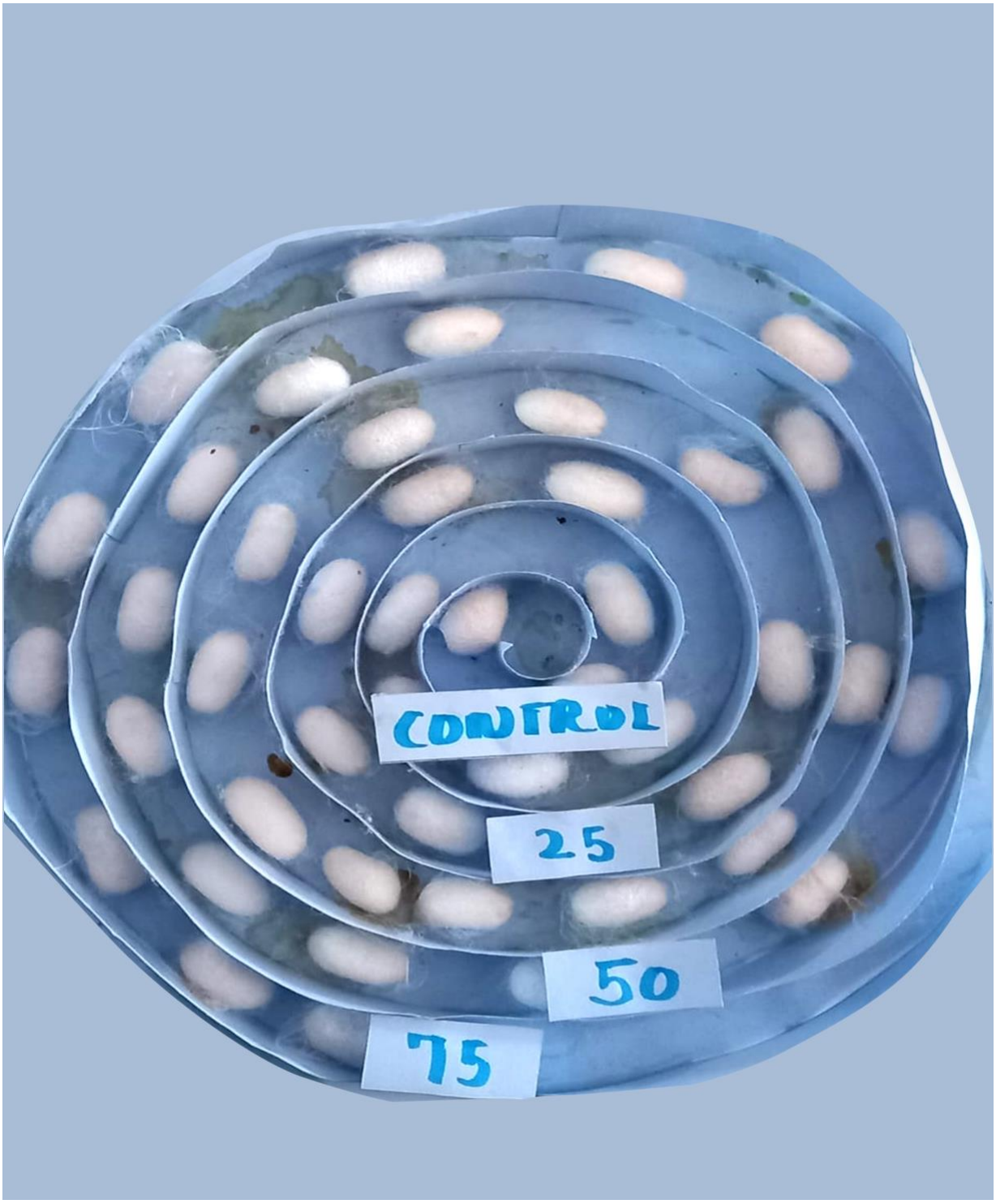


PLATE 3: COCOONS ON PAPER MOUNTAGE

COCOON CHARACTERS

WEIGHT OF GREEN COCOON

The result of the present study indicates that the honey has promoted the weight of green cocoon only in higher concentration (75%) but in lower concentration the weights were lower than the control.(Table IV , Fig 4)

SHELL WEIGHT

Shell weight of the cocoons from honey supplemented larvae exhibited a negative trend in lower concentration but gradually the shell weight increased as the concentration of honey supplementation increased . The shell weight was 2.781±0.28gm /10 larvae control .but it was 2.329±0.29 , 2.633±0.27 and 2.992±0.30 in 25% 50% and 75% honey supplementation. (Table IV , Fig 4)

SILK RATIO

As recorded in Table IV the silk ratio in control was 21.57. It was low (21.57) in 25% and started to increase as the concentration of honey increased . It was 20.91 and 21.72 in 50% and 75%.(Table IV,Fig 4)

PUPAL WEIGHT

The pupal weight was 10.01 gm in control .It was reduced to 9.84 gm in 25% honey supplementation . the Larval weight was 9.96 gm in 50% and 10.78 in 75% honey supplementation (Table IV,Fig 4)

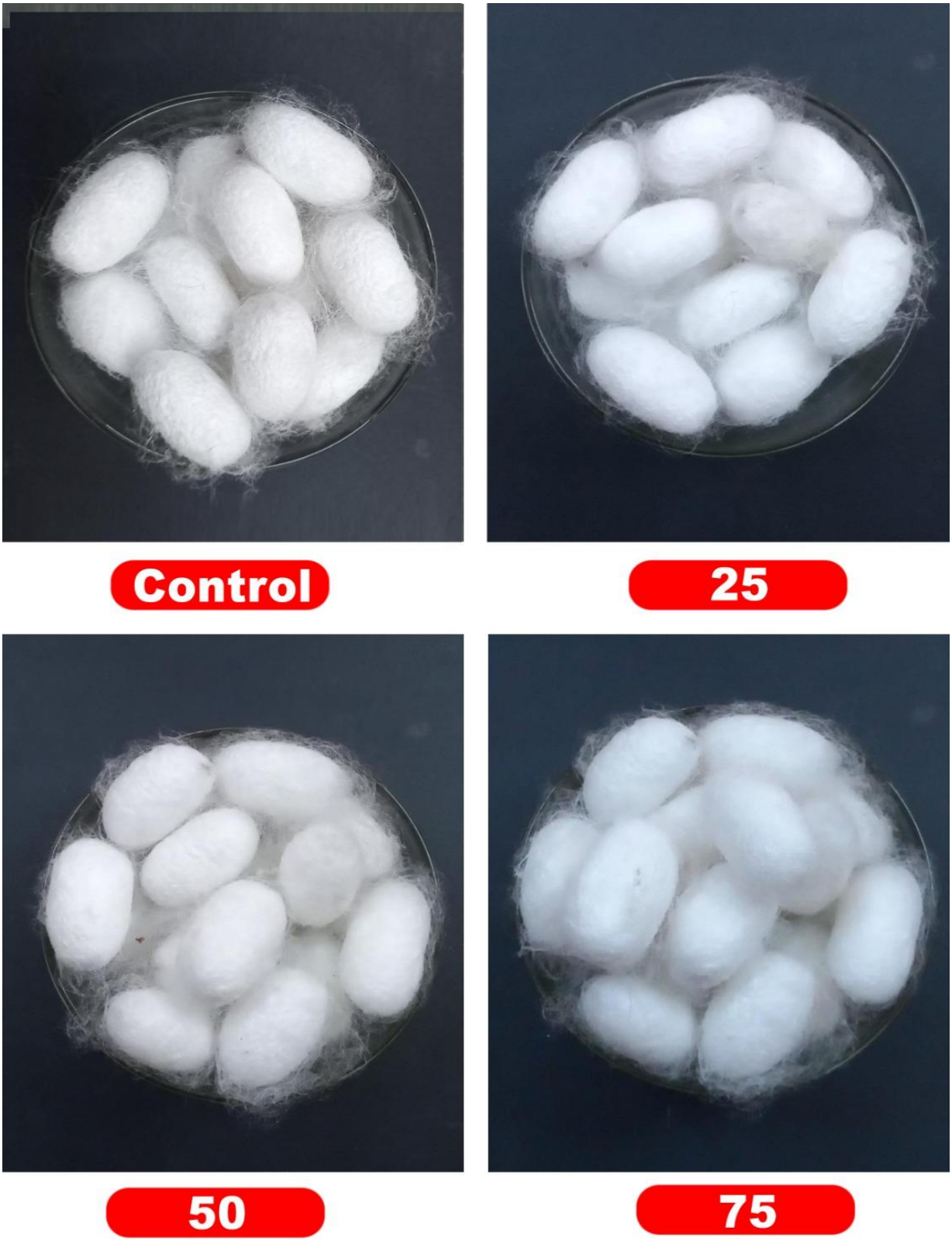


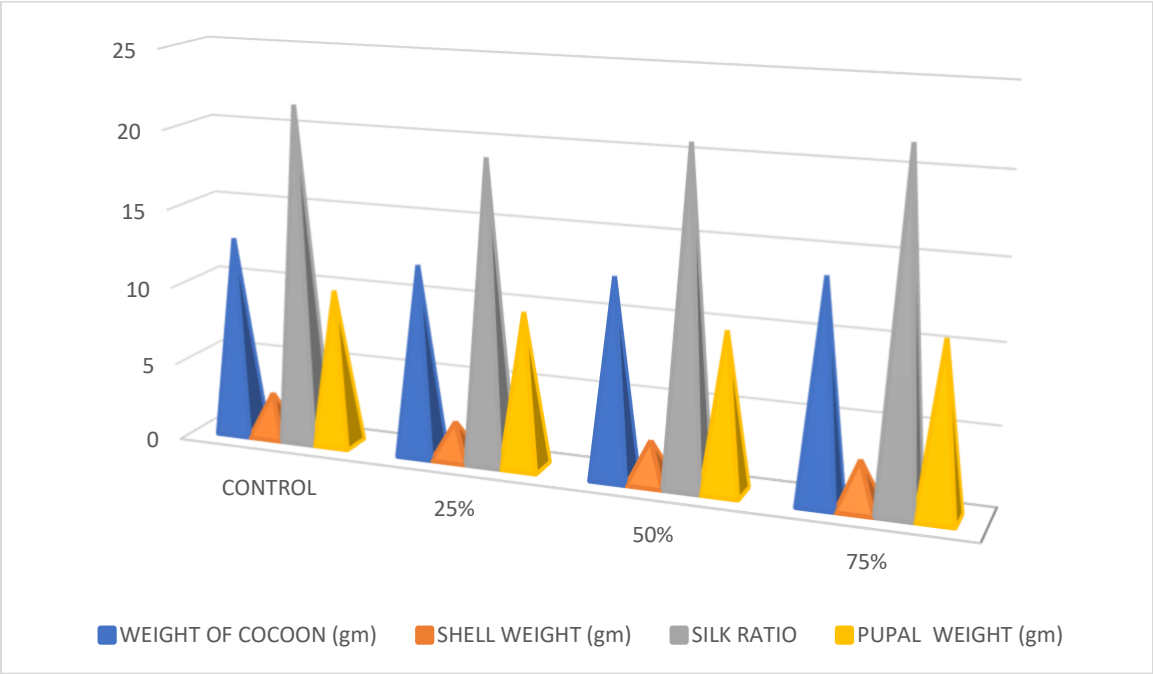
PLATE 4 : COCOONS OBTAINED FROM THE HONEY SUPPLEMENTED LARVAE

Table .IV Cocoon characters of *Bombyx mori* L fed on honey supplemented mulberry leaves.

(Values are expressed in mg /10 cocoon)

CONCENTRATION OF HONEY	WEIGHT OF COCOON (gm)	SHELL WEIGHT (gm)	SILK RATIO	PUPAL WEIGHT (gm)
CONTROL	12.79±0.37	2.781±0.28	21.57	10.01
25%	12.16±0.21	2.329±0.29	19.15	9.84
50%	12.59±0.43	2.633±0.27	20.91	9.96
75%	13.77±0.49	2.992±0.30	21.72	10.78

Fig 4 : Cocoon characters of Bombyx mori L.fed on honey supplemented mulberry leaves



SILK QUALITY

LENGTH OF SILK FILAMENT

The length of silk filament from 10 cocoons was 34.6 m in control .A slight reduction was noticed in the length (27.95) towards the 25% honey supplementation but the filament length started action increasing to 35.2 m and then to 37.3 m in 50% and 75 % honey supplemented larvae respectively (Table V,Fig 5)

WEIGHT OF SILK FILAMENT

Weight of the silk filament was 1.5gm/10 cocoon in control. There was a reduction in weight of the silk filament (1.15gm) in lower concentration of honey (25%). But the weight of the filament increased to 1.55 gm in 50% honey and the maximum length of 1.71 gm was obtained in75% honey supplementation. (Table V,Fig 5)

DENIER

The denier of the silk filament was enhanced by high percentage honey supplementation .the denier was 390 in control .it was 3.70.2, 3.96.3 and 412.5 in 25%, 50% and 75% of honey supplementation respectively (Table V,Fig 5)

PERCENTAGE OF RAW SILK

The present study indicator the enhancing effect of honey on the percentage of raw silk . the raw silk percentage was 12.17% in control .the percentage of raw silk value was 10.44 in 25% honey and 12.68 in 50% honey supplementation . maximum raw silk was obtained in 75% honey supplementation (Table V,Fig 5)

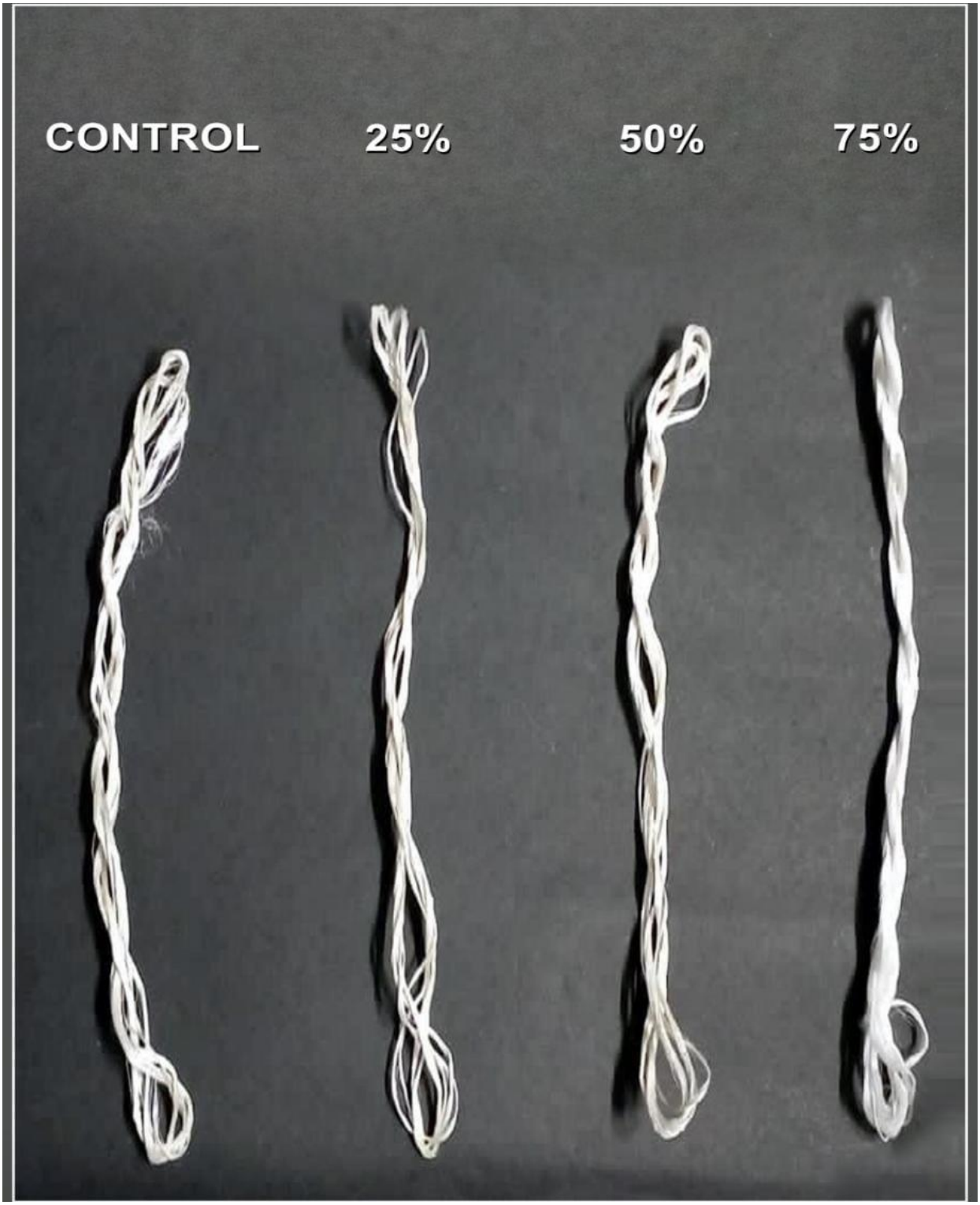
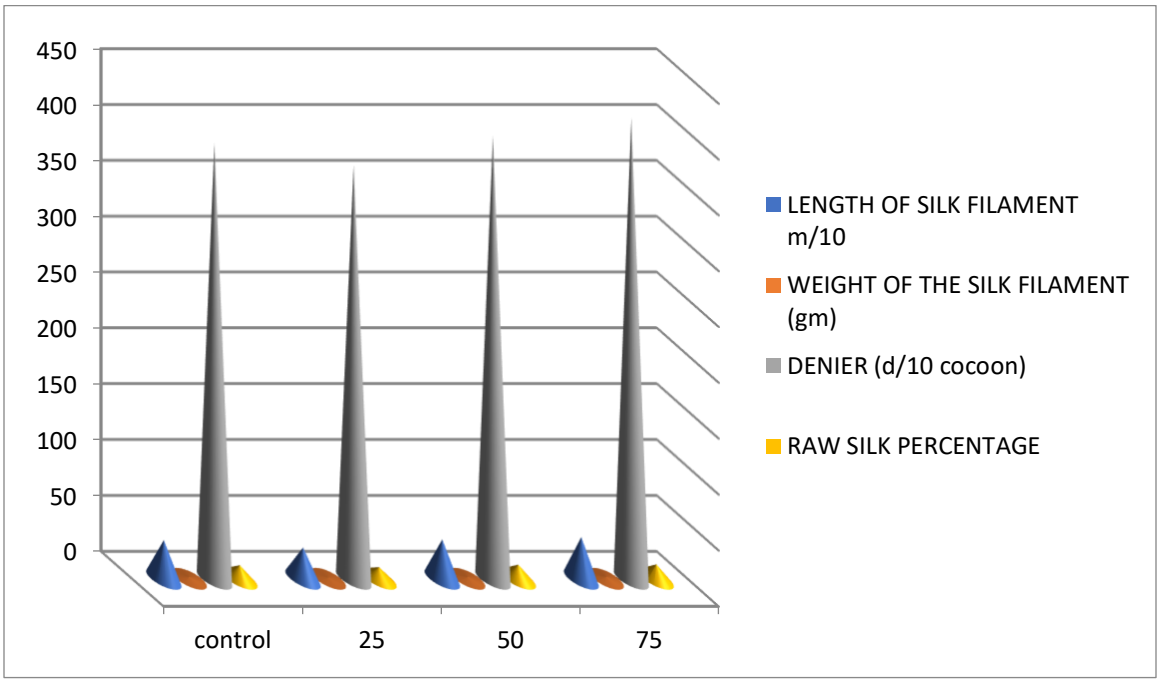


PLATE 5 : SILK FILAMENTS FROM HONEY SUPPLEMENTED LARVAE

Table V. Impact of honey supplementation on silk quality

CONCENTRATION	LENGTH OF SILK FILAMENT(m)	WEIGHT OF SILK FILAMENT (GM)	DENIER(d/10 cocoon)	RAW SILK PERCENTAGE
Control	34.6	1.5	390	12.17
25	27.95	1.15	370.2	10.44
50	35.2	1.55	396.3	12.68
75	37.3	1.71	412.5	12.97

Fig 5 : Impact of honey supplementation on silk quality



DISCUSSION

The growth and development of larvae and subsequent cocoon production are greatly influenced by the nutritional quality of mulberry leaves. Consumption of mulberry leaves enriched with nutritional supplement influence the silkworm larval characters and the silk output. (Ramesh *et al*., 2018)

Larval Growth

Feeding trials conducted by several workers proved that the level of nutrients in different varieties of mulberry have significant influence on the growth and development of silkworm (Ganesh prabhu *et al*., 2012; Ganesh prabhu *et al* , (2011).

In the present study honey supplementation to IV and final instar larvae of *Bombyx mori* was found to accelerate the growth in the higher concentration however in lower concentrations it had negative effect on it. This is in agreement with the findings of masthan *et al*., 2011 who reported that the blue green algae spirulina and yeast favoured the growth of the larvae of *Bombyx mori*. But comparatively the growth and development of V instar larvae were more enhanced by honey supplementation then IV instar larvae. It is supported by the report of Balasundharam *et al.,* (2013) and Ganesh prabhu *et al*., (2013) where the amino acid supplementation had more effects on larval growth.

CONSUMPTION & RATE OF CONSUMPTION

The present investigation the consumption of the IV and V instar larvae was found to decrease in lower concentration of honey. But the maximum concentration of honey has elevated the larval consumption in both the IV and V instar larvae. Supplementations of dietary nutrients with promising complementary additives increase the moisture content of the leaves which might have lead to higher consumption and consumption rate. (Trivedy *et al*., 2003).

The low consumption rate in lower concentration may be attributed to the fact that the taste of food in more important for silk worms to consume the diet. It might have had a phago repellent effect in low concentration of honey. (Balasundaram *et al*., 2013*)*

Cocoon characters

The commercial characters like cocoon weight, shell weight and silk ratio have decreased in lower concentrations and increased in the higher concentration of honey. This is in agreement with the report of Khyade and shendage, (2002) and Radjabi (2010) who have indicated the same effect of aloe vera juice and amino acid supplementation in silk worm. The result way be attributed to the fact that the enhanced consumption with high digestion and assimilation of food energy into

traval biomass and cocoon. This might have induced the upgrading of economic parameters (Badjabai, 2010) Minerals are the major constitutents of the diets of silkworm (Surgeon *et al* 2000) (Murugesh *et al*., (2020) has reported that *Bombyx mori* requires calcium , iron, magnesium , potassium and zinc for growth and development . Many studies have shown that the mulberry leaves with varied concentration of minerals have considerable positive influence on cocoon related parametering of *Bombyx mori* (Kaliwal and Hugar (2003)

This also coincide with the result of venkataramana (2003) who reported that mulberry leaf supplemented with spirulina as a feed to oraly was effective in enhancing the larval and cocoon characters. This is also supported by the result of feeding ascorbic acid enriched mulberry leaf to silk worm *Bombyx mori* carried out by Etebari K., Ebedi, R.and Mattindoost L. (2004). The results of the present study is supported by with Rayvanetbari and Lailiaamatindoost, 2005. This is in correlation with the result of Raj *et al*., (2002) and Etabari (2002) who have reported an increase in shell weight on administration of folic acid to *Bombyx mori L.*

Silk Quality

Various extracts of medicinal plants have been tested by supplementation to silkworm *Bombyx mori L*. and were seem to influence the length of silk thread Laskar and Datta (2000), Etabari *et al*., (2004), and Venkataramana (2003).

Spirulina supplementation has showed tremendous effect on the filament length in the present study similar observations were made by Venkateshkumar *et al.,* (2007) who has reported that mulberry leaf supplemented with spirulina as a feed to *Bombyx mori* was effective in enhancing the cocoon character and silk filament length.

Investigations revealed that fortification of mulberry leaves with potasium chloride elucidated significant improvement in the silk quality. (Bhattacharya and kaliwal,(2004) In the present the length denier and weight of raw silk and law silk percentage are elevated by honey supplement in high concentration which might be due to the high range of potassium and calcium and other minerals in honey.

SUMMARY

The present study revelated the enhancing effect of honey in higher concentration on the larval growth of both IV and V instar larvae of *Bombyx mori*.

The honey supplementation in *Bombyx mori* larvae showed profound effect on the consumption of and rate of consumption in *bombyx mori* larvae showed profound effect on the consumption of and rate of consumption .

The weight of silk gland was observed to be enhanced by the honey supplementation in higher concentration .

The cocoon characters in honey supplemented larvae was improved in high concentration of honey though it was low concentration

Positive changes were noticed in the filament length , denier and weight of silk were reported in the present study

Honey supplementation to Bombyx mori in our present study indicates that higher concentration has profound effect on the growth consumption silkgland cocoon characters and the silk quality

CONCLUSION

The present work has contributed to our knowledge that enrichment of mulberry leaves by honey has profound effect on food consumption , silk quality and economic characters of silkworm bombyx mori L .observation has made it clear that high concentration of honey produce more impact on improvement of silk quality

Silk is royal , exotic and sensuous in its radiance . the export potential of silk goods are promising in india

Nutrition plays an important role in improvement of growth and development of silkworm bombyx mori .for the past few years silk industry has received recognition through greater research on the nutritional aspects.nutritional supplementation and searching alternate food plants have been on way with varying degrees of success. So future researches could be done on silkworm rearing on artificial food .

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ANTIMICROBIAL AND LARVICIDAL ACTIVITIES OF MARINE PUFFER FISH LAGOCEPHALUS SPADICEUS FROM THOOTHUKUDI COAST

A dissertation submitted to

ST. MARY'S COLLEGE (Autonomous), THOOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

Master of Science in Zoology

by

S. SAMILA NISHA

Reg. No. 21APZO06

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 this dissertation entitled **"ANTIMICROBIAL AND LARVICIDAL ACTIVITIES OF MARINE PUFFER FISH LAGOCEPHALUS SPADICEUS FROM THOOTHUKUDI COAST"** submitted by **S. SAMILA NISHA Reg. No. 21APZO06** to St. Mary's College (Autonomous), Thoothukudi, affiliated to Manonmaniam Sundaranar University, Tirunelveli in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2022-2023 under my guidance and supervision. It is further certified that this dissertation or any part of this has not been submitted elsewhere for any other degree.

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DECLARATION

I do hereby declare that this dissertation entitled **"Antimicrobial and larvicidal activities of puffer fish *Lagocephalus spadiceus* from Thoothukudi coast"** submitted by me for the award of the degree of Master of Science in Zoology is the result of my original independent research work carried out under the guidance of **Dr. P. J. Joslin M.Sc., M.Phil., Ph.D.,** Associate Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi, and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date: :05.04.2023

S. Samila Nisha

Signature of the Candidate

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CONTENT

INTRODUCTION

1.0 INDRODUCTION

Marine ecosystem covers nearly 70% of the earth's surface. Most of the marine organisms live in a hostile environment, having developed a well defense mechanism for their survival (Garson, 1989). Ocean offers a large biodiversity of fauna and flora which is estimated to be over 5,00,00 species or more than double of land species (Kamboj, 1999).

The marine environment is an exceptional reservoir in 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 bioactive compounds. The number of natural products isolated from marine organisms increases rapidly, and now exceeds with hundreds of new compounds being discovered every year (Faulkner, 2002 and Proksch and Muller, 2006). As the evolution progresses, microbes have evolved themselves and became resistant to available antimicrobial drugs. Thus modern technologies have opened vast areas of research for the extraction of biomedical compounds from ocean and seas to treat the deadly diseases. Many classes of natural products from marine products exhibiting antitumour, anti-leukaemia, antibacterial and antiviral activities have been reported worldwide (Khora 2013).

Fish live in intimate contact with an environment containing pathogenic organisms. The slow adaptive immune response of fish makes innate immunity, which is fast acting and temperature independent - predominant system of fish host defense (Ellis, 2012). The defense includes many elements such as antimicrobial peptides, lipids and polypeptides (Ravichandran *et al.,* 2010). An antimicrobial substance that kill or inhibits the growth of microbes such as bacteria, fungi and viruses.

Fishes are a vital component of marine habitats. Fisheries play an important role in Indian economy and it is one of the most important activities along the coastal areas (FAO, 2002). Fishes are one of the diverse source of natural products and bioactive compounds with over 40,000 known species. They combat infections caused by viruses, bacteria, fungi, and parasites that are similar to those of humans and other vertebrates. Many species of marine fish have been reported as thyocrinotoxin (Halstead, 1978), releasing into the water toxic secretions.

Puffer fishes are commonly distributed in the 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, class Osterichthyes, order Tetradontiformes (Oliveira *et al*., 2006). It has a widespread distribution throughout the tropical Indian and Pacific oceans; for Japan, Australia and Hong Kong in the east to Mozambique and Southern African shores, as well as

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Red Sea (Smith and Heemestra, 1986; Watson *et al.*, 2003). They are usually found solitary in tropical waters, not more than 300m deep, mainly in areas of coral reefs.

The puffer fishes are commonly known of all type of fish poisoning and has been recognized from ancient times. It is probably the most common fish intoxication along the coasts of Asia (Hwang *et al.*, 2002). Puffer fishes are the second most poisonous vertebrate in the word, the first being a "Golden Poison Frog" (Keilichi *et al.*, 1998). The puffer fish takes its name from its tendency to puff its threat with water or air when predator's approach or a threatening situation arises, giving the fish a balloon-like appearance. In different parts of the world the puffer fish has different name such as blowfish, toadfish, swellfish, globe fish, bubble fish, balloon fish and sea squab (Torda *et al.*, 1973).

Puffer will eat all type of food such as shrimp, fish, clams, molluscs and crustaceans etc. It is also important that they consume hard shelled crabs, mussels and shell fish in their diet to wear down their teeth and prevent them from overgrowing. Further more toxicity changes with age, sex, season and geographical variations (Homaira *et al.*, 2010).

Puffer fishes are able to move their eyes independently and many species can change the colour or intensity of their patterns in response to environmental changes. They are somewhat similar to the terrestrial chameleon. Although most

puffers are dark and many have bright colour and distinctive markings and make no attempt to hide from predators (Keilichi *et al.*, 1998). Many marine puffers have a pelagic or open life stage. Spawning occurs after males slowly push female to the water surface or join females already present. The eggs are spherical or buoyant. Hatching occurs roughly after four days. The fry are tiny. They have a functional mouth and eyes and must eat within a few days (Han and Kim, 1998).

The Indo-Pacific originated *Lagocephalus spadiceus* (Richardson, 1845) is one of the most abundant non-indigenous puffer fishes of the eastern Mediterranean Sea, distributing along the entire Levantine basin coasts from Port Said to southern Aegean Sea (Golani *et al.*, 2002). Habitat of the species is mainly benthic, including sandy and muddy substrates of shallow coasts with depths less than 50 m. Largest specimen captured from the Levant coast was 24.5 cm standard length (Ben-Tuvia, 1966), however, a maximum size of 40 cm was mentioned (Golani *et al.*, 2002). The species has long been used as food in many localities of the IndoPacific, such as Japan, Thailand, Malaysia etc. (Brillantes *et al.*, 2003), but it does not have any commercial value in the Mediterranean and frequently discarded from the bottom trawl catch.

The toxin was first isolated and named in 1909 by Japanese scientist Yoshizumi tahara. Tetrodotoxin can be found in liver, gonads, intestine and skin of

these fish and can cause death in approximately 60% of persons who ingest it (Ellenhorn and Barceloux, 1988).

Awareness of the toxin contained in puffer fishes made the ancient Chinese and Japanese to consume the poisonous organs of the fish as a general health tonic (Kumaravel *et al.*, 2011). Tetrodotoxin is reported to reduce the symptoms of withdrawl in heroin addicts and serve as analgesic to minimize pain in cancer patient (Alonso *et al.*, 2003). Tetrodotoxin is the best known marine toxin due to its frequent involvement in fatal food poisoning unique chemical structure and specific action of blocking sodium channels of excitable membranes (Seltzer, 1990 and Lau, 1995). During the World War 2, the crude puffer fish extracts have been used for treating migraines and menstrual cramps. The bacteria associated with some puffer fish produce powerful neurotoxin in their internal organs making them an unpleasant, possibly and lethal meal for any predators (Chun-Fai *et al.*, 2004).

For the past two decades, the major obstacle to treat microbial infections is the emergence microbial resistance towards majority of synthetic compounds and also evolution of multiple mechanisms towards microbial resistance as well as, confined the use of antimicrobial compounds (Tran *et al.*, 2015).

Puffer fish are able to produce toxin with associate microbes on the mucus of their body. The antimicrobial function of epidermal mucus appears to result

from its mechanical properties. The mucus layer on the surface of fish is continuously replaced which possibly prevents stable colonization by parasites, bacteria and fungi (Pickering, 1974). Antibacterial proteins are assumed to form ion channels in bacterial membrane and kill both Gram positive and Gram negative bacteria (Ebran *et al.*, 2000).

The evolution of antibiotic resistant pathogenic bacteria has stimulated the search for alternative antimicrobial agents from natural source. Many antimicrobial peptides shows a high specificity for prokaryotes and a low toxicity for eukaryotic cells and their mode of action is considered unlikely to lead to the development of resistance. These properties have favoured their investigation as potential new antibiotics (Bax *et al.*, 2000).

Antimicrobial peptides have direct microbicidal effects against bacterial, fungal, viral, and protozoan pathogens (Hancock and Diamond, 2000). They also play roles in inflammatory responses such as recruitment of neutrophils and fibroblasts, promotion of mast cell degranulation, enhancement of phagocytosis and decreasing fibrinolysis (Boman, 2003; Zasloff, 2002). To prevent tissue injury, antimicrobial peptides stimulate apoptosis of infected or activated cells, decrease cytokine production, and some, such as cationic peptides, have the ability to bind and neutralize bacterial lipopolysaccharide (LPS) (Hancock and Diamond, 2000).

Secondary metabolites of marine organisms differ from that of terrestrial organisms. Bioactive compounds isolated from marine organisms exhibited various biological activities such as anti-cancer, anti-inflammatory, antifungal, antimicrobial and mosquito larvicidal properties (Gul and Hamann, 2005).

Mosquitoes transmit many tropical and subtropical diseases such as dengue fever, yellow fever, malaria, filariasis and Japanese encephalitis (Service, 2004). *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus* are the vector mosquitoes of malaria, dengue and lymphatic filariasis, respectively. Vector control is definitely the best method of protecting the community against the vector borne diseases (Sharma, 2001). Personal protection from biting mosquitoes and other haematophagous Arthropods is the first choice of defense against the infectious disease (WHO, 2004). Over two billion people in tropical countries are at risk from mosquito borne diseases and the search for effective vaccines against these diseases is still in progress (WHO, 2008). Many types of fish have been used in mosquito control programmes worldwide (Walton, 2007). Various indigenous fish species have been used for mosquito control in different parts of the world (Kim *et al*., 1994).

Gas Chromatography-Mass Spectrometry (GC-MS) is a hyphenated analytical technique that combines the separation properties of gas-liquid chromatography with the detection feature of mass spectrometry to identify

different substances within a test sample. GC is used to separate the volatile and thermally stable substitutes in a sample whereas GC-MS fragments the analyte to be identified on the basis of its mass. Further addition of mass spectrometer in it leads to GC-MS/MS. Superior performance is achieved by single and triple quadrupole modes (Sahil *et al.,* 2011; Jenke, 1996; Rowley, 2001).

The discovery of novel drugs from established sources has motivated the evaluation of new sources of chemically diverse compounds. In the last century, there has been a huge progress in the field of marine natural products and its chemistry (Plouffe *et al*., 2005). So far approximately 7000 marine natural products have been reported from the marine organism (Venkataraman and Wafer 2005).

In India studies on the puffer fish are very limited and it remains unexploited. Hence the aim of the present study is to evaluate the antimicrobial activity and larvicidal activity of skin, muscle and liver of puffer fish *Lagocephalus spadiceus* collected from Thoothukudi coast.

OBJECTIVES

2.0 OBJECTIVES

The objectives of the present study are

- to investigate the antibacterial activity (MIC) of acetic acid extract of skin of *Lagocephalus spadiceus*.
- to determine the antifungal activity of acetic acid extracts of skin, muscle and liver of *Lagocephalus spadiceus.*
- to estimate the larvicidal activity of acetic acid extracts of skin, muscle and liver of *Lagocephalus spadiceus*.
- to identify the bioactive compounds present in the methanol extract of skin using by GC-MS analysis.

REVIEW OF LITERATURE

3.0 REVIEW OF LITERATURE

A natural product plays an important role in the modern chemotherapy. The term "natural products" is applied to materials derived from higher plants, microorganisms, invertebrates and vertebrates. They may be used in eliminating pain, controlling, suffering or in the prevention or treatment of diseases (Bohonos and Piersma 1966).

Sato (1996) and Grabley (1999) reported that marine biota is the largest source of novel discovery of natural products or bio similarities such as pharmaceutical metabolites and medicines. There has been an extensive research showing that vast bioactive substance were identified and characterized from marine organisms.

Suvarna Devi, (2016) reported that the first record of half-smooth golden puffer *Lagocephalus spadiceus* (Richardson, 1845) from West Coast of India.

Mandal and Khora (2013) reported that the assessment of toxicity in puffer fish *Lagocephalus lunaris* from South East Indian Coast. This study was conducted along the South East Indian Coast. He suggested that *L.lunaris* collected from the South East Indian Coast is toxic, especially the muscle and therefore, it is not fit for consumption.

Venmathi Maran *et al.,* (2007) isolated and identified the bacteria from the copepod *Pseudocaligus fugu* ectoparasitic on the panther puffer *Takifugu pardalis* with the emphasis on TTX.

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

Selvi and Joslin (2016) reported that antibacterial activity of marine puffer fish *Chelonodon patoca* from Thoothukudi coast. The result revealed that the liver and muscle extracts exhibited potent antibacterial activity.

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

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

Kumaravel *et al.,* (2011) reported that antimicrobial activity of tissue extracts of puffer fish *A.immaculatus* against clinical pathogens. The result confirms that puffer fish is a source of antimicrobial potence.

Knouft *et al.,* (2003) have reported that the endogenous peptides with antimicrobial activity from fish skin and its secretions. In this study, a pronounced antimicrobial activity was shown against various bacterial and fungal strains.

Xia *et al*., (2008) studied the antimicrobial activity of brown-spotted grouper *Epinephelus fario.*

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

Rafat *et al.,* (2008) investigated antifungal and antibacterial activities of some marine organisms using disc diffusion method.

Kanthimathi and Joslin (2021) reported that antimicrobial, anticancer properties from various tissues extract of puffer fish *Arothron stellatus* from Thoothukudi coast.

Sasikala and Ravindran (2013) reported that antimicrobial activity of scorpion fish *Scorpaenopsis venosa* toxic extracts and its structural elucidation.

Omar *et al.,* (2012) focused on the antimicrobial activity of *Enteromorpha prolifera* and *Ulva reticulate*.

Abareethan (2021) reported that hemolytic activity and antibacterial activity of fish epidermal mucus from *Labeo rohita*.

Ong Yeong Wei *et al.,* (2010) reported that antibacterial activity of mucus extract of Snakehead fish, *Channa striatus* (Bloch).

Maheswari and Jagadish Naik, (2020) reported that antibacterial activity of marine fish *Chelonodon patoca* from Visakhapatnam coast.

Bragadeeswaran *et al*., (2010) revealed that *Arothron immaculatus* from Parangipettai coast showed antibacterial activity against pathogens and that extracts not shown any activity against fungal pathogens.

Chitrasom and Radhakrishnan (2010) examined the antimicrobial activities of polyunsaturated fatty acid extracts from *Sardinella longiceps* and *Sardinella fimbriata.* Polyunsaturated Fatty Acids (PUFA) content of two species of fishes was examined and their antibacterial activities were compared. The difference between the antibacterial activities of the two extracts could be attributed to the disparity of DHA content.

Chandra Sekhara Rao *et al.,* (2015) reported that larvicidal efficacy of four indigenous ornamental fish species of Lake Kolleru, India.

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

Anielle Espiegle *et al., (*2019) stated that water and methanol were used for the extraction of the sea cucumber body wall. All extracts showed larvicidal potential against 3rd instar wild Aedes larvae with the highest toxicity observed in *Holothuria atra* extract.

Gupta and Banerjee (2013) compared the mosquito biocontrol efficiency of *Poecilia reticulata* and *Aplocheilus panchax,* two popular fish species which so far have been used for mosquito biocontrol in India.

Samidurai and Mathew (2013) reported that mosquito larvicidal and ovicidal activity of puffer fish extracts against *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*.

Reegan *et al.,* (2013) demonstrated the larvicidal, ovicidal, and repellent activities of marine sponge *Cliona celata* (Grant) extracts against *Culex quinquefasciatus* and *Aedes aegypti*.

Mohamed Yacoob Syed Ali *et al.,* (2013) focused on the mosquito larvicidal activity of seaweeds extracts against *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus.*

Indumathi *et al.,* (2016) reported that antimicrobial and larvicidal activities of the tissue extracts of Oblong Blowfish (*Takifugu oblongus*) from South - East Coast of India.

Che Nin Man *et al.,* (2010) revealed that screening of tetrodotoxin in puffers using gas chromatography–mass spectrometry.

Bhuvaneswari and Babu Rajendren (2012) reported that GC-MS analysis of organochlorine pesticides (OCPs) in fish from river Cauvery and Veeranam Lake.

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

Jal *et al*., (2014) studied the bioactive potential of puffer fish *Arothron stellatus* collected from South East Coast of India.

Mzoughi *et al*., (2010) reported that polycyclic Aromatic Hydrocarbons $(PHH)_3$ and aliphatic hydrocarbons in fish by GC-MS.

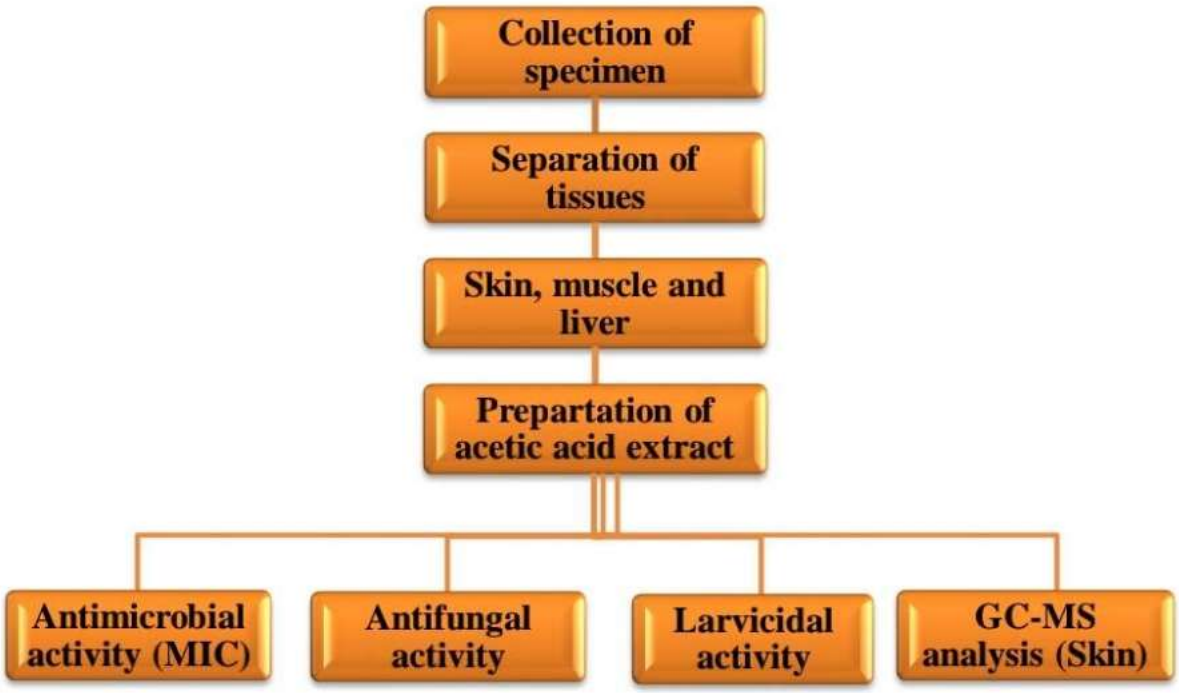
Bratu *et al*., (2013) reported that the quantitative determination of fatty acids from fish oil using GC-MS method and H-NMR spectroscopy.

Mottaleb *et al*., (2015) determined the use of LC-MS and GC-MS methods to measure emerging contaminants pharmaceutical and personal care products (PPCPs) in fish.

Wei Wu *et al.,* (2013) identified that volatile compounds in cooked meat of farmed obscure puffer (*Takifugu obscurus*) using SDE and HS-SPME combined with GC-MS.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN



4.0 MATERIALS AND METHODS

4.1. Systematic position of experimental animal

Kingdom : Animalia

Phylum : Chordata

Class : Actinopterygii

Order : Tetradontiformers

Genus : *Lagocephalus*

Species : *spadiceus*

Lagocephalus spadiceus (Plate 1).

It is commonly known as the half-smooth golden pufferfish. It is a species of fish in the family Tetraodontidae which includes 184 species distributed in tropical and temperate seas and fresh waters around the world. It is a common fish in the Red Sea, as well as the Indian Ocean, but can be found also in the Mediterranean, where it arrived from its natural habitat by Lessepsian migration. It is found over various types of substrata but mainly sandy and in depths ranging from 3 to 200m but most frequently in depths less than 50 m.

Plate : 1

Lagocephalus spadiceus

Belly of the Half-smooth Golden Puffer covered with spinules; nasal organ with 2 openings and slightly concave caudal fin. It has grayish brown dorsal half of the body; silver white side and white belly; white caudal fin in the ventral lobe and dark yellow in the dorsal lobe.

Both sexes of *L. spadiceus* exhibited almost similar growth rate and are homogenously distributed for major part of the year, excepting post-monsoon breeding season. It measures around 9 cm size and breeds twice in a year-once during February to March with smaller (9 to 15 cm) females and again from September to November with larger (11 to 28 cm) females.

4.2. Collection of specimen

The specimens of the puffer fish *L. spadiceus* were collected from the fish landing centre at fishing harbor Thoothukudi. Immediatedly after collection, the fresh samples were kept in an ice box and transported to the laboratory and stored in a deep freezer at -20^{0}C until they are used.

4.3. Preparation of Acetic acid extract:

The preparation of extract was carried out following the method described by Kawabata (1979). Specimens of *Lagocephalus spadiceus* were thawed and dissected out into tissues like skin, muscle and liver. Each tissue was homogenized with 10ml of acetic acid and 90ml of methanol and were kept in the refrigerator for

24 hours in a sterile condition, as incubation period. Once slurry of the tissue is ready, filter the materials with the Whatman filter paper. The filtrated sample is transferred to a rotatory evaporator and the crude extract has been obtained. Then it was stored in the deep freezer for further use at -20^{0}C.

4.4 Antibacterial activity

Bacterial strains

The reference strains of pathogens used to test antibacterial activity are

Vibrio cholerae (01) VC (01)

Escherichia coli (EC)

Aeromonas hydrophila (AH)

Shigella flexneri (SF)

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 - 100ml

2 to 3ml of sterilized broth medium was taken in the 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:

Beef infusion - 30g

Casein acid hydrolysate - 17g

Agar - 17g

Distilled water - 1000ml

Muller Hinton agar was suspended in 1000ml 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 120^0C for 15 minutes.

Agar diffusion technique

The antibacterial activity was evaluated using agar well diffusion method (Seedevi, *et al*., 2016). The 24 h old cultures were swabbed in Muller-Hinton Agar plates using a sterile cotton swab aseptically. The wells were punched on swabbed

plates using a sterile well cutter. The skin extract with three different concentrations such as 3µl, 4µl and 5µl were loaded into the respectively labeled wells. The plates were incubated at 37ºC for 24 hours, and the results were obtained by measuring zone of inhibition for each well and expressed in millimeter.

Minimum Inhibitory Concentration (MIC) determination

Determination of minimum inhibitory concentration of skin extract of *Lagocephalus spadiceus* by broth dilution method (D'amato *et al.,* 2015). The skin extract was diluted in sterile broth and then test organism was inoculated under aseptic condition. Dilutions of broth made from 0 - 120µl with sample, then the broth was incubated at 35 – 37ºC until the growth reaches the turbidity equal to or greater than the standard. Then the turbidity was calorimetrically observed at 625nm. Distilled water and streptomycin were used as negative and positive controls respectively. The culture from the tube showed 0. OD value was streaked on the agar plate to confirm the minimum inhibitory effect and the result was noted.

4.5. Antifungal activity

Media preparation

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

Potato dextrose agar

Potato - 200gm

Dextrose - 20gm

Agar - 15gm

Distilled water - 1000 ml

pH - 5.6±2

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

Antifungal activity screening test

The antifungal activity of skin, muscle and liver of puffer fish *Lagocephalus spadiceus* 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 skin, muscle and liver extracts (50µg), positive and negative controls were poured into each well. The antifungal drug (Fluconazole) was used as a positive control and water was used as negative control. 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. Larvicidal Activity

Larvicidal activity was determined by following the method of Samidurai and Mathew, (2013). Mosquito larvae were collected from stagnant rain water in the local rural areas. They were transferred to the plastic trays containing tap water and cultured. The larvae were maintained at 27°C ± 2°C under photoperiod of 14:10 hours (Light/Dark) during the period of experiment. The laboratory maintained larvae were used for larvicidal activity. The stock solution was used to make different concentrations of the acetic acid extract of skin, muscle and liver of *Lagocephalus spadiceus*. Triplicates were maintained for each concentration ranging from 10 - 40%. Batches of twenty larvae were transferred into each cup containing 10ml of water. The mortality rate of larvae was studied by treating the larvae with 10, 20, 30 and 40 % extract at 27 °C ± 2 °C. Tap water is maintained as control. The larval mortality was observed till the 24 hours of exposure and percentage of mortality was calculated from the following formula:

Percentage mortality = No. of dead larvae/ No. of larvae introduced X 100.

4.7. Statistical Analysis

The mean and standard deviations were calculated according to the standard methods for all the parameters. The P values were calculated by One-way ANOVA

by SPSS Statistics Software Program. Data expressed in the tables are as mean ± S.D. The value of $P<0.05$ was considered to be statistically significant.

4.8. GC-MS Analysis

GC-MS analysis of methanol extract of skin 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. Analysis was performed by using a GC, Varian CP 3800 and MS, Saturn 2200 (VF 5 ms 30 X 0.25 system) equipped with Elite-1, fused silica capillary column composed of 5% Phenyl Arylene – 95% Dimethyl poly siloxane. The system comprising a COMBIPAL auto sampler set under the following condition: 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 250ºC; the oven temperature was programmed from 100-2700ºC at the rate of GC-MS was done by using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

RESULTS

5.0 RESULTS

5.1. Antibacterial activity of acetic acid extracts of various tissues of *Lagocephalus spadiceus* against bacterial strains

The skin extract produced zone of inhibition with 8mm, 11mm and 12mm in *V. cholerae* at 3µl, 4µl and 5µl respectively. *E. coli* was sensitive with zone of inhibition 8mm, 12mm and 14mm at 3µl, 4µl and 5µl concentrations. The zone of inhibition extended upto 13mm in *Shigella flexneri* at 5µl concentration and 9mm at 3µl and 12mm at 4µl. The zone inhibition ranged from 9 to 11mm in *A. hydrophila*. The skin extract inhibited the growth of *S. aureus* with zone of inhibition 8mm, 10mm and 11mm at 3µl, 4µl and 5µl respectively (Plate 2, Fig 1).

5.2. Minimum inhibitory concentration (MIC)

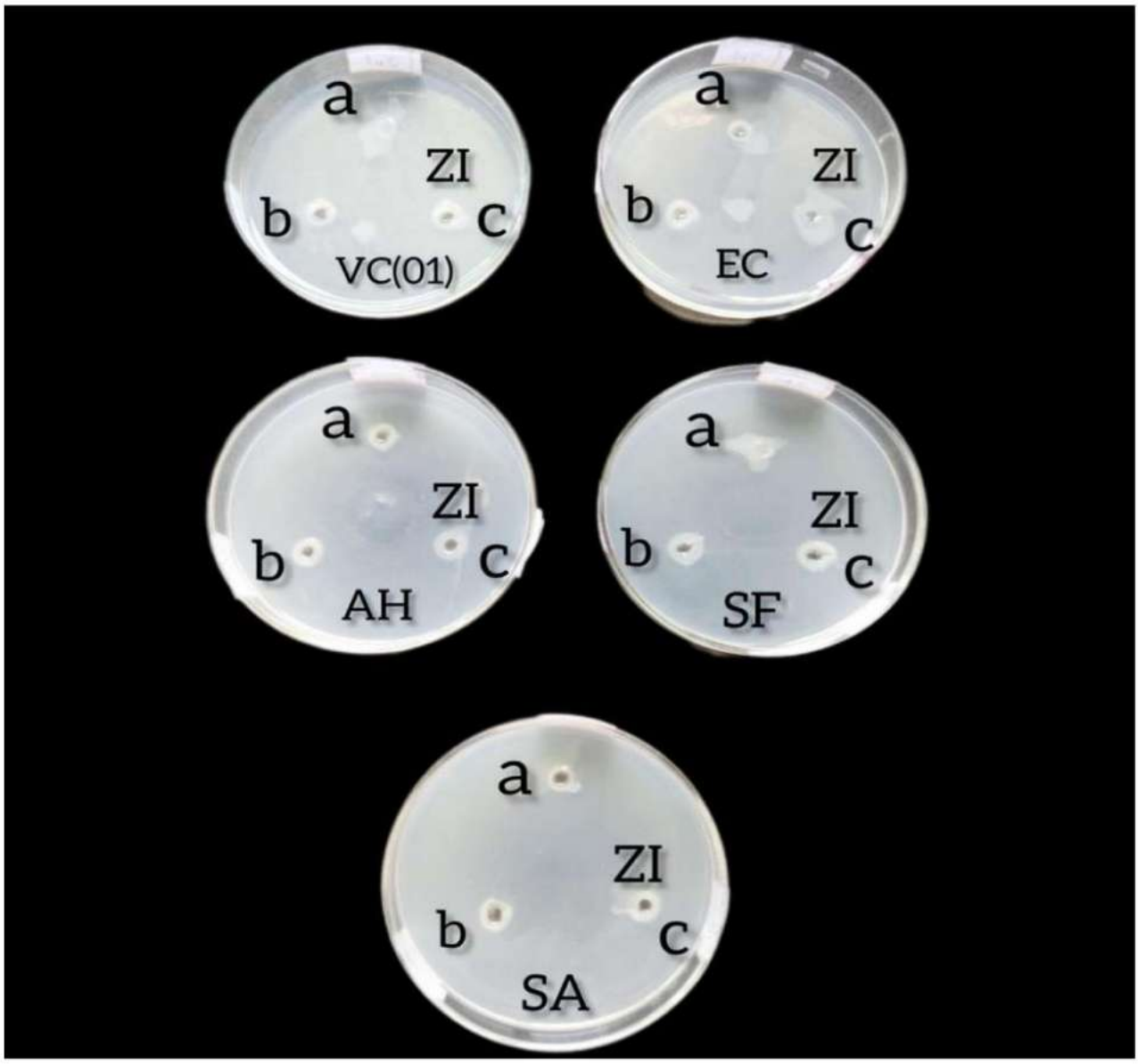
The MIC value of skin extract against *E. coli* was found to be 100µl (Plate 3).

5.3. Effect of acetic acid extracts of various tissues of *Lagocephalus spadiceus* on *Aspergillus niger*

Acetic acid extracts of skin, muscle and liver of *L. spadiceus* were subjected to antifungal activity against *A. niger* at 50µg concentration. The antifungal activity of various extracts of tissues was compared with standard antibiotic

Plate : 2

Muller Hinton Agar plates showing antibacterial activity of skin extract of *Lagocephalus spadiceus* against bacterial strains



ZI – Zone of inhibition; **a -** 3µl; **b -** 4µl; **c -** 5µl

Fig.1 - Antibacterial activity of acetic acid extract of skin of *Lagocephalus spadiceus* against bacterial strains

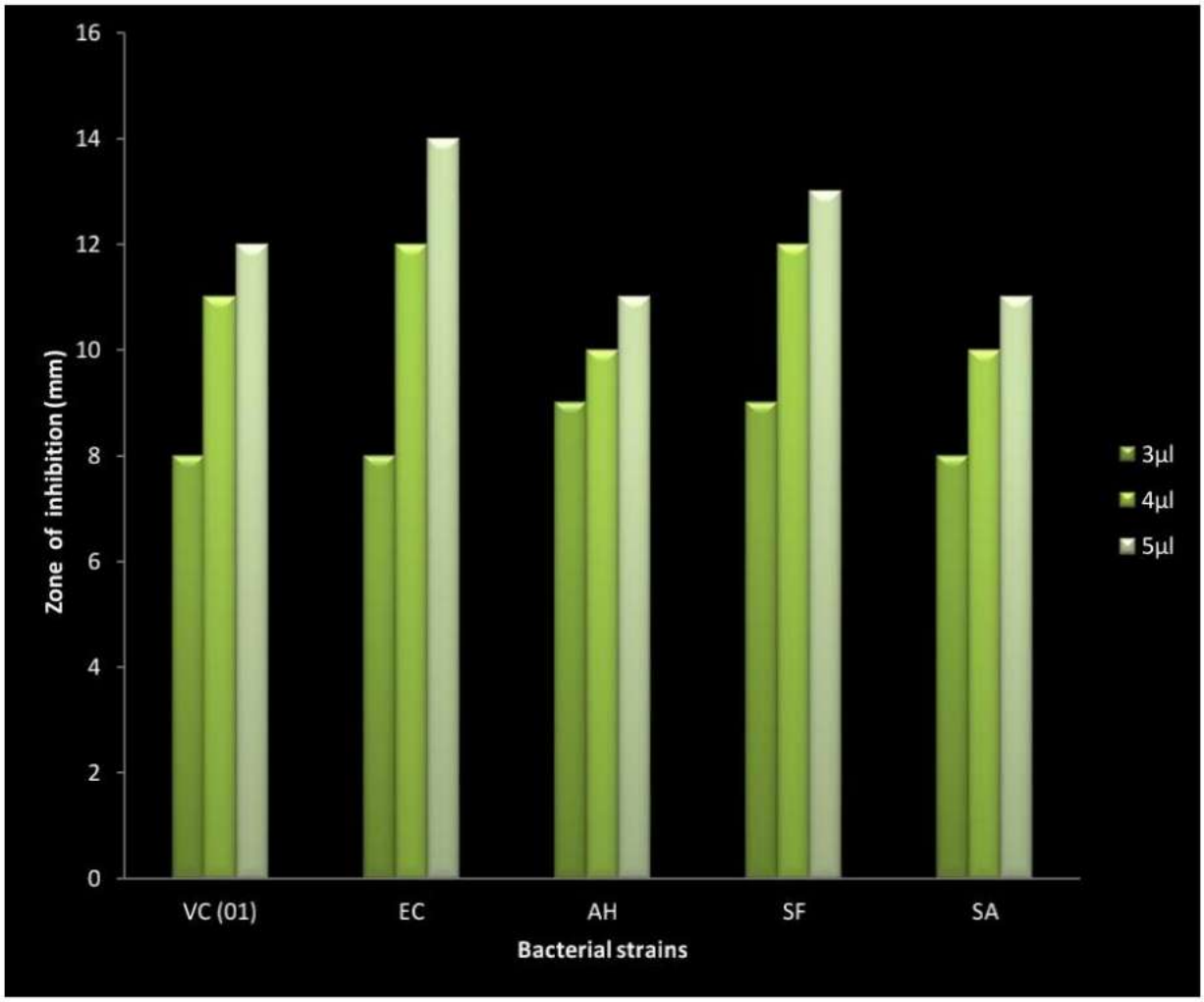
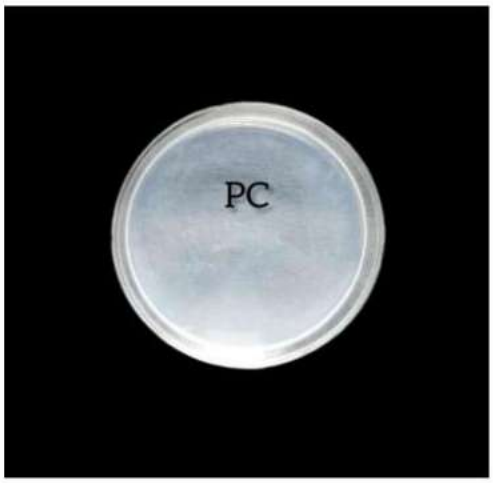


Plate : 3

Streak Agar Plates showing inhibitory activity of acetic acid extract of skin of *Lagocephalus spadiceus* (MIC)



S - Skin

PC – Positive control (Streptomycin)

NC – Negative control

Fluconazole at 50µg. Acetic acid extracts of skin, muscle and liver produced zone of inhibition with 14mm, 7mm and 13mm respectively (Plate 4, Fig. 2).

5.4. Larvicidal activity of acetic acid extracts of various tissues of *Lagocephalus spadiceus*

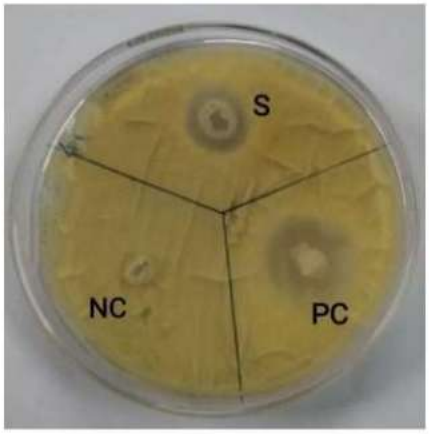
Acetic acid extracts of skin, muscle and liver of *L. spadiceus* were used to determine the larvicidal activity (Plate 5). Maximum death rate was observed at 40% concentration of all the extracts, while liver extract showed highest mortality at 30% and 20% too. Skin extract showed exceptional larvicidal activity at 20%, 30% and 40% concentrations, producing 100% death of all the larvae tested (Fig.3). Muscle extract showed 100% mortality of all the larvae at 40% concentration (Fig.4). Maximum death rate was recorded in the liver extract i.e., 100% mortality at 20%, 30% and 40% concentrations respectively (Fig.5).

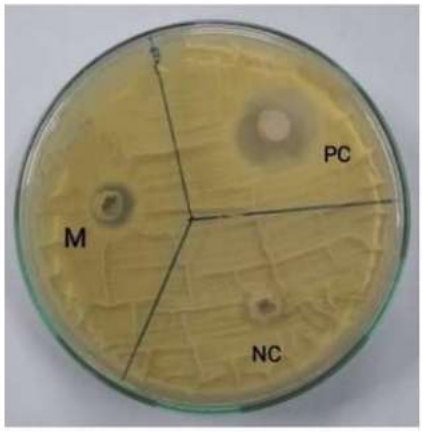
5.5. Statistical analysis

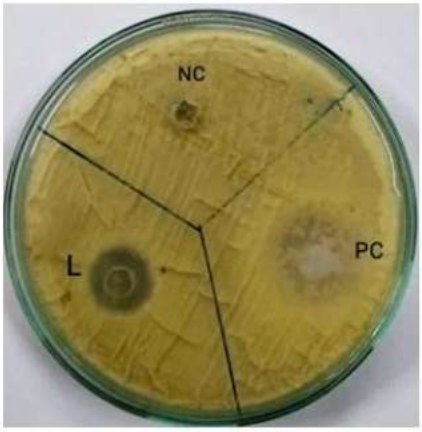
Larvicidal activity was calculated by one way ANOVA. The calculated P value is less than 0.05 level of significance in the skin, muscle and liver extracts of *Lagocephalus spadiceus*. It is statistically significant. The percentage mortality were expressed as Mean ± Standard deviation for triplicate experiment. Statistical analysis showed significant values ($P<0.05$). (Table 1)

Plate : 4

Antifungal activity of skin, muscle and liver extracts of *Lagocephalus spadiceus* against *Aspergillus niger*







PC - Positive Control (Fluconazole)

NC - Negative Control

S - Skin

M - Muscle

L - Liver

Fig.2 - Antifungal activity of skin, muscle and liver extracts of *Lagocephalus spadiceus* against *Aspergillus niger*

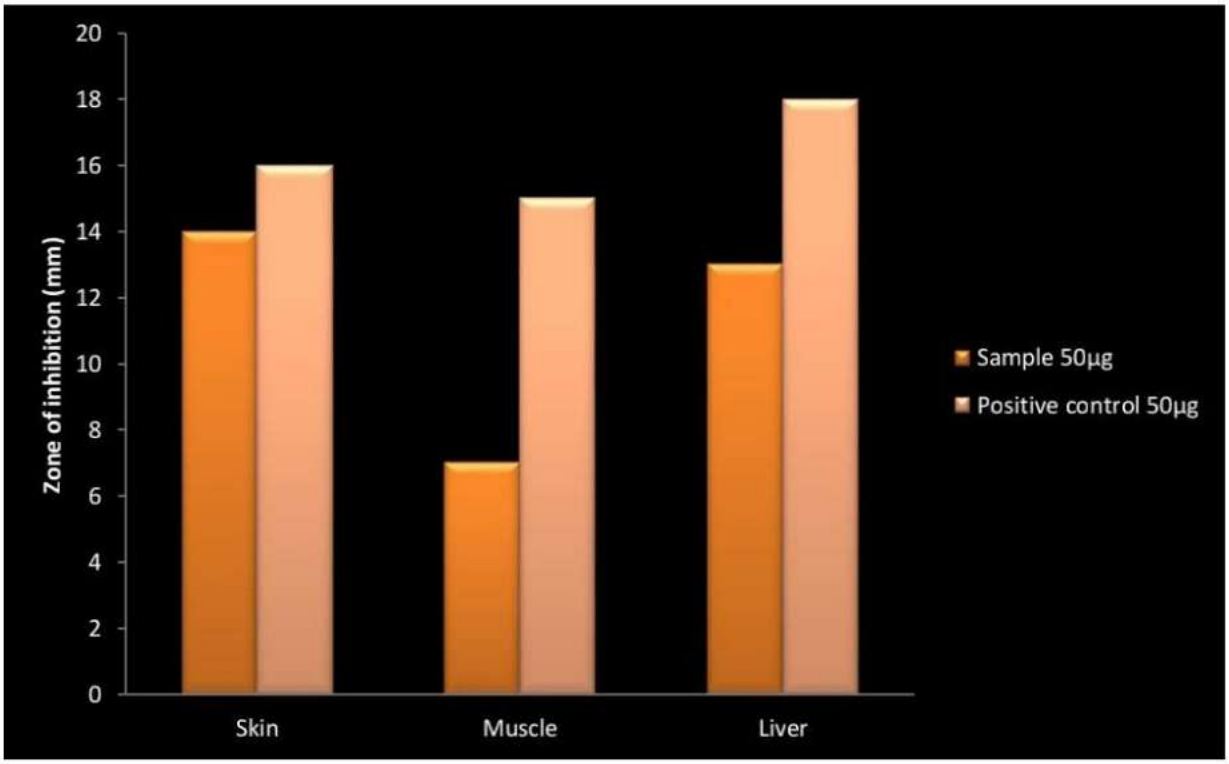
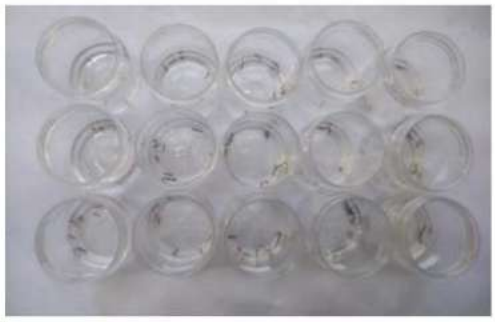


Plate : 5

Photographs showing experimental set up of larvicidal activity of *Lagocephalus spadiceus*

Skin extract

Muscle extract



Liver extract

Fig.3 - Larvicidal activity on mosquito larvae by skin extract of *Lagocephalus spadiceus*

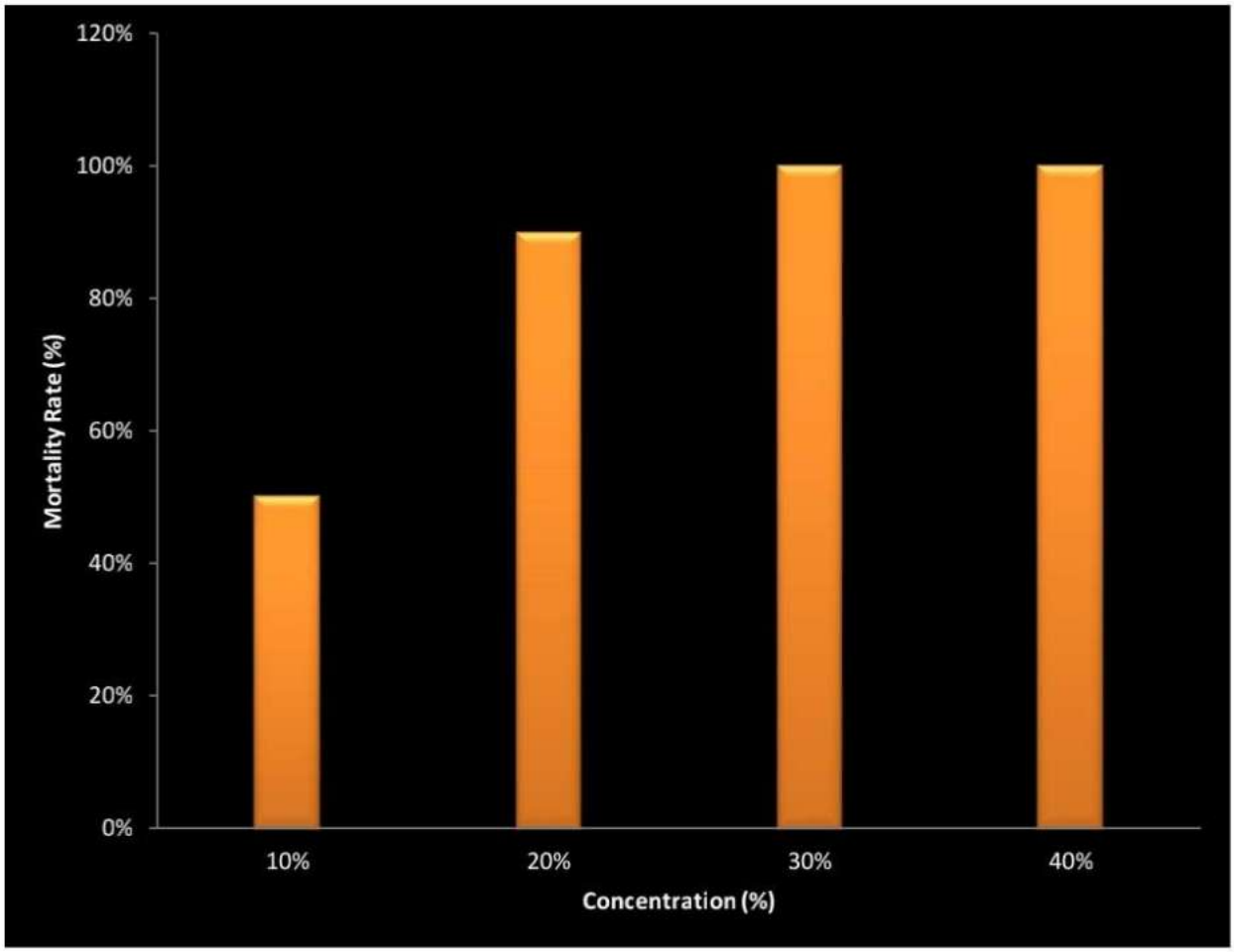


Fig.4 - Larvicidal activity on mosquito larvae by muscle extract of *Lagocephalus spadiceus*

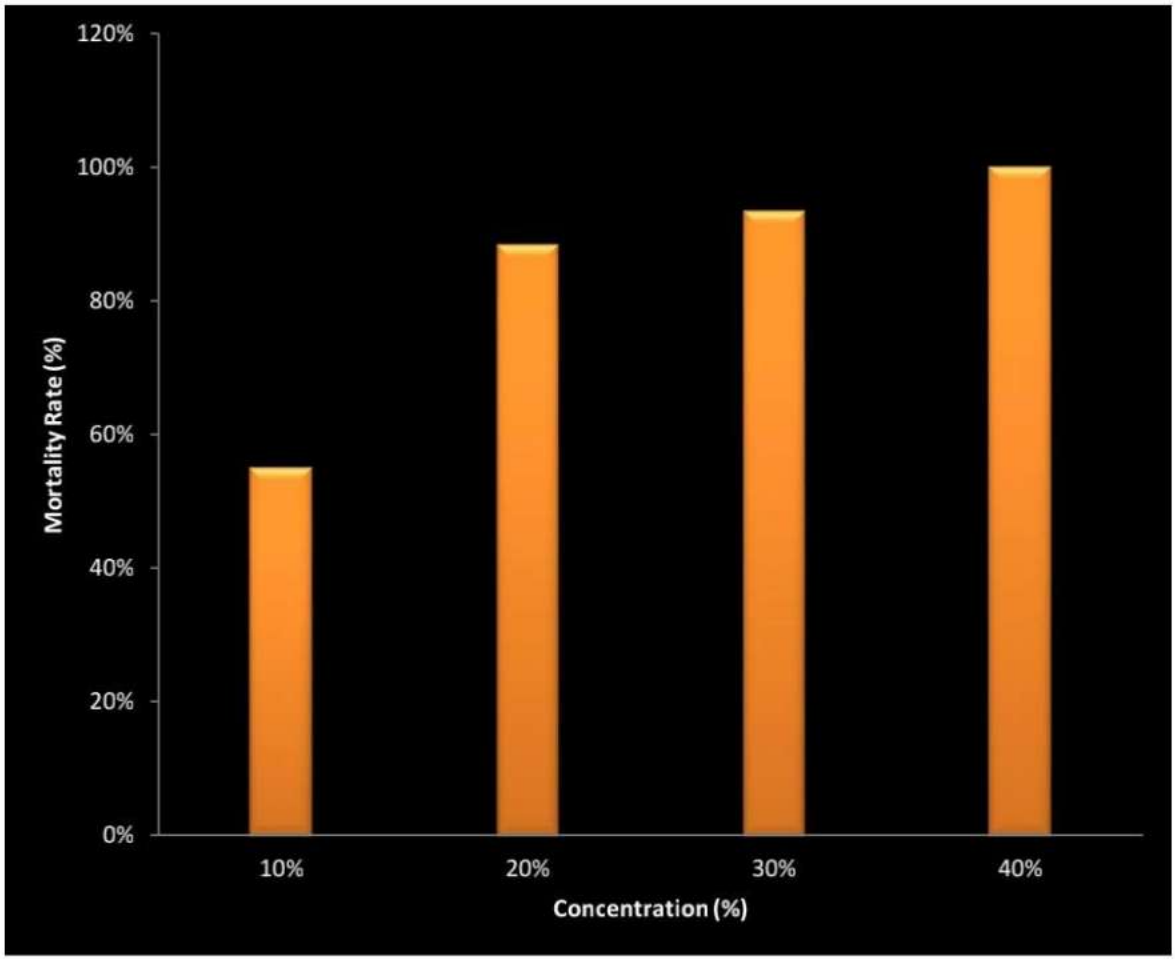


Fig.5 - Larvicidal activity on mosquito larvae by liver extract of *Lagocephalus spadiceus*

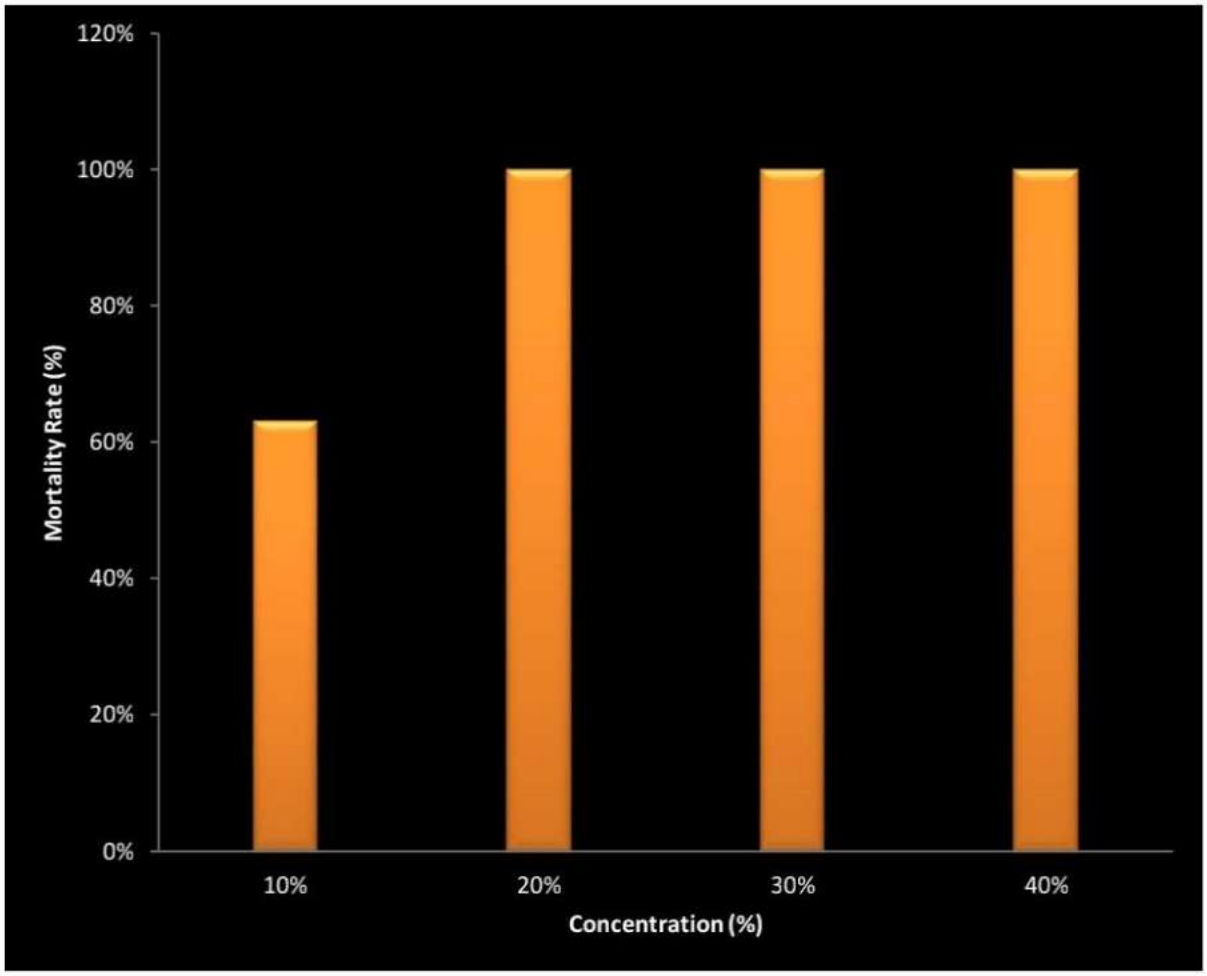


Table 1 - Larvicidal activity of various tissue extracts of *Lagocephalus spadiceus* against mosquito larvae

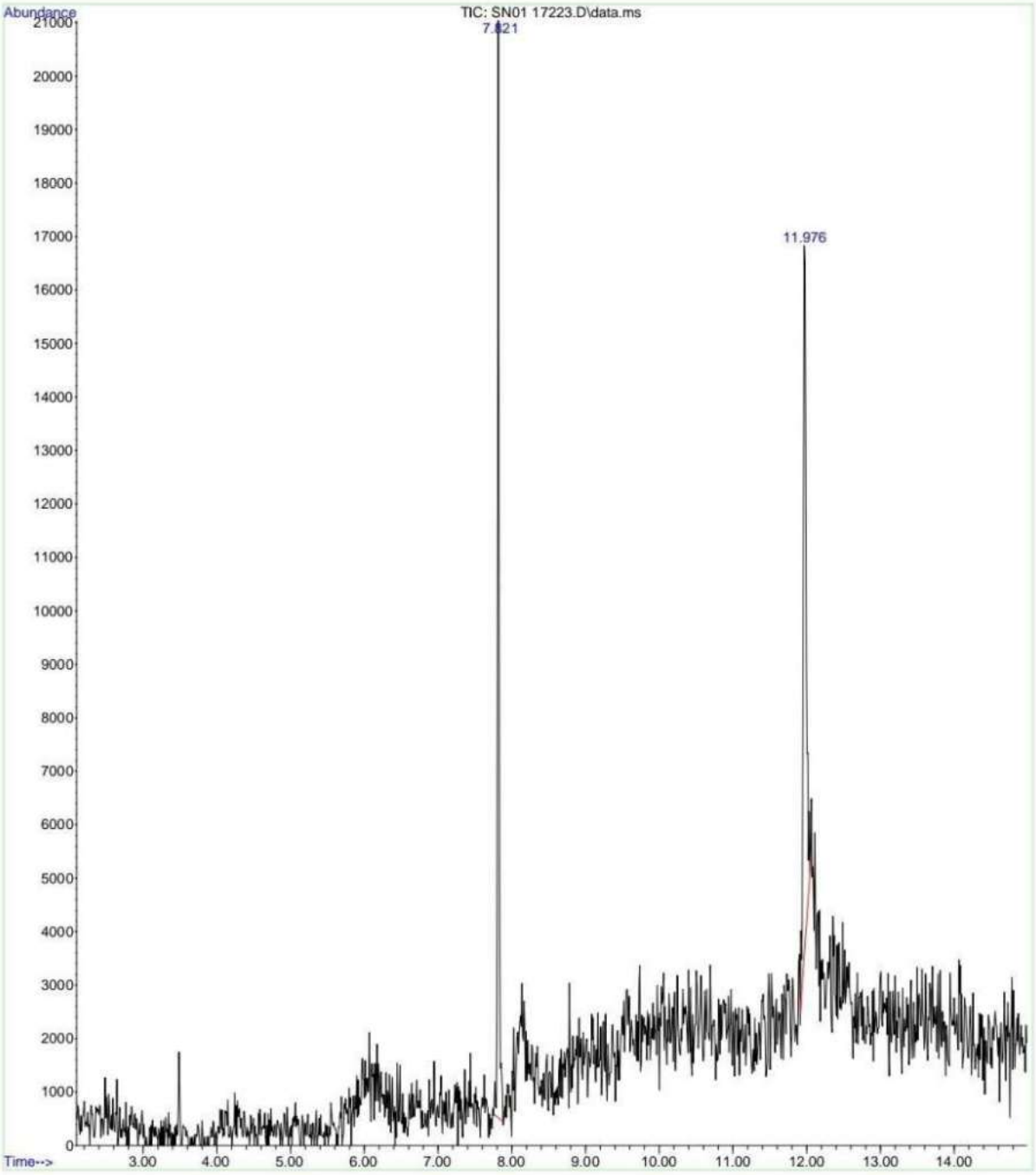
Extract	Concentration				F value	P-value
	10%	20%	30%	40%		
Skin	10±2	18±3.46	20±0	20±0	17	0.000785
Muscle	11±2	17.3±1.15	18.7±1.15	20±0	28.58333	0.000126
Liver	12.7± 3.06	20±0	20±0	20±0	17.28571	0.000742

Mean ± S.D, P<0.05 significant

5.6. GC-MS analysis

The GC-MS analysis of methanol extract of skin of *Lagocephalus spadiceus* revealed the presence of two compounds. They are Diethyl phthalate and n-Hexadecanoic acid (Table 2) (Fig.6-8). The compounds identified by GC-MS analysis was found to have antimicrobial, insecticidal, antioxidant, cancer preventive, nematicide, lubricant and Hyper Cholesterolemic activity.

Fig.6 – Chromatogram – methanol extract of skin of *Lagocephalus spadiceus*



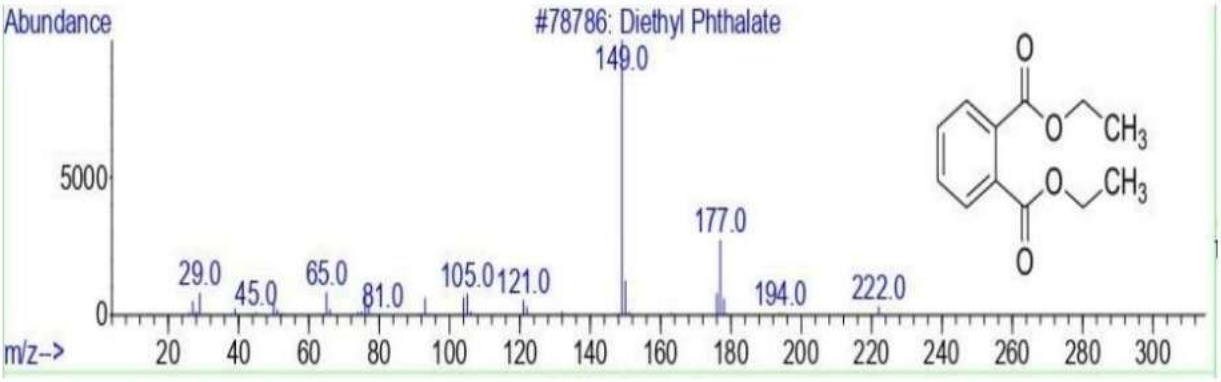


Figure: 7 **Molecular Formula :** $C_{12}H_{14}O_4$

Name: Diethyl phthalate **Molecular Weight** : 222.24 g/mol

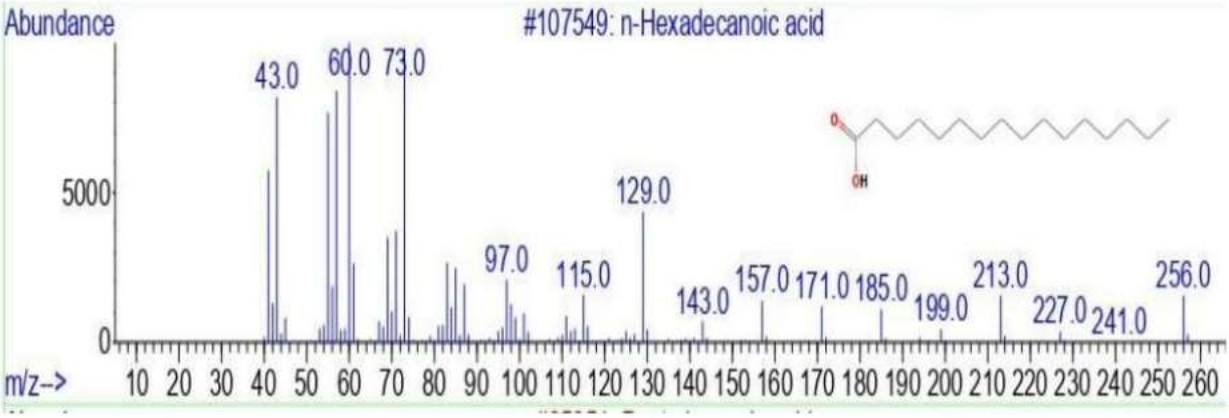


Figure: 8 **Molecular Formula :** $C_{16}H_{32}O_2$

Name: n-Hexadecanoic acid **Molecular Weight** : 256.42 g/mol

Table 2 - Activity of components identified in the skin sample (methanol) of *Lagocephalus spadiceus*

NO	RT	Name of the compound	Molecular formula	MW	Area %	Compound nature	**activity
1.	7.817	Diethyl Phthalate	C12H14O4	222.24 g/mol	44.74	Phthalate ester	Antimicrobial, Insecticidal
2.	11.977	n-Hexa decanoic acid	C16H32O2	256.42 g/mol	55.26	Fatty acid	Antioxidant, Cancer preventive, Nematicide, Lubricant, Hyper Cholesterolemic.

DISCUSSION

6.0 DISCUSSION

The rationale of searching for drugs from marine environment stems from the fact that marine plants and animals have adapted to all sorts of marine environments and these creatures are constantly under tremendous selection pressure including space competition, predation, surface fouling and reproduction. More than 100 antibiotic substances have been isolated from invertebrates. Among these 50 have found widespread use in the prevention and treatment of bacterial disease in animal and man (Gale and Kiser 1967). The first attempt to locate antimicrobial activity in marine organisms was initiated around 1950's (Berkholder and Burkholder 1958, and Nigrelli *et al.*, 1959). Since this time, a large number of marine organisms from a wide range of phyla have been screened for antimicrobial activity (Shaw *et al.*, 1976 and Rinehart *et al.*, 1981). Fish is the earliest and the largest class vertebrate with its innate immune system being considered as the predominant mechanism for host defense (Indumathi *et al.*, 2016) which includes excretion of antimicrobial peptides, polypeptides, non-classical complement activation, cytokine release, inflammation and phagocytosis (Fernandes & Smith, 2002; Magnadóttir, 2006). In recent years, different toxins derived from marine sources have been identified as having potential antimicrobial activities. Precisely, fishes evolved several innate immune mechanisms to defend microbial infection

(Indumathi *et al.*, 2016). In addition, the present study also helps identify the antibacterial and antifungal activity from the puffer fish *Lagocephalus spadiceus*.

We have done antibacterial activity using the acetic acid extract of skin, muscle and liver. In *L. spadiceus*, three samples studied were showed antibacterial activity against the tested five organisms. In the five bacteria tested *E. coli* was more sensitive than the remaining organisms. *Shigella flexneri* and *V. cholerae* were in the next place of showing inhibition activity. Maheswari and Jagadish Naik (2020) discussed that protein extract of skin of marine fish *Chelonodon patoca* performed better antibacterial activity than the liver and ovary extracts tested. Minimal Inhibition Concentration (MIC) values were varied (230µg/ml - 320µg/ml) with each extract. Lower MIC values (230µg/ml) were found for skin protein extract in case of *Salmonella* organisms. On the other hand 320µg/ml MIC was found for the skin protein and skin lipid extracts against *S. aureus*. In the present study, the Minimal Inhibition Concentration (MIC) value of skin extract was found be 100µl against *E. coli*.

Mohana Priya *et al.*, (2013) discussed that skin, muscle and liver extracts of *A.hispidus* showed activity against fungal strains. The skin extract has shown maximum zone against *A. niger* and the liver extract has shown minimum zone against *T. viridae* .Our results also in conjunction with Mohana Priya et al., The skin extract of *L. spadcieus* produced maximum inhibitory zone against *A. niger*.

Similar study was carried out by again Mohana Priya *et al.*, in 2014 using skin extract of *Arothron stellatus* against fungal strains. The antifungal activity was observed maximum against *A. niger* and *A. flavus* and minimum activity against *T. viridae*.

In the present study, larvicidal activities of the puffer fish *L. spadiceus* was evaluated and illustrated. Samidurai *et al.*, in 2013 reported maximum larvicidal activities by the liver and gonads extracts and minimal activity by the skin extract of puffer fish. Indumathi *et al.*, 2016 assessed larvicidal activity of skin, muscle, liver and gonad extracts of puffer fish *Takifugu oblongus* by analyzing the mortality rate of all tested mosquito larvae. They recorded maximum activity by the skin extract, followed by liver and gonads extracts. In the present study, maximum activity was observed in the liver extract, followed by skin and muscle extracts.

Wei Wu *et al.*, (2013) reported a total of 91 volatile compounds were identified under 3 different conditions by GC-MS analysis in cooked meat of puffer fish *Takifugu obscurus*. Neşe Kırımer *et al.*, (2016) discussed that TTX was determined by LC-MS/MS analysis in intestine, liver, ovary and muscle of puffer fish *Lagocephalus sceleratus*. 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 the present study only two compounds were identified in skin

extract of *Lagocephalus spadiceus.* These compounds exhibited antimicrobial, insecticidal, antioxidant, anticancer and nematicide activities.

SUMMARY

7.0 SUMMARY

The present study has been carried out to establish the occurrence of antibacterial, antifungal, larvicidal activities and the characterization of chemical compounds from skin of puffer fish *L. spadiceus*. The highlights of the study are summarized below.

Antibacterial activity of acetic acid extract of skin of marine puffer fish *L. spadiceus* against five bacterial strains viz. *Escherichia coli, Vibrio cholerae, Shigella flexneri, Aeromonas hydrophila* and *Staphylococcus aureus* has been investigated.

The skin extract produced maximum zone of inhibition with 14mm in *E. coli* at 5µl concentration.

MIC value of skin extract was found to be 100µl in *E. coli.*

Antifungal activity of skin, muscle and liver extracts was assessed using *Aspergillus niger* at 50µg. The skin extract exhibited maximum antifungal activity with zone of inhibition 14mm.

Larvicidal response of acetic acid extract of skin, muscle and liver was evaluated against mosquito larvae. Statistical analysis showed significant value $P<0.05$.

GC-MS analysis is a useful tool for chemical analysis. Results obtained suggest that skin extract possess compounds with antibacterial, antifungal, insecticidal, antioxidant, anticancer, lubricant, hyper cholesterolemic and nematicidal activities.

CONCLUSION AND SUGGESTIONS

8.0 CONCLUSION AND SUGGESSIONS

Marine natural products provide a novel and rich source of chemical diversity that can contribute to design and development of new bioactive molecules. As per the concluding remark from the present study skin extract of puffer fish *Lagocephalus spadiceus* shows significant antibacterial effect. The strong antibacterial effect was brought out by skin extract as which may be due to puffer fish skin being an effective source for tetrodotoxin. This study also shows crude extracts of skin, liver and muscles of puffer fish are effective in killing the mosquito larvae. The crude tissue extracts of the puffer fish *Lagocephalus spadiceus s*howed noteworthy bioactivities and so further studies may be recommended to identify the natural bioactive compounds, so that they could be promising candidates for drug development.

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9.0 BIBLIOGRAPHY

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MOLECULAR PHYLOGENETIC ANALYSIS AND BIOSYNTHESIS OF PALLADIUM NANOPARTICLES FROM *CHICOREUS RAMOSUS* (Linnaeus, 1758) AND *STROMBUS CANARIUM* (Linnaeus, 1758)

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THOOTHUKUDI-628001

April - 2023

CERTIFICATE

This is to certify that this dissertation entitled, **"MOLECULAR PHYLOGENETIC ANALYSIS AND BIOSYNTHESIS OF PALLADIUM NANOPARTICLES FROM *CHICOREUS RAMOSUS* (Linnaeus, 1758) AND *STROMBUS CANARIUM* (Linnaeus, 1758)"** submitted by **S. SORNA REVATHI, Reg. No. 21APZO08** to St. Mary's College (Autonomous), Thoothukudi affiliated to Manonmaniam Sundaranar University, Tirunelveli in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2022-2023 under my guidance and supervision. It is further certified that this dissertation or any part of this has not been submitted elsewhere for any other degree.

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DECLARATION

I do hereby declare that this dissertation entitled, **"MOLECULAR PHYLOGENETIC ANALYSIS AND BIOSYNTHESIS OF PALLADIUM NANOPARTICLES FROM *CHICOREUS RAMOSUS* (Linnaeus, 1758) AND *STROMBUS CANARIUM* (Linnaeus, 1758)"** submitted by me for the award of the degree of Master of Science in Zoology is the result of my original independent research work under the guidance of Dr. P. Subavathy M.Sc., M.Phil., SET., Ph.D., Assistant Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

Place: Thoothukudi

Date: 05.04.2023

S. Soorna Revathi.

Signature of the Candidate

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CONTENTS

1. INTRODUCTION

India is one of the twelve mega biodiversity countries. However, the marine fauna of India are not same everywhere. The phylum Mollusca constitutes dominant group of animals and includes familiar forms such as clams, oysters, squids, octopus and snails belonging to seven classes namely Aplacophora, Monoplacophora, Polyplacophora, Gastropoda, Bivalvia, Scaphopoda and Cephalopoda. Among these, Gastropoda, Bivalvia and Cephalopoda are considered as major classes which include commercially important species. Molluscs are the most diverse phylum of marine life, with more than 50,000 described species, coupled with a shortage of taxonomists (Bouchet, 2006).

Marine mollusc shells enclose a wealth of information on coastal organisms and their environment. Their life history traits as well as (palaeo-) environmental conditions, including temperature, food availability, salinity, and pollution, can be traced through the analysis of their shell (micro-) structure and biogeochemical composition. Adding to this list the DNA entrapped in shell carbonate bio minerals potentially offers a novel and complementary proxy both for reconstructing palaeo environments and tracking mollusc evolutionary trajectories (Clio Der Sarkissian *et al*., 2017).

Shells are bio mineral exoskeletons offering protection from predation

and desiccation to the soft bodies of invertebrates (Marin *et al*., 2012). Molluscs are prominent producers of shells, which despite microstructure variation among species, all consist of both a calcified and an organic layer (0.01-5.00% in weight) containing pigments, polysaccharides, lipids and proteins (Marin *et al.*, 2012). Marine mollusc shells survive in the fossil record and provide invaluable information about the past. Their morphology can for instance be used for phylogenetic inference (Ponder & Lindberg, 2008), while their microstructure can reveal important life history traits, including growth rates (Chauvaud *et al*., 2012) and diseases (Paillard *et al*., 2004 and Trinkler *et al*., 2010). Their internal structure is also commonly examined for sclerochronological dating (Gröcke and Gillikin, 2008 and Butler *et al*., 2013) or uncovering past changes in seawater temperature and salinity (Hiebenthal *et al*., 2012 and Reynolds *et al*., 2016).

DNA extracted from marine mollusc shells can potentially offer an extremely informative and complementary proxy. As DNA from both inside and outside the shell could be entrapped during bio mineral formation, shell DNA might be used not only for marine mollusc species identification, phylogeo graphic and demographic reconstructions, but also for bacterial community and environmental DNA profiling. Despite such potential, no work has evaluated the long-term preservation of DNA in marine mollusc shells and

hence, their potential for ancient DNA studies (Clio Der Sarkissian *et al*., 2017).

DNA barcoding can be useful in identifying specific taxonomic groups or developmental stages of various invertebrates in situations where species determination based on morphology is extremely problematic (Boyer *et al*., 2011; Meyer *et al*., 2013), as well as in cases where a damaged organism is present or just a tissue fragment is available (Schander *et al*., 2005).

DNA barcoding employs sequence diversity in a 648 base pair region of the cytochrome c oxidase subunit I (COI) gene to distinguish species (Hebert PDN *et al*., 2003; Hebert *et al*., 2004; Carr *et al*., 2010). Past work has shown that sequence divergences are generally much greater between than within species (Hebert *et al*., 2003). Because of this fact, DNA barcoding aids both the identification of known species and the discovery of over looked taxa (Witt *et al*., 2006). The latter application has revealed that the incidence of sibling species is often high enough to lead to serious inaccuracies in estimates of biodiversity (Carr *et al*., 2010 and Knowlton, 2000). In light of this, it is increasingly recognized that molecular approaches need to be incorporated into biodiversity surveys.

In addition, molluscs exhibit complex larval stages frequently have

cryptic taxa, and substantial phenotypic plasticity, all factors that impede morphological approaches to species identification (Drent *et al*., 2004 and Marko & Moran 2009). Because morphological analysis confronts so many challenges, it is imperative to integrate molecular diagnostics into the identification of molluscs.

Several prior studies have validated the efficacy of DNA barcoding in the discrimination of mollusc species, but most of this work has targeted a particular family or order. In recent years, increasingly violent and vigorous impacts of global climate change, coastal environment deterioration and anthropogenic activities have resulted in marked decline of biodiversity, and the numbers of endangered marine molluscs species have been distinctly increased. Moreover, the marine molluscs present a significant challenge for morphological approaches to specimen identification because they exhibit differences in life stage, frequently have morphologically cryptic taxa, and substantial phenotypic plasticity (Drent *et al*., 2004; Marko & Moran, 2009), which hampered the conservation and management of the richest diversity of this taxa. In this sense, reliable specimen identification and biodiversity monitoring of organism in the field is quite necessary.

Nanotechnology and nano science have undergone a remarkable revolution in the current century by exploring this mysterious area of research.

Nanotechnology is implemented in all disciplines of science including engineering, information technology, material science, and life science, along with clarifying astonishing facts regarding human health, specifically in cancer treatment. The array of atoms on a 1–100 nm scale, nano devices, systems, and structures makes them feasible in the research area (Bayda *et al*., 2019).

Currently, growing environmental concern requires the development of green and environmentally sustainable strategies for the preparation of metal nanoparticles (Kanchi and Ahmed, 2018 and Shafey, 2020). Metal nanoparticles (MNPs) with a high surface to volume ratio; serve an important role in a wide range of disciplinary field such as catalysis, bio-diagnostics, medicine, drug- delivery, pharmacology, energy production and environmental remediation.

Among the various MNPs, due to their unique electronic and chemical properties, palladium nanoparticles (PdNPs) have attracted major attention for their high efficiency as heterogeneous nano catalysts for various organic transformations such as C-C coupling reactions, hydrogenation of alkenes and alkynes, oxidation reactions, reduction of nitroarenes and degradation of dyes (Bej *et al*., 2016; Luo, *et al*., 2019; Hong *et al*., 2020; Li Astruc, 2018 and Saldan *et al*., 2015)

The catalytic and biological activities of palladium nanoparticles are closely related both in their shape and size and in the nature of the dispersing/capping agent. The increased interest in environmental issues has led to the development of various green approaches for the preparation of efficient, low-cost and environmentally sustainable palladium nano catalysts. Environmentally friendly solvents, non-toxic reducing reagents, biodegradable capping and stabilizing agents and energy-efficient synthetic methods are the main aspects that have been taken into account for the production of Pd nanoparticles in a green approach (Oriana Piermatti *et al*., 2021).

Palladium, is a chemical element with atomic number 46. It is a silvery-white lustrous rare metal that was discovered in 1803 by the English chemist William Hyde Wollaston. Pd was named after the asteroid Pallas that had previously been discovered, in 1802. Pd, together with platinum (Pt), rhodium (Rh),ruthenium (Ru), iridium (Ir), and osmium (Os) form a group of metals that are referred to as Platinum Group Metals (PGMs). These elements have similar chemical properties overall, but Pd is the least dense and has the lowest melting temperature amongst them (Nadeem Joudeh *et al*., 2022). Palladium nanoparticles and Pd-based alloy clusters have been extensively investigated in the last decades. Palladium nanoparticles form a versatile catalyst that has a wide range of applications in organic reactions such as in hydrogenation (Favier

et al., 2019).

In addition to the catalysis of organic reactions, applications of Palladium nanoparticles include fuel cells (Antolini, 2009) and hydrogen sensing and storage (Adams and Chen, 2011). Potential electronics applications also include sensors (Silva *et al*., 2011). Moreover, several applications in biomedical therapies including photo thermal, antibacterial, and anticancer therapies have been developed or discussed (Phan *et al*., 2020).

Recently, the biogenic synthesis of nanoparticles has attracted attention from the scientific community as it could in principle provide simple, rapid, cost-effective, and potentially more environmentally friendly alternative processes for the synthesis of nanoparticles. These processes are based on the use of naturally occurring bio molecules or metabolites from different organisms as reducing and stabilizing agents. In addition, these biogenic processes have been shown to offer very high levels of control over the properties of NPs, such as the size and shape (Qazi *et al*., 2016; Vishnukumar *et al*., 2017; Fahmy *et al*., 2020 and Manjare *et al*., 2021).

In addition to having a wide range of antimicrobial and anticancer properties, palladium nanoparticles have also shown promising results in several biomedical therapy approaches. Due to the diversity in their size and shape, photo thermal conversion efficiency, and photo stability, Palladium

nanoparticles are emerging as potentially efficient photo thermal therapy agents (Phan *et al*., 2020). NP-mediated photo thermal therapy (cancer cell death by heat generated in tumor tissue) has shown great potential for the treatment of cancer. Pd NPs could also play an important role in improving current cancer treatment technologies such as chemo- and radiotherapy (Song *et al*., 2018) have demonstrated that porous hollow Pd NPs can deliver 131I (a radio isotope that is commonly used in radio therapy) and DOX (a chemotherapy drug that is commonly used in chemotherapy) *in vivo* in mice injected with MCF-7 breast cancer cells. Moreover, Palladium nanoparticles have also shown promising results in other biomedical areas such as gene therapy (Kang *et al*., 2018), drug delivery (Gil *et al*., 2018), pro drug activation (Weiss *et al.,* 2014), and biosensors applications (Yi *et al.*, 2017).

Traditionally, Pd NPs are synthesized via various physical or chemical methods with the aid of toxic and hazardous reducing and stabilizing agents, however, over the years, in the quest of 'going green', there has been a paradigm shift towards bio-inspired strategies for the synthesis of metal NPs (Narayanan KB, Sakthivel, 2011; Khan *et al.,* 2014 and Basavegowda *et al.*, 2015). The biocompatibility and environmentally benign properties attributes to these biological techniques to supersede the conventional physical and wet-

chemical methods. The field of biological synthesis of Palladium nanoparticles encompasses the use of plant extracts, microorganisms, marine organisms, *etc* as green-reductants. Amongst those methods, the tapping of bio-resources, particularly the plant extracts for the synthesis of the NPs seems promising owing to their ready availability, rapid process, better cost-effectiveness, and the ability to use in large-scale biosynthesis (Akhtar *et al.,* 2013).

Antioxidants play a major role in balancing the free radicals since these free radicals mostly attack macromolecules (lipids, proteins and nucleic acids) which lead to cell damage. The higher accumulation of reactive species leads to diverse chronic pathologies, such as development of cancer, cardiovascular and neurodegenerative diseases (Dauthal Mukhopadhyay, 2013; Ajitha Reddy, 2015; Dipankar Murugan, 2012 and Mata *et al.,* 2015). Metal-based nanoparticles werediscovered 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 (Akhtar *et al.,* 2017; Mohammad *et al.,* 2008; Lushchak *et al.,* 2018; Zhang *et al.,* 2021). 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.

Green synthesized metal nanoparticles have an incredible aptitude against diabetes and regulate the functioning of diabetes by α-amylase release from pancreases, colonic, α-glucosidase, insulin levels, glycemic absorption, and other histochemistry characteristics during *in-vivo* and *in vitro* studies (Bhardwaj *et al.,* 2020). A metabolic disorder known as "diabetes" is usually considered as elevated glucose level in the blood presently affecting more than 100 million people worldwide (Guariguata *et al.,* 2014). Thus, there is an urgent need to develop nano medicine which can facilitate significant inhibition of carbohydrate-hydrolysing enzymes with high degree of specificity as well as to achieve maximal therapeutic efficacy with minimal side effects (Etxeberria *et al.,* 2012).

Inflammation and diabetes mellitus is the most prevalent clinical issues worldwide (Bornstein *et al.,* 2020; 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; 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; Narayanan *et al*., 2021b).

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).

The phylum mollusca is considered important among all the marine organism. By considering its importance, the marine gastropod *Chicoreus*

ramosus and *Strombus canarium* were selected to study the molecular phylogenetic analysis and biogenic synthesis, characterization and applications of palladium nanoparticles. The tissue extract of marine gastropods revealed the biosystematics and pharmacological efficacy of synthesized palladium nanoparticles.

2. REVIEW OF LITERATURE

DNA barcoding of marine organism has been ongoing around the world for more than a decade to distinguish cryptic and invasive species as well as to help explain different puzzling lifecycles, making it a convenient tool for assessment of marine biodiversity (Barco *et al.,* 2016; Trivedi *et al,* 2016; Ramiez *et al.*, 2020 and Yang Zhang, 2020).

Teske *et al.* (2007) reported that the sympatric intertidal limpets (Siphonariidae) off coastal southeast Africa lacked barcode differences, suggesting they are morphotypes of a single species. It has been reported that only one single study has reported successful PCR amplification of nine nuclear microsatellites and a single mitochondrial (mtDNA) barcode from shells of freshwater pearl mussels *Margaritifera margaritifera* (Geist *et al.,* 2008).

Aside from enabling identifications for whole specimens, barcode analysis opens up new possibilities- it can provide identifications during any stage of development. Puillandre *et al.* (2009b) clearly demonstrated the ability of barcodes to identify gastropod larvae, although barcode data are sparse and taxonomic coverage is biased toward shallow water species.

Many studies have validated the efficacy of DNA barcoding in specimen identification and species discovery for molluscs. Zou *et al.*

(2011) demonstrates the effectiveness of the character-based barcoding method for specimen identification in Neogastropoda. Two clams of the genus *Donax* showed no significant barcode variation and were found to represent one species. Barcodes have also revealed lack of genetic differentiation among some species of molluscs, given that not all morphological differences are the result of cladogenesis. Several prior studies have established the value of DNA barcoding in resolving morphologically cryptic species complexes in several molluscan families.

Ghiselli *et al*. (2012) studied de movo assembly of the Manila clam Ruditapes philippinarum transcriptome provides new insights into expression bias, mitochondrial doubly uniparental inheritance and sex determination. Audzijonyte *et al*., (2012) investigated the molecular taxonomy reveals broads trans-oceanic distribution and high species diversity of deep-sea clams. Zou *et al*. (2012) analyzed multigene barcoding and phylogeny of geographically widespread muricids along the coast of china.

Ratnasingham *et al.* (2013) showed the DNA – based registry for all animal species: the Barcode Index Number (BIN) system. Zouros *et al.*, (2013) analyzed biparental inheritance through uniparental transmission: the doubly uniparental inheritance (DVI) of mitochondrial DNA. Milani *et al.*, (2013) reported the comparative analysis of mitochondrial ORFans: new

clues on their origin and role in species with doubly uniparental inheritance of mitochondria.

Boyle *et al.* (2013) evaluated heteroplasmy in a deep-sea prosobranch bivalve suggests an ancient origin of doubly uniparental inheritance of mitochondria in Bivalvia. 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.

Layton *et al.* (2014) showed the patterns of DNA barcode variation in Canadian marine molluscs. 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 micro fouling activity. Knebelsberger *et al.* (2015) showed the molecular diversity of Germany's freshwater fishes and lampreys assessed by DNA barcoding. Monaliben Shah *et al*. (2015) synthesized metallic nanoparticles via biological entities.

Safaa Ali *et al*. (2015) investigated the applications of biosynthesized silver nanoparticles for the control of land snail *Eobaniavermiculata* 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) synthesized palladium nanoparticles using gum ghatti (*Anogeissus latifolia*) and studied their applications as an antioxidant and catalyst. Barco *et al.* (2016) identified North Sea molluscs with DNA barcoding.

Bera and Belhaj (2016) reported green synthesis of silver nanoparticles using plant extracts and their antimicrobial activities. The interaction of Ag NPswith known commercial antibiotic drugs has also been comprehensively studied. Dmitri Talapin and Elena Shevchenko (2016) gave an introduction to nanoparticles chemistry. Khwaja Salahuddin Siddiqui and Azamal Husen (2016) studied the green synthesis and investigated the characterization and uses of palladium and platinum nanoparticles.

Pejovic *et al.* (2016) studied DNA barcoding for assessment of exotic molluscs associated with maritime ports in Northern Iberia. Shao Sun *et al.* (2016) evaluated the DNA barcoding reveal patterns of species diversity amongnorthwestern Pacific molluscs. Furfaro *et al.* (2016) reported a DNA barcoding approach to the phenotypic diversity of mediterranean species with a preliminary phylogenetic analysis. Sun *et al.* (2016) analyzed the DNA barcoding reveal patterns of species diversity among Northwestern Pacific molluscs.

Yu Ioni *et al*. (2016) synthesized graphene with noble metals nanoparticles on its surface. 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 effort highlighted the nanohybrid systems with synergistic antibacterial properties to overcome the emerging antibiotic resistance as well as to define fruitful applications in biomedicine.

Nanoparticles synthesis using biological organisms by green synthesis technology is biologically safe, cost-effective, and environment-friendly. The recent development in the field of nanotechnology brought the identification of unexpected beneficial properties of metal nanoparticles such as gold, platinum, palladium, graphene and silver nanoparticles (Maria Arshad *et al*., 2018). 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. Liu *et al.* (2018) reported the DNA barcoding for species identification in deep-sea clams. 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.

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).

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. Ran *et al.* (2020) reported the DNA barcoding for identification

of marinegastropod species from Hainan Island, China.

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) synthesized palladium nanoparticles fabricated magnetic Fe_3O_4 nanocomposites over *Fritillariaimperialis* 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. Moira Bursic *et al.* (2021) evaluated the DNA barcoding of marine molluscs associated with *Corallina officinalis* Turfs in Southern Istria (Adriatic sea).

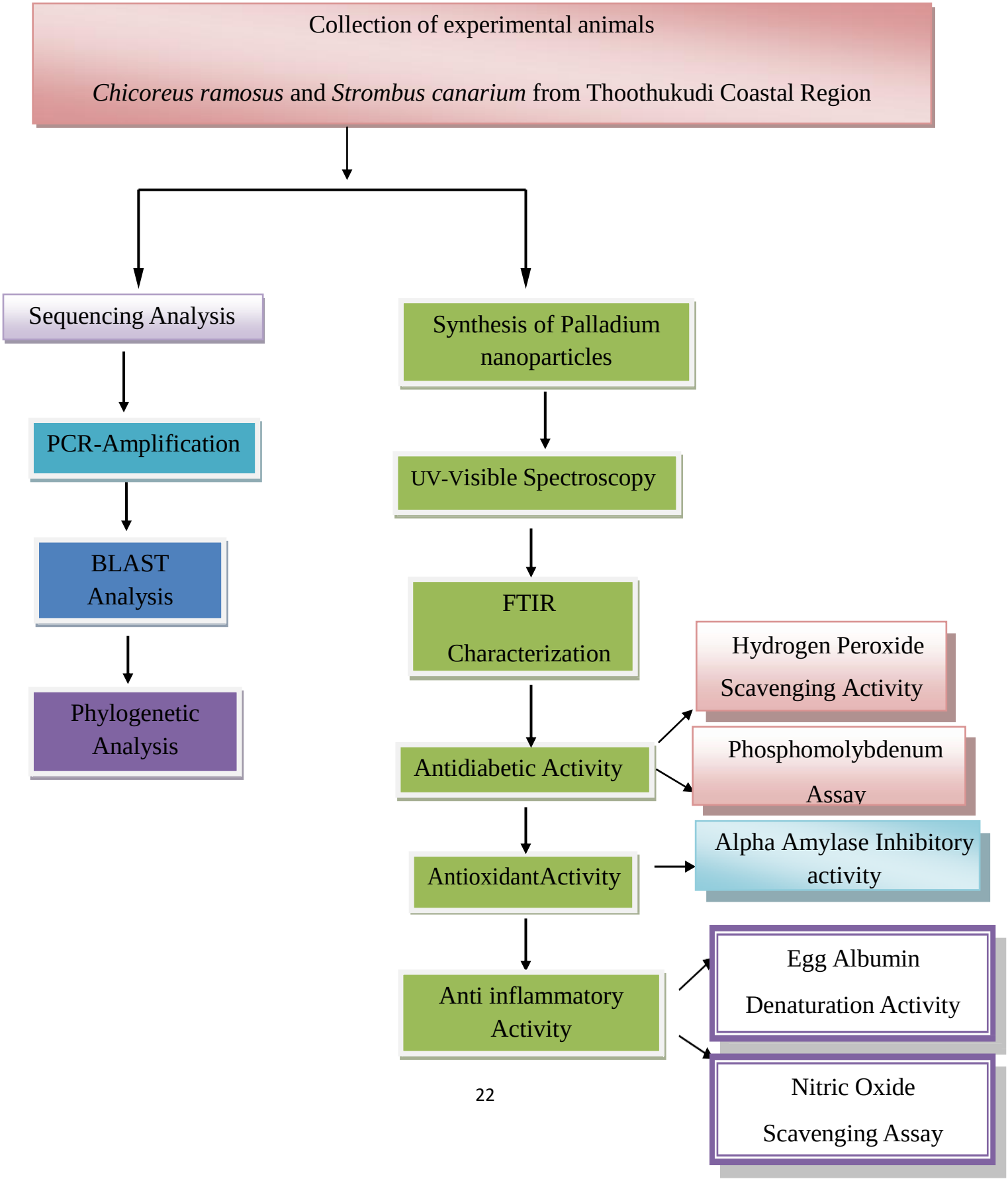
From the above review it was understand that the phylum mollusca, considered to be important and abundant among marine organism. So, the current study has been carried out to investigate the molecular phylogeny and synthesis of palladium nanoparticles using the tissue extract of marine gastropod *C. ramosus* and *S. canarium.*

3. OBJECTIVES

Molluscs are the largest of the phyla represented in the marine realm, even though currently the numbers of species are likely to be highly underestimated. Within the largest class of molluscs, the Gastropoda, the order Neogastropoda is the most species – rich, with over 10,000 extant species. Over the past few decades, with the advent of molecular techniques, there is growing information available with regard to the phylogenetic affinities among different groups of gastropods by means of widely used mitochondrial (COX I) & nuclear markers (18S, 28S) in reconstructing their phylogenetic affinities. However, there is a need for new nuclear markers in studying gastropodan phylogeneies especially with regard to deeper divergences. The present study has been carried out with the following objectives:

- To evaluate the phylogenetic affinities in the different groups of gastropodspecies *Chicoreus ramosus* and *Strombus canarium*
- To study the utility of COX I gene to understand the phylogeny of *Chicoreus ramosus* and *Strombus canarium* especially with regard to divergence of internal nodes.
- To analyze the antioxidant, antidiabetic and anti-inflammatory activities of palladium nanoparticles using the marine gastropods *Chicoreus ramosus* and *Strombus canarium*.

4. EXPERIMENTAL DESIGN



5. MATERIALS AND METHODS

5.1 Description of the study area

The Gulf of Mannar is located between India and Srilanka, stretches fromthe 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 animals

In the present study the gastropods *Chicoreus ramosus and Strombus canarium* were collected from the Gulf of Mannar coastal region (Plate 1). The neogastropod *Chicoreus ramosus* and Caenogastropoda *Strombus canarium* were 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 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, tissues were removed 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 *Chicoreus ramosus* (Linnaeus, 1758)

Phylum	: Mollusca
Class	: Gastropoda
Subclass	: Caenogastropoda
Order	: Neogastropoda
Super family	: Muricoidea
Family	: Muricidae
Genus	: *Chicoreus*
Species	: *ramosus*

The shell is moderately large, solid, rough and heavy, globose shaped, spire short, body whorl slightly inflated, sculptured with thick foliaceous spines on varices, siphonal canal moderately long, colour half white, aperture whitish with light rose pink colour along the aperture margin, outer lip conspicuously crenulated, columella rose pink.

Shell is large upto 254 mm in length, fusiform, protoconch rounded and smooth. It consists of six rounded whorls, suture impressed and body whorl large and globose. Aperture large, ovately rounded, anal silcus shallow and broad with a parietal ridge. The outer lip has coarse dentations and a prominent tooth on the lower half, inner side lirate. The columella is detached anteriorly but partly adherent posteriorly forming a callus. The siphonal canal is of moderate size, broad and narrowly open. The shoulder spine is prominent followed by five of equal length with spine lets in between and canal with three spines decreasing in size anteriorly. The colour of the shell is light brown to dark with pinkish tinge on spiral cords.

5.3.2 Systematic position of *Strombus canarium* (Linnaeus, 1758)

Phylum : Mollusca

Class : Gastropoda

Subclass : Caenogastropoda

Order : Littorinimorpha

Super family : Stromboidea

Family : Strombidae

Genus : *Strombus*

Species : *canarium*

Laevistrombus canarium (commonly known as the dog conch or by its better-known synonym, *Strombus canarium*) is a species of edible sea snail,

a marine gastropod mollusc in the family Strombidae (true conches). The shell of adult individuals is coloured from light yellowish-brown to golden to grey. It has a characteristic inflated body whorl, a flared, thick outer lip, and a shallow stromboid notch. The shell is valued as an ornament, and because it is heavy and compact, it is also often used as a sinker for fishing nets. The external anatomy of the soft parts of this species is similar to that of other strombid snails.

The animal has an elongated snout, thin eyestalks with well-developed eyes and sensory tentacles, and a narrow, strong foot with a sickle-shaped operculum. *S. canarium* lives on muddy and sandy bottoms, grazing on algae and detritus. It is gonochoristic and sexually dimorphic, depending on internal fertilization for spawning. Larvae of this species spend several days as plankton, undergoing a series of transformations until they reach complete metamorphosis. The maximum life span is 2.0 to 2.5 years. The dog conch is an economically important species in the Indo-West Pacific, and several studies indicate that it may be suffering population declines due to overfishing and overexploitation.

5.4 Genomic DNA isolation:

DNA isolation from tissue samples were done using the Expure Tissue DNAisolation kit developed by Bogar Bio Bee stores Pvt Ltd.,

Protocol:

1. Lysis/homogenization: Grind approx., 50mg of sample with 500 µl of lysis buffer in a 2 ml micro centrifuge tube and lyse the cells by repeated pipetting.

2. Add 4 µl of RNAs and 500 µl of neutralization buffer into it.

3. Vortex the content and incubate the tubes up to 1 hour at 65˚C in water bath. To minimize shearing the DNA molecules, mix DNA solutions by inversion.

4. Centrifuge the tubes for 10 minutes at 10,000 rpm.

5. Following centrifugation, transfer the resulting viscous supernatant into a fresh 2 ml micro centrifuge tube without disturbing the pellet.

6. Add 600 µl of Chloroform Isoamly Alcohol and do hand mixing vigorously.

7. Centrifuge the tubes for 10 minutes at 10,000 rpm. Carefully, transfer 600 µl of aqueous phase into a fresh 2ml micro centrifuge tube.

8. Binding Add 600 µl of binding buffer to the content and mix thoroughly by pipetting and incubate the content at room temperature for 5 minutes.

9. Transfer 600 µl of the contents to a spin column placed in 2 ml collection tube.

10. Centrifuge for 2 minutes at 10,000 rpm and discard flow-through.

11. Reassemble the spin column and the collection tube then transfer the remaining 600µl of the lysate.

12. Centrifuge for 2 minutes at 10,000 rpm and discard flow-through.

13. Washing: Add 500 µL washing buffer I to the spin column. Centrifuge at 10,000 rpm for 2 mins and discard flow- through.

14. Reassemble the spin column and add 500µl washing buffer II and Centrifuge at 10,000 rpm for 2mins and discard flow-through. Dry spin the tube for 5 minutes at 10,000 rpm.

15. Transfer the spin column to a sterile 1.5-ml micro centrifuge tube

16. Elution**:** Add 100 µl of Elution buffer at the middle of spin column. Care should be taken to avoid touch with the filtrate.

17. Incubate the tubes for 2 minutes at room temperature and Centrifuge at 10,000 rpm for 2 minutes. The buffer in the micro centrifuge tube contains the DNA.

18. DNA concentrations were measured by Qubit flurometer or 1% Agarose GelElectrophoresis.

5.4.1 PCR Protocol

Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single stranded DNA template. DNA

polymerase adds nucleotides to the 3`end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA.

Composition of the Taq Master Mix

- Taq DNA polymerase is supplied in 2X Taq buffer
- 0.4mM dNTPs
- 3.2mM $MgCl_2$ and
- 0.02%bromophenolblue

5.4.2 Primer Details

Primer Name	Sequence Details	Number of Base
LCO1490	5'GGTCAACAAATCATAAAGATATTGG3'	25
HCO2198	5'TAAACTTCAGGGTGACCAAAAAATCA3'	26

Add 5 µL of isolated DNA in 25 µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). Perform PCR using the following thermal cycling conditions.

1. **Denaturation:**

The DNA template is heated to 95°C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA.

2. **Annealing:**

The mixture is cooled to anywhere from 40℃. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

3. **Extension:**

The reaction is then heated to 72°C, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

5.4.3 PCR Condition

STAGES	TEMPERATURE	TIME	CYCLE
Initial Denaturation	95˚C	2 min	25
Denaturation	95˚C	30 sec	
Annealing	40˚C	30 sec	
Extension	72˚C	2 min	
Final extension	72˚C	10 min	
Hold	4˚C	∞	

Purification of PCR Production

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).The PCR product was sequenced using the primers. Sequencing reactions were performed using a ABI PRISM® Big DyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

5.4.4 Sequencing Protocol

Single-pass sequencing was performed on each template COX1 universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

5.4.5 Bioinformatics Protocol

1. The sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

2. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar, 2004). The resulting aligned sequences were cured using the program G blocks 0.91b.This G blocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana, 2007). Finally, the

program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

3. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering (Dereeper *et al*., 2008).

5.5 Synthesis of Palladium Nanoparticles

Synthesis of Palladium nanoparticles is carried out using 0.01M Palladium chloride in double-distilled water using *Chicoreus ramosus and Strombus canarium*. Palladium chloride and the tissue extract of *Chicoreus ramosus and Strombus canarium* 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 orange color within 1 hr. The whole reaction was carried out in the dark. The obtained suspension was centrifuged at 15,000 rpm for 15 min. The pellet containing palladium 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.

5.5.1 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 palladium nanoparticles by using *Chicoreus ramosus and Strombus canarium* 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 palladium 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 palladium 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

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 4mM 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

5.8 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 (40 μl at concentrations ranging from 50 μg/ml to 500 μg/ml) were mixed in 96-well microplates with 40 μl of amylase solution (100 U/ml in 0.1M sodium 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 distilled water) were added and the absorbance was measured at 580 nm. Results were expressed as IC_{50} values (µg/ml). A carbose was used as the standard.

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

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, and 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 Assay

The anti-inflammatory activity of synthesized palladium nanoparticles of tissue extract of *Chicoreus ramosus* and *Strombus canarium*, were 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, 2 ml of sample extract (concentrations ranging from 50 to 500 µg/ml)or standard and 2.8 ml of

phosphate buffered saline (pH7.4) were mixed together to form a reaction mixture of total volume 5 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:

$$\text{Percentage Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance control}} X100$$

5.9.3 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 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 an 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:

$$\%\text{NO scavenged} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance control}} \text{X100}$$

6. RESULTS

6.1 PCR Amplification of DNA Barcoding Region

DNA barcoding region of COX 1 gene in *Chicoreus ramosus and Strombus canarium* were successfully amplified. From the gel analysis, the highly intense bands without smearing strongly indicated the amplification of COX 1 gene. All the species were given good amplified products. The bands of size 550bp was observed in *C. ramosus*, 620bp was observed in *S. canarium* (Plate 2). There was no overlapping of the bands in the case of the test organisms and that way the bands were clear (Plate 2).

6.2 Sequencing

The PCR amplified products were successfully read using an ABI 3730 genetic analyzer following manufacturer's instructions. The sequences with good peak clarities were selected for further analysis. The sequences of about 512 bases were observed in *C. ramosus* and 577 bases were reported in *S. canarium* (Figure.2a, 2b, 3a and 3b).

6.3 Sequences Producing Significant Alignments from NCBI

The sequences were checked for considerable alignments from NCBI. In *C. ramosus* and *S. canarium* samples of about 8 and 9 sequences showed significant alignments of which the maximum identity ranged from 98% to 88%

and 97% to 87%. The maximum score ranged from 881 to 625 in *C. ramosus*, 990 to 643 in *S. canarium* respectively. The query coverage was found to be97% in *C. ramosus* and 100% in *S. canarium* respectively (Fig. 2c and 3c).

6.4 Accession numbers of sequences

All the submitted sequences were assigned accession numbers by Genbank. The accession numbers are OQ449645 and OQ449646 (Table 1).

6.5 Phylogenetic Analysis

Phylogenetic tree of the two species were constructed by using Tree Dyn 198.3 program. In *C.ramosus* two major clades were formed, one major clade comprising 2 species and the next clade comprising of 5 species. In *S. canarium* topologically two major clades were formed, one major clade comprising 2 species and the next clade comprising of 4 species. *Chicoreus ramosus* SV01 was phylogenetically closer to the clade of FJ784239.1_*Chicoreus ramosus*. *Laevistrombus canarium* SV02 was phylogenetically closer to the clade of NC_053786.1 *Laevistrombus canarium*. The genus and species level discrimination can be ascertained by strong bootstrap support of over 100%. Throughout the tree, several clades have moderate 61% in *C. ramosus* and high 88% in *S. canarium* levels of bootstrap support (Fig.2d and 3d).

6.6 Synthesis and Characterization of Palladium Nanoparticles

6.6.1 UV-Visible Spectroscopic Analysis

UV-Visible spectroscopic analysis confirmed the formation of the biosynthesized palladium nanoparticles using the marine molluscan tissue extract of *Chicoreus ramosus and Strombus canarium* (Figure 4). The above solutions were subjected to optical measurements by UV-Visible spectrophotometer. In *C. ramosus*, the wavelength obtained around 480 nm suggested the presence of palladium nanoparticles in the solution (Figure 4a). This is the specific wavelength which indicates synthesized palladium nanoparticles. The maximum absorption was obtained around 480nm. In *S. canarium*, the wavelength obtained around 430 nm suggested the presence of palladium nanoparticles in the solution (Figure 4b). This is the specific wavelength which indicates synthesized palladium nanoparticles. The maximum absorption was obtained around 430 nm. The occurrence peak at absorption intensity between 200 to 900 nm indicated the presence of surface plasmon resonance.

6.6.2 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 bio molecules in *C.ramosus* and *S.canarium* tissue extracts which are 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 tissue extract.

The results of FTIR analysis of this study show different stretches of bonds shown at different peaks in *C.ramosus*; 3476.87, 3145.97, 1637.82, 1560.50, 1400.81, 1119.18, 1048.96, 842.61, 683.49, 612.49, 473.52 cm^{-1}. The image shows a strong absorption peak around 3476.87cm^{-1} to 1400.81cm^{-1} which shows the presence of C-H stretching vibration. A peak around 400cm^{-1} to 1100 cm^{-1} shows the presence of C-O stretching frequency. A peak around finger print region confirms the presence of palladium nanoparticles (Figure 4c).

In *S. canarium* different stretches of bonds are shown at different peaks in 3455.36, 3150.06, 2067.20, 1636.93, 1400.48, 1111.01, 1025.20, 686.04, 538.81 cm^{-1}. The image shows a strong absorption peak around 3455.36cm^{-1} to 1400.48cm^{-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 palladium nanoparticles (Figure 4d).

6.7 Antioxidant Activity

6.7.1 Hydrogen Peroxide Radical Scavenging Activity

The hydrogen peroxide radical scavenging activity of marine molluscan tissue extract of *C. ramosus* 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 79.28% was observed at 500 µg/ml followed by 56.64% at 250 µg/ml, 48.93% at 100 µg/ml and 32.46% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 76.27% was observed at 500 µg/ml followed by 52.73% at 250 µg/ml, 43.12% at 100 µg/ml and 31.28% at 50 µg/ml respectively. The IC_{50} value of 53.29 µg/ml was noted for *C. ramosus* and 56.13 µg/ml for *S. canarium* 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 5).

6.7.2 Phosphomolybdenum Scavenging Assay

The phosphomolybdenum scavenging assay of marine molluscan tissue extract of *C. ramosus* 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 86.27% was observed at 500 µg/ml followed by 74.29% at 250 µg/ml, 63.91% at 100 µg/ml and 50.29% at 50 µg/ml

respectively. In *S. canarium* the highest percentage inhibition of 75.23% was observed at 500 µg/ml followed by 67.98% at 250 µg/ml, 55.67% at 100 µg/ml and 43.24% at 50 µg/ml respectively. The IC_{50} value of 86.01 µg/ml was noted for *C. ramosus* and 56.15 µg/ml for *S. canarium* 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).

6.3 Antidiabetic Activity

The α-amylase activity of marine molluscan tissue extract of *C. ramosus* was observed at various concentrations of 500 µg/ml, 250 µg/ml, 100 µg/ml and50 µg/ml respectively. The highest percentage inhibition of 69.34% was observed at 500 µg/ml followed by 58.63% at 250 µg/ml, 47.82% at 100 µg/ml and 39.27% at 50 µg/ml respectively. In S. canarium the highest percentage inhibition of 67.83% was observed at 500 µg/ml followed by 54.64% at 250 µg/ml, 46.29% at 100 µg/ml and 35.26% at 50 µg/ml respectively. The IC50 value of 21.27 µg/ ml was noted for C. ramosus and 24.06 µg/ ml for S. canarium 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 7).

6.4Anti-inflammatory Activity

6.4.1 Egg Albumin Denaturation Activity

The egg albumin denaturation activity of marine molluscan tissue extract of *C. ramosus* 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 85.39% was observed at 500 µg/ml followed by 78.26% at 250 µg/ml, 65.91% at 100 µg/ml and 51.02% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 81.92% was observed at 500 µg/ml followed by 73.26% at 250 µg/ml, 61.09% at 100 µg/ml and 44.23% at 50 µg/ml respectively. The IC_{50} value of 13.23 µg/ ml was noted for *C. ramosus* and 29.31 µg/ ml for *S. canarium* 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 8).

6.4.2 Nitric Oxide Scavenging Assay

The nitric oxide scavenging assay of marine molluscan tissue extract of *C. ramosus* 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 77.84% was observed at 500 µg/ml followed by 69.82% at 250 µg/ml, 58.67% at 100 µg/ml and 49.26% at 50 µg/ml respectively. In *S.*

canarium the highest percentage inhibition of 71.86% was observed at 500 µg/ml followed by64.28% at 250 µg/ml, 53.18% at 100 µg/ml and 45.27% at 50 µg/ml respectively. The IC_{50} value of 41.5 µg/ ml was noted for *C. ramosus* and 45.34 for *S. canarium* 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).

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

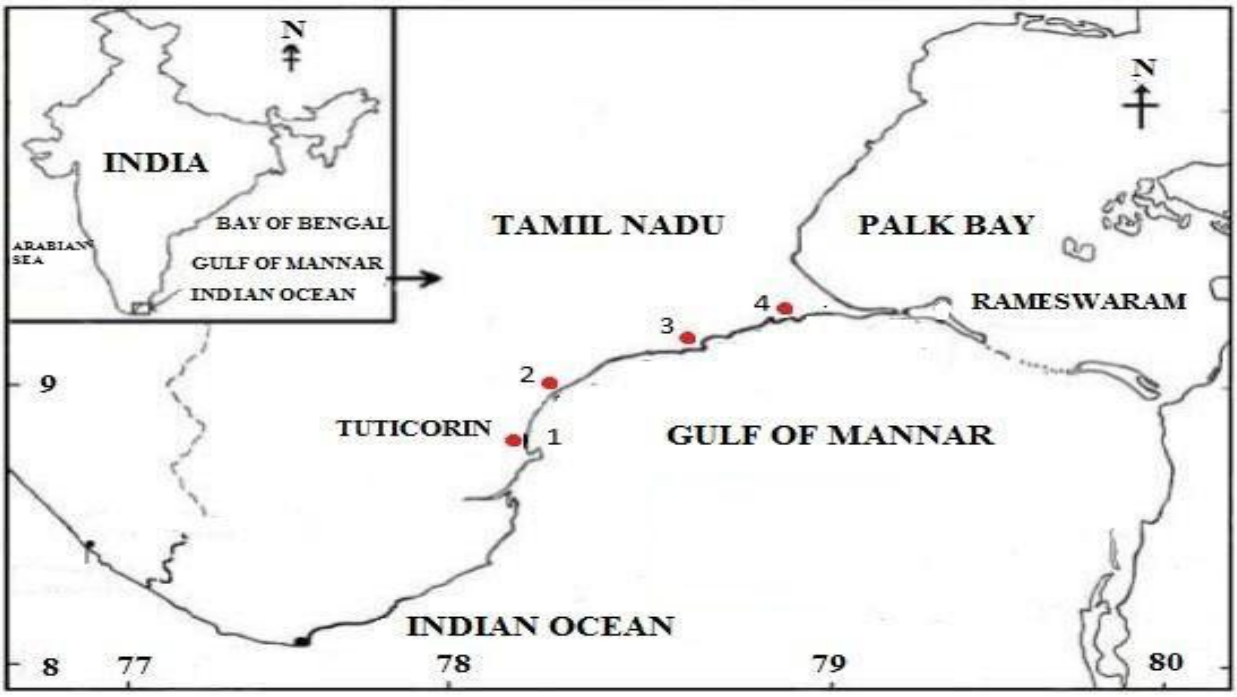
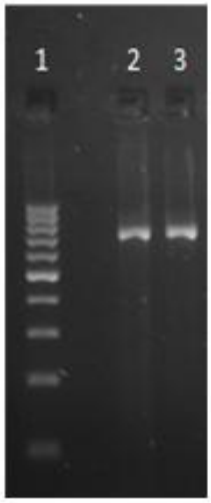


Plate 1: Dorsal and ventral view of *Chicoreus ramosus* and *Strombus canarium*

Plate 2: Agarose gel electrophoresis image of PCR



WELL NO	SAMPLE ID
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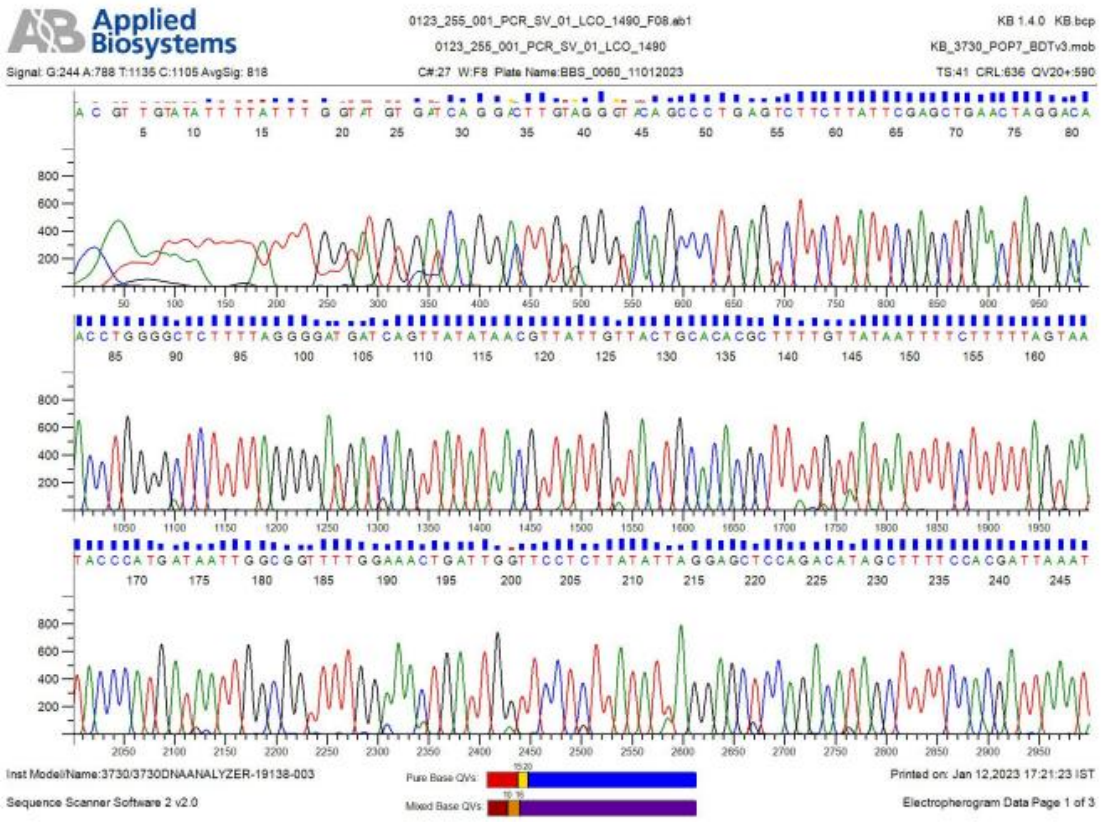
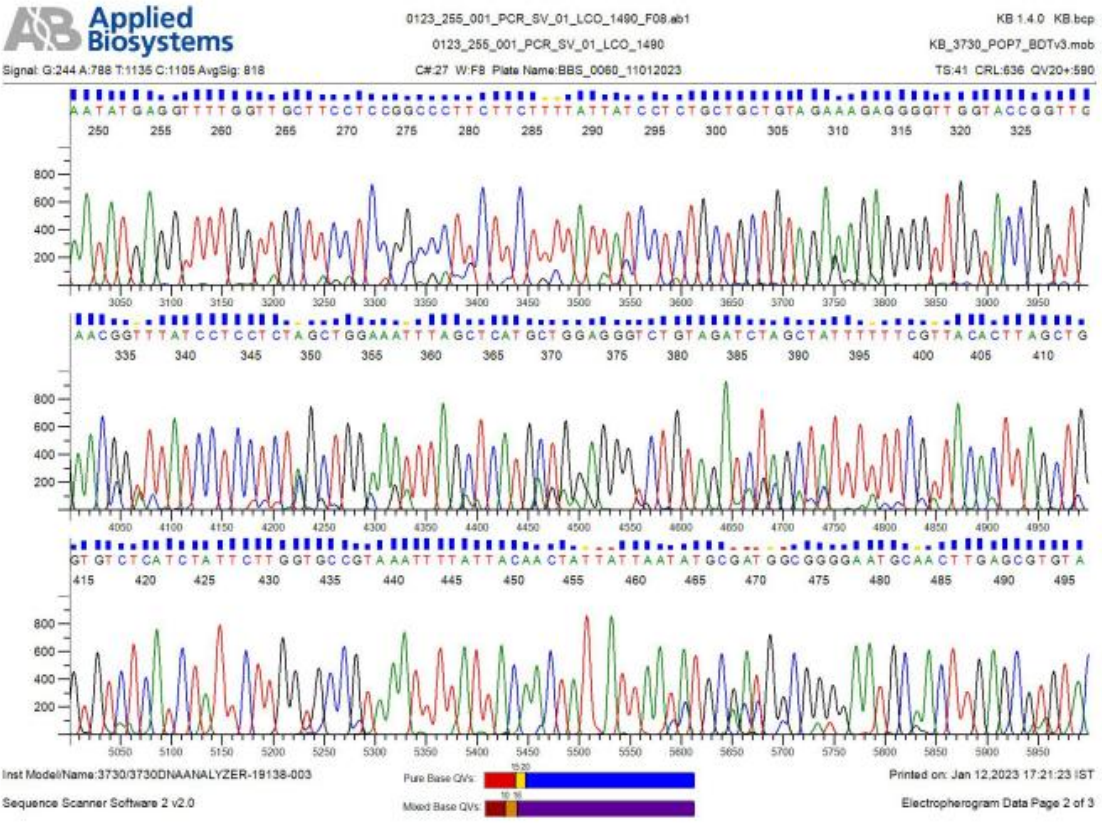
2	*Chicoreus ramosus*
3	*Strombus canarium*

Figure 2a: Chromatogram of Multiple Sequence Alignment – *C. ramosus*

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Mixed Base QVs:
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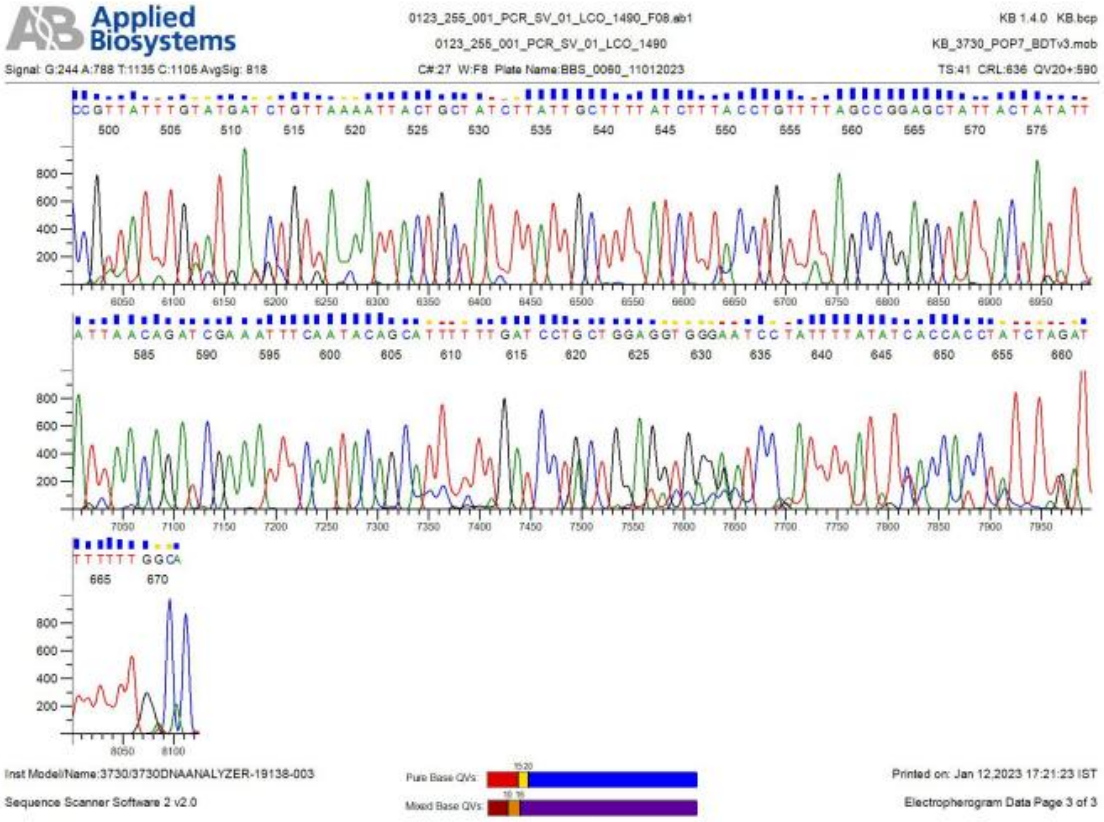
Electropherogram Data Page 2 of 3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Sequence Scanner Software 2 v2.0
Pure Base QVs
Mixed Base QVs
Printed on: Jan 12,2023 17:21:23 IST
Electropherogram Data Page 3 of 3

Figure 2b: Sequence of *Chicoreus ramosus*

Chicoreus ramosus isolate SVO1 cytochrome c oxidase subunit I (COX1) gene, partial cds: mitochondrial

AGTTATATAACGTTATTGTTACTGCACACGCTTTTGTTATAATTTTCTTTTTAGTAATACCCAT
GATAATTGGCGGTTTTGGAAACTGATTGGTTCCTCTTATATTAGGAGCTCCAGACATAGCTTT
TCCACGATTAAATAATATGAGGTTTTGGTTGCTTCCTCCGGCCCTTCTTCTTTTATTATCCTCT
GCTGCTGTAGAAAGAGGGGTTGGTACCGGTTGAACGGTTTATCCTCCTCTAGCTGGAAATTTA
GCTCATGCTGGAGGGTCTGTAGATCTAGCTATTTTTTCGTTACACTTAGCTGGTGTCTCATCTA
TTCTTGGTGCCGTAAATTTTTATTACAACTATTATTAATATGCGATGGCGGGGAATGCAACTT
GAGCGTGTACCGTTATTTGTATGATCTGTTAAAATTACTGCTATTTTATTGCTTTTATCTTTAC
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ATCCT

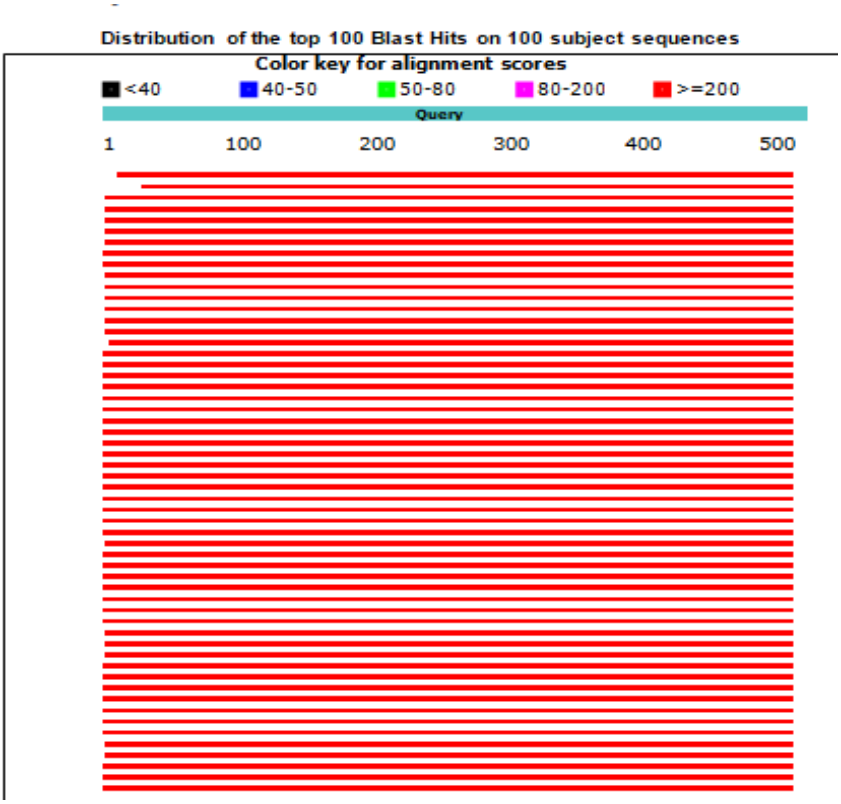


Figure 2c: Sequences producing significant alignments

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Laevistrombus canarium mitochondrion, complete genome	990	990	100%	0.0	97.76%	NC_053788.1
Tridacnidus dactylus voucher MNHN-IM-2019-1738 mitochondrion, complete genome	671	671	99%	0.0	87.91%	NC_059923.1
Strombus labiatus voucher USNM:1467137 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	654	654	97%	0.0	87.92%	MZ559454.1
Doxander vittatus isolate LSGB_M_075 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	649	649	97%	0.0	87.63%	MN389054.1
Macrostrombus obscurus isolate XN25 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	649	649	97%	0.0	87.63%	JF893438.1
Macrostrombus obscurus isolate X3112 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	649	649	97%	0.0	87.63%	JF893428.1
Mycistrombus vokdalis mitochondrion	647	647	100%	0.0	87.09%	MW244824.1
Strombus urceus cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	645	645	96%	2e-180	87.81%	DQ525237.1
Strombus labiatus voucher USNM:1467136 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	643	643	97%	9e-180	87.57%	MZ559787.1
Strombus labiatus voucher USNM:1467138 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	643	643	97%	9e-180	87.57%	MZ559455.1
Strombus labiatus voucher USNM:1467135 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	643	643	97%	9e-180	87.57%	MZ559453.1
Macrostrombus obscurus isolate X3111 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	643	643	97%	9e-180	87.46%	JF893427.1
Strombus labiatus cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	640	640	96%	1e-178	87.63%	DQ525230.1
Doxander vittatus isolate LSGB_M_076 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	638	638	99%	4e-178	86.85%	MN389055.1

Figure 2d: Phylogenetic Tree of *Chicoreus ramosus*

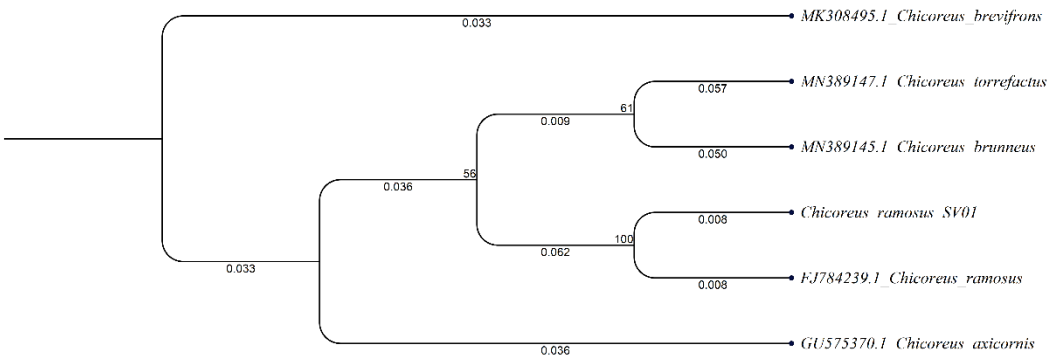
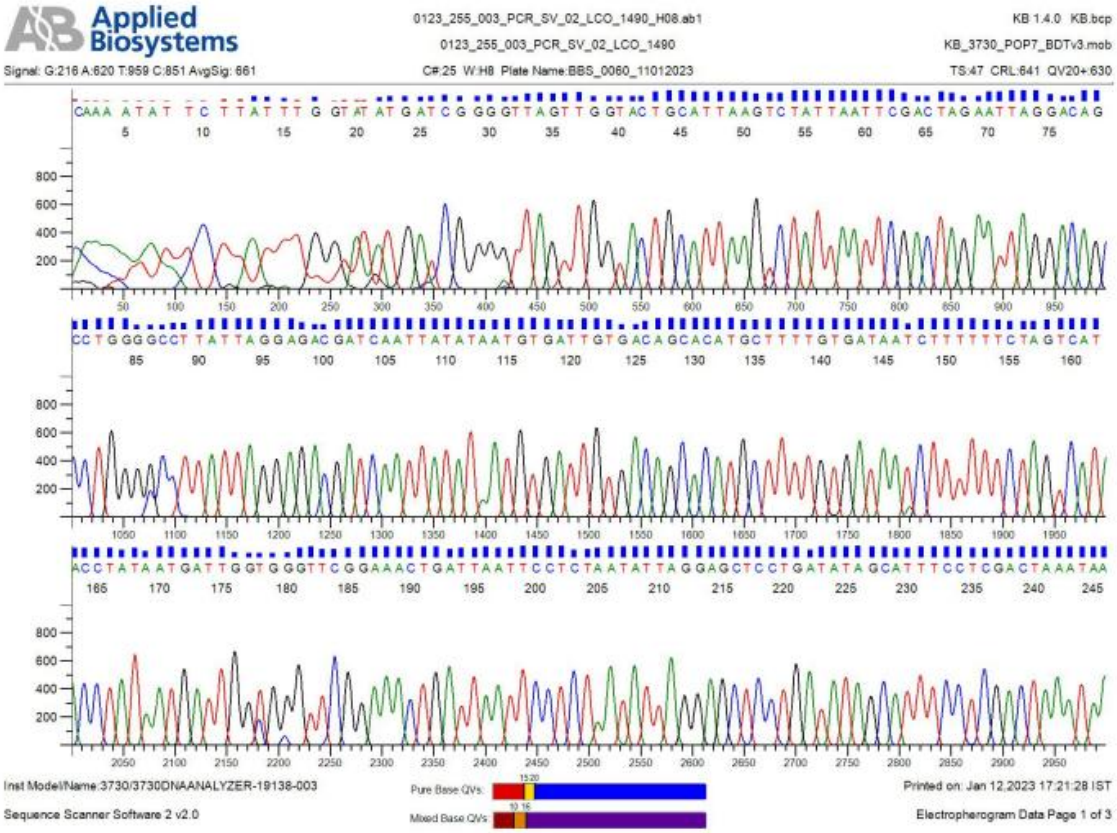
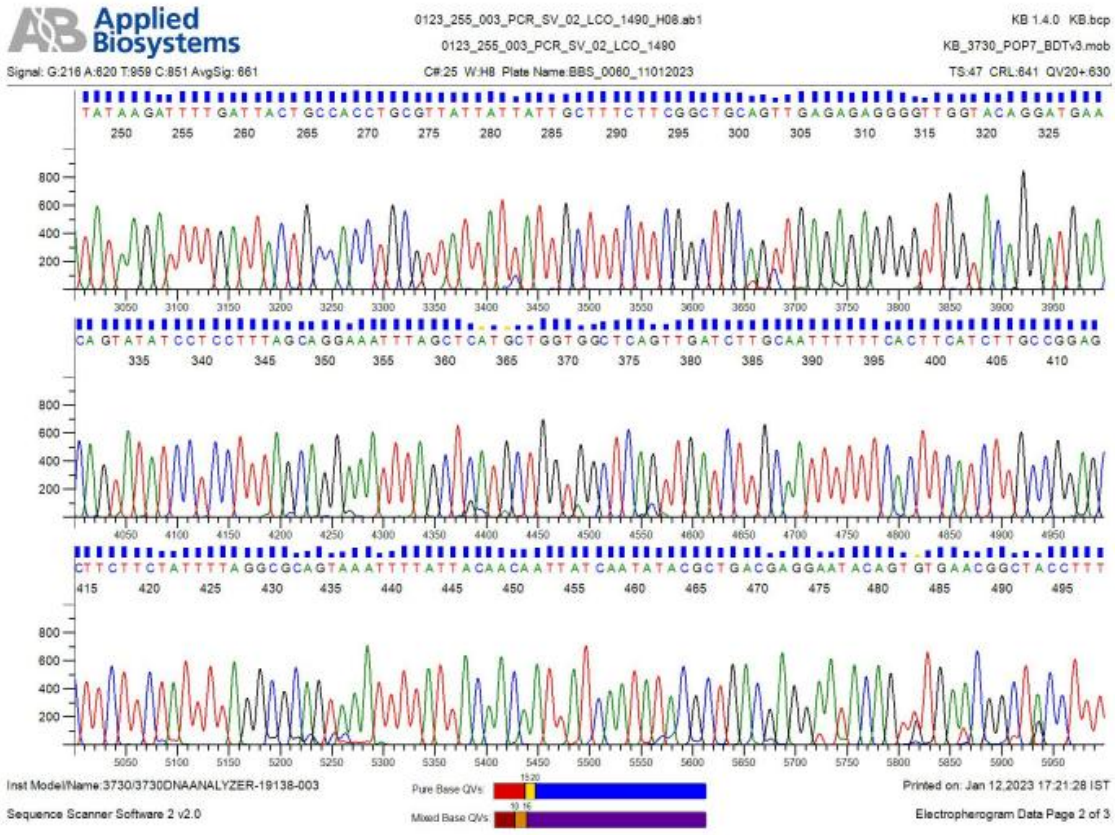


Figure 3a: Chromatogram of Multiple Sequence Alignment – *S. canarium*

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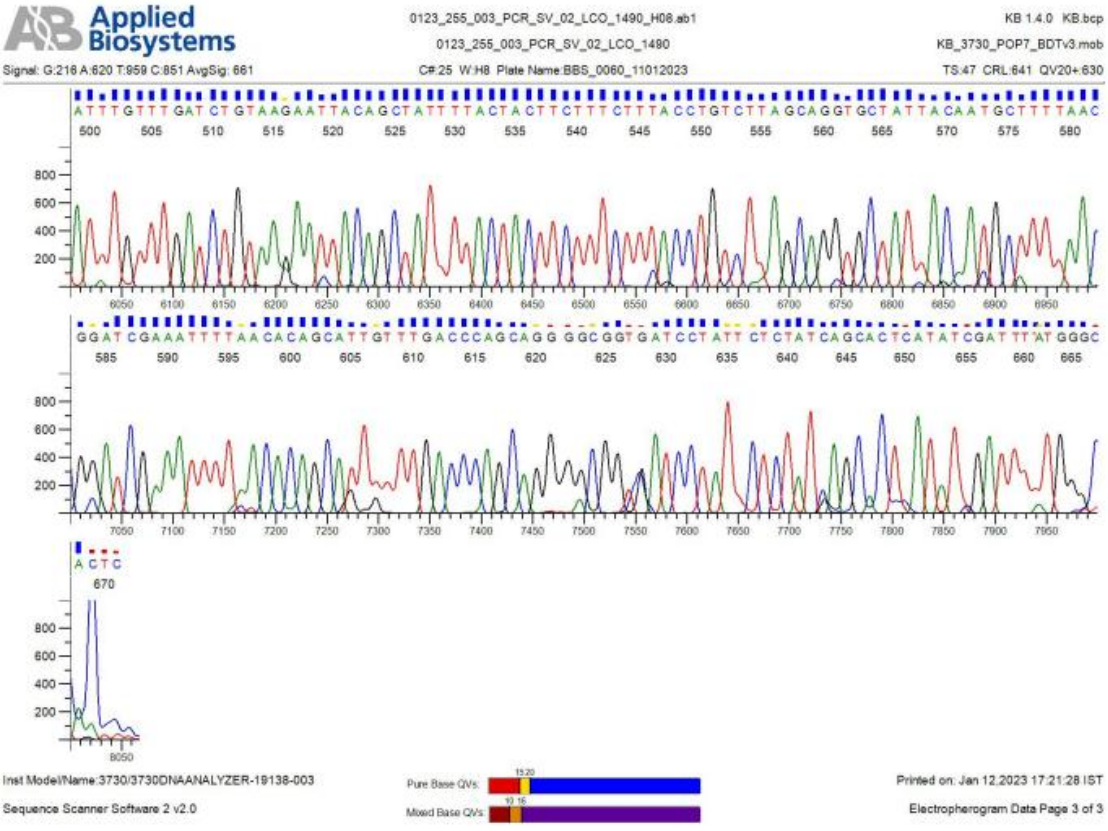
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Pure Base QVs:
Mixed Base QVs:
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Electropherogram Data Page 3 of 3

Figure 2 b: Sequence of *Laevistrombus canarium*

Laevistrombus canarium isolate SVO2 cytochrome c oxidase subunit I (COX1) gene, partial cds: mitochondrial

GGAGATGATCAATTATACAATGTAATTGTTACAGCTCACGCTTTTGTTATAATTTTTTTCTTA
GTTATGCCTATAATAATTGGTGGCTTTGGAAACTGGTTAGTTCCGCTAATATTAGGAGCTCC
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TTACTTTCATCTGCAATGCTGTCGAAGGAGCGGAGTTGGTACAGGATGAACAGTATATCCTC
CGAGCTGGAAACCTTAGCTCATGCTTTGGGGCTCAGTTGATTTGGCTATTTTTTCATTACACT
TAGCTGGTGTATCTTCTATTTTAGGTGCAGTTAATTTTATTACTACAATTATTAATATGCGAT
GACGAGGGATACAATTTGAACGCTTCCTTTATTTGTTTGATCGGTAAAAATTACTGCTGTGC
TCTTATTACTTTCTTTACCTGTTTTAGCTGGAGCTATTACAATGCTTTTAACTGATCGAAATTT
TAACACTGCATTTTTTGACCCTGCTGGAGGGGGAGATCCTATTTTATATCAACATCTATTCTG
ATTTTTTGGCC

Figure 3c: Sequences producing significant alignments

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
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Tridentarius dentatus voucher MNHN-IM-2019-1738 mitochondrion, complete genome	671	671	99%	0.0	87.91%	NC_058923.1
Strombus labiatus voucher USNM:1467137 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	654	654	97%	0.0	87.92%	MZ559454.1
Dolomena swainsoni isolate LSGB_M_075 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	649	649	97%	0.0	87.63%	MN389054.1
Margistrombus robustus isolate XNZ5 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	649	649	97%	0.0	87.63%	JF893438.1
Margistrombus robustus isolate X3112 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	649	649	97%	0.0	87.63%	JF893428.1
Ministrombus variabilis mitochondrion	647	647	100%	0.0	87.09%	MW244824.1
Strombus urceus cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	645	645	96%	2e-180	87.81%	DQ525237.1
Strombus labiatus voucher USNM:1467136 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	643	643	97%	9e-180	87.57%	MZ559787.1
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Strombus labiatus voucher USNM:1467135 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	643	643	97%	9e-180	87.57%	MZ559453.1
Margistrombus robustus isolate X3111 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	643	643	97%	9e-180	87.46%	JF893427.1
Strombus labiatus cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	640	640	96%	1e-178	87.63%	DQ525230.1
Doxander vittatus isolate LSGB_M_076 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	638	638	99%	4e-178	86.85%	MN389055.1

Figure 3d: Phylogenetic tree of *Strombus canarium*

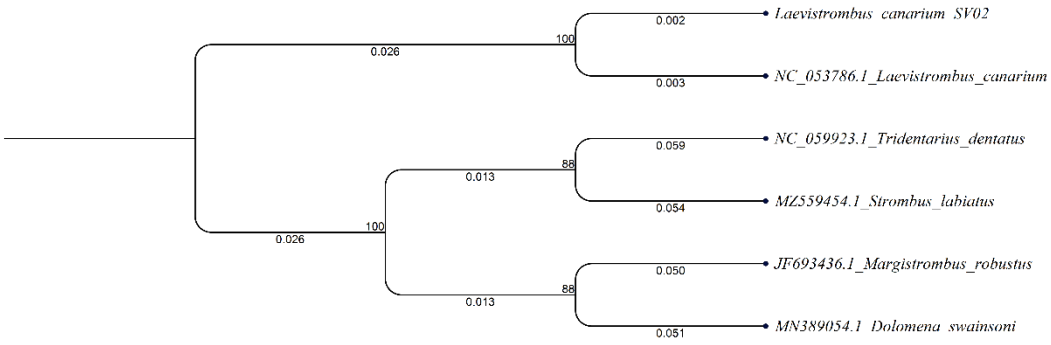

Figure 4: Synthesis of Palladium nanoparticles using the tissue extract of *C.ramosus* and *S. canarium*

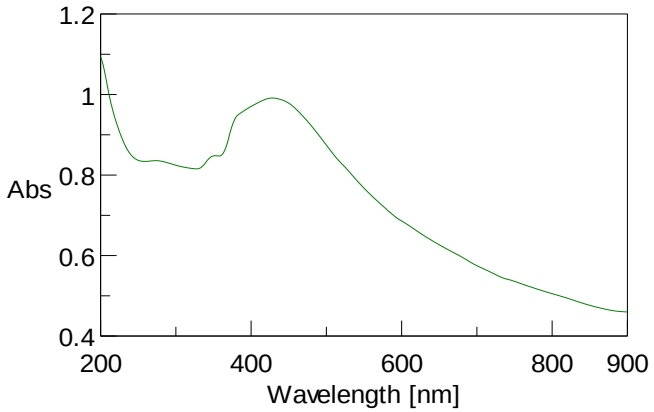

Figure 4a: UV-Visible Spectra of Palladium Nanoparticles using *Chicoreus ramosus*

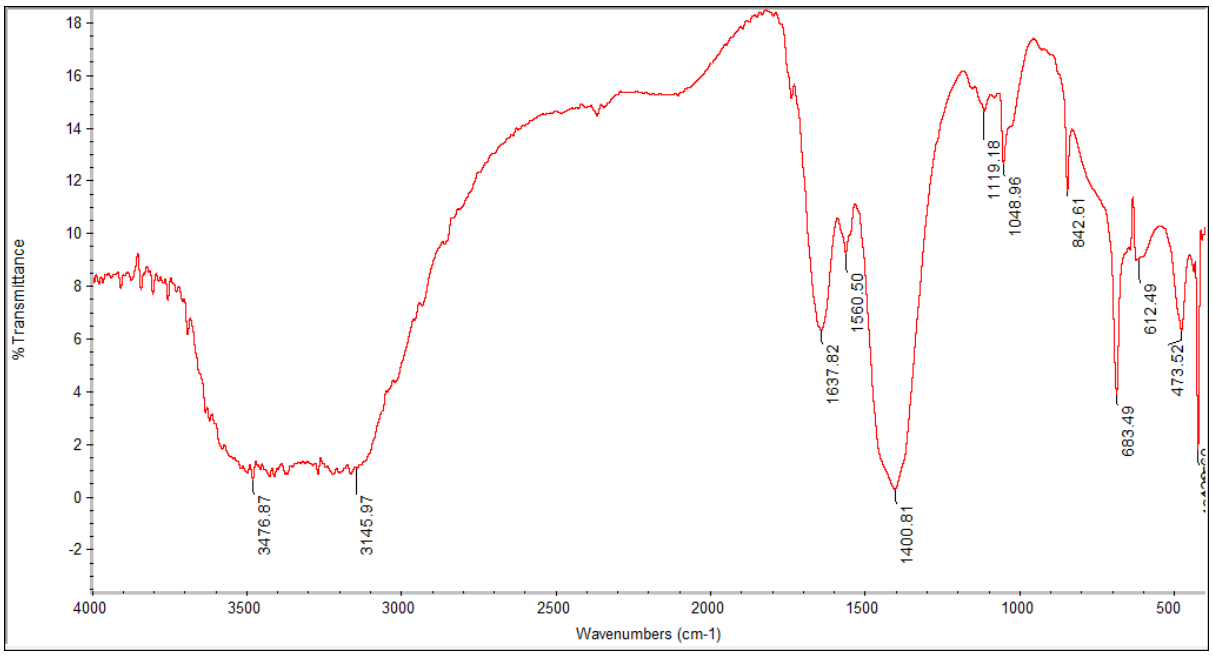

Figure 4b: Infra Red Spectra of Palladium Nanoparticles using *Chicoreus ramosus*

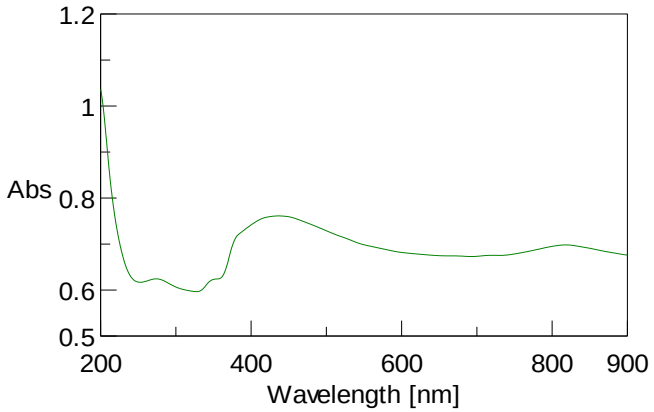

Figure 4c: UV-Visible Spectra of Palladium Nanoparticles using *Strombus canarium*

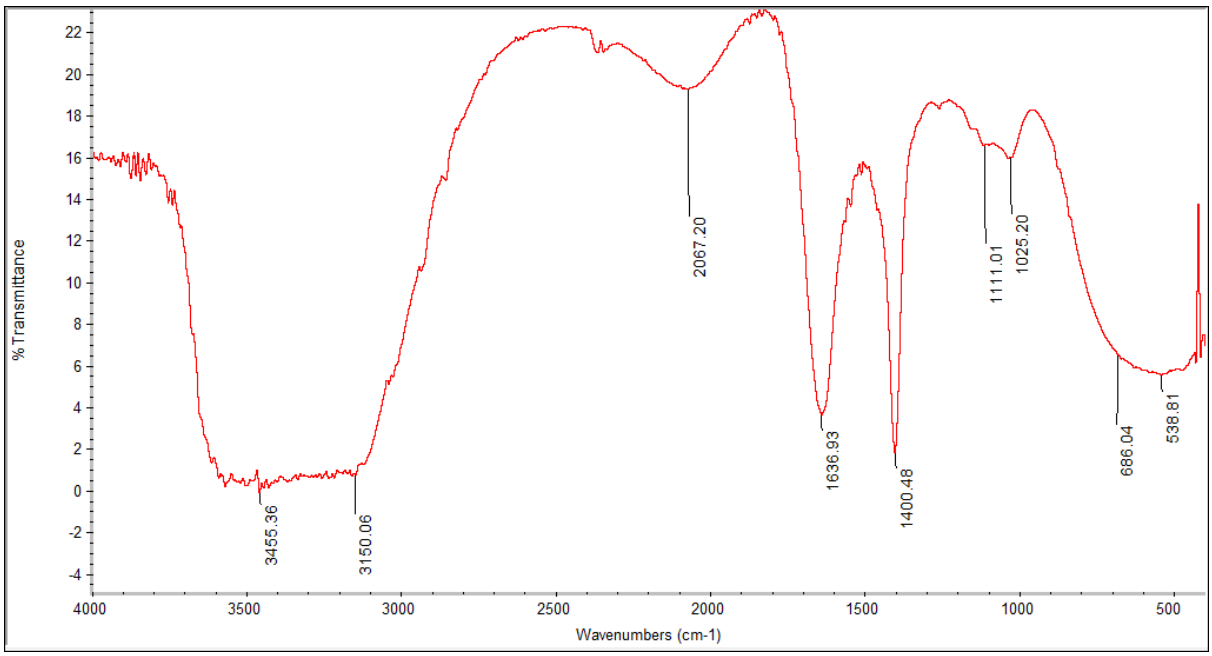

Figure 4d: Infra Red Spectra of Palladium Nanoparticles using *Strombus canarium*

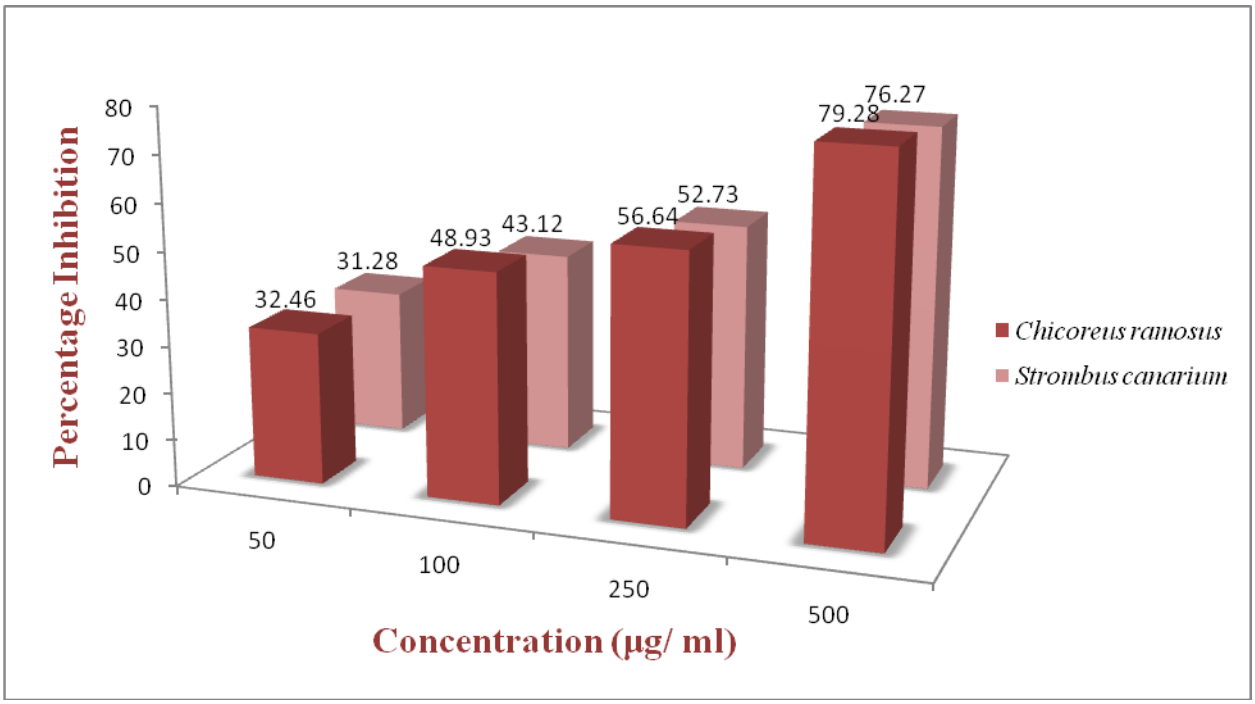

Figure 5: Hydrogen peroxide scavenging activity of palladium nanoparticles using *Chicoreus ramosus* and *Strombus canarium*

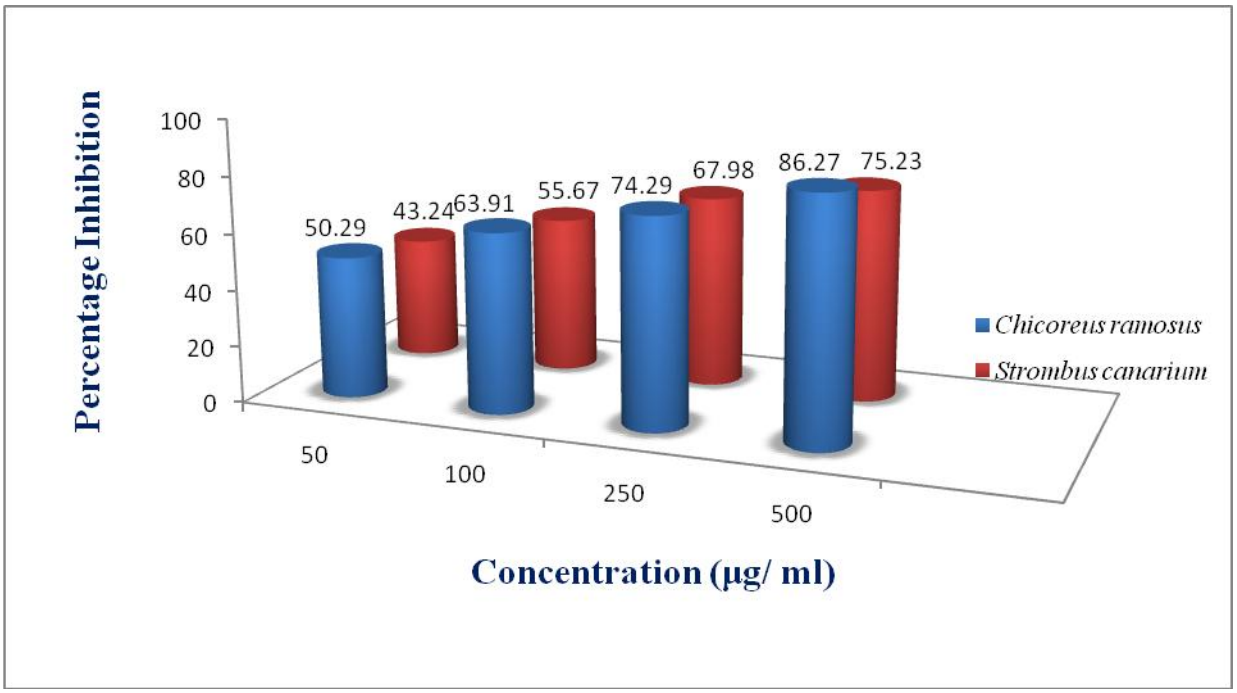

Figure 6: Phosphomolybdenum assay of palladium nanoparticles using *Chicoreus ramosus* and *Strombus canarium*

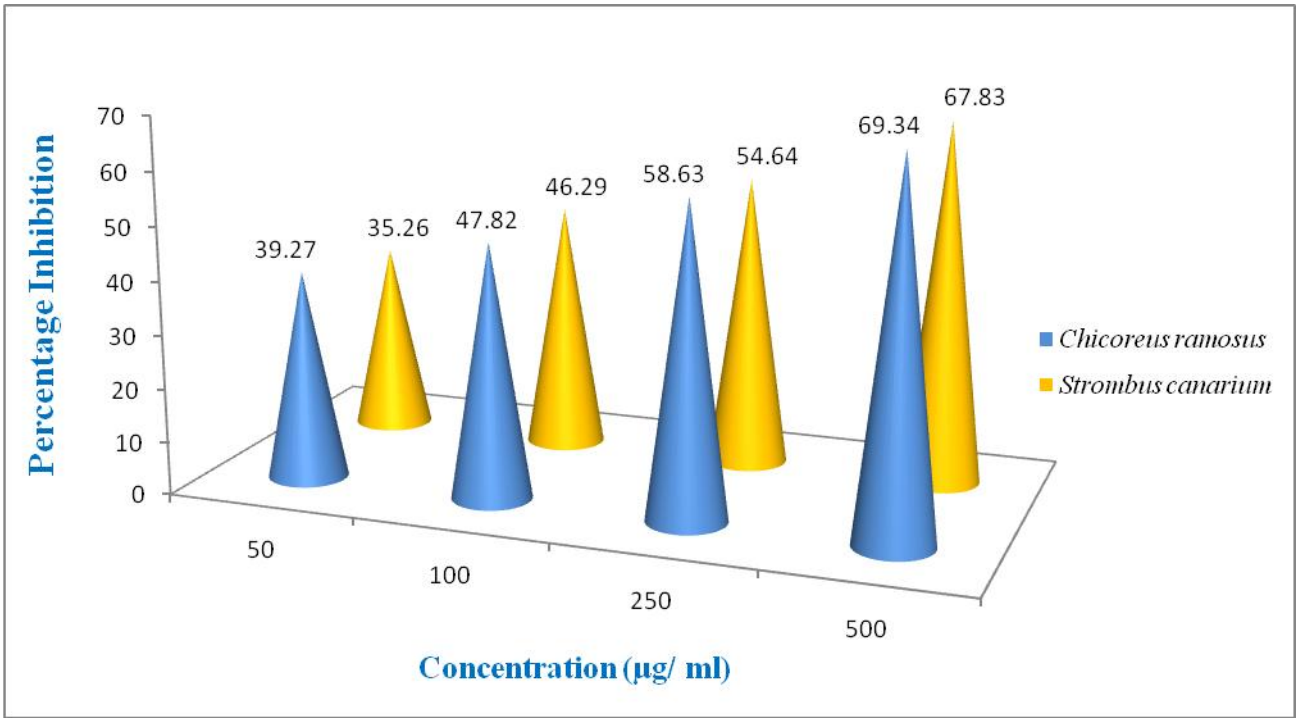

Figure 7: Alpha amylase activity of palladium nanoparticles using *Chicoreus ramosus* and *Strombus canarium*

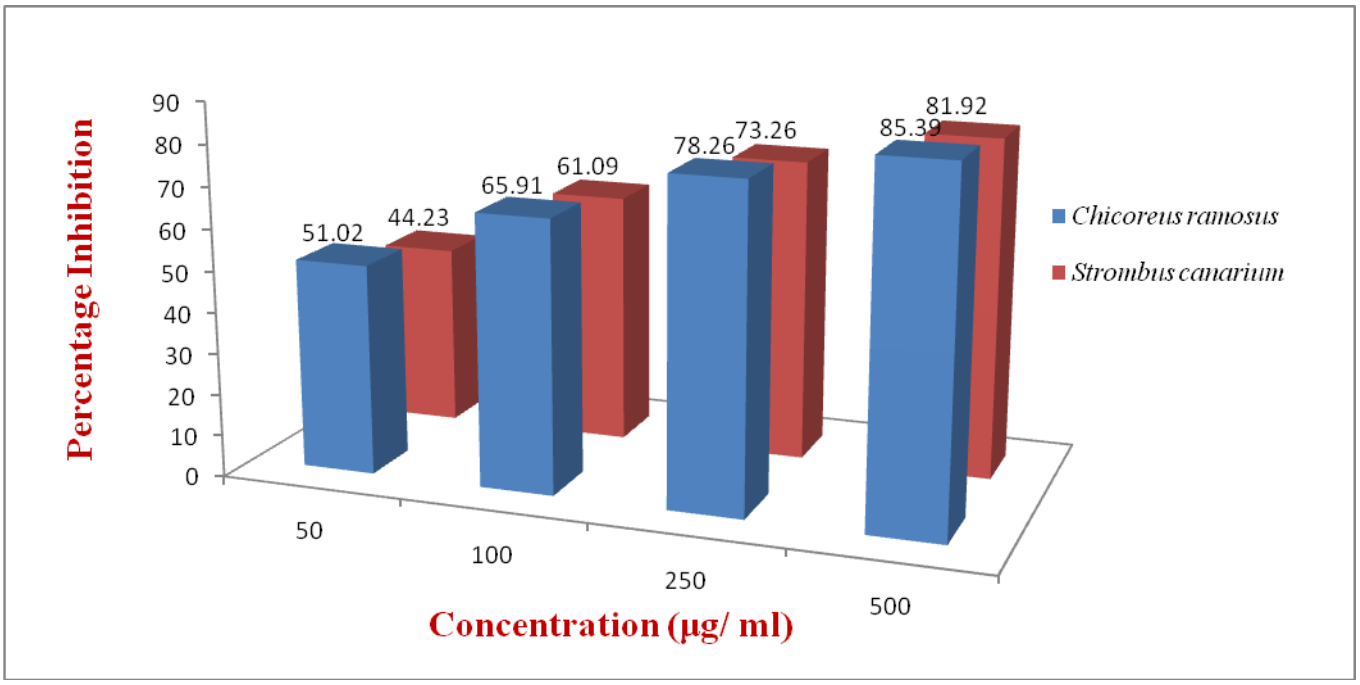

Figure 8: Egg albumin Denaturation Activity of palladium nanoparticles using *Chicoreus ramosus* and *Strombus canarium*

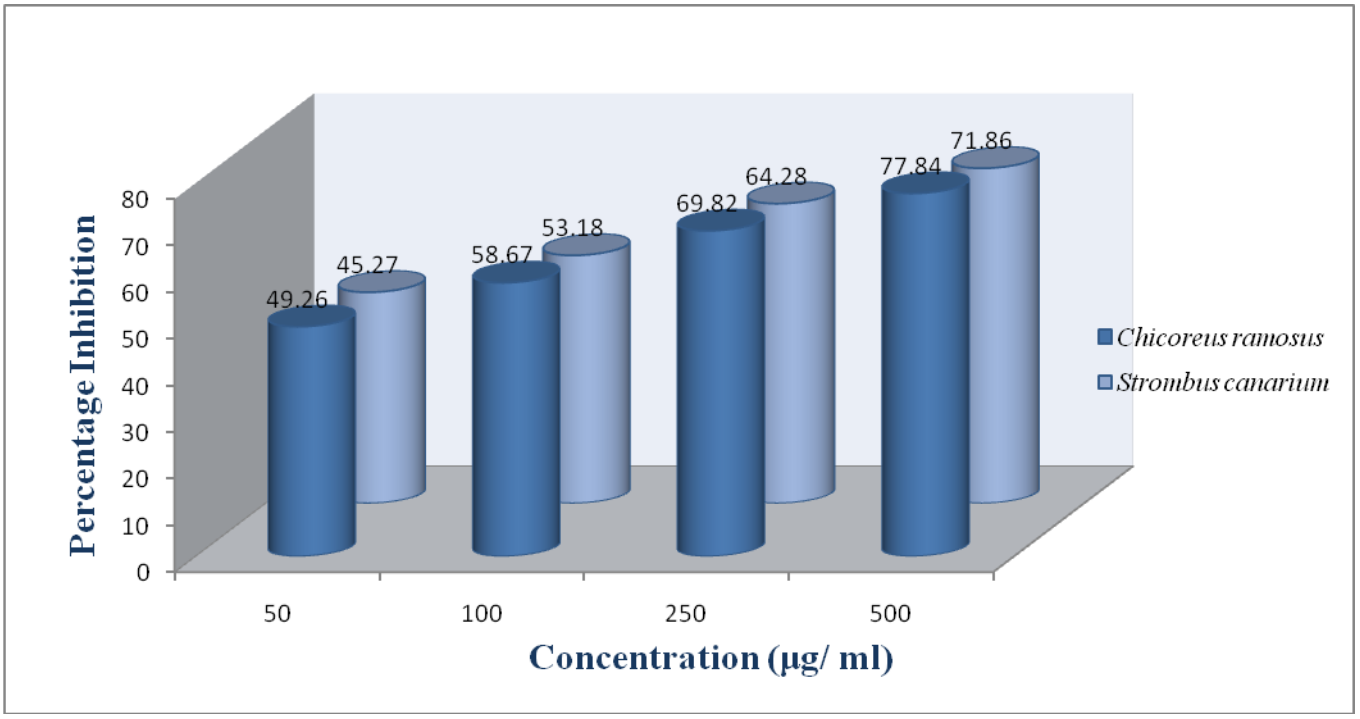

Figure 9: Nitric Oxide Scavenging Assay of palladium nanoparticles using *Chicoreus ramosus* and *Strombus canarium*

7. DISCUSSION

Marine mollusc shells survive in the fossil record and provide invaluable information about the past. Their morphology can for instance be used for phylogenetic inference (Ponder and Lindberg 2008), while their microstructure can reveal important life history traits, including growth rates (Chauvaud *et al.* 2012) and diseases (Paillard *et al.*, 2004 and Trinkler *et al.*, 2010a).

In the future, sequence alignments resulting from targeted mapping against reference nuclear and mtDNA sequences could be used to reconstruct the evolutionary and demographic trajectories of molluscs (Leonardi *et al.*, 2016). In the present study, DNA barcoding region of COX 1 gene in *Chicoreus ramosus and Strombus canarium* were successfully amplified. From the gel analysis, the highly intense bands without smearing strongly indicated the amplification of COX 1 gene. All the species were given good amplified products (Plate 2). The bands of size 550bp were observed in *C. ramosus*, 620bp was observed in *S. canarium*. The sequences with good peak clarities were selected for further analysis. The sequences of about 512 bases were observed in *C. ramosus* and 577 bases were reported in *S. canarium*. All the submitted sequences were assigned accession numbers by Genbank. The

accession numbers are OQ449645 and OQ449646.

In *C. ramosus* two major clades were formed, one major clade comprising 2 species and the next clade comprising of 5 species. In *S. Canarium* topologically two major clades were formed, one major clade comprising 2 species and the next clade comprising of 4 species. *Chicoreus ramosus* SV01 was phylogenetically closer to the clade of FJ784239.1_*Chicoreus ramosus*. *Laevistrombus canarium* SV02 was phylogenetically closer to the clade of NC_053786.1 *Laevistrombus canarium*. The genus and species level discrimination can be ascertained by strong bootstrap support of over 100%. Throughout the tree, several clades have moderate 61% in *C. ramosus* and high 88% in *S. canarium* levels of bootstrap support.

Very similar to the present study Liana *et al*. (2010) studied the phylogenetic analysis of *Biomphalaria tenagophila*. *B. tenagophila* and *B. glabrata* were located in the same branch. However, both are more closely related with opisthobranchia than with other pulmonata, supported by high bootstrap values (98% and 100%). Tanaka and Aranishi (2013) showed a monophyletic clade including *Scapharca*, *Anadara* and *Tegillarca* using a neighbour-joining tree. The nucleotide and amino acid sequences of the mtDNA COI gene indicated a monophyletic clade of closely related genera

that belong to Anadarinae.

Journey *et al*. (2014) reported the phylogenetic tree with 13-protein encoding mt gene sequences of *Physella acuta*. Tony Kess*et al*. (2015) described the DNA extraction and sequences from a family of ten *Littorina saxatilis*. Uribe *et al*. (2016) studied the phylogenetic relationships of four extant super families Neritopsoidea, Hydrocenoidea, Helicinoidea and Neritoidea.

Future studies thus hold the potential to provide invaluable information to research in archaeology, evolutionary biology, marine community ecology and climate change. DNA analyses of molluscs could also be applied to study living communities, including the development of conservation programmes for threatened species, the genetic monitoring of economically important mollusc species, as well as the biological surveillance of aquaculture and early detection of pathogen outbreaks.

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 straightforwardness of the systems and adaptability (Popescu *et al*., 2010).

The synthesis of 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. Amid other metal nanoparticles, palladium is gaining special mention because of its profound applications as catalyst in large number of organic transformations which include various types of carbon-carbon cross-coupling, oxidation and reduction reactions (Aditya *et al*., 2015 and Pramanik *et al*., 2017). Hence, the present study was an attempt to synthesize palladium nanoparticles from the tissue extracts of marine gastropod *C. ramosus* and *S. canarium*.

UV-Visible spectroscopic analysis confirmed the formation of the biosynthesized calcium nanoparticles using the marine molluscan tissue extract of *Chicoreus ramosus and Strombus canarium*. The above solutions were subjected to optical measurements by UV-Visible spectrophotometer. In *C.ramosus,* the wavelength obtained around 480 nm suggested the presence of palladium nanoparticles in the solution. In *S. canarium,* the wavelength obtained around 430 nm suggested the presence of palladium nanoparticles in the solution. Munmi Hazarika *et al*., (2017) evaluated the biogenic synthesis of palladium nanoparticles and their applications as catalyst and antimicrobial agent. A simple *in-situ* process of synthesizing highly dispersed palladium nanoparticles (PdNPs) using aqueous leaf extract of *Garcinia pedunculata* Roxb as bio-reductant and starch (0.3%) as bio-stabilizer. The palladium

nanoparticles are characterized by techniques like FTIR, TEM, SEM-EDX, XRD and XPS analysis. The present study agrees well with the above findings.

Fourier Transform Infra Red Spectroscopy (FTIR) is a technique which isused to analyze the chemical composition of many organic chemicals, polymers, paints, coatings, adhesives, lubricants, semiconductor materials, coolants, gases,biological samples, inorganic, and minerals. FTIR can be used to analyze a widerange 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 in *C.ramosus*; 3476.87, 3145.97, 1637.82, 1560.50, 1400.81,1119.18, 1048.96, 842.61, 683.49, 612.49, 473.52 cm^{-1}. The image shows a strong absorption peak around 3476.87cm^{-1} to 1400.81cm^{-1} which shows the presence of C-H stretching vibration. A peak around 400cm^{-1} to 1100 cm^{-1} shows the presence of C-O stretching frequency.

The results of FTIR analysis of this study show different stretches of bonds shown at different peaks in *S.canarium*; 3455.36, 3150.06, 2067.20,

1636.93, 1400.48, 1111.01, 1025.20, 686.04, 538.81 cm^{-1}. The image shows a strong absorption peak around 3455.36cm^{-1} to 1400.48cm^{-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 palladium nanoparticles (Figure 2f and 3f).

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 thedevelopment 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 AgNPs before its use *in vivo* models and also human applications.

The hydrogen peroxide radical scavenging activity of marine molluscan tissue extract of *C. ramosus* 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 79.28% was observed at 500 µg/ml followed by 56.64% at 250 µg/ml, 48.93% at 100 µg/ml and 32.46% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 76.27% was observed at 500 µg/ml followed by 52.73% at 250 µg/ml, 43.12% at 100 µg/ml and 31.28% at 50 µg/ml respectively. The IC_{50} value of 53.29 µg/ml was noted for *C. ramosus* and 56.13 µg/ml for *S. canarium* which shows the good antioxidant activity (Figure 5).

The phosphomolybdenum scavenging assay of marine molluscan

tissue extract of *C. ramosus* 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 86.27% was observed at 500 µg/ml followed by 74.29% at 250 µg/ml, 63.91% at 100 µg/ml and 50.29% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 75.23% was observed at 500 µg/ml followed by67.98% at 250 µg/ml, 55.67% at 100 µg/ml and 43.24% at 50 µg/ml respectively. The IC_{50} value of 86.01µg/ml µg/ml was noted for *C. ramosus* and 56.15 µg/ml for *S. canarium* which shows the good antioxidant activity (Figure 6).

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.

In the present study, the α-amylase activity of marine molluscan tissue extract of *C. ramosus* 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 69.34% was observed at 500 µg/ml followed by 58.63% at 250 µg/ml, 47.82% at 100 µg/ml and 39.27% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 67.83% was observed at 500 µg/ml followed by54.64% at 250 µg/ml, 46.29% at 100 µg/ml and 35.26% at 50 µg/ml respectively. The IC_{50} value of 21.27 µg/ml was noted for *C. ramosus* and 24.06 µg/ml for *S. canarium* which shows the good antidiabetic activity (Figure 7).

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. Our results are confirmatory with the reports of Pretsch *et al*. (2014) and Tomer *et al.* (2019).

The egg albumin denaturation activity of marine molluscan tissue extract of *C. ramosus* 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 85.39% was observed at 500 µg/ml followed by 78.26% at 250 µg/ml, 65.91%at 100 µg/ml and 51.02% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 81.92% was observed at 500 µg/ml followed by 73.26% at 250 µg/ml, 61.09% at 100 µg/ml and 44.23% at 50 µg/ml respectively. The IC_{50} value of 13.23 µg/ml was noted for *C. ramosus* and 29.31µg/ml for *S. canarium* which shows the good anti-inflammatory activity (Figure 8).

The nitric oxide scavenging assay of marine molluscan tissue extract of *C. ramosus* 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

77.84% was observed at 500 µg/ml followed by 69.82% at 250 µg/ml, 58.67% at 100 µg/ml and 49.26% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 71.86% was observed at 500 µg/ml followed by 64.28% at 250 µg/ml, 53.18% at 100 µg/ml and 45.27% at 50 µg/ml respectively. The IC_{50} value of 41.5 µg/ml was noted for *C. ramosus* and 45.34 µg/ml for *S. Canarium* which shows the good anti-inflammatory activity

(Figure 9).

Synthesis of nanoparticles using a biological agent is eco-friendly and of low cost. The palladium nanoparticles significantly showed antioxidant, anti diabetic andanti- inflammatory activities. The outcome of the research confirms that the presence of active compounds are responsible for the formation of palladium nanoparticles and also prove to be a good antioxidant, anti diabetic and anti- inflammatory agent.

8. SUMMARY

- DNA barcoding region of COX 1 gene in *Chicoreus ramosus and Strombus canarium* were successfully amplified. The bands of size 550bp was observed in *C. ramosus*, 620bp was observed in *S. canarium*.
- The sequences of about 512 bases were observed in *C. ramosus* and 577 bases were reported in *S. canarium*.
- The maximum score ranged from 881 to 625 in *C. ramosus*, 990 to 643 in *S. canarium* respectively. The query coverage was found to be 97% in *C. ramosus* and 100% in *S. canarium* respectively.
- All the submitted sequences were assigned accession numbers by Genbank. The accession numbers are OQ449645 and OQ449646.
- In *C.ramosus* two major clades were formed, one major clade comprising 2 species and the next clade comprising of 5 species. In *S. canarium* topologically two major clades were formed, one major clade comprising 2 species and the next clade comprising of 4 species.
- *Chicoreus ramosus* SV01 was phylogenetically closer to the clade of FJ784239.1_*Chicoreus ramosus*. *Laevistrombus canarium*SV02 was phylogenetically closer to the clade of NC_053786.1 *Laevistrombus canarium*.

- In *C. ramosus*, the wavelength obtained around 480 nm suggested the presence of palladium nanoparticles in the solution. In *S. canarium*, the wavelength obtained around 430 nm suggested the presence of palladium nanoparticles in the solution.
- The results of FTIR analysis of this study show different stretches of bonds shown at different peaks in *C.ramosus*; 3476.87, 3145.97, 1637.82, 1560.50, 1400.81, 1119.18, 1048.96, 842.61, 683.49, 612.49, 473.52 cm^{1}.The results of FTIR analysis of this study show different stretches of bonds shown at different peaks in *S.canarium*; 3455.36, 3150.06, 2067.20, 1636.93, 1400.48, 1111.01, 1025.20, 686.04, 538.81 cm^{-1}.
- In hydrogen peroxide scavenging activity of *C. ramosus* the highest percentage inhibition of 79.28% was observed at 500 µg/ml followed by 56.64% at 250 µg/ml, 48.93% at 100 µg/ml and 32.46% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 76.27% was observed at 500 µg/ml followed by 52.73% at 250 µg/ml, 43.12% at 100 µg/ml and 31.28% at 50 µg/ml respectively.
- In phosphomolybdenum assay the highest percentage inhibition of 86.27% was observed at 500 µg/ml followed by 74.29% at 250 µg/ml, 63.91% at 100 µg/ml and 50.29% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 75.23% was observed at 500 µg/ml

followed by 67.98% at 250 µg/ml, 55.67% at 100 µg/ml and 43.24% at 50 µg/ml respectively.

- In antidiabetic activity the highest percentage inhibition of 69.34% was observed at 500 µg/ml followed by 58.63% at 250 µg/ml, 47.82% at 100 µg/ml and 39.27% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 67.83% was observed at 500 µg/ml followed by 54.64% at 250 µg/ml, 46.29% at 100 µg/ml and 35.26% at 50 µg/ml respectively.
- In egg albumin denaturation assay the highest percentage inhibition of 85.39% was observed at 500 µg/ml followed by 78.26% at 250 µg/ml, 65.91% at 100 µg/ml and 51.02% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 81.92% was observed at 500 µg/ml followed by 73.26% at 250 µg/ml, 61.09% at 100 µg/ml and 44.23% at 50 µg/ml respectively.
- In nitric oxide scavenging assay the highest percentage inhibition of 77.84% was observed at 500 µg/ml followed by 69.82% at 250 µg/ml, 58.67% at 100 µg/ml and 49.26% at 50 µg/ml respectively. In *S. canarium* the highest percentage inhibition of 71.86% was observed at 500 µg/ml followed by 64.28% at 250 µg/ml, 53.18% at 100 µg/ml and 45.27% at 50 µg/ml respectively.

9. CONCLUSION AND SUGGESTIONS

Relationships among the major lineages of Mollusca have long been debated. The phylogenetic studies, which focused on few of the widely used marker COI. The results provide important resolution of mollusc phylogeny and offer new insights into ancestral character states of major mollusc clades. However, limited genomic resources spanning molluscan diversity has prevented use of a phylogenomic approach.

In the present study, the molecular phylogeny of *C. ramosus* and *S. canarium* were analysed to study the barcoding and sequencing of cytochrome c oxidase subunit I (COX 1) gene. The results revealed the good amplified products with 512 bases in *C. ramosus* and 577 bases in *S. canarium*.

The synthesized palladium nanoparticles showed the wavelength around 480 nm in *C. ramosus* and 430 nm in *S. canarium* indicated the presence of surface Plasmon resonance. The biogenic nanoparticles showed good antioxidant, antidiabetic and anti-inflammatory activities.

The continuous growth of Palladium nanoparticle applications have made it imperative to exploit novel methodologies for the efficient and environmentally friendly synthesis of Palladium nanoparticles. Biological synthesis of nanoparticles using a broad range of species and bio molecules

has emerged as a powerful alternative to the classic methodologies. These processes can potentially result in more environmentally friendly approach and might become a key part of a cyclic palladium economy when included in recycling and waste water treatment processes.

Today, the available knowledge on the mechanisms of palladium nanoparticles formation from different biological sources is insufficient, and more dedicated research is necessary to exert even better control over important parameters such as nanoparticles size and shape. The vast number and diversity of possible application of palladium nanoparticles in catalysis, medicine, and biomedical therapies highlights the importance of such Studies in the area of research.

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A BASELINE STUDY ON THE PRESENCE OF MICROPLASTIC AND HEAVY METAL DISTRIBUTION ALONG THE TUTICORIN COASTAL SALT PAN STATIONS, GULF OF MANNAR, SOUTH INDIA.

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By

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April 2023

CERTIFICATE

This is to certify that this dissertation entitled, "**A BASELINE STUDY ON THE PRESENCE OF MICROPLASTIC AND HEAVY METAL DISTRIBUTION ALONG THE TUTICORIN COASTAL SALT PAN STATIONS, GULF OF MANNAR, SOUTH INDIA.**" submitted by **P.SRUTHI, Reg No. 21APZO09** to St. Mary's College (Autonomous), Thoothukudi, affiliated to Manonmaniam Sundaranar University, Tirunelveli in partial fulfilment for the award of the degree of Master of Science in Zoology is done by her during the period of 2022 - 2023 under my guidance and supervision. It is further certified that this dissertation or any part of this has not been submitted elsewhere for any other degree.

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1. INTRODUCTION

In India, nearly 24 million tonnes of raw salt are produced in a year, out of which 20% is exported mainly to China, Japan, Indonesia, and the United States. The major salt-manufacturing states in India are Gujarat, Tamil Nadu, Rajasthan, Maharashtra, Andhra Pradesh, Orissa, and West Bengal according to their production scale. In India, Tuticorin (Tamil Nadu) is the second leading producer of salt with an average estimated production of 25 lakh tonnes of salt every year .Of the total area, 25,000 acres of land are covered under salt pans, and small-scale manufacturing is carried out on 10,000 acres of land. Thus far, there are no detailed studies conducted in India (Amdouni, R. 2009)

Natural salt pans or salt flats are flat expanses of ground covered with salt and other minerals, usually shining white under the sun. They are found in deserts and are natural formations (unlike salt evaporation ponds, which are artificial). A salt pan forms by evaporation of a water pool, such as a lake or pond. This happens in climates where the rate of water evaporation exceeds the rate of precipitation that is, in a desert. If the water cannot drain into the ground, it remains on the surface until it evaporates, leaving behind minerals precipitated from the salt ions dissolved in the water. Over thousands of years, the minerals (usually salts) accumulate on the surface.

These minerals reflect the sun's rays and often appear as white areas (Briere Peter R, 2002).

Table salt (NaCl) is an important place in our food consuming. The taste of our foods is changed by the addition of table salt. Salt used for our foods are generally two kinds, refined or unrefined. The usage of unrefined salts is at high ratio in developing countries. Salt is a mineral composed primarily of sodium chloride (NaCl), a chemical compound belonging to the larger class of salts (Westphal, 2010). It flavors food and is used as a binder and stabilizer. It is also a food preservative, as bacteria can’t thrive in the presence of a high amount of salt. (Denton, 1986)

The human body requires a small amount of sodium to conduct nerve impulses, contract and relax muscles, and maintain the proper balance of water and minerals. It is estimated that we need about 500 mg of sodium daily for these vital functions. Sodium is an irreplaceable ion of the solute base of living organisms and of the functioning and communication of cells. Life cannot be sustained if bodily sodium levels are not maintained and animals dwelling in domains where sodium needs to be foraged are endowed with a robust sodium appetite, first recognized, studied and described by Curt Richter, founder of our science over 70 years ago . Hyponatremia is a term for having a blood sodium level that is lower than normal. Kidney failure, congestive heart failure,diuretics, antidepressants and pain

medication severe vomiting or diarrhea and excessive thirst are symptoms of Hyponatremia(Arieff 1986).

Chloride is a mineral naturally found in various foods, but our main dietary source is sodium chloride, otherwise known as table salt. Chloride carries an electric charge and therefore is classified as an electrolyte, along with sodium and potassium. It helps to regulate the amount of fluid and types of nutrients going in and out of the cells. It also maintains proper pH levels, stimulates stomach acid needed for digestion, stimulates the action of nerve and muscle cells, and facilitates the flow of oxygen and carbon dioxide within cells. Chloride is absorbed in the small intestine and remains in the body's fluids and blood. Any excess amount is excreted in urine. A loss of chloride in the body usually accompanies conditions that cause sodium losses. These include conditions that remove too much fluid from the body, such as prolonged diarrhea, vomiting, or excessive sweating. Low levels of chloride may be a sign of: Heart failure, Lung disease, Addison disease, a condition in which your body's adrenal glands don't produce enough of certain types of hormones. It can cause a variety of symptoms, including weakness, dizziness, weight loss, and dehydration. Metabolic alkalosis,a condition in which you have too much base in your blood. It can cause irritability, muscle twitching, and tingling in the fingers and toes. (Roy *et al*, 2015).

Pollution is a 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. Plastic pollution has been a topic of rising environmental concern in recent years. Model estimates show that between 0.8 and 30 million metric tonnes of plastic waste enter the aquatic environment annually around the globe. The majority of plastic pollution is generated on land and transported through rivers to the marine environment. It can have adverse effects in all three of these systems, which include mortality of fauna through ingestion or entanglement, reduction of livelihoods of those dependent on ecosystem health (e.g. fishing and tourism), contamination of seafood with microplastics with implications for food safety and human health;(Chowdhary *et al*,2020)

Microplastics become more threatening than large plastic materials because they could be swallowed and concentrated by humans causing adverse side effects. A lower limit to Microplastic size study has not yet been defined, although most investigations have focused on the 0.3–5 mm size range. Microplastic can be classified into two basic groups, namely primary and secondary, depending on their origin. Primary microplastic is industry-made particles, mainly used in commercial formulations, from cosmetics and toothpaste to micro-additives in synthetic paints and

coatings.Secondary microplastic results from the fragmentation of larger plastics (Cole *et al*, 2014 and Zhang *et al*, 2017).

When it comes to microplastic pollution, salts for consumption are not exempt from the problem. Table salts are obtained from mining mineral rock or the evaporation of water sources at sea. During the production process, saltwater undergoes different physical processes. It is first pumped into evaporation ponds, subsequently concentrated and crystallized by the action of the sun and wind, then being cut and packed for sales. Accordingly, the final product may contain concentrates of the anthropogenic contaminants already present in the saltwater. (Nicole *et al*, 2009)

Many researchers have reported that microplastics affect various marine and freshwater products (clams, fulmars, mussels, marine and freshwater fish etc.).Microplastics could transfer to humans through products acquired from the aquatic environment. One of the most important transport routes is table salt. Water drawn from seas or lakes to manufacture salt, can contain microplastics, organic materials and sand particles. With regard to microplastic contamination, table salts do not carry contamination that comes from aquatic sources alone. In addition to this, there is a high probability of microplastics contamination of table salt during the manufacturing process. (Amelineau *et al*, 2019).

Heavy metals are defined as metallic elements that have a relatively high density compared to water. With the assumption that heaviness and toxicity are inter-related, heavy metals also include metalloids, such as arsenic, that are able to induce toxicity at low level of exposure. In recent years, there has been an increasing ecological and global public health concern associated with environmental contamination by these metals. Also, human exposure has risen dramatically as a result of an exponential increase of their use in several industrial, agricultural, domestic and technological applications. Reported sources of heavy metals in the environment include geogenic, industrial, agricultural, pharmaceutical, domestic effluents, and atmospheric sources.(He et al,2005)

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. 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. 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 (Tchounwou *et al*, 1999)

Contamination of table salt could results in creating the health hazard for human. Since most of salt used around the globe comes from mines and solar salterns, it is estimated that heavy metal contamination might be a concern for table salt. Contamination of heavy metals creates a toxic effect on the surrounding biological environment.They reported that continuous intake of heavy metal contaminated salt and salted foods could leads to stomach cancer and pre-cancerous effects in human.The enrichment of heavy metals in salt pan sediments and salt potentials biological risk for the salt pan ecosystem and the consumers of salt.(Boppel ,1976)

Cadmium would be acutely toxic when an animal dies from exposure to a high concentration over a short period of time. It would be chronically toxic when the animal is exposed to cadmium over a long period of time at a lower concentration. While chronic exposure can lead to mortality, sublethal effects such as reduced growth and reproductive success are more common. Cd of brine may be due to the discharge of industrial effluents and this metal is also concentrated in salt samples and harmful to human (Soylak *et al*, 2008).

Copper is an essential mineral that supports various body functions, such as enzyme production and neurological functions. However, exposure to high levels of copper in water or food can lead to copper toxicity. Too much copper in the body can damage the liver, kidney, heart, and brain. If left untreated, copper toxicity can have severe health effects and even result in death. Pb content in Tuticorin saltpans was found and its contamination is almost certainly from petrochemical industries, metal smelting units, various fertilizer and salt chemical industries, dredging and dumping of sediments from harbors.(Linder *et al*,1996)

Concentrations of Zn salt pans are mainly due to effluents because of the ore handling in the harbor, heavy water plant and solar alkali plant. The untreated industrial waste water can form complexes with humic substances which affects the groundwater and sediments. Oral uptake of zinc affect the gastrointestinal tract before it is distributed through the body. Therefore, multiple gastrointestinal symptoms after oral uptake of zinc have been reported (Muthu Raj *et al*, 2008)

Plastics can play a key role as vectors for heavy metal ions in the marine system. Metal pollution is common within harbors and marinas, and is originated from multiple sources such as the usage of metal based antifouling paints, industrial waste and fuel combustion. Antifouling paints in particular, are one of the major sources of heavy metals into the marine

environment, especially in harbors and marinas, through paint deterioration and consequent diffusion.The most modern marine antifouling paints contain a copper based biocidal pigment and are applied to ship hulls and several other fixed structures. (Amdouni, R. 2009)

Tuticorin is the largest salt producing district in Tamil Nadu, harvesting around 20 million tons of salt annually. Halite (salt) is one of the major mineral sources for human consumption as well as industrial usages. In Tuticorin, the major units of thermal power plants and chemical industries are close to the salt pans. They discharge untreated waste into the ground and the nearest water bodies, which finally reach the ocean. Various disposable materials, fly ash, and some petroleum products are discharged onto the ground (Asha, *et al*., 2010) and they mix with groundwater and the sediment of salt pans. Heavy metal can be introduced as a dissolved or particulate matter due to natural processes or anthropogenic contributions (Marengo *et al*. 2006, Tsai *et al*., 1998, Duzzin*et al*., 1988, Hamid Reza Pakzad*et al*., 2014, Santhanakrishnan*et al*., 2015). Metal contamination of surface sediments can directly affect the seawater and groundwater quality, resulting in potential consequences to the sensitive lowest levels of the food chain and ultimately to human health (Christophoridis*et al*., 2009). According to Qasim*et al*., (1988), while Mn, Cu, Fe and Zn are considered as essential micronutrients, mercury, cadmium and lead are not required for

any important biological functions of organisms. As such, these heavy metals, if found in abnormal concentrations in salt, can lead to thyroid, liver damage and other harmful effects in consumers (Munoz *et al*., 2005). Hence, the present investigation is aimed at determining the concentration of microplastics and accumulation of heavy metals such as Cd, Cu, Pb, and Zi in the sediment samples collected from various ponds of salt pans in Tuticorin district.

REVIEW OF LITERATURE

2. REVIEW OF LITRATURE

Various studies have been carried out on the distribution pattern of microplastics in a regional geographical area while several other studies have brought out the impacts of microplastics on certain marine organisms. However, this information on the global scale is incomplete due to limitations in the number of studies. Prior to 2010, there were scanty literatures available on microplastics; the main aim of this article is to critically review the studies on microplastic abundance and heavy metal distribution from 2002.

Eriksen et al.,(2014) analyzed the plastic pollution in the world's oceans and concluded that more than 5 trillion plastic pieces weighing over 250,000 tons float at sea. Song.,(2014) observed large accumulation of micro-sized synthetic polymer particles in the sea surface microlayer. Barboza (2015) analyzed microplastics in the marine environment: Current trends and future perspectives. Brennecke *et al*.,(2016) examined microplastics as vector for heavy metal contamination from the marine environment .Lee *et al*., (2016) analyzed natural sea salt consumption confers protection against hypertension and kidney damage in Dahl salt-sensitive rats. Karami *et al*., (2017) analyzed the presence of Microplastics in commercial salts from different countries. Gundogdu *et al*.,(2018) examined the contamination of table salts from Turkey with microplastics.

Kumar *et al*., (2018) examined about occurrence of microplastics in fishes from two landing sites in Tuticorin, south east coast of India.

Prata *et al*.,(2018) reviewed the methods for sampling and detection of Microplastics in water and sediment. Pecher *et al*.,(2019) analysed effects of road salt on microbial communities, Halophiles as biomarkers of road salt pollution and the impact on microbial communities in soils exposed to urban road salt runoff using both culturing and 16S amplicon sequencing. Ajith *et al*.,(2019) analyzed about global distribution of Microplastics and its impact on marine environment. Barletta *et al*., (2019) studied distribution, sources and consequences of nutrients, persistent organic pollutants, metals and Microplastics in South American estuaries.

Campanale *et al*., (2019) investigated about a detailed review study on potential effects of Microplastics and additives of concern on human health. Sathish *et al*., (2019) observed the microplastics in Salt of Tuticorin, Southeast Coast of India. Selvama *et al*.,(2020) surveyed microplastics presence in commercial marine sea salts along Tuticorin coastal salt pan stations, Gulf Of Mannar, south India. Deng *et al*., (2021) analyzed the microplastics pollution in mangrove ecosystems: a critical review of current knowledge and future directions.

Elgarahy *et al*., (2021) examined microplastics prevalence, interactions, and remediation in the aquatic environment. Motallaei *et al*.,

(2021) analyzed evaluation of cytotoxic and antimicrobial properties of Iranian Sea Salts. Nithin *et al*.,(2021) examined microplastic contamination in salt pans and commercial salts on the salt pans of Marakkanam and Parangipettai, Tamil Nadu, India. Rakib *et al*., (2021) analysed Microplastics pollution in salt pans from the Maheshkhali Channel, Bangladesh.

The development of urban infrastructure, fisheries, maritime cultivation, transport and tourism, deficient wastewater treatment has caused major disturbance to the coastal environment. Bioaccumulation level of heavy metals in marine organism is very important, with many implications in various domains, like environment protection, public health, control of standards compliance or risk assessment. The ability of marine biota to accumulate metals from their environment, their utilization as marine pollution bio indicators has been confirmed by numerous examples. Heavy metal concentration was determined by variables such as water contamination, mining activities and effluent treatment activities in the fishing region (Martin, 1975).

Jenne *et al*., (1968) observed controls on Mn, Fe Co, Ni, Cu and Zn concentrations in soils and water. Crecelius,*et al*.,(1985)analyzed fly-ash disposal in the ocean: an alternate worth considering. Duzzin *et al*.,(1988) examined macro invertebrate communities and sediments as pollution

indicators for heavy metals in the River Adige, Italy.Wurbs *et al*., (2002) examined natural salt pollution control in the southwest and this article provides a general overview of natural salt pollution in the Permian Basin Region as well as studies and projects dealing with its control or mitigation.

Baskaran, *et al*., (2002) analyzed metal pollution in Tuticorin coastal waters due to fly ash of thermal power plant. Jarup (2003) examined hazards of heavy metal contamination. Marengo *et al*., (2006) investigated the anthropogenic effects connected with metal ions concentration, organic matter and grain size in Bormida river sediments.

Morillo *et al*., (2007) observed potential mobility of metals in polluted coastal sediments in two bays of southern Spain. Khaniki *et al*.,(2007) examined about determination of trace metal contaminants in edible salts in tehran (iran) by atomic absorption spectrophotemetery. Zukowska *et al*.,(2008) examined the methodological evaluation of method for dietary heavy metal intake. Soylak *et al*.,(2008) analyzed heavy metal contents of refined and unrefined table salts from Turkey, Egypt and Greece.

Amdouni, *et al*., (2009) observed behavior of trace elements during the natural evaporation of sea water along the Tunisian coast. Asha (2010) observed heavy metal concentration in sea water, sediment and bivalves off Tuticorin. Cheraghali *et al*.,(2010) analyzed heavy metals contamination of

table salt consumed in Iran. Zarei *et al*.,2011 examined determination of heavy metals content of refined table salts.

Wang *et al*., (2012) study on the pollution characteristics of heavy metals in seawater of Jinzhou bay. Pourgheysari *et al*., (2012) examined heavy metal content in edible salts in Isfahan and estimation of their daily intake via salt consumption. Krishna *et al*.,(2012) analysed trace element concentration in groundwater, Tuticorin, Tamil Nadu. Hamid *et al*.,(2014) examined heavy metal distribution in the salt pan of Gavkhuni Playa lake (southeast of Isfahan, Iran). Eftekhari *et al*.,(2014) examined content of toxic and essential metals in recrystallized and washed table salt in Shiraz, Iran. Diya *et al*., (2014) observed status of heavy metal pollutants around the south east coast of India. Santhanakrishnan *et al*., (2016) studied heavy metal distribution in the salt pans of Tuticorin, Tamil Nadu, India. Zhang *et al* .,(2017) studied heavy metals in seawater and sediments from the northern Liaodong Bay of China and their levels, distribution and potential risks and also surveyed on the concentrations of heavy metals (Cd, Pb, Hg, As, Cu, and Zn) in surface seawater and sediments in the northern Liaodong Bay of China.

OBJECTIVES

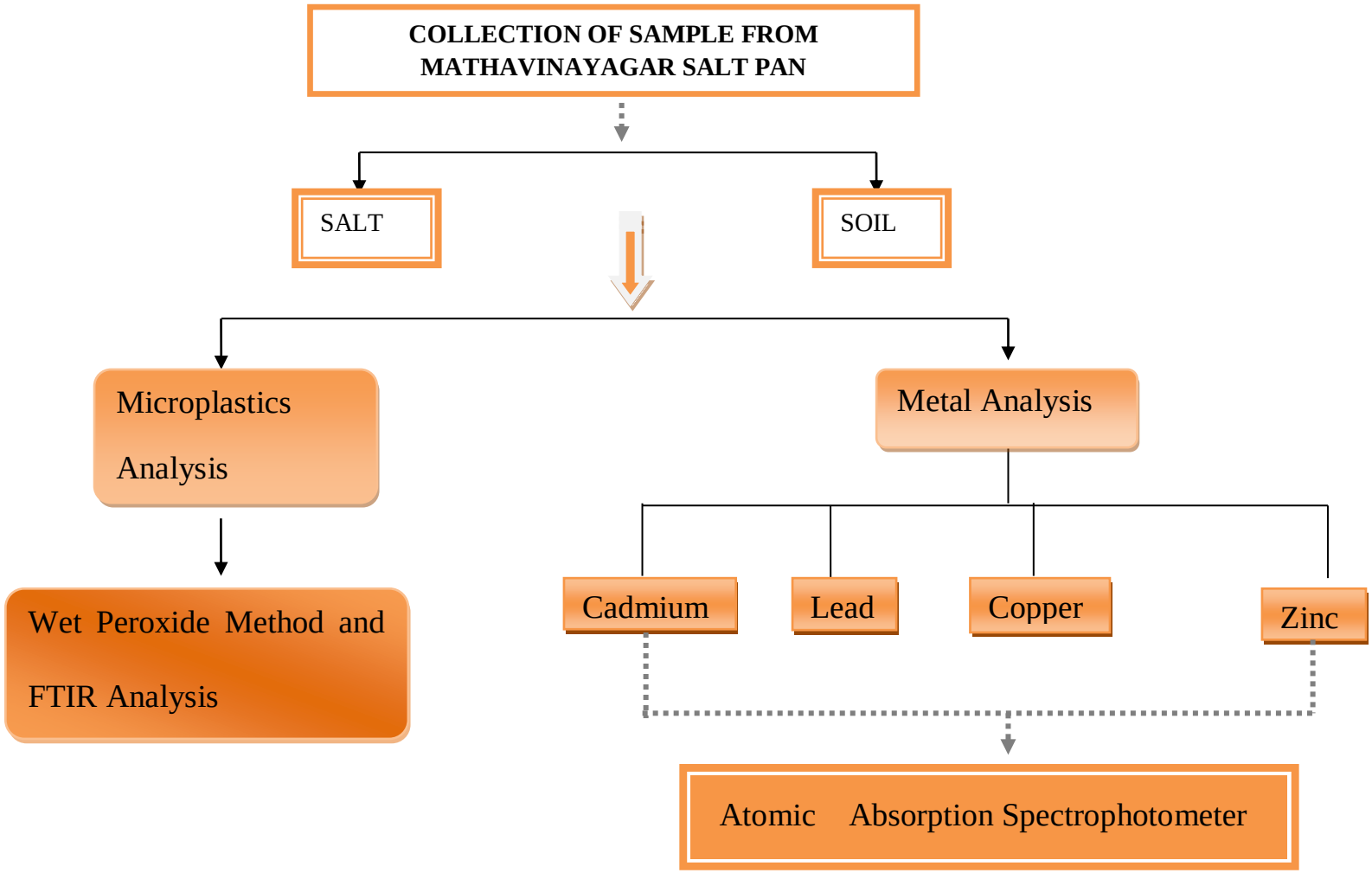
3. OBJECTIVES

The objectives of the present study are

- To study the pollution contents in edible salt collected from salt pan
- To analyze the microplastics content in the salt and its substratum (soil) by wet peroxide method.
- To identify the appearance, group and compound class of that microplastics by using FTIR analysis.
- To estimate heavy metals such as cadmium, copper, lead and zinc in the same sample by Atomic Absorption Spectrophotometer.

EXPERIMENTAL DESIGN

4. EXPERIMENTAL DESIGN:



MATERIALS AND METHODS

5. MATERIALS AND METHODS

5.1. STUDY AREA:

The Gulf of Mannar is located between India and Srilanka (Long.78’8 " to 79'30 " E and Lat 8 ' 35 " to 925 " N). It is a part of the southward extension of the Bay of Bengal and it meets in the Indian Ocean. This geographical area runs from Pamban Island including Rameshwaram to Cape Comarin along the Southeast coast of India to a distance of about 170 nautical miles. This coast maintains a rich biological diversity of flora and fauna largely due to diversified microhabitats such as mangroves, corals, seaweed beds, sea grasses, sandy, rocky and muddy shore etc.

Tuticorin district is located on south east of Tamil Nadu state. The district covers an area of 4621 sq.km and is bounded by the districts of Virudhunagar and Ramanathapuram on the east and by Gulf of Mannar on the south-east and by Tirunelveli district on the West and South-west. Its geographical co-ordinates are 8047’0” North, 7808’0” East. For the present study the salt and its substratum (soil) were collected from a salt pan which is located in Mathavinayagar colony, Thoothukudi.

Tamil Nadu ranks second in country’s salt production. Gujarat tops the list with almost 80% of the national production. Covering about 30,000 acres Tuticorin salt fields contribute to about 10% of the national salt

production. The process of salt making starts at the beginning of the year. First the salt pans are prepared during January – March. The salt pans are shallow pools with elevated mud borders and prepared solely by manual labour. The harvested salt is left to dry by evaporation in mounds and is finally sent to the nearby salt refiners for cleaning and idolization process. Apart from edible salt the salt pans of Tuticorin also produces industrial salts. The harvesting takes place during October after which the Tuticorin receives rains from the retarding north east monsoon. Interestingly Tuticorin region hardly receives any rainfall during the monsoon season of June – August. Long stretches of dry period makes the Tuticorin region an ideal for salt production, but however non seasonal rains can jeopardize the entire process.

5.2. Sample collection and Preparation:

Salt samples were collected directly from salt pan. The salt were crushed into a fine powder by using a porcelain motor and pestle The soil samples were also collected and dried in the oven at 200°C. and both samples analyzed for Microplastics and heavy metals.

5.3. Wet Peroxide Oxidation (WPO)

Procedure

Take 20 ml of aqueous 0.05 M Fe (II) solution to the beaker containing the 0.3 mm size fraction of collected solids. Add 20 ml of 30%

hydrogen peroxide. Let mixture stand on lab bench at room temperature for five minutes prior to proceeding to the next step. Add a stir bar to the beaker and cover with a watch glass.

Heat to 75°C on a hotplate. As soon as gas bubbles are observed at the surface, remove the beaker from the hotplate and place it in the fume hood until boiling subsides. If reaction appears to have the potential to overflow the beaker, add distilled water to slow the reaction. Heat to 75°C for an additional 30 minutes. If natural organic material is visible, add another 20 ml of 30% hydrogen peroxide. Repeat until no natural organic material is visible. Add ~6 g of salt (NaCl) per 20 ml of sample to increase the density of the aqueous solution (~5 M NaCl). Heat mixture to 75°C until the salt dissolves.

Density Separation

Transfer the WPO solution is transferred to the density separator. A sample is depicted in a glass funnel to separate plastic. Rinse the WPO beaker with distilled water to transfer all remaining solids to the density separator. Cover loosely with aluminum foil. Allow solids to settle overnight. Visually inspect settled solids for any microplastics. If any are present, drain the settled solids from the separator and remove microplastics using forceps. Archive or discard the settled solids. Drain settled solids

from the separator and discard. Collect floating solids in a clean 0.3-mm custom sieve .Rinse the density separator several times with distilled water

to transfer all solids to the 0.3-mm sieve. Allow the sieve to air dry while loosely covered with aluminum foil for 24 hours. A prepared sample is ready for microscope examination.

Microscopic Examination

• Weigh a clean and dry 4-mL vial. Include the label and cap.

• Under a dissecting microscope at 40X magnification, use forceps to collect identifiable Microplastics from the 0.3-mm sieve and transfer them to the tarred vial

5.4. Analysis of Lead, Cadmium, Copper and Zinc Metals:

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 per Chloric 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.

RESULTS

6. RESULT

Plastic products like container bags and packing materials have been phased out thanks to solar energy and wave tidal movement. The textile and synthetic apparel businesses in the region could also be connected to the fibers. The Buckle Canal receives solid waste from about 18 textile workshops. Also, the generated solid waste is discharged into the ocean while the fishing net and boat are regularly prepared at the beach. Additional elements that may have had an impact on the morphology include residence time, original plastic form, photo-degradation, mechanical abrasion of erosional elements, and biological activity.

A preliminary characterization was done by a combination of visual identification under microscope, fragmentation test with tweezers, and application of the hot needle test. The identification of the microplastics found was done by Fourier Transform Infrared Spectroscopy (FTIR), which is one of the most popular methods used to confirm the composition of microplastics. Using this technique, several types of microplastics were identified and it revealed the presence of polymers namely Polyethylene (PE), Polypropylene (PP), Polystyrene (PS), Polycarbonate (PC), Polyvinyl chloride (PVC), Nylon. PTFE (Polytetrafluorethylene) ,EVA (Ethylene vinyl acetate) ,PU(Polyurethene) ,Nitrile and Latex

In general, polymers undergo degradation through physical and chemical processes as well as through photo- and biodegradation. In salt sample, FTIR spectra showed different characteristic peak that varied from 691.43 to 3691.43. FTIR spectra of PE (Polyethylene) showed a characteristic peak at 1746.42 cm^{-1} and 2360.71 cm^{-1}, PS(Polystyrene) showed 691.43 cm^{-1} and 1046.31 cm^{-1},PP(polypropylene) showed 854.41 cm^{-1} and 1384.79 cm^{-1},Nylon showed 1282.57 cm^{-1},1544.88 cm^{-1},1642.27 cm^{-1},2883.38 cm^{-1},3330.84 cm^{-1} and 3738.75 cm^{-1},PVC(Polyvinyl Chloride) showed 1339.47 cm^{-1} and PC(Polychloride) showed 1510.16 cm^{-1} and 2822.63 cm^{-1} in salt sample.

FTIR spectra for various types of polymers are shown in (Figure 3). These spectra have been evaluated using five different spectral ranges, such as stretching vibration of C=C bending,C-Cl stretching,CO-O-CO stretching,C-O stretching,C-N stretching,O-H bending,C-F stretching,N-O stretching,C=C stretching,C=O stretching,C-H stretching,O-H stretching in salt sample are represented in (Table 2 and Figure 1).

Comparing FTIR spectra of unknown MPs to a commercially available polymer library of known recorded polymer spectra can efficiently identify the unknown MPs (Renner, Nellessen, *et al.*, Citation2019). 2 microplastics of PE, 2 microplastics of PP, 2 microplastics of PP, 6 microplastics of Nylon and 2 microplastics of PC were detected from the IR

image produced by the spectral range of 691.43 cm^{-1} to 3738.75 cm^{-1}. The IR picture was counted, and one PVC particle was discovered. Due to this, a total of 15 microplastics were found using FTIR imaging are shown in the salt sample (Table 2)

In soil sample, FTIR spectra showed different characteristic peak that varied from 468.67cm^{-1} to 3420.52cm^{-1}. FTIR spectra of PTFE (Polytetrafluorethylene) showed a characteristic peak at 468.67,518.82 cm^{-1} and 580.53 cm^{-1}, Nylon showed 691.43 cm^{-1} and 1641.31cm^{-1}, EVA (Ethylene vinyl acetate) showed 780.15 cm^{-1},PC(Polycarbonate) showed 1029.92 cm^{-1},1315.36 cm^{-1} and 2818.77,PU(Polyurethene) showed 1510.16 cm^{-1} and Nitrile showed 2360.71 cm^{-1},Latex showed 2883.38cm^{-1} and Polystyrene showed 3420.52 cm^{-1} (Table 4).

FTIR spectra for various types of polymers are shown in (Figure 3). These spectra have been evaluated using five different spectral ranges, such as stretching vibration of C-I stretching, C=C bending, C-F stretching, O-H bending, N-O stretching,C=C stretching ,C=O stretching ,O-H stretching ,N-H stretching sample are represented in soil sample (Table 4 and Figure 2).

Comparing FTIR spectra of unknown MPs to a commercially available polymer library of known recorded polymer spectra can efficiently identify the unknown MPs (Renner, Nellessen, *et al.*, Citation2019). 3 microplastics of PTFE, 2 microplastics of Nylon,1 microplastic of EVA,4

microplastics of PC,2 microplastics of PU and 1 microplastic of PS were detected from the IR image produced by the spectral range of 468.67cm^{-1} to 3420.52cm^{-1}. The IR picture was counted, and one Latex and Nitrile particle was discovered. Due to this, a total of 15 microplastics were found in soil sample using FTIR imaging is shown in the soil sample (Table 4). FTIR spectra for each peak have represented with Intensity, Correlation Intensity, Base (H), Base (L), Area and Correlation Area for each sample separately. (Table 1 and Table 3).

The results on the concentration of heavy metals namely cadmium, Lead, Copper and Zinc in salt and soil are represented in the (Table 7).Salt had a cadmium concentration of 1.15µg/g, while soil had a cadmium concentration of 1.01 µg/g. The highest concentration was found in salt. Copper levels in soil and salt were both 2.19 µg/g and 2 µg/g, respectively. The highest concentration was visible in the salt. Lead concentrations in salt and soil are 0.79µg/g and 0.73 µg/g, respectively. When compared to its substratum (soil), salt was the most contaminated species (0.79µg/g), as seen in (Figure 3 and Table 5).

When compared to all the other elements, zinc was found to be abundant in all the samples that were gathered for the current investigation. A low concentration of 2.98 g/g of zinc occurred in the substratum (soil) of salt, which has a high concentration of 3.11 g/g of zinc (table 7). The levels of the components extracted from various species in order

Zn > Cu > Cd > Pb.

Plates showing the different types of polymer in salt samples

Plate 1

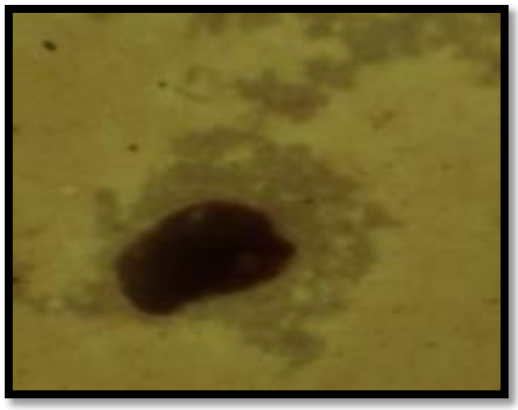


Plate 2

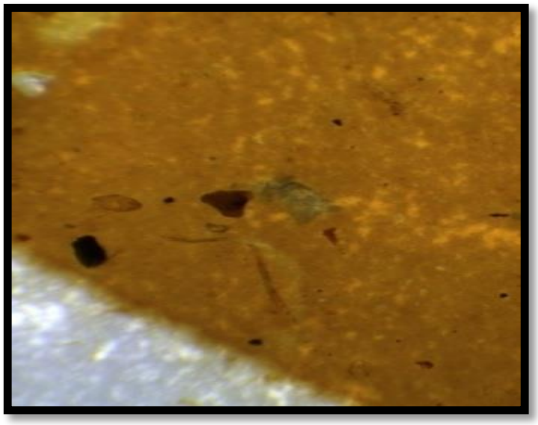


Plate 3

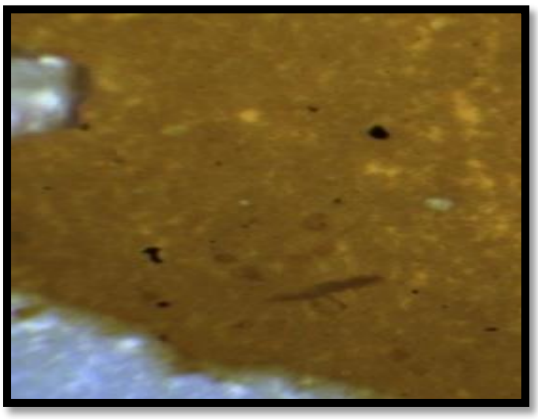


Plate 4

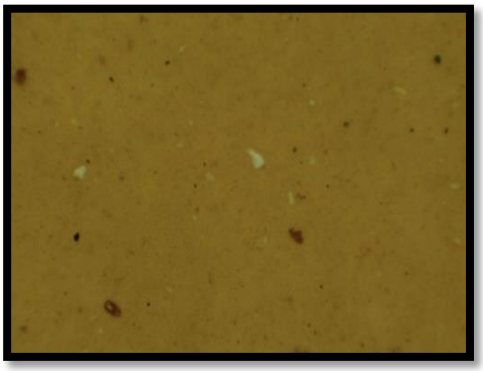


Plate 5

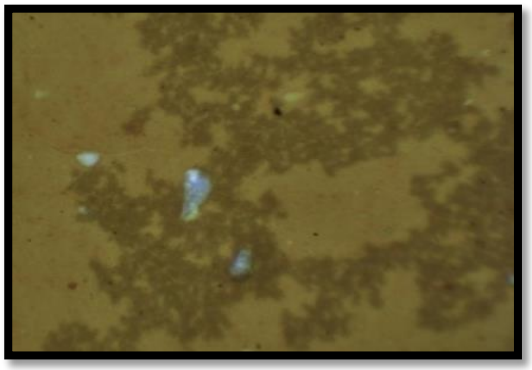


Plate 6

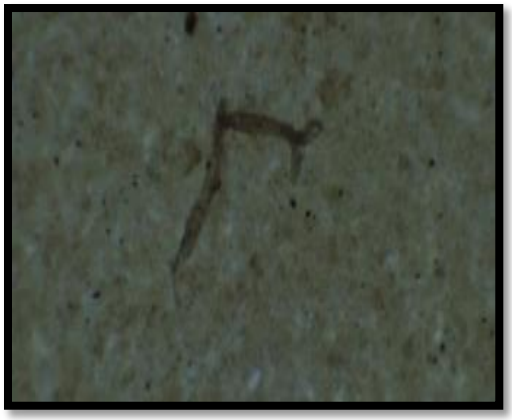


Plate 7

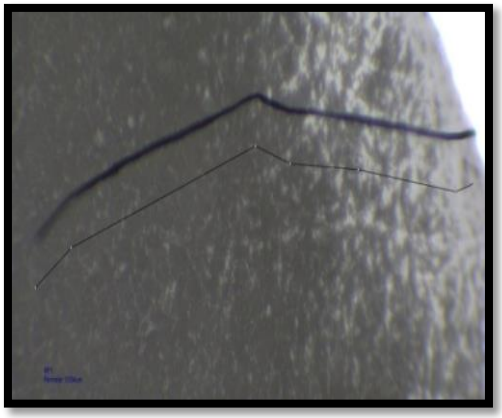


Plate 8

Figure 1: FTIR spectra showing different types of polymer in salt samples.

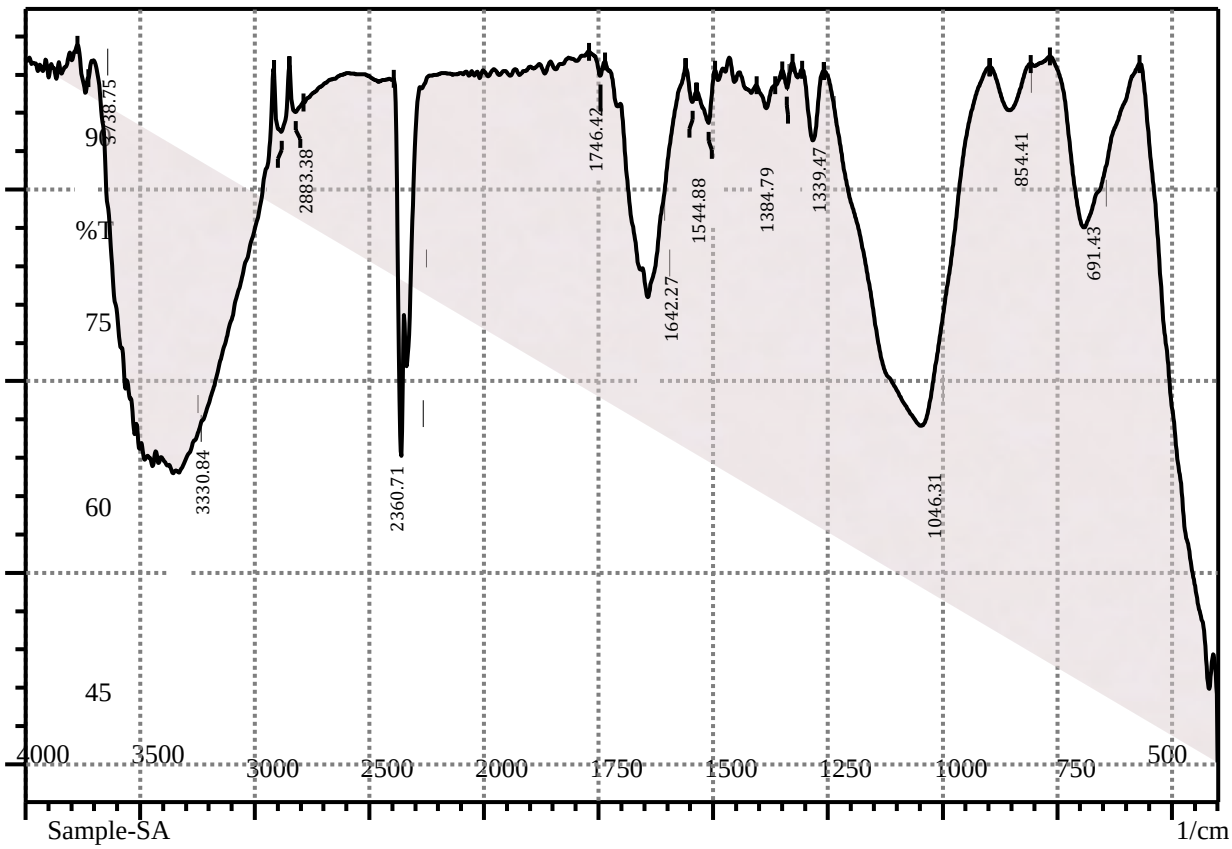


Table 1: Table showing peaks and intensity of various microplastics from reflectance of FTIR-spectroscopy in salt samples.

	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	691.43	87.028	13.175	765.69	570.89	5.391	5.503
2	854.41	96.224	3.498	897.8	808.12	0.833	0.726
3	1046.31	71.497	27.978	1258.47	897.8	27.758	26.89
4	1282.57	93.845	5.5	1306.68	1258.47	0.692	0.554
5	1339.47	97.961	1.641	1349.11	1327.9	0.109	0.075
6	1384.79	96.354	1.819	1406.01	1364.54	0.475	0.143
7	1510.16	95.23	3.556	1536.2	1496.66	0.565	0.309
8	1544.88	96.87	1.506	1560.3	1536.2	0.228	0.085
9	1642.27	81.574	4.222	1653.85	1560.3	4.369	0.709
10	1746.42	98.914	1.343	1771.5	1735.81	0	0.064
11	2360.71	69.013	16.689	2392.53	2347.21	3.983	1.681
12	2822.63	96.073	2.423	2848.67	2786.95	0.899	0.429
13	2883.38	94.539	5.045	2917.13	2848.67	1.248	1.124
14	3330.84	67.849	1.059	3343.37	2917.13	44.96	8.731
15	3738.75	97.585	1.783	3773.47	3727.18	0.184	0.186

Table 2: Showing characteristics of peaks ,functional groups of polymers present in salt sample

PEAK cm^{-1}	FUNCTION GROUP	FUNCTIONAL GROUP NAME	APPEARANCE	VIBRATIONS	POLYMERS
691.43	C=C	Alkene	Strong	Bending	Polystyrene
854.41	C-Cl	Halo Compound	Strong	Stretching	Polypropylene
1046.31	CO-O-CO	Anhydride	Strong, Broad	Stretching	Polystyrene
1282.57	C-O	Aromatic Ester	Strong	Stretching	Nylon
1339.47	C-N	Aromatic Amine	Strong	Stretching	Polyvinyl chloride
1384.79	O-H	Phenol	Medium	Bending	Polypropylene
1510.16	C-F	Fluoro Compound	Strong	Stretching	Polycarbonate
1544.88	N-O	Nitro Compound	Strong	Stretching	Nylon
1642.27	C=C	Alkene	Strong	Stretching	Nylon
1746.42	C=O	Δ-Lactone	Strong	Stretching	Polyethylene
2360.71	C-H	Alkene	Medium	Stretching	Polyethylene
2822.63	C-H	Alkane	Medium	Stretching	Polycarbonate
2883.38	C-H	Alkane	Medium	Stretching	Nylon
3330.84	C-H	Alkyne	Strong, Sharp	Stretching	Nylon
3738.75	O-H	Alcohol	Medium, Sharp	Stretching	Nylon

Plates showing different types of polymers in soil samples

Plate 9

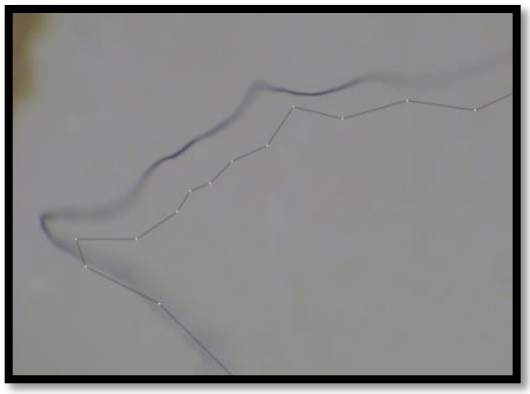


Plate 10

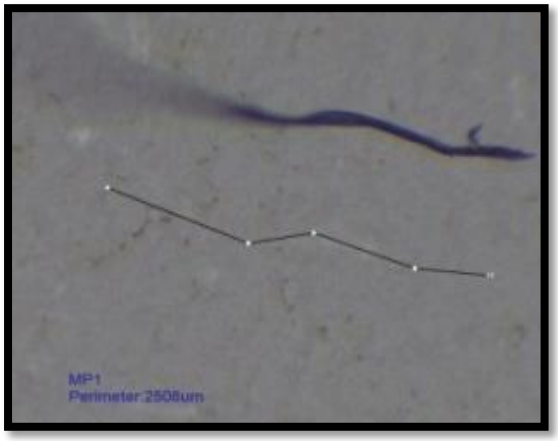


Plate 11

Plate 12

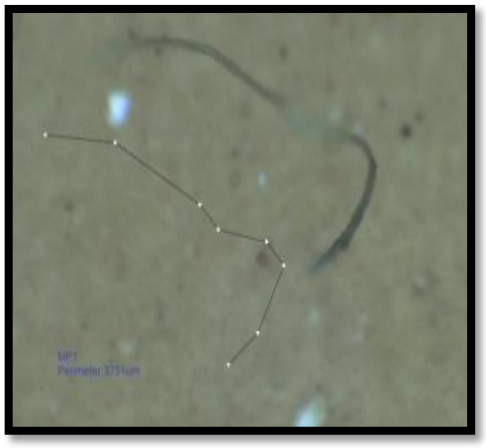


Plate 13

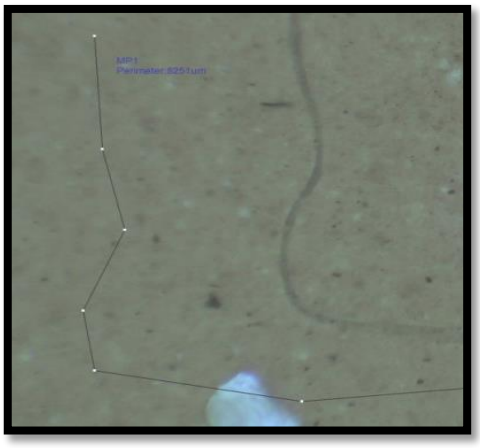


Plate 14

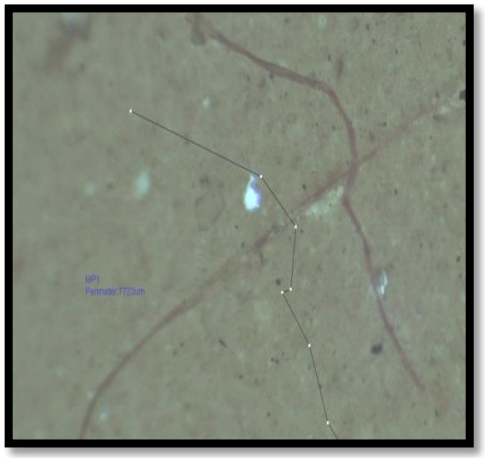


Plate 15

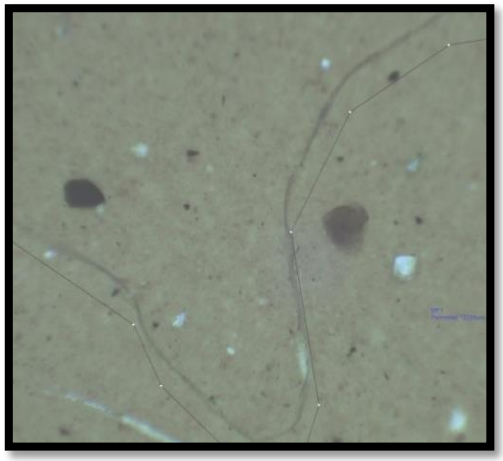


Plate 16

Figure 2: FTIR spectra showing different types of polymers in soil sample

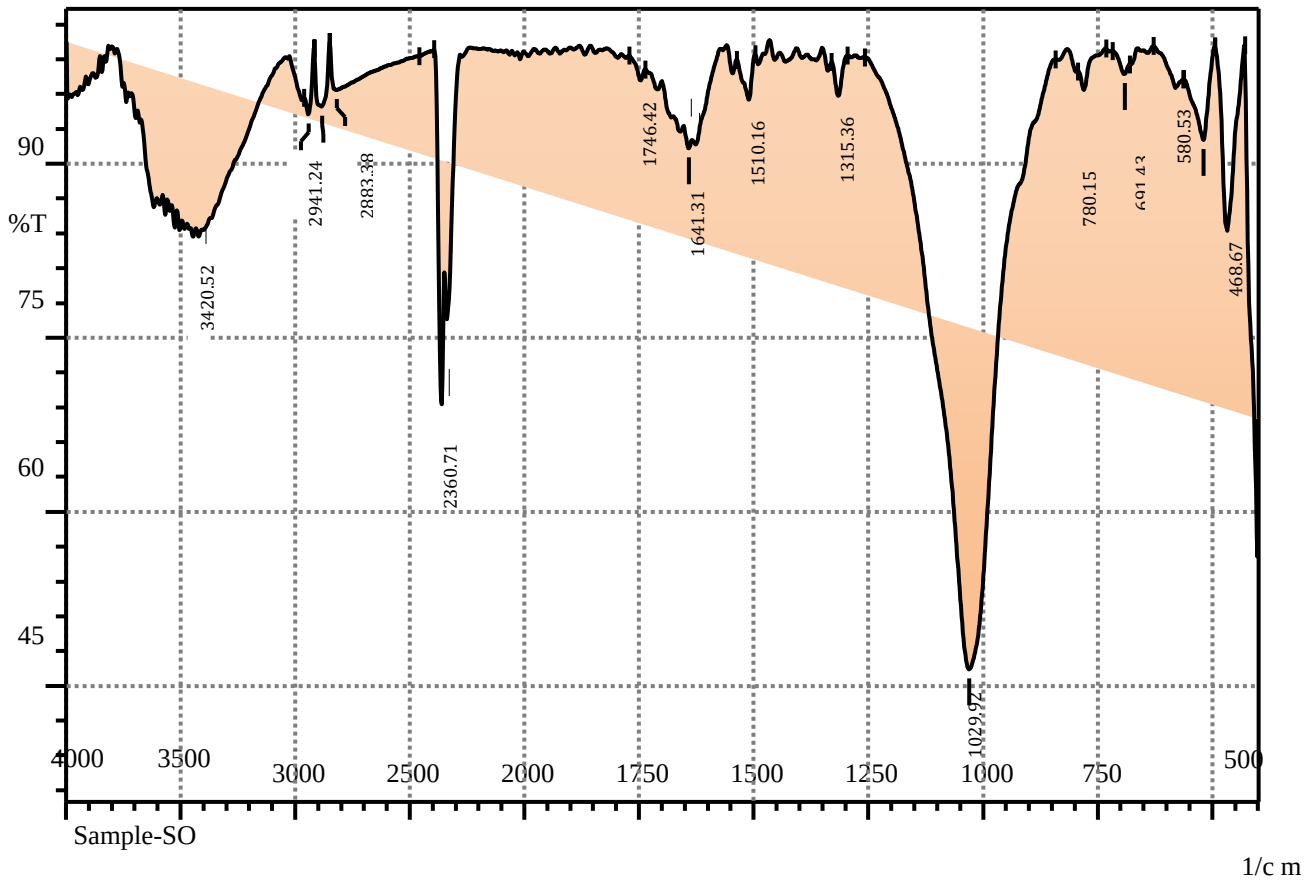


Table 3: Table showing peaks and intensity of various microplastics from reflectance of FTIR-spectroscopy in soil sample

	Peak cm^{-1}	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	468.67	84.15	15.995	494.71	429.13	2.192	2.237
2	518.82	92.045	7.048	562.21	494.71	1.385	0.995
3	580.53	96.527	1.534	628.75	562.21	0.514	0.134
4	691.43	97.72	1.187	717.47	679.86	0.224	0.082
5	780.15	96.353	2.01	792.69	731.94	0.385	0.103
6	1029.92	46.424	52.648	1257.5	841.87	44.932	43.264
7	1315.36	95.854	3.146	1329.83	1295.11	0.358	0.214
8	1510.16	95.509	3.783	1537.16	1496.66	0.475	0.335
9	1641.31	91.303	1.21	1652.88	1634.56	0.663	0.057
10	1746.42	97.168	1.317	1771.5	1735.81	0.276	0.076
11	2360.71	69.125	16.929	2392.53	2348.17	3.782	1.693
12	2818.77	96.327	4.074	2848.67	2457.14	3.549	3.363
13	2883.38	94.934	5.268	2917.13	2848.67	1.131	1.192
14	2941.24	94.279	3.227	2959.56	2917.13	0.779	0.364
15	3420.52	83.706	0.661	3433.06	3407.02	1.962	0.04

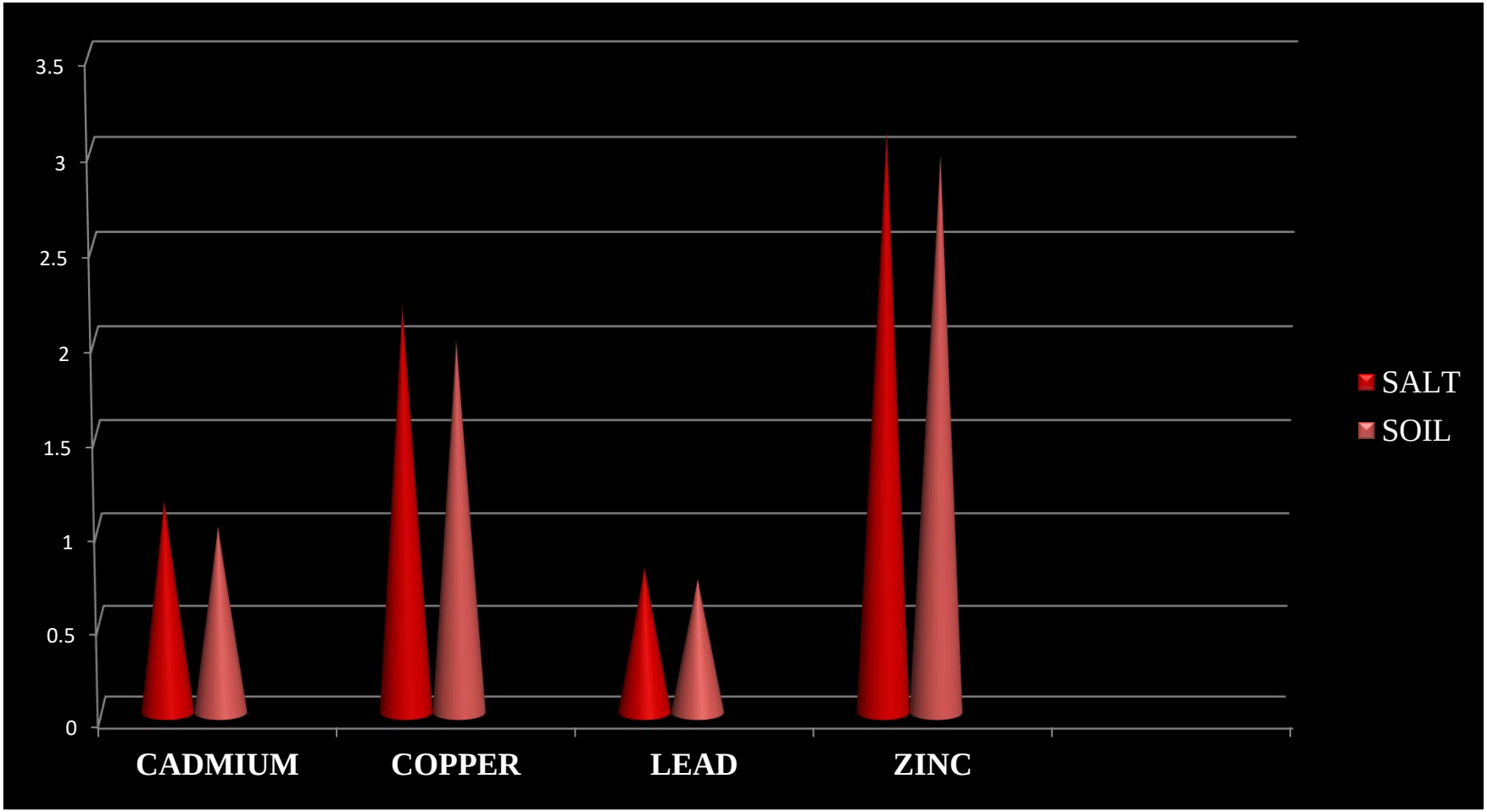
Table 4: Showing characteristic of peaks,functional group of polymers present in soil sample

PEAK cm^{-1}	FUNCTION GROUP	FUNCTIONAL GROUP NAME	APPEARANCE	VIBRATIONS	POLYMERS
468.67	C-I	Halo compound	Strong	stretching	Polytetrafluorethylene
518.82	C-I	Halo Compound	Strong	stretching	Polytetrafluorethylene
580.53	C-I	Halo Compound	Strong	stretching	Polytetrafluorethylene
691.43	C=C	Alkene	Strong	bending	Nylon
780.15	C=C	Alkene	Medium	bending	Ethylene vinyl acetate
1029.92	C-F	Fluoro Compound	Strong	stretching	Polycarbonate
1315.36	O-H	Phenol	Medium	bending	Polycarbonate
1510.16	N-O	Nitro Compound	Strong	stretching	Polyurethane
1641.31	C=C	Alkene	Strong	stretching	Nylon
1746.42	C=O	Δ-Lactone	Strong	stretching	Polyurethane
2360.71	O-H	Alcohol	Strong, Broad	stretching	Nitrile
2818.77	N-H	Amine Salt	Strong, Broad	stretching	Polycarbonate
2883.38	N-H	Amine Salt	Strong, Broad	stretching	Latex
2941.24	N-H	Amine Salt	Strong, Broad	stretching	Polycarbonate
3420.52	O-H	Alcohol	Strong, Broad	stretching	Polystyrene

Table 5: Heavy Metal Accumulation in Salt and Soil Samples

METALS	SALT SAMPLE (µg/g)		SOIL SAMPLE (µg/g)	WHO Standard (µg/g)
Cadmium	1.15		1.01	0.003
Copper	2.19		2	1.5
Lead	0.79		0.73	1.01
Zinc	3.11		2.98	5.0

Figure 3: Graph represents the heavy metals analysis in salt and soil sample



DISCUSSION

7. DISCUSSION

Salt is one of the most important and commonly used food additives throughout the world. Contamination of table salt could results in creating the health hazard for human. Since most of salt is used around the globe comes from mines and solar salterns, it is estimated that microplastic and heavy metal contamination might be a concern for table salt. Contamination of microplastic and heavy metals in salt creates a toxic effect on the surrounding biological environment (Jarup, 2003).Yamaguchi and kakzoai, 2001, Christophe Kaki *et al.*, 2011 reported that continuous intake of contaminated salt and salted foods could leads to stomach cancer and pre-cancerous effects in humans.

Over 14 million tonnes of microplastics have accumulated on the world's ocean floor according to research estimates. The amounts are increasing every year causing harm to ecosystems, animals and people. Globally, about 16-35% of microplastics are estimated to release into the oceans are from synthetic textiles. Between 200,000 and 500,000 tonnes of microplastics from textiles enter the global marine environment each year. The total amount of Microplastic intake by humans through contaminated salts, therefore, is expected to increase. Johnson *et al.* (2017) reported that an average Indian consumer takes 10.98 g of salt per day, which is twice the limit of 5 g per day recommended by the WHO (2012). Marine debris produces a wide variety of negative environmental, economic, safety, health

and cultural impacts. The presence of microplastics in the sea water has been revealed as hazardous which further gets accumulated in the salt pans. In literature, three possible toxic effects of plastic particle have been indicated: first due to the plastic particles themselves, second to the release of persistent organic pollutant adsorbed to the plastics and third to the leaching of additives of the plastics. Coastal environments contain keystone species and are a source of food supply. Microplastics might absorb these contaminants from the seawater and transfer them to the sea products and salt pans. So the presence of microplastics in the sea salt might pose a threat to food safety. However, microplastics can reach the human organism through other type of food from sea, such as fish and mussels.

FTIR is a well-recognized, rapid and quite reliable method to identify polymer types of different microplastics by comparing the resulting FTIR spectra with known plastic polymers in the spectral library and also used to confirm the composition of microplastics. Using this technique, several types of microplastics were identified in the present study, being the most common ones are Polystyrene (PS), Polycarbonate (PC), polyethylene (PE), Nylon, Polypropylene (PP) and Polyvinyl Chloride (PVC).

Polyethylene is the most common element found in packaging material and floaters that are used during fishing practices. Recently, MPs were also recorded from Arctic sea ice, fish, sea birds and sea salts in highly contaminated surface waters. Until now, only a limited number of global

surveys have been conducted on the quantity and distribution of MPs in marine sea salts (Soylak and Yilmaz, 2006; Van Cauwenberghe *et al.*, 2013;Jambeck *et al.*, 2015; Mason *et al.*, 2017; Schirinzi *et al.*, 2017). Domestic waste materials were found to be the main source of poly-ethylene and polypropylene contaminants. Every day, large volumes of polyethylene covers in the form of tea covers, milk covers, cooking covers, shop covers, medicine covers, and bust plastic materials are left abandoned due to lack of proper garbage disposal.

The results obtained in this study have been compared with other salt studies worldwide. The MP concentrations found in the salts are similar to those reported by studies in sea salt samples that were collected from eight representative salt pans located in the country's largest salt farming area, in the Maheshkhali Channel, along the Bay of Bengal. Microplastics were detected in all samples, with mean concentrations ranging from 78 ± 9.33 to 137 ± 21.70 particles kg^{-1}, mostly white and ranging in size from 500–1000 µm. The prevalent types were: fragments (48%) > films (22%) > fibers (15%) > granules and lines (both 9%). Fourier transform mid-IR and near-IR spectra (FT-MIR-NIR) analysis registered terephthalate (48%), polypropylene (20%), polyethylene (17%), and polystyrene (15%) in all samples.

Andrea Kappler *et al*, (2016) analysed the environmental microplastics by vibrational micro spectroscopy like FTIR and Raman. FTIR

(spectroscopy and chemical imaging) techniques that are used to describe different polymer kinds of MPs showed Polyethylene (PE), Polypropylene (PP), Polystyrene (PS), Polycarbonate (PC), Polyvinyl chloride (PVC), and Nylon are the predominant polymer types found in all environmental matrices, according to the review analysis. Other polymer types that have been covered in the studies include polypropylene (PP), nylon, and polyethylene (PE).

In the present study, spectral ranges from 691.43cm-1 to 3738.75 cm^{-1} were reported in salt and from 468.67 cm-1 to 3420.52 cm-1 were reported in soil samples. Andrea Kappler *et al*, (2016) examined spectral range of 2780–2980 cm−1, 22 particles of PE (including PE copolymers and oxidized PE), 5 particles of PP and 9 particles of PVC were identified. In the Raman image generated by the spectral range of 1580–1640 cm−1, three microplastics of PC, one microplastics of PS and six microplastics of PET were determined. Using the Raman image of 709–759 cm−1, three particles of PTFE could be detected. Hence, the total number of microplastics caught by Raman imaging was 49 for the selected sample area (1000 × 1000 μm) of the marine sample.

Heavy metals are naturally occurring elements that have a high atomic weight and a density at least 5 times greater than that of water. Their multiple industrial, domestic, agricultural, medical and technological applications have led to their wide distribution in the environment; raising

concerns over their potential effects on human health and the environment. Their toxicity depends on several factors including the dose, route of exposure, and chemical species, as well as the age, gender, genetics, and nutritional status of exposed individuals. Because of their high degree of toxicity, arsenic, cadmium, chromium, lead, and mercury rank among the priority metals that are of public health significance. These metallic elements are considered systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure.

Contamination of heavy metals creates a toxic effect on the surrounding biological environment (Jarup, 2003).Yamaguchi and kakzoai, 2001, Christophe Kaki *et al.*, 2011 reported that continuous intake of heavy metal contaminated salt and salted foods could leads to stomach cancer and pre-cancerous effects in humans.

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 and Pb. 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). Cd is one of the most toxic metals to

aquatic biota as well to humans (Neff, 2002). In the present study concentration of Cd in salt is 1.15µg/g, while soil had a cadmium concentration of 1.01µg/g. The highest concentration was found in salt.

Cu contents of Veppalodai and Tharuvaikulam saltpan sediments reflects the various industrial activities and fly ash dumps of Tuticorin Thermal Power plant (Baskaran*et al.*, 2002).The trace amount of this element is also found associated with white salt because Cu tends to be absorbed by organic matter and is also a coprecipitate with carbonates (Hamid Reza Pakzad *et al.*,2014, Morillo*et al.*, 2007, Jenne, 1968, Alloway, 1994,Amdouni, 2009). As stated earlier and by Brookins (1988), Cu could precipitate as Cu, Cu2S and CuS when suitable Eh–pH conditions prevail in mud sediments. Therefore, a noticeable difference is observed between the amounts of this component in the salt samples relative to its average concentration in the mud flat sediments of Tuticorin salt pans (Hamid Reza Pakzad *et al.*, 2014). In our present study copper levels in soil and salt were both 2.19µg/g and 2 µg/g, respectively. The highest concentration was visible in the salt.

In the present study, Lead concentrations in salt and soil were 0.79 and 0.73 µg/g, respectively. Krishna Kumar *et al.*, 2012 examined the Pb content in Tuticorin salt pans was found to be 15.15 to 17.18 mg/ kg with an of average 15.97 mg/ kg in Roche Park and SPIC Nagar area salt pans. Pb contamination is almost certainly from petrochemical industries, metal

smelting units, various fertilizer and salt chemical industries, dredging and dumping of sediments from harbors (Ramachandran *et al.*, 1991) and fly-ash (SiO_2, Al_2O_3, Fe_2O_3 and insoluble residues) from the nearby thermal power plant (Smith and Anderson, 1981., Crecelius, 1985).

Krishna Kumar *et al.*, 2012 reported the concentration of Zn in Tharuvaikulam and Veppalodai salt pans was 5.07 - 0.45 mg/ kg and in Roche Park and SPIC Nagar salt pans it was 19.5 - 2.16 mg/ kg. These higher concentrations of Zn, recorded in Roche Park and SPIC Nagar salt pans. In the present findings, a concentration of 2.98 g/g of zinc occurred in the substratum (soil) of salt and a high concentration of 3.11 g/g of zinc were observed in salt. These may be mainly due to effluents because of the ore handling in the harbor, heavy water plant and solar alkali plant (Diya *et al.*, 2014). When compared to all the other elements, zinc was found to be abundant in all the samples that were gathered for the current investigation.

Soylak *et al.*, (2008) reported nearly 1.64µg/g Pb content in Turkey, Egypt, Greece and Brazil and 0.01-0.03, 0.18-0.22 and 0.18-0.19 µg/g Cd in edible solar salt consumed by humans. As nickel and zinc are also deposited in the sediment of salt pans; toxic recurrence is imminent due to direct intake of solar salt by humans.

The results were compared with the standard limits of the World Health Organisation (WHO). Lead and Zinc where within the permissible

limit while Cadmium and Copper were slightly above the permissible limit. This may be due to anthropogenic activities, discharging of industrial sewages and fly ash dumps from the nearby Tamil Nadu Thermal Power Plant etc.

CONCLUSION AND SUGGESTIONS

8. CONCLUSION AND SUGGESTIONS

The Tuticorin solar salt pans may receive traces of microplastic and heavy metal accumulation due to new industrial developments. Further, present values of microplastic and heavy metal concentrations can be used as baseline data for future comparisons with regard to heavy metal pollution. Although MP contamination levels are now modest, they could eventually rise due to rising plastic usage and persistently inappropriate disposal techniques. To fully comprehend the process by which MPs bind to human tissue and their potential effects, more research is required. For the safety of food and human health, MPs in various sea resources must be regularly monitored.

As a measure to prevent contamination 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 microplastic and heavy metal concentration.
- Programmes on the protection of Gulf of Mannar biosphere reserve and Pollution control may be periodically broadcast and telecast to raise awareness among the public.

SUMMARY

9. SUMMARY

- Microplastics and heavy metals were present in salt and soil samples.
- In salt samples,2 microplastics of PE, 2 microplastics of PP, 2 microplastics of PP, 6 microplastics of Nylon and 2 microplastics of PC were detected from the IR image produced by the spectral range of 691.43 cm^{-1} to 3738.75 cm^{-1}. Total of 15 microplastics were found in salt sample using FTIR imaging.
- Likewise 3 microplastics of PTFE, 2 microplastics of Nylon,1 microplastic of EVA,4 microplastics of PC,2 microplastics of PU and 1 microplastic of PS were detected from the IR image produced in soil sample by the spectral range of 468.67cm^{-1} to 3420.52cm^{-1}.
- The IR picture was counted and one Latex and Nitrile particle was discovered in soil sample.
- Due to this, a total of 15 microplastics were found in soil sample using FTIR imaging. In terms of MP forms, this study found a greater proportion of fibre and fragment. The morphologies of the fragments and fibres identified in this investigation suggest that the microplastics are secondary microplastics.
- Next to microplastics analysis, some of the heavy metals like cadmium, Lead, Copper and Zinc in salt and soil were analyzed.

- According to this investigation, Copper and Cadmium are over the limit, Zinc and Lead are below the WHO allowed level. With the industrial expansion along this stretch of the Thoothukudi coast, this baseline data can be used for routine ecological monitoring.
- According to the bioanalysis of microplastics in salt , humans who are exposed to them may suffer from oxidative stress, cytotoxicity, neurotoxicity, immune system disruption, and the transfer of MPs to other tissues. These microplastics are caused by plastic that is not properly disposed of and left on the streets, as it can end up in canals, rivers, and drainage systems as salt come from the evaporation method of those plastic contaminated sea water.

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11. BIBLIOGRAPHY

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ANALYSIS OF GUT MICROBES FROM *PENAEUS MONODON*FROM THOOTHUKUDI COAST

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Thoothukudi

April 2023

CERTIFICATE

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Date: 06.04.2023

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CONTENTS

INTRODUCTION

1. INTRODUCTION

Penaeus monodon, is a marine prawn that is a common sea food. It is also commonly referred to as the giant tiger prawn, Asian tiger shrimp, or black tiger shrimp, *Penaeus monodon* mature and breed only in tropical marine habitats and spend their larval, juvenile, adolescent and sub-adult stages in coastal estuaries, lagoons or mangrove areas. In the wild, they show marked nocturnal activity, burrowing into bottom substratum during the day and emerging at night to search for food as benthic feeders. Under natural conditions, the giant tiger prawn is more of a predator than an omnivorous scavenger or detritus feeder than other penaeid shrimp. After moulting, the new shell is still soft which causes prawns to become vulnerable and they may subsequently be eaten by their predators or companions. Adults are often found over muddy sand or sandy bottoms at 20-50 m depth in offshore waters.

Wild males posses spermatozoa from around 35 g body weight and females becomes gravid from 70 g. Mating occurs at night, shortly after moulting while the cuticle is still soft, and sperm are subsequently kept in a spermatophore (sac)inserted inside the closed the lycum of the female. There are five stages in ovarian maturation; undeveloped, developing; nearly ripe; ripe; and spent. *P. monodon* females are highly fecund with gravid females producing as many as 500 000 to750000 eggs. Spawning occurs at night and fertilization is external with females

suddenly extruding sperm from the lycum as eggs are laid in offshore waters. Hatching occurs 12-15 hours after fertilization. The larvae, termed nauplii, are free swimming and resemble tiny aquatic spiders. This first stage in larval development does not feed but lives on its yolk reserve and passes rapidly through six moults.

The next larval stages [protozoa, mysis and early post larvae (PL) respectively] remain planktonic for some time and are carried towards the shore by tidal currents. Protozoa, which have feathery appendages and elongated bodies, moult three times and then metamorphose into the mysis stage. Mysis, which have segmented bodies, eyestalk and tails characteristic of adult shrimp, also moult three times before metamorphosing into PL with similar characteristics to adult shrimp.

Penaeus monodon is found at depths from 0 to 110 m, inhabiting bottom mud and sand. Giant tiger prawn live in brackish, estuarine (juveniles) and marine (adults) environments (FAO, 1980). In its natural range, *P. monodon* frequents water temperatures of 18–34.5°C and salinities of 5–45 ppt. It is even grown commercially at salinities of 1–5 ppt. Penaeus monodon appears to select muddy mangrove channels and often associates with marginal or floating vegetation.

Penaeus monodon are generally dark coloured, with the carapace and abdomen transversely banded with black and white **(Figures 1**). The rest of the body

is variable, ranging from light brown to blue or red, while some smaller specimens show a dull red dorsal strip from the rostrum to the sixth abdominal segment

Penaeus monodon is the most prominent farmed crustacean product in international trade and has driven a significant expansion in aquaculture in many developing countries in Asia. Frozen head-on, head-off, and peeled shrimp used to be the major products for export to the main markets, which are USA, EU and Japan. Later, value-added products, such as microwavable or ready-to-cook tempura, sushi, shaomei, hargao, straightened, skewered, battered and breaded, spring roll and balls mainly processed in Thailand, have become increasingly popular. This has been because tight economic conditions in many developed countries limit frequent dinner in restaurants, and the time for cooking at home is scarce. Chilled product, which is sold in domestic markets, is generally non-exportable grade and shares less than 10percent of all markets. Live product, which is mainly for domestic Chinese restaurants with some exports to Hong Kong and China, also shares less than 2percent. (Chaitiamvong et al ,1992).

In financial value, Penaeus monodon is the most important traded aquaculture commodity in Asia. C & amp; F prices in Japan, whose market mainly requires large headless (16/20 size) shrimp from extensive and semi-intensive farms in Indonesia, India and Viet Nam, varied from USD 9-14/kg during 2001-2004. The US market purchased mainly small headless (21/25 size) shrimp (both peeled and shell-on) from

intensive farms in Thailand and India at C & amp; F prices ranging from USD 7-13/kg during the same period. The EU market, which mainly requires small head-on shrimp (31/40 size) from South East Asian intensive farms, paid C & amp; F prices between USD 4.7 and 9.0/kg during 2001-2004. (Kongkeo et al.,1997).

The prawn is a potent source of protein, minerals (magnesium, phosphorous, calcium and manganese), vitamins (A and D), and long-chain unsaturated fatty acids, including eicosapentaenoic acid (EPA, C20:5, and DHA, C20:5) (Ferdose & Hossain, 2021; Bhavan et al., 2018). It also contains an elevated level of phospholipid (36–42% of the total lipid) mostly concentrated in the ovary, which has a vital role in brain and mental health (Ahammed, Tian, et al., 2020).

Aquaculture has evolved as one of the fastest growing food production systems in the world, contributing significantly to global food security, and after finfish, a major share of the global aquaculture production is contributed by crustaceans.

Animals live in intimate association with diverse communities of symbiotic microorganisms (Hamady and Knight,2009). The intestinal microbial communities are particularly abundant and diverse, and the microbes contribute several important functions to their hosts, such as promoting host development, nutrition, and immunity (Bolnick 2014; Lathrop 2011), The intestinal microbes contribute important functions to their hosts, such as fermenting unused energy substrates,

training the immune system, preventing growth of pathogenic bacteria, regulating the development of the gut, and producing vitamins for the host (Hooper et al., 2002; Ye et al., 2014).

Establishing and maintaining beneficial interactions between the host and its associated microbiota are key requirements for host health. Furthermore, the sum of the genetic information of host and its microbiota is defined as the "hologenome", and the hologenome theory suggests that the adaptation and evolution of higher organisms cannot be well described without considering their microbial symbiont. (Zilber-Rosenberg and Rosenberg E (2008).

The gut microbiota is a complex microbial ecosystem with important roles in health and development of organisms. Some deterministic and stochastic processes are thought to shape the gut microbiota. These processes are generally driven by environmental and biological factors. Factors such as host immune system, pH in the gut, and dietary composition are considered to be the dominant factors in shaping the gut microbiota. Biological factors, for example, interspecies interactions (competitive, mutualistic, and some synergistic interactions) may further affect the composition of the microbiota.

Bacteria have a very important role in aquaculture because they develop naturally and contribute to organic matter degradation, nutrient cycling, and

productivity (Martinez-Porchas et al., 2012). Some bacteria can be added to culture systems with the purpose of improving water quality, controlling diseases, improving nutrition of cultured animals, and reducing the environmental impact of aquaculture effluents (De Schryver et al., 2008). A recent trend to in aquaculture today is formulation of probiotics that will improve the farm yields by decreasing the incidences of diseases. Probiotics play an important role to improve the growth, feed efficiency, control microbiota, water quality and confer resistance to diseases in Aquaculture industries (Tuan et al.,2013).

Penaeid shrimp aquaculture is an important source of economic gain for many Asian and Latin American countries (Hernández-Rodríguez et al., 2001) and shrimp research has subsequently dominated the field of marine-based invertebrate gut microbiomes. However, in comparison with mammals and terrestrial invertebrates, relatively very little is known about the bacteria living in the gut of aquatic invertebrates such as penaeid shrimp.

A knowledge the gut microbiota is essential to formulate probiotics as feed for the animals in culture. The microbiome analysis is not only for probiotic formulation, but is also an index for analyzing the environmental niche. The importance to study the intestinal microbiota of the prawns, it contributes to the knowledge of the resident bacteria with probiotic capacities that can be applied in

aquaculture, because the isolated bacteria from the fish are always used as an efficient probiotic (Ferreira et al., 2015).

Gut microbiome are also considered as indices of general health of the prawns in aquaculture. Gut-inhabiting microbes are recognised as important drivers of several metabolic processes in the host. As such, the characterization and subsequent manipulation of this microscopic community is an attractive proposition for aquaculture research. The shrimp gut microbiota is fundamental to the host's nutrition, growth, pathogen resistance and maintenance of the internal steady state or homeostasis. Feeding characteristics of shrimp, such as actively grazing and cannibalism, make the host vulnerable to pathogen invasion. Colonization by alternative microorganisms destabilize the intestinal microbiota, which leads to infections and co-infections in a taxonomically diverse gastrointestinal (GI) tract.

Recent studies have shown that the host genetics was a main contributor to the divergence of shrimp gut microbiomes between two closely related species, while the host habitat type seemed to play a critical role when comparing the microbial variation of hosts belonging to different lineages (Tzeng et al., 2015).

The culturable gut microbiome is studied by isolation and culture techniques using appropriate culture media and culture techniques. The unculturable gut microbiome is analysed by metagenomic analysis. A recent trend in microbiome

analysis is by massive parallel sequencing of gut microbiome using next generation sequencing technologies. In the present study, an attempt was made to analyse the composition of the culturable gut microbiome.

REVIEW OF LITRATURE

2. REVIEW OF LITERATURE

Shrimp aquaculture is an important industry that provides important source of seafood for the growing world's population. The black tiger shrimp (*Penaeus monodon*) is one of the major species cultivated in Southeast Asia and the coasts of Australia (FAO, 2016; Stentiford et al., 2012), with high market demand. However, shrimp production is facing major challenges, mainly due to the increase in disease outbreaks that are causing mass mortalities and inflicting severe economic losses on shrimp aquaculture (Huang et al., 2020; Stentiford et al., 2012). Intestinal bacteria play an important role in promoting host health by enhancing nutrient absorption, metabolic processes, physiological adaptation, immune response, and disease resistance in aquatic animals (Hanning and Sanchez, 2015; Rajeev et al., 2020; Xiong et al., 2019). For instance, bacteria of the phylum Firmicutes can synthesize short-chain fatty acids and lactic acid-derived compounds, providing important metabolites for growth performance and health in many aquatic animals such as common carp (*Cyprinus carpio*) (Hoseinifar et al., 2015) and Atlantic salmon (*Salmo salar* L.) (Reveco et al., 2014). A previous study reported that *Brevibacillus*, which belongs to the phylum Firmicutes and has the ability to produce essential amino acid (Liang et al., 2020), was found in high abundance in the intestine of *P. monodon* and correlated with shrimp growth performance (Pitogo et al., 1998). In

addition, when given as feed additives, *Bacillus* sp. and *Lactobacillus* sp. have been reported to enhance shrimp resistance to *V. parahaemolyticus*, a causative pathogen of acute hepatopancreatic necrosis disease (AHPND) (Kewcharoen and Srisapoome, 2019) or *V. harveyi* in *Litopenaeus vannamei* (Kongnum & Hongpattarakere, 2012).

Microbiome is a term that describes the genome of all the microorganisms, symbiotic and pathogenic, living in and on all vertebrates (Taneja, 2017). The gut microbiome is comprised of the collective genome of microbes inhabiting the gut including bacteria, archaea, viruses, and fungi. Gut-inhabiting microbes are recognized as important drivers of several metabolic processes in the host. As such, the characterization and subsequent manipulation of this microscopic community is an attractive proposition for aquaculture research. The importance of the gut microbiome to health and wellbeing of humans and livestock is well understood, but this topic has only recently gained attention in aquaculture, particularly in prawn farming. The major challenges in prawn farming are availability of the right brood stock, low productivity, low culture technology and high dependencies on imported live foods during larval stages.

With rapid increases in the global shrimp aquaculture sector, a focus on animal health during production becomes ever more important. Animal productivity is intimately linked to health, and the gut microbiome is becoming increasingly recognised as an important driver of cultivation success. The microbes that colonise

the gut, commonly referred to as the gut microbiota or the gut microbiome, interact with their host and contribute to a number of key host processes, including digestion and immunity. Gut microbiome manipulation therefore represents an attractive proposition for aquaculture and has been suggested as a possible alternative to the use of broad-spectrum antibiotics in the management of disease, which is a major limitation of growth in this sector. Microbiota supplementation has also demonstrated positive effects on growth and survival of several different commercial species, including shrimp. Development of appropriate gut supplements, however, requires prior knowledge of the host microbiome. Little is known about the gut microbiota of the aquatic invertebrates, but penaeid shrimp are perhaps more studied than most.

The diversity prawn gut microbiome is influenced by various factors like environmental change, phylogeny of the host, developmental stage of the crustacean, exposure of the host to pathogens etc.

One of the biggest threats to shrimp aquaculture is the onset of disease and subsequent mortality in cultured stocks (Seibert & Pinto, 2012; Stentiford et al., 2012). Even in cases where the clinical signs of disease are well described, little is known about how the presence of a pathogen may impact or interact with the microbial communities in the gut and subsequently influence the metabolic processes within the host. On the other hand, it is unclear whether changes to the gut

microbiome may predispose the gut to invasion by (a) pathogen(s). Changes in gut microbiome structure could also facilitate the progression of enteric pathogens that rely on translocation through the gut epithelia to initiate infection in the target tissue. The notion of a 'one pathogen-one disease' scenario is being increasingly challenged (Dai et al., 2018; Bass et al., 2019; Huang et al., 2020b). The 'pathobiome' concept argues that the interactions between free-living microbes in the environment, host-associated symbionts (including the gut microbiota) and the host itself likely drive both beneficial and detrimental impacts on host health (Bass et al., 2019).

In humans, changes to the gut microbiota have been implicated in a wide range of health conditions. Characterization of the interplay between the microbiota and the host immune system is becoming increasingly well-defined (Sekirov et al., 2010). Pattern recognition receptors (PRRs) such as Toll-like receptors on the surface of the gut epithelia are in close proximity to microbial associated molecular patterns (MAMPs) of the microbiota such as lipopolysaccharides (Chu & Mazmanian, 2014). Although there are key differences between the vertebrate and invertebrate immune system, the gut microbiota likely has important roles to play in maintaining the health of the shrimp. The presence alone of symbiotic microbiota could itself provide a kind of immunity. A general theory true of all hosts is that space and resources within the gut are ultimately finite and colonization resistance may limit the proliferation of pathogenic organisms through competitive exclusion (Lawley &

Walker, 2013). Furthermore, colonization resistance may be further supported through microbiota-derived antimicrobial compounds, which may limit the establishment and proliferation of transient microbes in the digestive tract (Kobayashi & Ishibashi, 1993). A more species-diverse microbiota in the gut may facilitate resistance to a greater degree of potentially problematic colonizers, as there is consequently a larger set of species-species antagonisms. Reducing the abundance of certain bacterial classes within the microbiota can allow previously symbiotic species to become pathogenic (Blumberg & Powrie, 2016).

Because of the links between the gut microbiota and the host immune system, it is often suggested that a reduction in bacterial diversity within the gut or the differential abundance of particular microbial taxa may be responsible for the onset of pathogenesis. However, without follow-up studies involving gut supplementation and/or gnotobiotic organisms (germ free animals and/or organisms that harbour a defined microbial community) it is often impossible to discern between cause and effect.

A study about the habitat and indigenous gut microbes in oriental river prawn suggested that it shows high plasticity when its host faces environmental changes, even over short timescales (Chen et. al 2017). Further, the changes in external environment might influence the gut microbiome not just by providing environment-

associated microbes directly, but also by interfering with the composition of indigenous gut bacteria indirectly.

Tzeng, et.al (2015) studied about **e**ffects of host phylogeny and habitats on gut microbiomes of oriental River Prawn (*Macrobrachium nipponense*). It was found that Proteobacteria is the major phylum in oriental river prawn, followed by Firmicutes and Actinobacteria. Hierarchical clustering also showed that host genetics had a greater impact on the divergence of gut microbiome than host habitats.

Gut microbial communities associated with the molting stages of the giant freshwater prawn *Macrobrachium rosenbergii* was investigated and the structural changes of the resident gut bacterial communities was studied using the diversity of the 16S rRNA gene by 454 pyrosequencing, in the freshwater prawn *Macrobrachium rosenbergii* during its four-stage molt cycle (Mente *et al.,* 2016). Developmental and gut-related changes to microbiomes of the cultured juvenile spiny lobster *Panulirus ornatus* samples were analyzed using 16S rRNA next-generation sequencing. Core gut microbiomes of *P. ornatus* comprised the phyla Tenericutes and Proteobacteria. Within class Gamma proteobacteria, families *Pseudoalteromonadaceae* and *Vibrionaceae* were dominant members across the majority of the gut microbiomes (Ooi *et al.,* 2017). (Holt et al., 2021)

Microbiome analysis of Pacific white shrimp gut with reference to the rearing water from Malaysia and Vietnam. Microbial diversity of the shrimp gut was found to be generally lower than that of the pond environments with a few ubiquitous genera representing a majority of the shrimp gut microbial diversity such as *Vibrio* and *Photobacterium*, indicating host-specific selection of microbial species (Zoqratt et al., 2018).

The change in gut microbial community in disease outbreaks was studied. The shrimp (*Penaeus monodon*) gut microbiome with Acute Hepatopancreatic Necrosis Disease (AHPND) out breaks was studied by 16S rRNA metagenomics analysis. The microbial communities of infected shrimp were enriched with Firmicutes and Bacteroidetes, but the number of Proteobacteria was reduced compared with the healthy shrimps. AHPND infection was positively correlated with the levels of SCFAs (butyrate and propionate) producing species such as Bacteroides, Bifidobacterium, Lactobacillus and Staphylococcus. Significant differences were found in the functional gene profile category between healthy and infected shrimp gut. These findings improve the current understanding of AHPND disease's impact on the assembly of shrimp gut microbiota (Hossain et al., (2021)).

The Effect of the probiotic Lactococcus lactis on the microbial composition in the water and the gut of freshwater prawn (Macrobrachium rosenbergii) cultivated in biofloc was analyzed the most abundant genera present were Alphaproteobacteria,

Gammaproteobacteria, Betaproteobacteria, Flavobacteria, Actinobacteria, and Clostridia which have different benefits in the prawns' health (Kathia et al., 2022). Analysis of microbiota in the stomach and midgut of two penaeid shrimps during probiotic feeding showed that a probiotic can affect the microbiota throughout digestive tract of penaeid shrimps and that probiotic can have a role in preventing disease (Imaizumi et al., 2021).

A study on the factors affecting the gut microbiome in a state of dynamic equilibrium showed that there is a high correlation between the development of shrimp intestinal microbiota and environmental changes and subsequently the health status of shrimp. The changes in aquaculture ecosystem across age, environment, diet, and diseases or the exposure to new habitat has a great impact on composition of shrimp microbiota (Miao et al., 2020).

Marine invertebrate meals and feed restriction influence the biological and gut microbiota of shrimp *Penaeus monodon.* Feed restriction had a stronger effect on hepatopancreas digestive enzymes and gut microbiota than addition of any feed ingredient. Total protease activity was positively correlated with feed efficiency, including FCR, RETL and REGE. Shrimp fed to satiety tended to have high levels of *Vibrio* whereas those on the restricted ration tended to have higher levels of bacteria in Rhodobacteraceae, Flavobacteriales and Bacteroidales. Feeding restriction was shown to be a useful strategy to improve *P. monodon* feeding

efficiency, digestive capacity and modulate gut microbiota (Simon et al., 2020). *Bacillus coagulans* supplemented feed to *Macrobrachium rosenbergii* helped in the exclusion of two pathogenic bacteria *Streptococcus spp*., and *Klebsiella spp*., from its gut (Manjula et al., 2019)

Studies also relate the phenotypic characters of the shrimps with the gut microbiota. Quanxin Characteristics of intestinal microbiota in male morphotypes of the giant freshwater prawn *Macrobrachium rosenbergii.* It was observed that *Lactococcus garvieae* was significantly predominant. This finding strongly suggests that the intestinal microbiota of the Giant fresh water prawn is tightly correlated with the morphotypic differentiation likely owing to its effects on nutrient metabolism in the gut (Gao et al., 2022).

Response of gut microbiota, digestive enzyme ability, and immune function to starvation in *Macrobrachium nipponense* was analyzed. The functional prediction of the metabolic pathways showed that the intestinal microbiota was enriched in the KEGG pathways of amino acid, carbohydrate, fatty acid, and lipid biosynthesis and degradation. This result implied that the microbiota of shrimp gut decreased nutrition metabolism under the stress of starvation. The results confirmed that the activities of digestive and immune enzymes were directly related to the gut bacterial community and notably affected prawn growth (Shen et al., 2022).

The gut microbiota of the shrimps also affects the host genetics and probiotic treatment. By high throughput sequencing of amplicons generated from the bacterial 16S rRNA gene analysis of aquaculture-raised Pacific Whiteleg Shrimp, *Litopenaeus vannamei* demonstrated differences in gut bacterial composition. The results suggest that host genetic background can be an important determinant of gut bacterial composition in aquaculture-raised white leg shrimp and indicate that development of strategies to manipulate the microbiome of this important seafood will likely need to be customized depending on the genetic line (Landsman et al., 2019)

The knowledge of the gut microbiome is also important to formulate probiotics for aquaculture feed. Host gut-derived probiotic *Lactobacillus* sp. improves resistance of giant freshwater prawn *Macrobrachium rosenbergii* against *Vibrio harveyi*. The probiotic bacteria enhanced weight gain, digestibility, and immune response in the experimental prawn and its resistance against bacterial diseases (Ahmmed et al., 2020).

A comparison of microbiome bacterial community composition between Wild, Aquaculture and AHPND/EMS outbreak affected Pacific White leg shrimp revealed was done using sequencing of seven hypervariable regions of the 16S rRNA gene. Bacterial taxa like Faecalibacterium prausnitzii and Pantoeaagglomerans were enriched in healthy shrimp, whereas bacterial communities such as *Aeromonas*

taiwanensis, Simiduiaagarivorans and *Photobacterium angustum* were predominant in diseased shrimps (Cornejo Granados et al., 2017). Prawn gut bacteria were analyzed for their efficacy against shrimp pathogens. *Bacillus* spp. isolated from the gut of giant freshwater prawn were found to be utilized as potential probiotics against pathogens causing Vibriosis and Aeromonosis and were successfully used to resolve the infections. The Bacillus spp. isolated displayed high survivability towards 0.3% bile salt and exhibited amylase, protease, and lipase activities. Thus, the isolated Bacillus spp. are considered safe based on the sensitivity analysis towards antibiotics and γ-haemolytic activity. (Sam On et al., 2022)

The study of potential probiotic and health fostering effect of host gut-derived *Enterococcus faecalis* on freshwater prawn, *Macrobrachium rosenbergii* against a pathogenic microorganism, *Vibrio harveyi* occurring in the giant freshwater prawn confirmed its effectiveness against the pathogen. It as recommended that *E. faecalis* could be recommended to be used against bacterial infections of the *M. rosenbergi* (Khushi et al., 2022).

The maternal microbiome is found to influence the gut microbiota of the off springs in various stages of development. (Liu et al., 2021) reports that maternal and environmental microbes dominate offspring microbial colonization in the giant freshwater prawn. The results showed that Proteobacteria and Firmicutes were the dominant phyla in the intestine, gonads, and hepato pancreas of maternal prawn. The

microbial colonization in embryonic and post-larval stages was found to be attributed to the maternal and environmental microbe community. This study provides a theoretical basis for microbial community manipulation to promote prawn growth and physiological health in aquaculture.

Dietary manganese requirement and its effects on antioxidant enzyme activities, intestinal morphology and microbiota in of shrimps was investigated. The dominant phyla in the gut of *M. nipponense* were Proteobacteria, followed by Firmicutes and Bacteroidetes, regardless of the level of Mn supplementation. The intestinal core microbiota were not affected by the dietary Mn level, although the microbiota richness was somewhat affected (Ding et al., 2020).

Effect of stock density on the microbial community in biofloc water and Pacific white shrimp (*Litopenaeus vannamei*) gut microbiota were studied. It was found that shrimp intestinal microbiota as well as bacteria aggregated in flocs assembled into distinct communities from different stock densities, and the intestinal communities were more similar with the surrounding environment as the increase of stock density and resulting high floc biomass. The high stock density changed the core gut microbiota by reducing the relative abundance of Paracoccus and increasing that of Nocardioides, which may negatively influence shrimp performance (Deng et al., 2019).

The effect of *Lactobacillus brevis* supplementation for growth promotion of the freshwater prawns post larvae on gut microflora was done through 16s rDNA. The biochemical confirmation tests revealed presence of *Escherichia coli*, *Pseudomonas* spp., *Streptococcus* spp. and *Klebsiella pneumoniae* in the gut of control organism, whereas in the gut of experimental organism, in addition to the presence of *E. coli*, *Pseudomonas* spp. and *Streptococcus* spp., *Lactobacillus* spp., *Bacillus* spp. and *Clostridium butyricum* were detected (Bhavan 2018).

Core gut microbiota of shrimp function as a regulator to maintain immune homeostasis in response to WSSV Infectio was analyzed. The data showed the role of metabolites responsible for maintaining the immune homeostasis of the host and prove the function of the gut microbiota and the related metabolome in antiviral immunity of shrimp (Zhang et al., 2022). Evidence of Phylosymbiosis and their observations of a parallelism in the taxonomy of the gut microbiota with host phylogeny for all shrimp groups examined and in the predicted functions for the penaeid shrimps indicate a tight host-microbial relationship during evolution (Tang et al., 2021).

Intestinal bacterial community composition of shrimp varies under low- and high-salinity culture conditions and is correlated with high risk of disease Dongwei (Hou et al., 2016).

OBJECTIVES

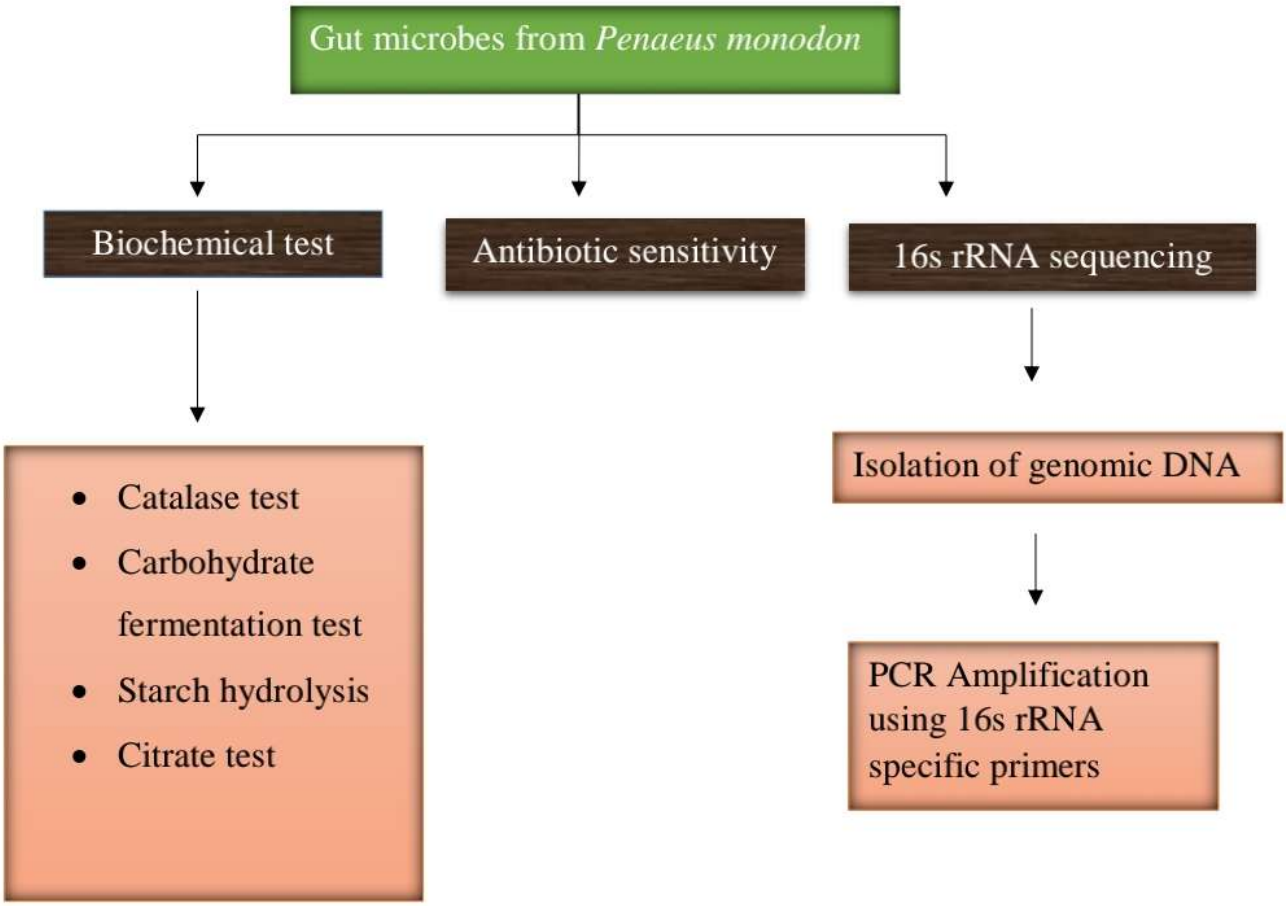
3.OBJECTIVES

The main objectives of this study are

1. To analyse the microbiome of the gut of giant tiger prawn *Penaeus monodon*
2. Isolate and characterize the culturable microbes.
3. Identification of the microbial population
4. Analyse the microbes for utilization as perspective probiotics.

EXPERIMENTAL DESIGN

4. EXPERIMENTAL DESIGN



MATERIALS AND METHODS

5. MATERIALSAND METHO

5.1 Sample collection

Fresh samples of the giant tiger prawn, *Penaeus monodon* were collected from the fishermen with their commercial captures landed at the fishing harbor. Samples were then immediately brought to the laboratory in insulated ice boxes. Specimen was identified by using the FAO Fishery identification key. At the laboratory, they are cleaned with water to remove all impurities. Samples were carefully dissected in order to separate the gut from prawn. Then the gut was homogenized using sterile mortar and pestle.

5.2 Isolation of the microbiome from the gut

Gut of the prawn was separated by cutting gently using a sterile forceps and scapel. The gut was then homogenized in a pestle and mortar using 1 ml of 1% saline solution. The homogenate was centrifuged at 12000 rpm and the supernatant was taken. The supernatant was serially diluted using 1% saline. Dilutions till 10^{-6} was done and 100 µl of the samples were plated from each dilution in Zobell Marine Agar. The plates were incubated at 37° C for 24 hours.

5.2.1 Isolation of pure bacterial cultures:

Isolated colonies were marked and numbered on the agar plates. Single colonies were picked and individual pure cultures were obtained by repeated

restreaking of the colonies. The colonies thus obtained were preserved in 4^0C for further analysis. The colonies were patched into fresh medium every 15 days.

5.3 Characterization of isolates:

Morphological characteristics of the colony of each isolated colony was studied. All the isolates were streaked on petriplates containing suitable media. After one day of incubation, morphological characteristics of colonies such as shape, size, pigmentation was studied under a compound microscope.

5.4 Staining of isolates:

Classification of Gram positive and Gram negative bacterial stain was done using the standard Gram staining protocol. A smear of bacterial cells was prepared on a clean class slide by gentle heat fixation. Heat fixed smear filled with crystal violet solution for one minute. The smear was washed with distilled water and then gram iodine was added. Smears were washed with 95% decolourisation and cleaned with water. Finally safranin was used as counter stains for 60-80 sec and washed with water. Then observed under a microscope.

5.5 Biochemical characterization:

Isolates were analysed for biochemical characters such as production of citrate production, catalase production, amylase production and glucose fermentation.

5.5.1 Citrate test:

About 15-20 ml of Simmon's citrate agar was transferred to the sterilized petridish. After the solidification of the medium, the culture was streaked and incubated for 24 hours at 37ºC. A colour no change of the media from green to blue indicates a negative result.

5.5.2 Catalase test:

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

5.5.3 Starch hydrolysis test:

Starch Agar medium was used to streak the cultures. The culture was inoculated and was incubated at 37^0C for 2 days. After the period of incubation flood the surface of the plate with Lugol's iodine solution. A zone of clearance around the colonies shows a positive test.

5.5.4 Carbohydrate fermentation test:

Carbohydrate fermentation medium was prepared. Durham's tube was inserted into the medium. Then the tubes were autoclaved at 121ºC for 15 minutes.

The selected isolates were inoculated and incubated at 37°C for 24 hours and observe the result.

5.6 Antibiotic sensitivity:

1 ml of 12 hours old cultures of the microbial isolates were spread plated on Marine Zobell agar. Sterilized paper discs (0.2 mm diameter) were prepared with different concentrations of antibiotics ampicillin and kanamycin.

The paper discs were loaded with different concentration viz 10 mg/ml, 25 mg/ml, 50 mg/ml, 100 mg/ml of ampicillin and kanamycin and placed on the medium inoculated with the cultures. The zone of inhibition of growth of microbe was measured after 24 hours.

5.7 Temperature sensitivity assay

The cultures were patched on Zobell Marine agar and the plates were incubated at different temperatures viz. 32^0C, 35^0C, 40^0C. The growth was observed after 24 hours of incubation.

5.8 Polymerase Chain Reaction

The genomic DNA of the cultures was isolated by lysing the bacterial cells, and precipitating out the DNA. The isolated genomic DNA was observed by loading it in 0.8% agarose gel and running the gel in 1X TBE. The concentration of the

DNA was estimated visually. 100 pg of each sample was taken for PCR analysis using 16s rRNA specific universal primers. The PCR was performed with the following conditions.

5.8.1 PCR conditions

95^0C – 3 min

94^0C -30 min
53^0C -1 min
72^0C -1 min
} 35 cycles

72^0C -8 min

RESULT

6.RESULT

6.1 Isolation of bacteria

Six bacterial strains were isolated from the mid gut of the black tiger shrimp (*Penaeus monodon*) (Fig. 1) gut. Out of the numerous colonies that were obtained on the Zobell Marine agar plates (Himedia™) six distinct colonies were taken and pure cultures of the colonies were obtained by quadrant streaking (Fig. 2). The colonies restreaked to obtain pure isolates. The purity of the samples was ensured by repeated streaking and observation through the compound microscope. The pure cultures were named as C1, C2, C3, C4, C5 and C6 and taken for further analysis. The pure culture were stored at 4° C for further study. The colonies preferentially grew on Marine Zobell Agar and they failed to grow on LB agar supplemented with high concentration of NaCl (15 g/l) (Fig. 3).

6.2 Temperature sensitivity

The cultures were tested for their temperature sensitivity. The optimal growth of the culture was found at 37^0C. None of cultures grew at 40^0C. Except for the colonies C3 and C6 all the other colonies were sensitive to temperatures 30^0C and 35^0C. (Fig.4)

6.3 Morphological characterization of isolated bacteria:

6.3.1 Morphological characterization

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

Taxonomic identity of bacterial strains were done by various tests based on the Bergy's manual of bacterial classification.

6.4 Gram staining of the isolates

Gram staining differentiates a bacteria as Gram positive or Gram negative, the two major divisions of bacterial classification. The six colonies were stained to differentiate as Gram positive or Gram negative. All the bacterial colonies were gram positive strain (Table 2, Fig.5)

6.5 Biochemical Analysis of isolate bacteria:

Biochemical test was performed for isolates and results are tabulated in Table2.

6.5.1 Citrate utilization test

Citrate test was done to identify the bacterial strains that can utilize citrate as their carbon source. The ability of the organisms to utilize citrate is observed by the

colour change of the medium from blue to green. The colony C6 was able to utilize citrate where as the other colonies were not able to utilize citrate as their carbon source. (Table 2, Fig. 6).

6.5.2 Catalase Test

Catalase test is done to identify strains that produce the catalase enzyme. The strains C1, C2, C3, C4, C5 and C6 are positive for catalase test (Table 2, Fig. 7).

6.5.3 Starch Hydrolysis test

In starch hydrolysis test is done to identify bacterial strains that produce amylase enzyme. None of the colonies grown on starch agar showed the zone of clearance with addition of iodine. All the colonies were negative for starch hydrolysis (Table 2, Fig. 9).

6.5.4 Carbohydrate fermentation test

Carbohydrate fermentation test was done to identify bacterial strains that ferment glucose. All the colonies (C1, C2, C3, C4, C5 and C6) fermented glucose, which was indicated by formation of air bubbles in Durham's tube. (Table 2, Fig. 8).

6.6 Antibiotic sensitivity:

The antibiotics like ampicillin and kanamycin were used to test the sensitivity of reference bacterial strains. Different concentrations of the antibiotics were used

to test their inhibitory activity and to find the minimum lethal concentrations. Concentrations 10 mg/ml, 25, mg/ml, 50 mg/ml and 100 mg/ml were used for the analysis. The zones of inhibition of the different colonies are tabulated in Table 3. While the colonies C1, C2, C4 and C6 were sensitive to both the antibiotics at 25 mg/ml, the colonies C3 and C5 were resistant to both the antibiotics, even at 100 mg/ml concentration (Fig .10).

6.7 PCR with 16s rRNA specific primers

In order to identify the species of the bacteria isolated PCR with the universal 16s rRNA primers were performed. Initially the genomic DNA from the six colonies were isolated and their presence was confirmed by running it on an agarose gel (Fig.12). After DNA estimation, about 500 pg of the DNA was taken for analysis with the 16s rRNA primers. A 700 bp amplicon was obtained in all the colonies (Fig. 13). The amplicons have to be taken for sequencing for confirming the species of the gut microbiome.

TABLE 1 MORPHOLOGY OF THE ISOLATES

Isolates	Colour	Shape	Margin	Elevation	Opacity
C1	Dull white	Irregular	Entire	Raised	Opaque
C2	Dull white	Irregular	Entire	Raised	Opaque
C3	Yellow	Irregular	Entire	Raised	Opaque
C4	Dull white	Irregular	Entire	Raised	Opaque
C5	Dull white	Circular	Entire	Raised	Opaque
C6	Yellow	Circular	Entire	Raised	Opaque

Note: C1 – Colony 1; C2- Colony 2; C3- Colony 3; C4 – Colony 4; C5 – Colony 5; C6 – Colony 6

TABLE 2 GRAM STAINING AND BIOCHEMICAL CHARACTERIZATION

Isolates	Gram staining	Citrate test	Catalase test	Starch hydrolysis test	Carbohydrate fermentation test
C1	Positive	Negative	Positive	Negative	Positive
C2	Positive	Negative	Positive	Negative	Positive
C3	Positive	Negative	Positive	Negative	Positive
C4	Positive	Negative	Positive	Negative	Positive
C5	Positive	Negative	Positive	Negative	Positive
C6	Positive	Positive	Positive	Negative	Positive

Note: C1 – Colony 1; C2- Colony 2; C3- Colony 3; C4 – Colony 4; C5 – Colony 5; C6 – Colony 6

TABLE 3 ANTIBIOTIC SENSITIVITY

Ampicillin sensitivity test for the isolates strains

Concentration of Ampicillin	Zone of Inhibition					
	Colony 1	Colony 2	Colony 3	Colony 4	Colony 5	Colony 6
10	0.6 cm	0.9 cm	-	0.5 cm	-	0.7 cm
25	1 cm	1 cm	-	0.7 cm	-	1 cm
50	1 cm	1.2 cm	-	0.7 cm	-	1.1 cm
100	1.3 cm	1.3 cm	-	1 cm	-	1.5 cm
Control	-	-	-	-	-	-

C – Water Control, 10-10 mg/ml Ampicillin; 25-25mg/ml Ampicillin;50-50mg/ml Ampicillin; 100-100mg/ml Ampicillin.

TABLE 3. KANAMYCIN SENSITIVITY TEST FOR THE ISOLATES

STRAINS

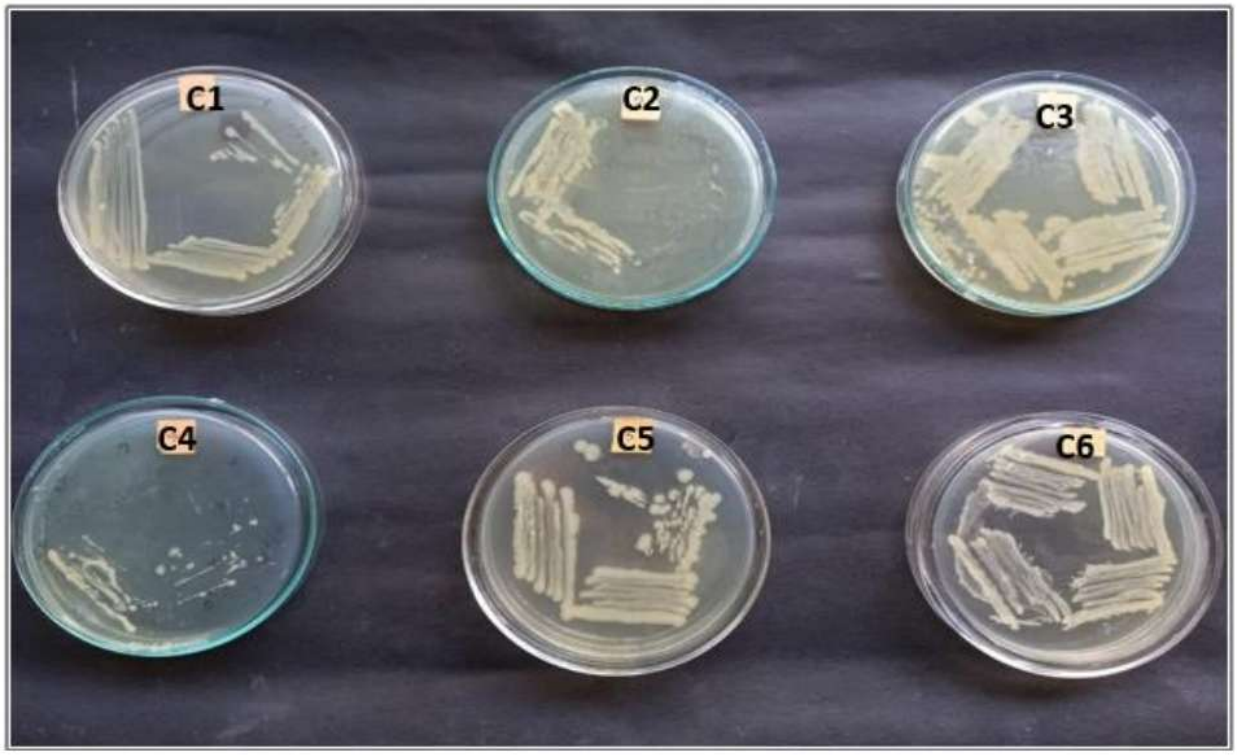
Concentration of Kanamycin	Zone of Inhibition					
	Colony 1	Colony 2	Colony 3	Colony 4	Colony 5	Colony 6
10	0.8 cm	0.5 cm	0.7 cm	0.5 cm	o.5 cm	0.6 cm
25	0.9 cm	0.6 cm	1 cm	0.7 cm	0.7 cm	0.7 cm
50	1 cm	1.2 cm	1 cm	0.9 cm	0.9 cm	0.8 cm
100	1.2 cm	1.2 cm	1.1 cm	1 cm	1.1 cm	1.2 cm
Control	-	-	-	-	-	-

C – Water Control, 10-10 mg/mlKanamycin; 25-25mg/ml Kanamycin;50-50mg/ml Kanamycin; 100-100mg/ml Kanamycin.

FIGURE 1 *penaeus monodon* – SCIENTIFIC CLASSIFICATION

Kingdom	-	Animalia
Phylum	-	Arthropoda
Subphylum	-	Crustacea
Class	-	Malacostraca
Order	-	Decapoda
Suborder	-	Dendrobranchiate
Family	-	Penaeidae
Genus	-	*Penaeus*
Species	-	*P. monodon*

FIGURE 2 PURE CULTURE FOR ISOLATED BACTERIAL STRAINS



Note: C1 – Colony 1; C2- Colony 2; C3- Colony 3; C4 – Colony 4; C5 – Colony 5; C6 – Colony 6

FIGURE 3 GROWTH OF THE BACTERIAL COLONIES ON LURIA BERTANI AGAR (LB AGAR) AND MARINE ZOBELL AGAR

Plate 1 – LB agar

Plate 2 – Marine Zobell agar

FIGURE 4 TEMPERATURE SENSITIVITY TEST

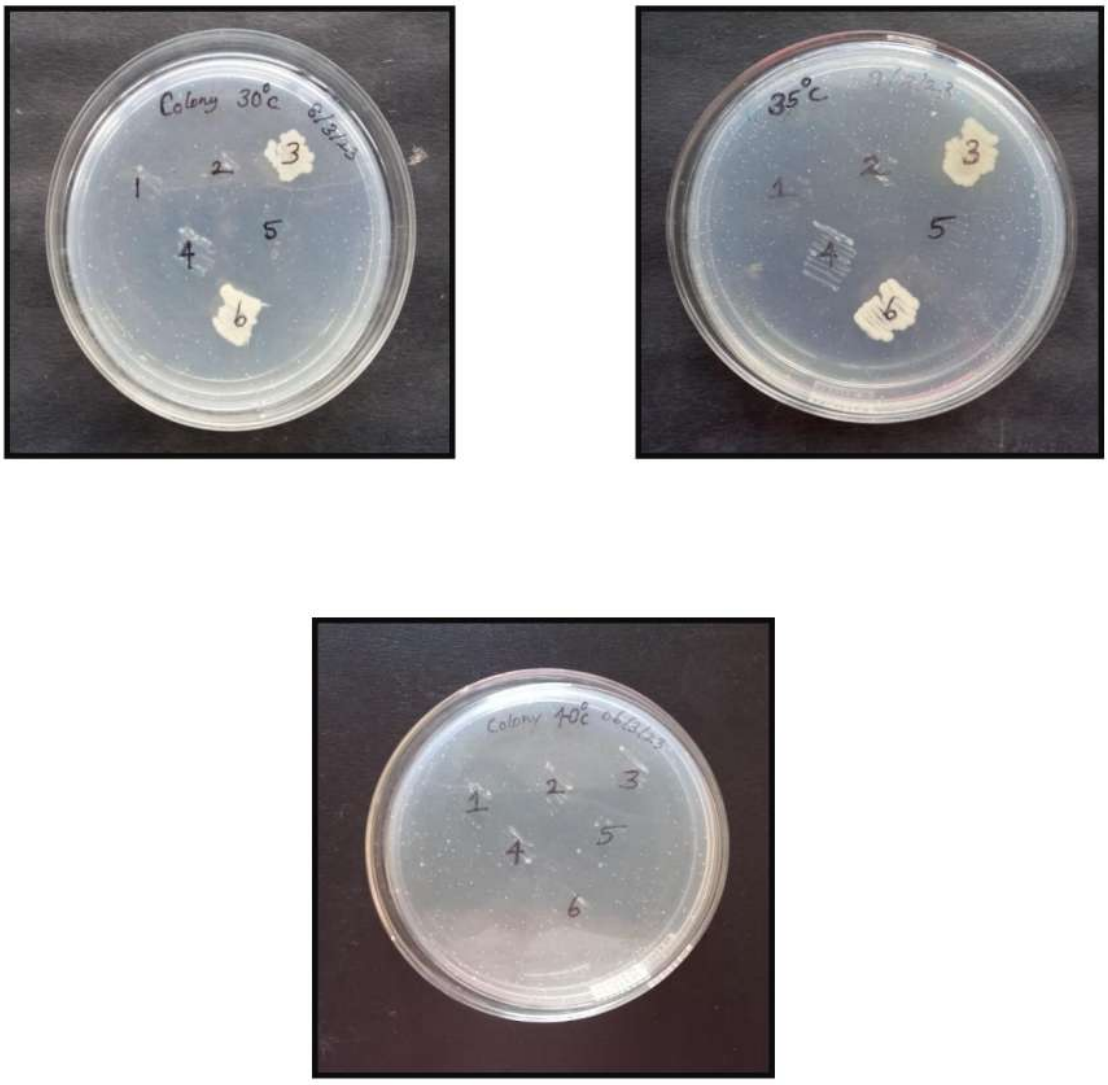
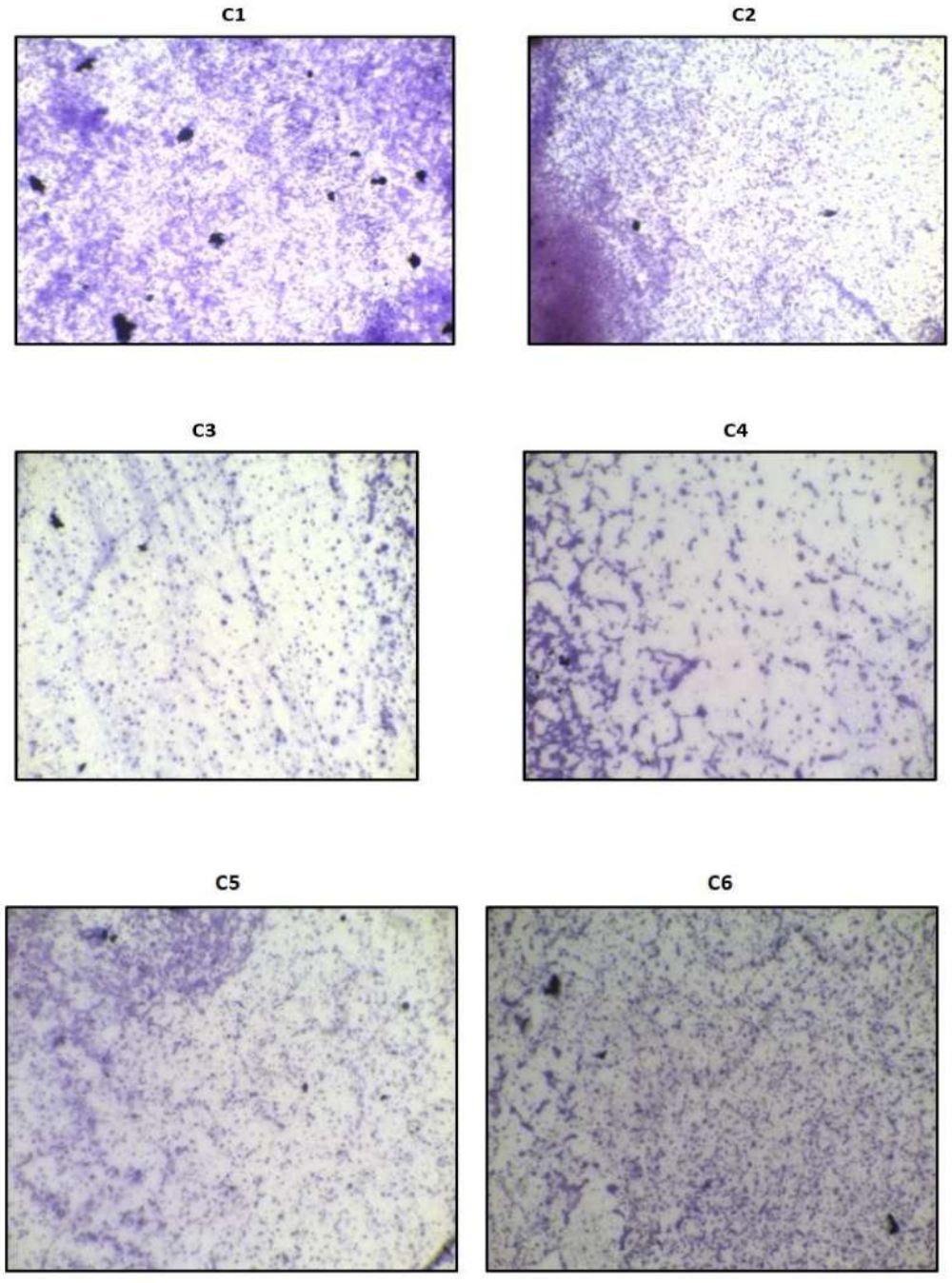
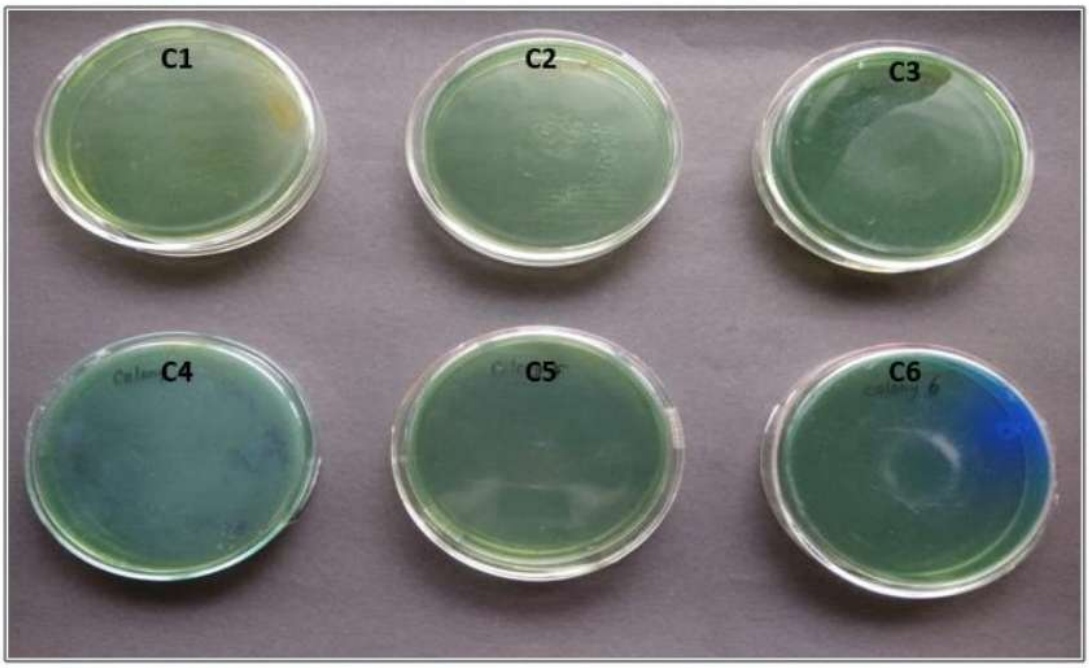


FIGURE 5 GRAM STAINING OF ISOLATED BACTERIAL STRAINS



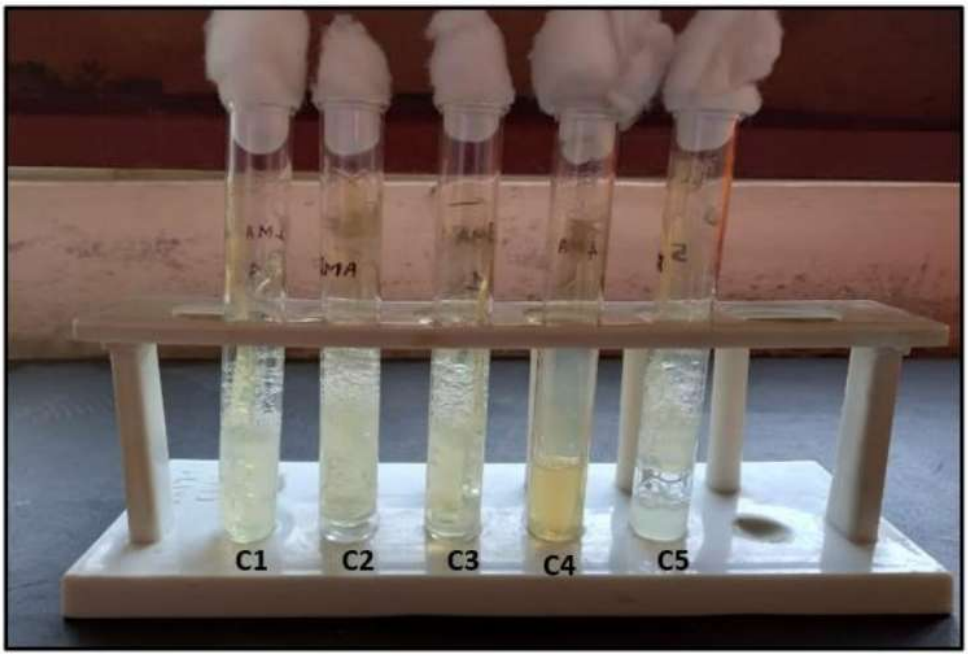
Note: C1 – Colony 1; C2- Colony 2; C3- Colony 3; C4 – Colony 4; C5 – Colony 5; C6 – Colony 6

FIGURE 6 CITRATE UTILIZATION TESTS OF THE BACTERIAL STRAINS



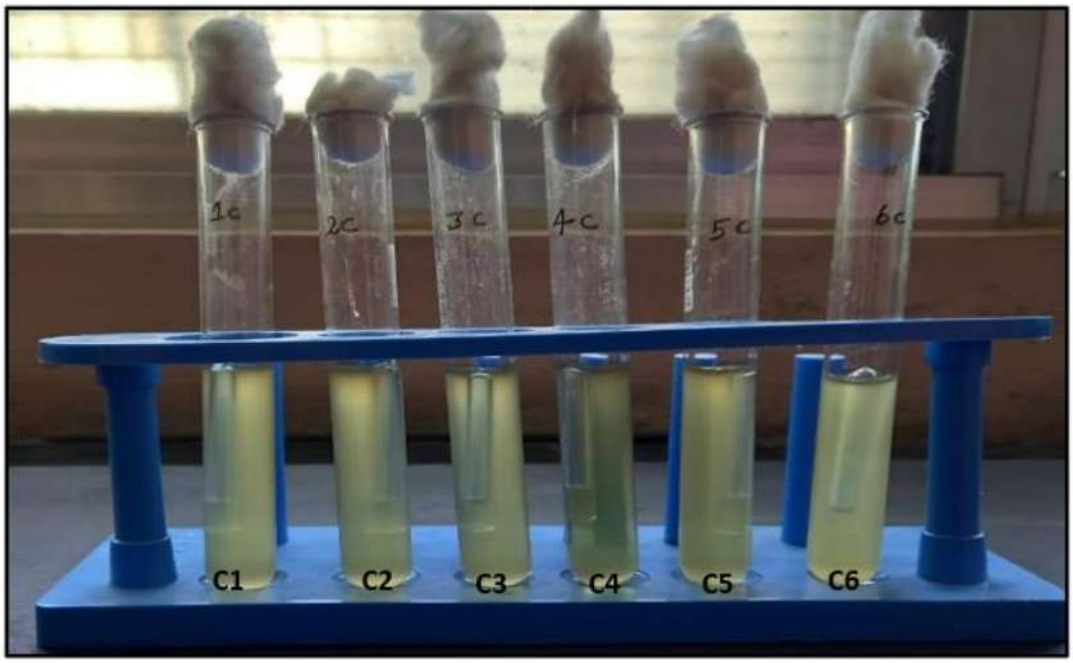
Note: C1 – Colony 1; C2- Colony 2; C3- Colony 3; C4 – Colony 4; C5 – Colony 5; C6 – Colony 6

FIGURE 7 CATALASE TEST FOR THE ISOLATED BACTERIAL STRAINS



Note: C1 – Colony 1; C2- Colony 2; C3- Colony 3; C4 – Colony 4; C5 – Colony 5

FIGURE 8 CARBOHYDRATE FERMENTATION TEST FOR THE ISOLATED BACTERIAL STRAINS



Note: C1 – Colony 1; C2- Colony 2; C3- Colony 3; C4 – Colony 4; C5 – Colony 5; C6 – Colony 6

FIGURE 9 STARCH HYDROLYSIS TEST FOR ISOLATED BACTERIAL STRAINS

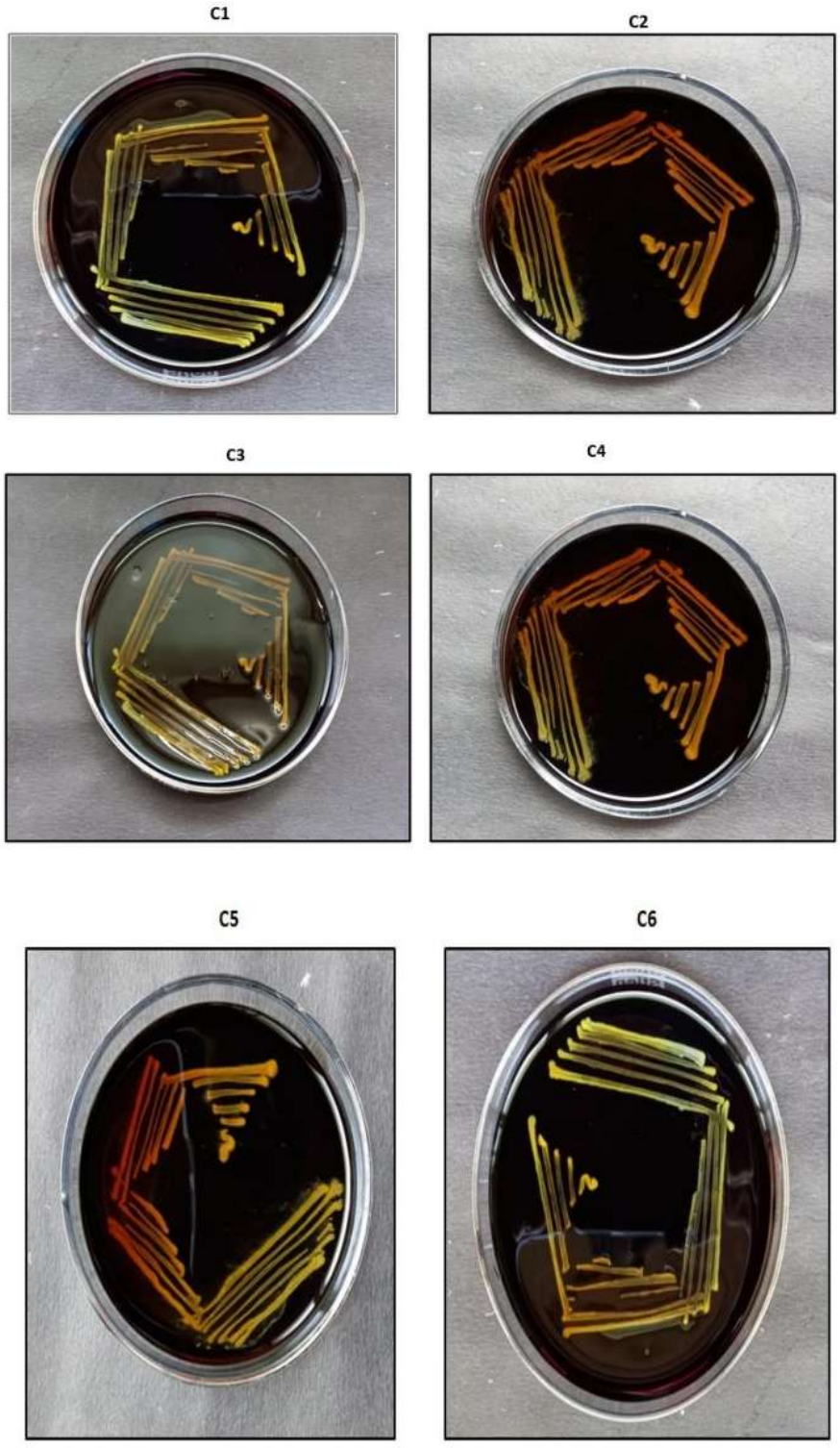


FIGURE 10.A AMPICILLIN SENSITIVITY OF ISOLATED STRAIN

FIGURE 10B KANAMYCIN SENSITIVITY

FIGURE 11 GRAPHICAL REPRESENTATION OF ZONE OF INHIBITION IN ANTIBIOTIC SENSITIVITY ASSAY -USING FOR AMPICILLIN SENSITIVITY

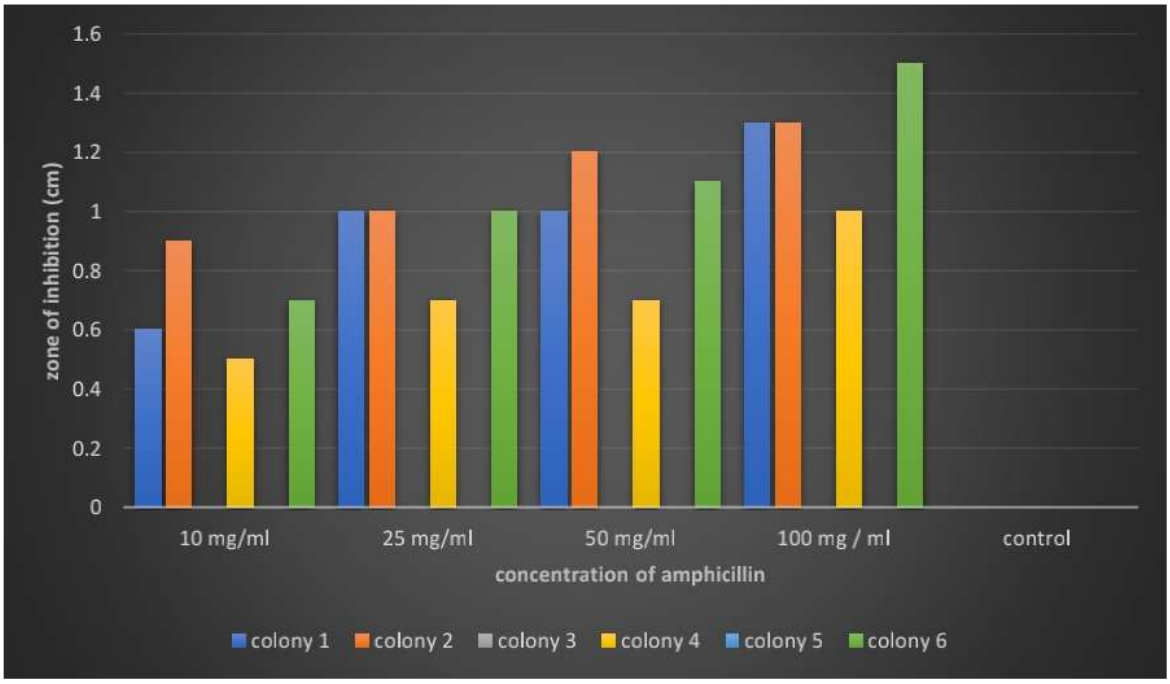


FIGURE 11 A GRAPHICAL REPRESENTATION OF ZONE OF INHIBITION IN ANTIBIOTIC SENSITIVITY ASSAY -USING KANAMYCIN SENSITIVITY

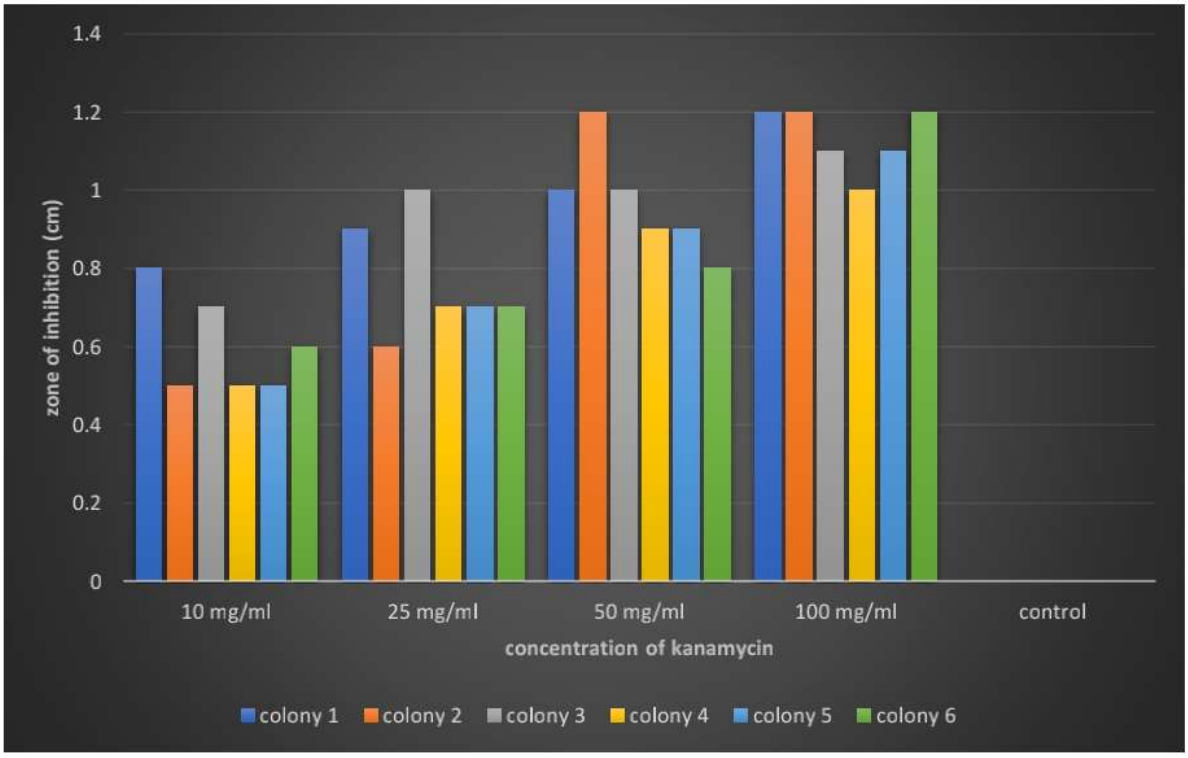
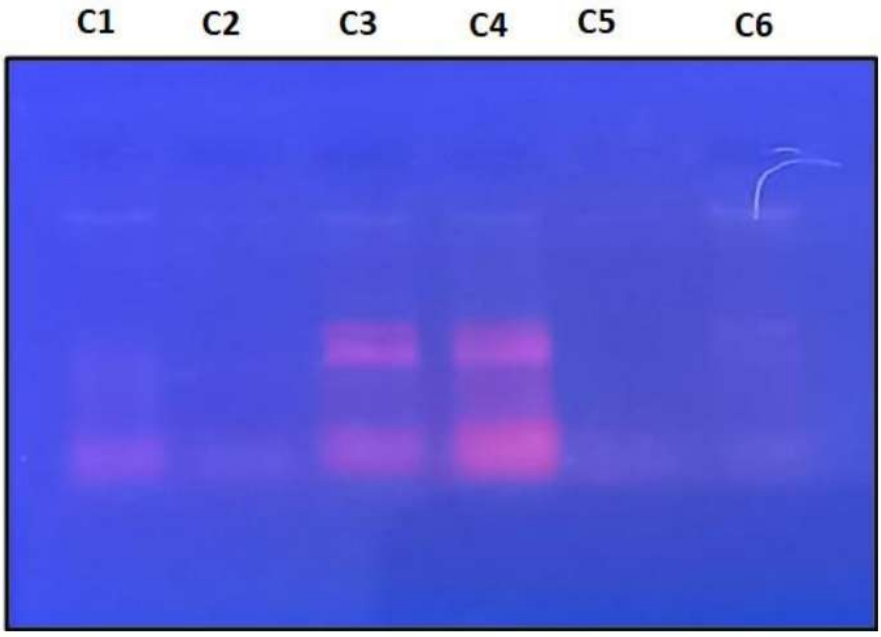


FIGURE 12 ISOLATION OF GENOMIC DNA

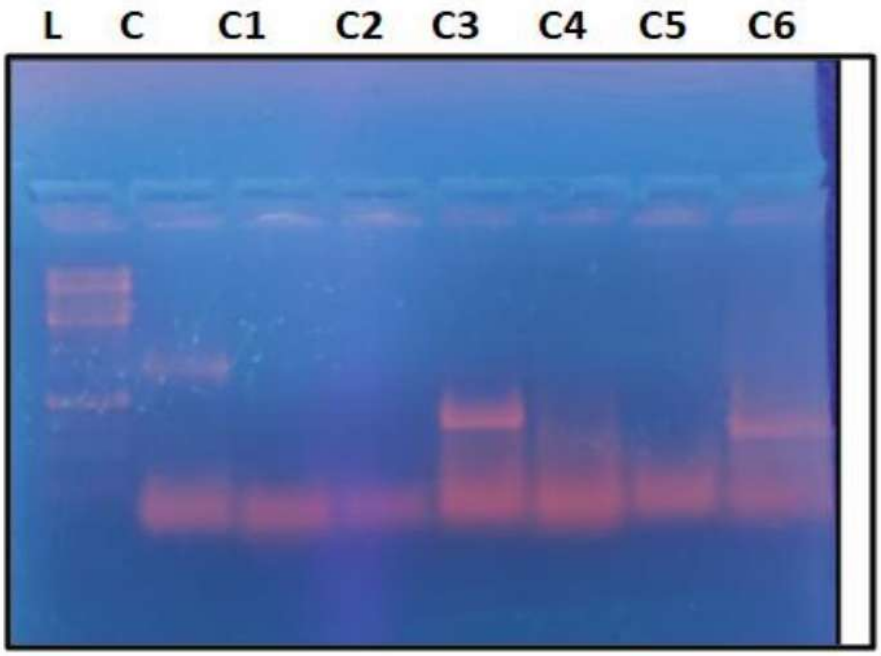


DNA Isolation

DNA was isolated from 6 colonies and 5µl of the sample was loaded in each lane.

Note: C1 – Colony 1; C2- Colony 2; C3- Colony 3; C4 – Colony 4; C5 – Colony 5; C6 – Colony 6

FIGURE 13 PCR USING 16S RRNA SPECIFIC PRIMERS



PCR: PCR was performed with 16s rRNA primers. 100pg of the bacterial DNA was used for the reaction.

Note: L- 1kb ladder, C- N template control,C1 – Colony 1; C2- Colony 2; C3- Colony 3; C4 – Colony 4; C5 – Colony 5; C6 – Colony 6

7.DISCUSSION

The major challenge in shrimp aquaculture is the disease out breaks that disrupts the production cycle very badly. Problem-related to disease outbreak caused by bacterial infection especially in large scale production can result in serious economic loss to farmers and investors. To overcome this problem, most of the farmers prefer to use chemical additive and medicine such as antibiotics and vaccination as disease control and prevention. The uncontrolled use of antibiotics, pesticides and disinfects in prevention and control of disease outbreak can lead to more serious problems which are food safety problems and the existence of antibiotic resistance bacteria (Wang et al. 2007). Due to the awareness and concern on antibiotic resistance bacteria, the use of probiotics might become a promising way to overcome this problem. Administration of probiotics have sought to be a new avenue for improving prawn culture.

Probiotic is defined as a live microbial supplemented in a feed that gives beneficial effects by balancing the host-microbial intestine (Fuller, 1989). Many studies were carried out to investigate the use of probiotics in improving the growth, survival and immune response especially in larval culture (Farzanfar, 2006). In the shellfish aquaculture sectors, several probiotics has been practically applied included of; *Lactobacillus, Enterococcus, Bacillus, Aeromonas, Alteromonas, Arthrobacter, Bifidobacterium, Clostridium, Microbacterium, Paenibacillus, Phaeobacter,*

Pseudoalteromonas, Pseudomonas, Rhodosporidium, Roseobacter, Streptomyces and *Vibrio* (Ringø, 2020a). Previous studied proved that the application of probiotic Bacillus sp. showed an improvement in the growth and digestive process of P. monodon, *P. vannamei* and giant freshwater prawn (Zhou et al., 2009). Probiotics also play an important role to improve the growth, feed efficiency, control microbiota, water quality and confer resistance to diseases in Aquaculture industries (Tuan et al., 2013). Concurrently, there is a need for the improvement on the disease resistance, growth performance, feed efficiency and safe of the aquatic animal organisms for human consumption that encourage on the development and applications of probiotics in aquaculture (Ringø, 2020a).

The use of probiotics added in feed and rearing tank or pond as disease control, increase Aquaculture production, avoiding antibiotics resistance and increase the immune response of the Aquaculture species are widely used due to its easy application and cost-efficient. In the present study, a total of 6 samples of bacteria were isolated from the gut of the giant tiger prawn *Penaeus monodon*. All the bacterial colonies were Gram-positive, rod shaped, showed fermentation of glucose, utilized citrate as carbon source and it produced catalase enzyme. The isolates preferred to grow in a Zobell marine agar which has a NaCl concentration of 19.45 gm/L of NaCl, whereas they did not grow on Luria Bertani Agar with 15 gm/L NaCl. All the six colonies which were isolated showed an optimal growth

temperature of 37^0C. Colonies C1, C2, C4 and C5 were sensitive to temperatures 30^0C and 35^0C. The colonies C3 and C6 grew well at these lower temperature. None of the colonies grew at 40^0C.

In an antibiotic sensitivity assay was done by plating the microbes on 10 mg/ml, 25 mg/ml, 50 mg/ml and 100 mg/ml of the antibiotics Ampicillin and Kanamycin. In the Kanamycin sensitivity test all the six colonies showed the zone of inhibition even at 10 mg/ml concentration of Kanamycin. In the ampicillin sensitivity test the colonies C3 and C6 were resistant to even 100 mg/ml of antibiotic. Presence of antibiotic resistant microbes in the prawn gut is a major environmental concern. The exposure of the animals to antibiotics would have resulted in the emergence of antibiotic resistant bacterial communities. The presence of antibiotic resistant bacteria in the gut of a edible prawn is a major concern of food quality as the resistance genes may get into the human gut microbiota and render them antibiotic insensitive. Further investigations have to done to analyse the location of the antibiotic resistance genes in the microbial genome. Earlier studies have also revealed presence of tetracycline and chloramphenical resistant isolates from cooked shrimps. Isolates from cooked shrimp showed higher resistance towards chloramphenicol (18.6%) and tetracycline (20%), while those from raw shrimp exhibited low levels of resistance towards nalidixic acid (10%) and tetracycline (8.2%) (Lakshmisharma et al., 2021). An attempt was made to identify the isolates

by 16s rRNA sequencing. Towards this the DNA was isolated and PCR using 16s rRNA specific primers. Amplicons of size 700bp were obtained and are given for sequencing. Molecular identity of the organism will be taken and the isolates can be used as candidates for probiotic formulations.

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CONCLUSION AND SUGGESTIONS

8. CONCLUSION AND SUGGESTIONS

The investigation into the gut microbiome of the aquatic invertebrates is a relatively new discipline. Therefore, any attempts to guide the field into a more consistent and reliable consensus, in terms of the information required for accurate reporting, should be encouraged. Given the increasing number of available sample preparations and bioinformatic tools, it is unrealistic to limit all future studies to one methodology or analytical pipeline.

The area of research on selective isolation of bacterial strains and their characterization is still at its infancy. The present work is an attempt to isolate and characterise the microbiome of the giant tiger prawn *Penaeus monodon*. A thorough analysis of the gut microbiota will give an idea of formulation of probiotics that can be included in the prawn feed. The current work also gives a lead to the antibiotic resistant microbes that evolve in the microbiome of edible organisms, which is a prospective peril for food safety.

Future prospective

Identification of the species of bacterial strains

Analysis of the probiotic capacities of the isolated organisms.

A thorough study of the origic of antibiotic resistance in the gut microbiota of *Penaeus monodon*

SUMMARY

9. SUMMARY

- Microbiome of the gut of the giant tiger prawn *Penaeus monodon* was analysed. The microorganism from the prawn gut were selectively isolated by plating the gut content on the selective Marine Zobell agar.
- From the colonies that were observed on the Marine Zobell Agar plate, six bacterial strains distinct morphological characteristic were taken for morphological, biochemical and molecular analysis.
- The colonies grew well at 37^0C The colonies were morphologically diverse.– circular, filamentous and are irregular too.
- Morphological analysis confirmed that the stains were Gram positive, catalase positive, and could ferment glucose. Colony C6 was able to utilize citrate as its carbon source. None of the colonies produced amylase enzyme.
- Except for the colonies C3 and C6 all the other colonies were sensitive to temperatures 30^0C and 35^0C . None of the colonies grew at 40^0C
- Antibiotic sensitivity was checked for the bacterial isolates. All the colonies were sensitive to the antibiotics kanamycin at different concentrations. The colonies C3 and C5 were resistant ampicillin at concentration 100 mg/ml.
- An attempt was made to identify the species of the bacterial strain by 16s rRNA sequencing. DNA from the bacterial strains was isolated and PCR with 16s rRNA specific primers was carried out.

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MOBILE PHONE DEPENDENCY AND HEALTH RELATED LIFESTYLE DISORDERS AMONG THE COLLEGE STUDENTS OF THOOTHUKUDI

A field work submitted to

ST.MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

MASTER OF SCIENCE IN ZOOLOGY

by

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

April 2023

CERTIFICATE

This is to certify that the field work entitled, "**MOBILE PHONE DEPENDENCY AND HEALTH RELATED LIFESTYLE DISORDERS AMONG THE COLLEGE STUDENTS OF THOOTHUKUDI** " submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Master of Science in Zoology** is done under my supervision. It is further certified that this field work report or any part of this has not been submitted elsewhere for any other degree by the following students.

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DECLARATION

We do hereby declare that this field work entitled, **"MOBILE PHONE DEPENDENCY AND HEALTH RELATED LIFESTYLE DISORDERS AMONG THE COLLEGE STUDENTS OF THOOTHUKUDI"** submitted by us for the award of the degree of Master of Science in Zoology is the result of our original independent research work carried out under the guidance of **Dr. Hermin Pasangha M.Sc., B.Ed., Ph.D.,** Associate Professor, Department of Zoology, St. Mary's College (Autonomous), Thoothukudi and it has not been submitted elsewhere for the award of any other degree.

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INTRODUCTION

1. INTRODUCTION

Mobile phones have become an integral part of human life. With the evolution of mobile communication from 1G to 5G, there is also an increase in the frequency of the radiations used for communication. The frequency of communication of mobile phones falls in the range of 900 MHz to 2.5 GHz. Continuous and long-term exposure to low-intensity electromagnetic radiations may have negative effects on the biological system of human beings affecting their memory and cognition (Zeqiri *et al.,* 2019). The mobile radiations utilize the frequency range from 3 kHz to 300 GHz, which consists of different wireless devices. For instance, a laptop connected to the Wi-Fi and placed on laps is very harmful (Oni *et al.,* 2011).

India ranks as the second largest mobile using country in the whole world. Tamil Nadu is the 7^{th} most populous and the 11^{th} largest state in India. It ranks as the second highest mobile subscribers state with an estimated population of around 72 million people using cell phones. It is one of the top 10 states in India with highest mobile users (The Indian blog, 2020).

With the rapid growth of mobile phone device, the technology developed an alarming situation for the normal functioning of the biological systems of the human body and lead to serious ailments such as: diseases of brain like cancer, brain tumor, Alzheimer's disease, Parkinson's disease and so on. It also causes short term effects like hormone disruption, sleep disruption, centration,

impairment of cognitive function, behaviour, attention and long-term effects like DNA damage as well as male infertility (Sage and Carpenter, 2009).

Mobile phone excessive use has been found to be associated with health problems such as impaired concentration, headache, dizziness, fatigue, thermal sensations in and around ear, facial dermatitis, stress, sleep disturbances owing to night time use, and frustration (Susila *et al.,* 2017).

Besides the number of cell phone calls per day, the length of each call and the amount of time people use cell phones are important factors which enhance the health-related risks. Scientists have reported adverse health effects of using mobile phones including changes in brain activity, reaction times, and sleep patterns. When mobile phones are used very close to some medical devices (including pacemakers, implantable defibrillators, and certain hearing aids) there is the possibility of causing interference with their operation (Naeem Z., 2014).

Self-reported symptoms associated with using mobile phones most commonly include headaches, earache, and warmth sensations and sometimes also perceived concentration difficulties and fatigue (Johansson et al., 2010). Musculoskeletal symptoms due to intensive texting on a mobile phone have been reported and techniques used for text entering have been studied in connection with developing musculoskeletal symptoms (Ming et al., 2006).

Muscle activity of upper trapezius, erector spinae and the neck extensor muscles are increased as well as head flexion angle, head tilt angle and forward

head shifting which increased during the smartphone use. Also, smartphone use in a sitting position seems to cause more shift in head-neck angle than in a standing position. Smartphone usage may contribute to musculoskeletal disorders (Eitivipart *et al.,* 2018).

Smartphones have made our life easy in many ways but we should also be aware of the negative effects of smartphone usage and the most concerning is the smartphone addiction. Addiction is considered as the dependence and continuous use of something for the sake of relief or stimulation which often causes cravings when it is absent (WHO, 2019).

Globally, the prevalence of mobile phone addiction is varying from 2.4% to as high as 60.3% among adolescents and school-going children. India is one of the largest and fastest-growing markets for digital consumers, with 560 million internet subscribers (nearly 41%) in 2018, second only to China. The average Indian social media user spends 17 hours on the platforms each week, more than social media users in China and the United States. Half of India's entry-level users for smartphones are between 15 and 24 years old and mostly students. Chat, video streaming, browsing, social networking, and image apps are the most engaging and account for more than 50% of the total time spent on smartphones. India has the highest number of adolescents (253 million) and they constitute one-fifth of the Indian population and 22% of them live in urban areas (Gangadharan *et al.,* 2022).

People spend their time more likely on social media, do business emails, academic search, finding answers to questions, and playing games. Such too much dependency makes us “Mobile addictive”. Mobile phones make peoples’ lives easier, but on the other hand, they tie us. Mobile addiction not only has physical effects but also psychological and academics effects at the same time. Sleep deficit, anxiety, stress, and depression which are all associated with internet abuse, have been related to mobile phone usage too (De-Sola et al., 2016).

Researchers found an intensive increase of cell phone usage among teenagers and the symptoms of depression, suicide risk factors and suicide rate in the year 2012. Cell phone addiction is negatively correlated with academic performance (Ng et al., 2017).

With the rapid development of communication technology, the smartphone, as an information carrier, has penetrated into people’s daily life, and mobile phone addiction (MPA) is its largest negative product (Puspitasari *et al*., 2016). MPA refers to addictive behavior in which individuals use their phones excessively and compulsively, thereby negatively affecting their psychological and social functions (Mei *et al*., 2022). Internet addiction is similar to drug addiction except behavioral addiction (internet addiction) doesn't involve a substance. In addition, the physical symptoms are absent in behavioral addiction, but if internet addiction continues, it will undergo the same results as alcohol

addiction (Alavi et al., 2012). The predictive factors for smart phone addiction were social networking and awareness of game overuse (Cha and Seo, 2018)

Cell phone usage badly affects mental health of adolescents and they look anxious, depressed and angry or sometimes commit suicide. The suicidal rate is increasing in this era. Some studies also showed a positive relation of cell phone addiction and physiological health. The other school of thought reveals an indirect relation between cell phone usage and psychological health. They say adolescents use cell phones at night, which leads to insomnia and insomnia ultimately results in anxiety, and depression. Cell phone addiction has no direct relation to mental health (Shoukat, 2019).

The mobile phones which make communication easy and accessible, form good carriers of pathogenic agents of disease transmission too. If care is not taken, they could be vehicles for the transmission of biological weapons. The constant handling of cell phones by different users exposes it to an array of micro-organisms and makes it a good carrier for microbes, especially those associated with skin. These mobile phones are ideal breeding sites for microbes because of the temperature and moisture. The contributory factors include age group, duration of cell phone usage and hygienic practices followed (Chaturwedi *et al*., 2022).

Mobile phones are constantly being used and handled by the owners in places such as toilets, hospitals, and kitchens which are typically loaded with

enteric pathogen (Bhoonderowa *et al.*, 2014). Being an electronic gadget, mobile phones are seldom cleaned. Microbes can persist on the phone's surfaces for weeks and the daily contact with face, ear and hands pose as a potential risk to our health (Chaturwedi *et al*., 2022).

The purpose of the present study is to investigate the relationship of smartphone use with anxiety, depression, stress and quality of sleep. With the growing popularity of smartphone technology among young adults, it is important to understand predictive factors of stress, depression, anxiety and quality of sleep to prevent negative outcomes. A survey study is conducted to investigate the possible effects of mobile phone on headache, dizziness, extreme irritation, shaking in the hands, speaking falteringly, forgetfulness, neuro-psychological discomfort, increase in the carelessness, decrease of the reflex and clicking sound in the ears.

OBJECTIVES

2. OBJECTIVES

The widespread use of mobile phones has been increased over the past decade; they are now an essential part of education, business, commerce and society. The use of mobile phones can cause various health problems. Therefore, this field work has been planned

- To collect an online survey on mobile phone usage and associated health effects among the college students of Thoothukudi, Tamil Nadu.
- To know the impacts of mobile phone addiction through analyzing their behavior and health problems.
- To assess their health awareness about these problems.
- To compare the addiction level between different age category of students using statistical analysis.
- To provide health and social awareness using experimental analysis.

REVIEW OF LITERATURE

3. REVIEW OF LITERATURE

Several studies have been conducted in recent years to find the consequences of smartphone use and the literature reviewed will be valuable for the researchers.

Hocking *et al.* (2004) reported that placing of mobile phone directly at the same position of body part, regularly leads to the stimulation of neurological problem which is basis for dysaesthesiae. Khlaiwi *et al*. (2004) investigated that the long-time use of mobile phones is a risk factor for health hazards like fatigue, headache, dizziness and sleep problem.

Higuchi *et al.* (2005) analyzed the combination of looking at a bright display and doing an exciting task (e.g., playing a shooting game) may change the secretion of melatonin and therefore the quality of sleep. Akinyemi *et al.* (2009) analyzed the potential role of mobile phones in the dissemination of diseases. Mobile phones may serve as vehicles of transmission of both hospital and community-acquired bacterial diseases.

Oshima *et al.* (2012) investigated the association between mobile phone use after lights out and poor mental health, suicidal feelings, and self-harm in both early and late adolescents. The non-ionising electromagnetic fields, cause an increased risk for brain tumours. The meta-analysis of glioma in the most exposed part of the brain proved that the use of cordless phones increased the risk for glioma and acoustic neuroma (Hardell *et al.*, 2013).

Schoeni *et al.* (2015) observed that nocturnal mobile phone use was associated with an increase in health problems such as tiredness, rapid exhaustibility, headache and physical ill-being, but not with memory and concentration capacity. Stalin *et al*. (2016) reported that prevalence of mobile phone usage was 70% and calling facility (94.2%) was used more than the SMS (67.6%). Health problems like headache, earache, tinnitus, painful fingers and restlessness etc., were found to be positively associated with mobile phone usage. There was negative association between hypertension and mobile phone usage.

Findings of Exelmans *et al.* (2016) suggest that bedtime mobile phone use is negatively related to sleep outcomes in adults, too. Sending/receiving text messages and/or phone calls after lights out cause longer sleep latency, worse sleep efficiency, more sleep disturbance and more daytime dysfunction, higher insomnia score and increased fatigue. Durusoy *et al.* (2017) studied that an association between mobile phone use and headache, concentration difficulties, fatigue, sleep disturbances and warming of the ear.

Parasuraman *et al*. (2017) found that one-fourth of the studied population were found having feeling of wrist and hand pain because of smartphone use which may lead to further physiological complication. Tamura *et al.* (2017) finding implies that long hours of mobile phone use were associated with insomnia, particularly in students using mobile phones for 5 h or more a day

and long hours spent using mobile phones for SMS or online chat was related to depression, particularly in students who spent 2 hour or more on SMS and online chat.

Ibrahim *et al.* (2018) studied the impact of mobile phone use which lowers academic achievers and significantly worse MP scores on financial problems. MP dependency was correlated with subjective sleep quality score, and sleep latency. Basu *et al.* (2018) investigated that mobile phone use with increasing adoption of smartphones promotes an addiction-like behavior that is evolving as a public health problem in a large proportion of Indian youth.

The potential impact of microwave radiofrequency electromagnetic fields (RF-EMF) emitted by wireless communication devices on neurocognitive functions of adolescents is controversial and a potential adverse effect of RF-EMF brain dose on cognitive functions that involve brain regions mostly exposed during mobile phone use (Foerster *et al.*, 2018).

Barrault *et al.* (2019) in their investigation linked the strong association between smartphone addiction and internet addiction. Smartphones may not be the object of the addiction but rather a medium facilitating internet access as it makes it possible to connect anywhere anytime. Jadia *et al.* (2019) clinical study implies that there is a significant moderate to severe hearing loss in those participants exposed to mobile phone usage of more than 4 h/day, for continues 4–6 years.

Paik *et al.* (2019) findings imply that prolonged bedtime smartphone use can be an important behavioral measure of problematic smartphone use and altered insula-centered functional connectivity may be associated with it. Derevensky *et al.* (2019) investigated that excessive use of the internet and smartphones can result in multiple mental and physical health issues. Gambling disorders, gaming disorders, internet use disorder, and excessive smartphone use often begin during childhood and adolescence.

Amiri *et al.* (2020) reviewed that problematic use of mobile phone adversely affects quality of life, quality of sleep, academic self-concept, academic engagement, achievement motivation, academic performance, psychological health, social interactions, and feeling of loneliness and increases academic burnout, aggression, anxiety, depression, family status (cohesion, relationships, parenting style, and support), attachment style, identity style, personality traits, general health, psychological health, feeling of loneliness, depression, and emotional intelligence.

Shih *et al.* (2020) investigated that excessive smartphone use significantly increased the risk of breast cancer, particularly for participants with smartphone addiction, a close distance between the breasts and smartphone, and the habit of smartphone use before bedtime. Basu *et al.* (2021) analyzed that using mobile phones with higher scores associated with sleep deprivation. A majority of

students were unable to reduce their time spent on social media despite wanting to do so, signifying the presence of tolerance and impaired control.

Siste *et al.* (2021) reported the occurence of psychological factors such as internalization, externalization, prosocial, and sleep problems that had correlations to Internet Addiction (IA) among adolescents in the COVID-19 pandemic. Sleep impairment might have resulted from the emotional and behavioral issues and directly contributed to IA development. Ali *et al.* (2021) investigated the factor structure, invariance, predictive validity, criterion validity, and reliability of the IAT (Internet Addiction Test) among Spanish women with eating disorders by excessive eating was associated with increased intensity of IA (Internet Addiction).

Al-Khlaiwi *et al.* (2022) found that the youngsters used mobile phones longer than others and insignificant decrease in emotional well-being was also noticed and life style parameters in social functioning also affected.

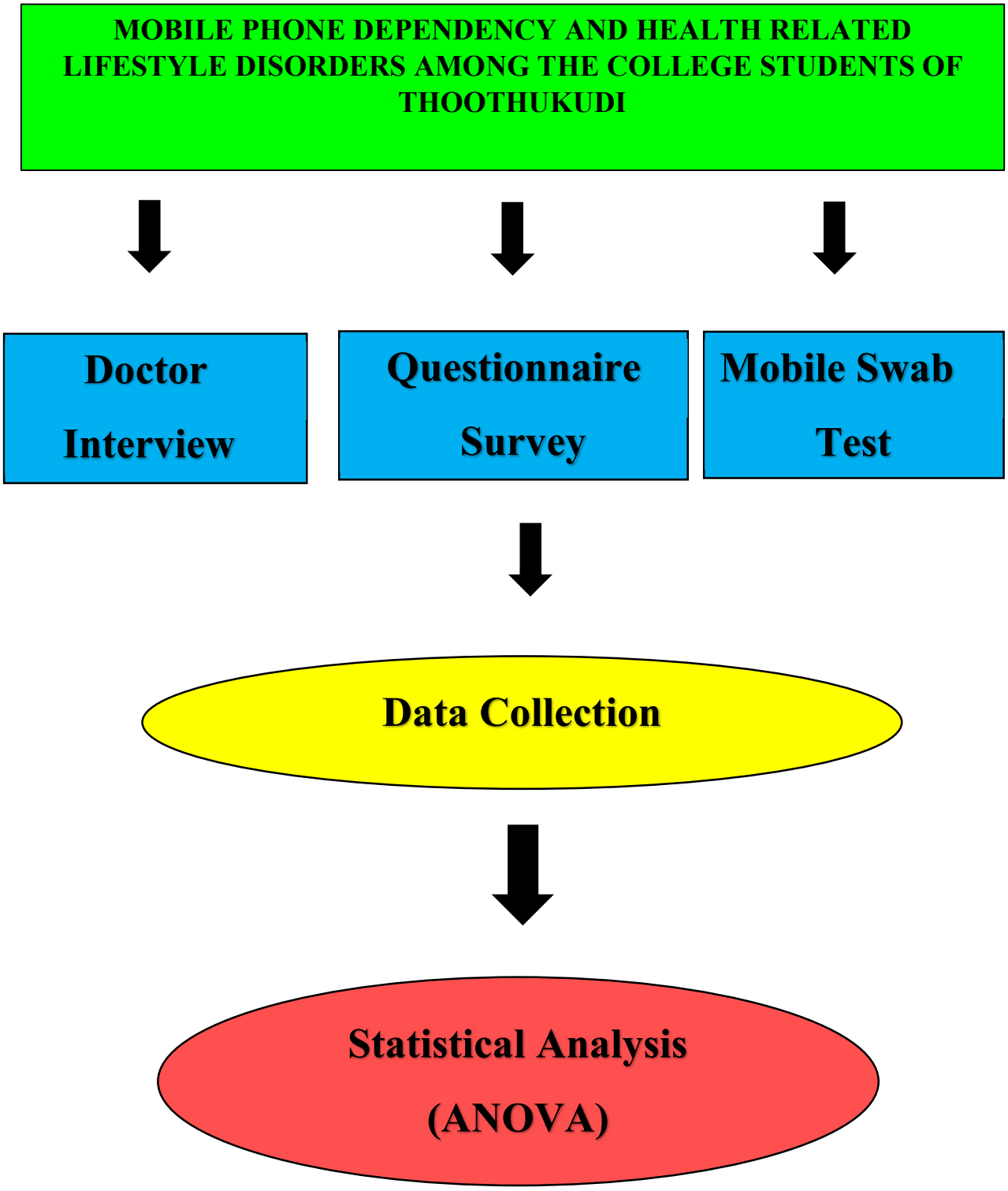
Punj *et al*. (2022) survey proved that there was significant aerobic bacterial contamination, including potentially pathogenic bacteria, seen in Mobile Phones (MP), hands, and Disinfected dominant hand after one minute of phone (DHM) of Health Personals in a tertiary care hospital of India. However, anaerobic bacteria (Clostridium sp.) were found only in MP. Similar bacterial microbes in MP and DHM point to probable transfer of aerobic bacteria from

MP to hands of HP which did not decrease when hand disinfectants are used in non-protocolized way, which was a point of concern.

Hasan *et al.* (2023) documented high prevalence of Smart phone Addiction (SA), Eating Disorder (ED) risk, and poor sleep quality was reported among university students in the UAE. Associations between poor sleep quality, evening chronotype, SA risk, and ED risk were further confirmed, with sleep quality predicting ED risk.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN



4. MATERIALS AND METHODS

An online questionnaire survey was conducted among the college students of Thoothukudi, Tamil Nadu to obtain data on their health status and the prevalence of subjective symptoms related to the mobile phone usage. Students between the age of 17 to 25 where selected and the Questionnaires were sent. 198 completed questionnaires were returned as 100% of response rate. We categorized ‘adolescents’ as below 19 years old and ‘adult stage’ as beyond 20. According to the criteria, 66 adolescents and 132 adulthoods participated in this study.

We conducted a cross-sectional survey on mobile phone usage and associated health effects between January 2023 and March 2023. College students were requested to fill out a questionnaire regarding mental health status, behaviors, and lifestyle through google form. Questions regarding mental health status included items from the sociodemographic status, habitual analysis, smartphone activities and length of sleep to detect addiction level.

4.1 QUESTIONNAIRE

TITLE: MOBILE PHONE DEPENDENCY AND HEALTH RELATED LIFESTYLE DISORDERS AMONG THE COLLEGE STUDENTS OF THOOTHUKUDI

SECTION 1: SOCIODEMOGRAPHIC STATUS

1. Age
2. Marital status
3. Accommodation
4. Place of residence

SECTION 2: HABITUATION ANALYSIS

1. What type of mobile phone do you use?
2. How many phones do you use?
3. How many years do you use mobile phone?
4. How many hours do you spend on mobile phone per day?
5. How many times do you check your mobile phone?
6. Do you check your mobile phone in-between sleep?
7. Do you use mobile phone in darkness?
8. Do you use mobile phone during a meal?
9. Is your eye protection mode is turned on?
10. What is the time interval between your mobile phone usage and sleep?

SECTION 3: SMARTPHONE ACTIVITIES

1. Do you use social media (Instagram, Twitter)?
2. Do you use communication-meeting (Whatsapp, Telegram)?
3. Do you use apps for listening to music/ watching film/ video (youtube, etc.)?
4. Do you use mobile for doing research/homework/ newspaper/ book reading?
5. Do you use mobile for playing games?
6. Do you use mobile for banking/ shopping (Amazon, etc.)?

SECTION 4: HEALTH PROBLEMS FACED DURING PROLONGED USAGE OF MOBILE PHONE

1. Do you suffer from headaches?
2. Do you have vision problems (Farsightedness, myopia, astigmatism)?
3. Do you suffer from dry eyes?
4. Do you suffer from pain in the ears?
5. Have you ever felt a rhythm ringing in your ears after long time usage of ear phones?
6. Do you feel exhausted after using phone for a long time?
7. Do you suffer from any neck pain?
8. Do you suffer from any fingers or hand pain or numbness in your fingers?
9. Do you have any problems with falling asleep?

10. Do you have any problems with your appetite?
11. Do you suffer from depression?

SECTION 5: COMMON SYNDROMES CAUSED BY EXCESSIVE USE OF MOBILE PHONES

1. Do you suffer from De Quervain Tenosynovitis?
2. Do you suffer from Cubital Tunnel Syndrome or Cell Phone Elbow?
3. Do you suffer from text neck syndrome?
4. Do you suffer from NOMOPHOBIA or NO MObile PHone PhoBIA?
5. Do you suffer from Phantom Vibration Syndrome (PVS)?

SECTION 6: SMARTPHONE COMPULSION STATUS

1. Do you find yourself spending more time on your smartphone than you realize?
2. Do you find yourself mindlessly passing time on a regular basis by staring at your smartphone even though there might be better or more productive things to do?
3. Do you seem to lose track of time when on your cell phone?
4. Do you find yourself spending more time texting, tweeting, or emailing as opposed to talking to real-time people?
5. Has the amount of time you spend on your cell phone been increasing?
6. Do you secretly wish you could be a little less wired or connected to your cell phone?

7. Do you sleep with your smartphone on or under your pillow or next to your bed regularly?
8. Do you find yourself viewing and answering texts, tweets, and emails at all hours of the day and night, even when it means interrupting other things you are doing?
9. Do you text, email, tweet, or surf the internet while driving or doing other similar activities that require your focused attention and concentration?
10. Do you feel your use of your cell phone actually decreases your productivity at times?
11. Do you feel reluctant to be without your smartphone, even for a short time?
12. When you leave the house, you ALWAYS have your smartphone with you and you feel ill-at-ease or uncomfortable when you accidentally leave your smartphone in the car or at home, or you have no service, or it is broken?
13. When you eat meals, is your cell phone always part of the table place setting?
14. When your phone rings, beeps, buzzes, do you feel an intense urge to check for texts, tweets, or emails, updates, etc.?
15. Do you find yourself mindlessly checking your phone many times a day even when you know there is likely nothing new or important to see?

4.1.1 Statistical analysis

The statistical analysis of the data was performed using Microsoft Excel. One way ANOVA test was applied to analyze qualitative values and statistical significance was considered for $p < 0.05$.

Analysis of Variance (ANOVA)

ANOVA method was used to find out the variance in the data and the significance was tested.

Steps in computation

The below mentioned formula represents one-way ANOVA test statistics:

$$F_s = \frac{MS_{\text{between groups}}}{MS_{\text{within groups}}}$$

$$MS_{\text{between groups}} = \frac{SS_{\text{between groups}}}{df_b}$$

$$MS_{\text{within groups}} = \frac{SS_{\text{within groups}}}{df_w}$$

$df_b = k-1$ (k = number of groups)

$df_w = n-k$ (n = Total number of observations)

$df_t = n-1$

Where,

F = Anova Coefficient

$MS_{\text{between groups}}$ = Mean sum of squares between the groups

$MS_{\text{within groups}}$ = Mean sum of squares within the groups

$SS_{\text{between groups}}$ = Sum of squares between the groups

$SS_{\text{within groups}}$ = Sum of squares within the groups

SS_{total} = Total Sum of squares

df = Degrees of freedom

df_b = Degrees of freedom between groups

df_w = Degrees of freedom within groups

df_t = Total degrees of freedom

ANOVA Summary

Source of Variation	***SS***	***df***	***MS***	***F statistical value***
Between Groups	$SS_{\text{between groups}}$	$df_b=k-1$	$MS=\frac{SSB}{df_b}$	$F_s=\frac{MS_{\text{between groups}}}{MS_{\text{within groups}}}$
Within Groups	$SS_{\text{within groups}}$	$df_w=n-k$	$MS=\frac{SSW}{df_w}$	
Total	SS_{total}	$df_t=n-1$		

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

4.2 Doctor interview questions:

1. What are some of the health risks associated with excessive usage of mobile phone?
2. Which age groups are most affected?
3. Which gender is most at risk of addiction?
4. Can the use of mobile phone also affect the functioning of the brain?
5. Is there any safe daily limit for mobile usage?
6. Do personality traits and conditions have any association with addiction?
7. What triggers the compulsive use of phone?
8. What are the symptoms of phone addiction?
9. Can you give us some tips to get rid of phone addiction?
10. Is there problem using mobile phones during meal?
11. Is it really necessary to undergo psychotherapy and addiction counselling for smartphone addiction?
12. Is it really necessary to take medications like Beta blockers and Benzodiazepines to help with severe symptoms of NOMOPHOBIA?
13. How to do a digital detox?
14. Is there any link between mobile phone usage and seizures?
15. What are some exercises that can be done by smartphone users?
16. According to your observation is there any major difference of mobile usage between before and after COVID-19?

4.3 Experimental analysis:

Sample collection:

The samples were collected from mobile phones of 10 devices randomly from the college students of Thoothukudi. Samples were collected aseptically with sterile swabs moistened with sterile normal saline and by rolling over the exposed surfaces of the mobile phones. Maximum care was taken to ensure that all the buttons of the keypad, screen, mouthpiece, earpiece, sides and back of the mobiles were properly swabbed since these areas are the most frequent spots, in contact with the fingers.

Sample inoculation:

After collection, the samples were immediately transported to the laboratory and inoculated on MacConkey's agar and plates were incubated aerobically at 37°C for 48 hours. After incubation, plates were examined for growth and colonial morphology of the isolates.

RESULT

5. RESULT

Constant usage and sort of addiction to smartphones has affected the people physically and psychologically by making them have aches and pains in some a disability too, lose their required number of hours sleep, get angry and scrap over trivial matters, and so on and so forth. Many people using a mobile phone in their activity have increased continuously. Many jobs are now dependent on the use of mobile phone. Among the health issues resulting from Visual Display tasks, there are visual stress and musculoskeletal disorder (MSDs). Various risk factors (physical, occupational, ergonomic, psycho-social, and individual) interact in the development of these symptoms and disorders.

5.1 Analysis of demographic details of the study participants

Demographic characteristics of mobile phone users are shown in Table.1. A total of 198 students participated in the study. Of them 68% of participants were days scholars and 32% of participants were hostellers. The study sample comprised of 184 (92.93%) single and 14 (7.07%) married students. Most of the students were from urban area than rural places and its ratio is 60:39. Equal number of participants (n=66) from each age groups including 17 to 19, 20 to 23 and 23 to 25 were observed. Comparatively, there was a higher participation rate of urban side, day scholars and unmarried mobile users than the rural side, hostellers and married users (Table.1).

Table 1: Demographic details of the study participants

Particulars	**Number of participants**
Number of participants enrolled	198
Age groups	
17 – 19	66
20 - 22	66
23 - 25	66
Marital Status	
Single	184
Married	14
Accommodation	
Home	135
Hostel	63
Place of residence	
Urban	120
Rural	78

5.2 Habituation analysis of mobile phone usage

Smartphone use was reported by 177 (89.40%) and both basic mobile phones as well as smart phones was used by 21 (10.60%) students. Among 198 participants, 20 participants (10.10%) using more than 1 mobile phones. Most of the students (75.25%) were using mobile phone for 1 to 5 years. In our analyses a large number of students (42.42%) were using mobile phones for 5 to 8 hours/ day and 20.71% were using mobile phones for more than 9 to 12 hours per day. 66.16% of students were using mobile phones in darkness, 44.44% checking mobile phone in between sleep and 49.49% were using mobile phones during meals which may cause eye problems, sleep disturbance and digestion problems respectively (Table.2). Based on age groups and duration of mobile usage Table.3 is constructed. It shows that as the age increases the duration of mobile phone usage decreases (Table.3 and Figure.1).

Table 2: Habituation analysis of mobile phone usage

Habituation	No. of participants	Percentage
Type of mobile phone usage		
Smartphone	177	89.40%
Normal phone	0	0%
Both	21	10.60%
Number of phones used by the participant		
1	178	89.90%
More than 1	20	10.10%
Number of years of mobile phone usage by study participant (years)		
1 - 5	149	75.25%
6 - 10	15	7.57%
11 - 15	34	17.18%
Duration of mobile phone usage per day (in hours)		
1 - 4	73	36.87%
5 - 8	84	42.42%
9 - 12	41	20.71%
Frequency of mobile phone checking (times)		
1 - 10	105	53.03%
11- 20	37	18.69%
21 - 30	31	15.66%
More than 30	25	12.62%

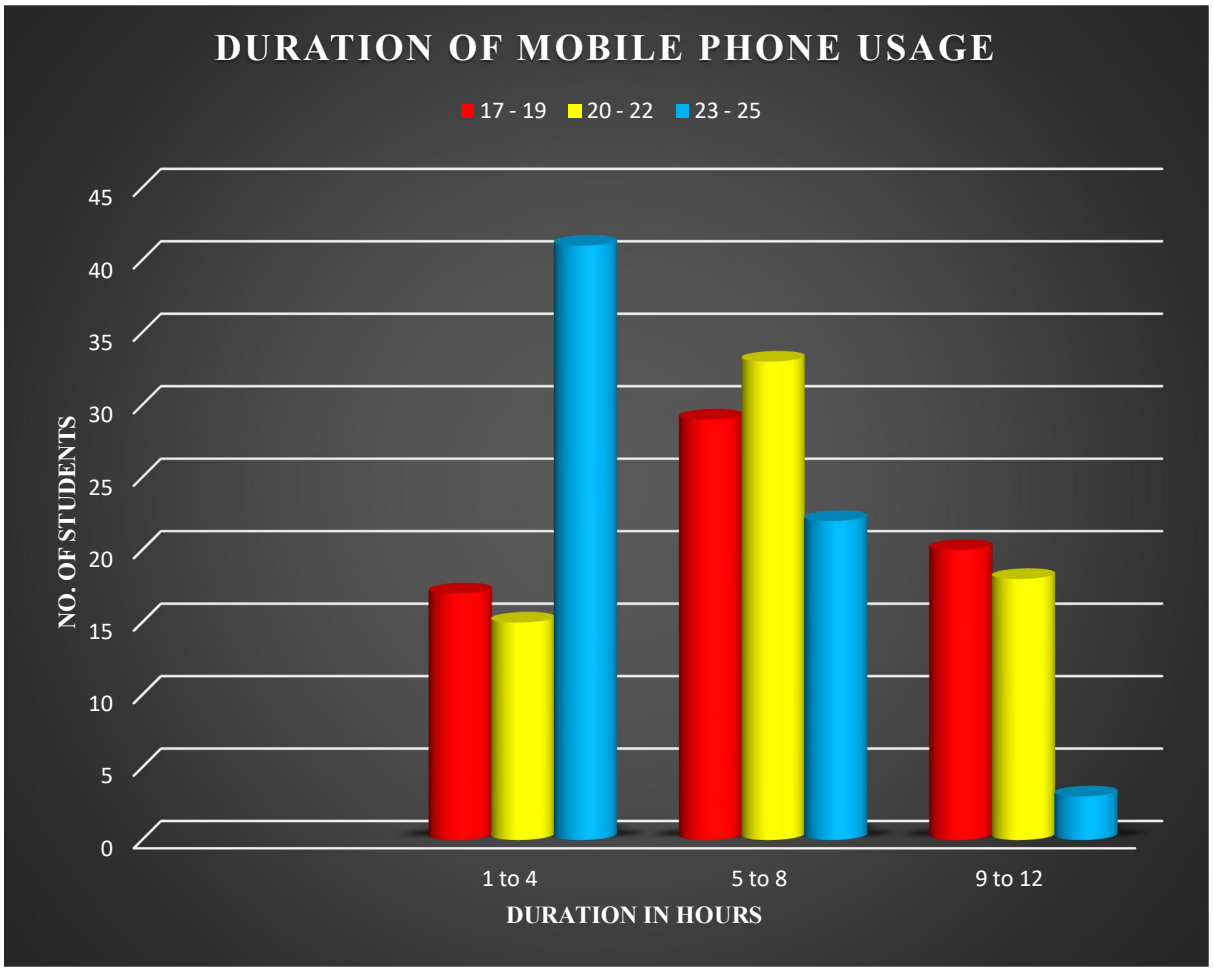
Table.2 continued on next page

Habituation	**No. of participants**	**Percentage**
Checking mobile phone in-between sleep		
Yes	88	44.44%
No	110	55.56%
Using mobile phone in darkness		
Yes	131	66.16%
No	67	33.84%
Using mobile phone during a meal?		
Yes	98	49.49%
No	100	50.51%
Eye protection mode is turned on		
Always	72	36.36%
Often	47	23.74%
Rarely	23	11.62%
No	56	28.28%
Time interval between mobile phone usage and sleep		
Before 2 hours of sleep	43	21.72%
Before 1 hour of sleep	37	18.69%
Before 15 mins of sleep	74	37.37%
Sleep during mobile phone usage	44	22.22%

Table 3: Distribution of student participants based on duration of mobile phone usage

S.No.	Duration in hours	Age groups			Overall
		17 - 19	20 - 22	23 - 25	
1.	1 - 4	17	15	41	73
2.	5 - 8	29	33	22	84
3.	9 - 12	20	18	3	41
				Total	198

Figure 1: Duration of mobile phone usage



5.3 Analysis of smartphone activities

The students reported that they use their smartphones for browsing social media networks (81.82%), communication and meetings (93.94%), listening to music/ watching video (96.46%), educational purpose (88.38%), playing games (60.61%) and banking / shopping (85.35%). All the adolescence were using mobile phone for listening to music/ watching film/ video as for their entertainment. 88.38% of adolescents used their mobile phones for academic purposes (Table.4 and Figure.2).

From the results of ANOVA (Table.5) it was found that variance between the age groups and smartphone activities was found to be not statistically significant ($p>0.05$). In this case, p value is 0.355025 which is greater than 0.05. F statistical value has two degrees of freedom (2, 15). F statistical value (1.110469) is less than F critical value (3.68232). It shows that there is no difference between smartphone activities and age. Every age group uses phone for various applications.

Table 4: Smartphone activities

S.No.	Smartphone activities	Age groups			Overall
		17 to 19	20 to 22	23 to 25	
1.	Social media	61 (92.42%)	49 (78.78%)	52 (74.24%)	162 (81.82%)
2.	Communication and meetings	63 (95.45%)	62 (93.93%)	61 (92.42%)	186 (93.94%)
3.	Listening to music/ watching film/ video	66 (100%)	64 (96.96%)	61 (92.42%)	191 (96.46%)
4.	Doing research/ homework/ newspaper/ book reading	59 (89.39%)	62 (93.93%)	54 (81.81%)	175 (88.38%)
5.	Playing games	52 (78.78%)	37 (56.06%)	31 (46.96%)	120 (60.61%)
6.	Banking/ shopping	57 (86.36%)	61 (92.42%)	51 (77.27%)	169 (85.35%)

Figure 2: Smartphone activities

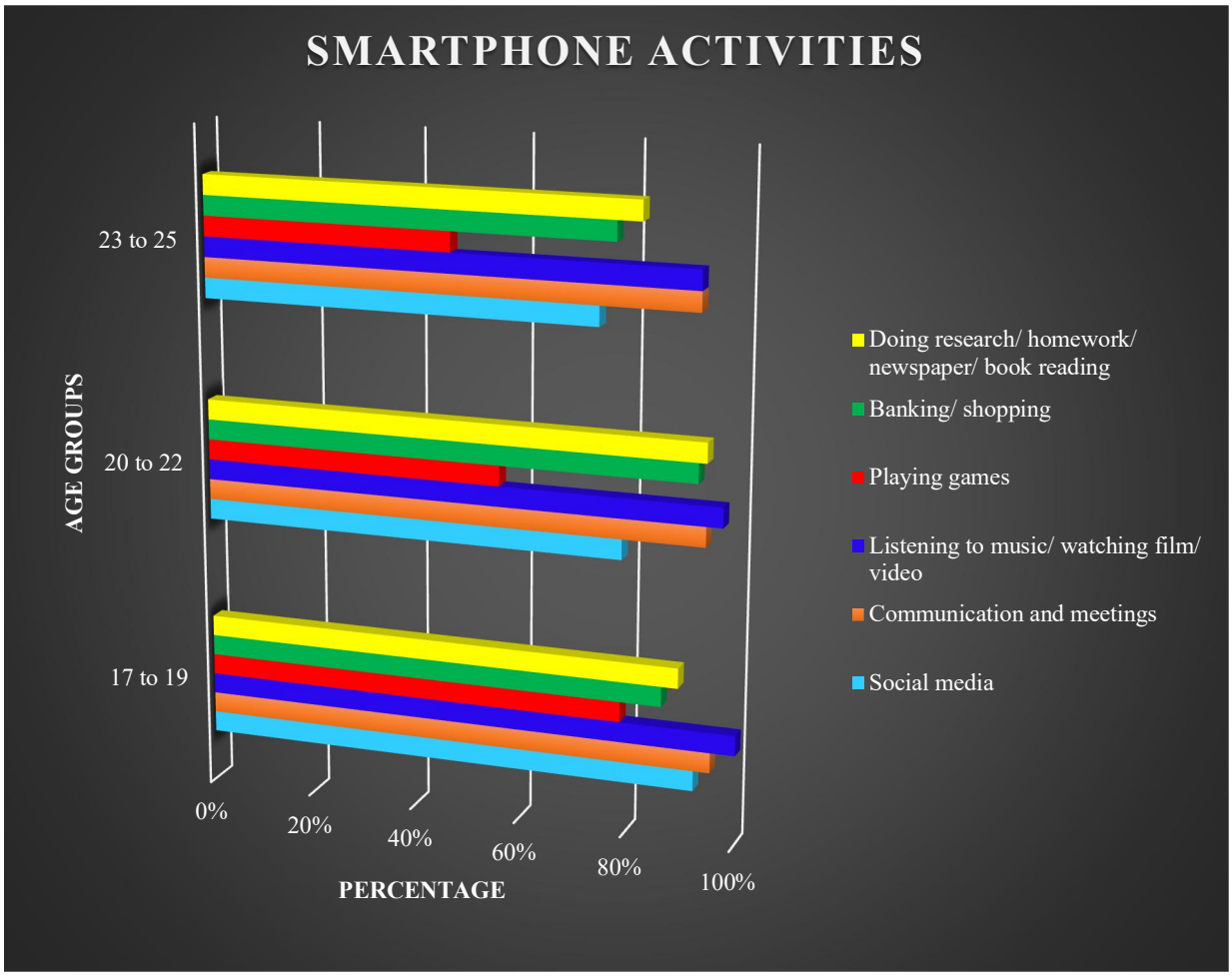


Table 5: Summary statistics for smartphone activities

Summary						
Age Groups	***Count***	***Sum***	***Average***		***Variance***	
17 to 19	6	358	59.66667		23.86667	
20 to 22	6	335	55.83333		114.1667	
23 to 25	6	310	51.66667		121.4667	
Result details						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	192.1111	2	96.05556	1.110469*	0.355025	3.68232*
Within Groups	1297.5	15	86.5			
Total	1489.611	17				

*p>0.05 statistically not significant

$F_{stat}<F_{crit}$

5.4 Analysis of health problems in students

Table.6 shows, the association between long time mobile phone usage and selected health problems like neck pain, ear pain, headache, numbness in finger and exhaustion. The prevalence of health problems was significantly higher in the long-time mobile phone users of adolescence (Figure.3).

From the results of ANOVA (Table.7) it was found that variance between the age groups and health problems was found to be statistically significant ($p \leq 0.05$). In this case, p value is equal to 0.005163. F statistical value has two degrees of freedom (2, 30). F statistical value (6.308964) is greater than F critical value (3.31583). It shows that there is statistically significant difference between age groups and health problems.

Table 6: Distribution of students by health problems

S.No.	Health problems	Age groups			Overall
		17 – 19	20 – 22	23 – 25	
1.	Headache	38 (57.57%)	40 (60.61%)	31 (46.96%)	109 (55.05%)
2.	Vision problems (farsightedness/ myopia/ astigmatism)	30 (45.45%)	21 (31.82%)	22 (33.33%)	73 (36.87%)
3.	Dry eyes	25 (37.87%)	23 (34.85%)	25 (37.87%)	73 (36.87%)
4.	Pain in the ears	25 (37.87%)	15 (22.73%)	14 (21.21%)	54 (27.27%)
5.	Rhythm ringing in ears after long time usage of ear phones	31 (46.97%)	23 (34.85%)	15 (22.73%)	69 (34.85%)
6.	Exhaustion	35 (53.03%)	37 (56.06%)	27 (40.91%)	99 (50%)
7.	Neck pain	43 (65.15%)	34 (51.52%)	19 (28.79%)	96 (48.48%)

Table.6 continued on next page

S.No.	Health problems	Age groups			Overall
		17 – 19	20 – 22	23 – 25	
8.	Fingers or hand pain or numbness in fingers	35 (53.03%)	28 (42.42%)	25 (37.88%)	88 (44.44%)
9.	Problem in falling asleep	30 (45.45%)	27 (40.91%)	22 (33.33%)	79 (39.90%)
10.	Problem with appetite	26 (39.39%)	12 (18.18%)	9 (13.64%)	47 (23.74%)
11.	Depression	29 (43.94%)	32 (48.48%)	20 (30.30%)	81 (40.91%)

Figure 3: Health problems

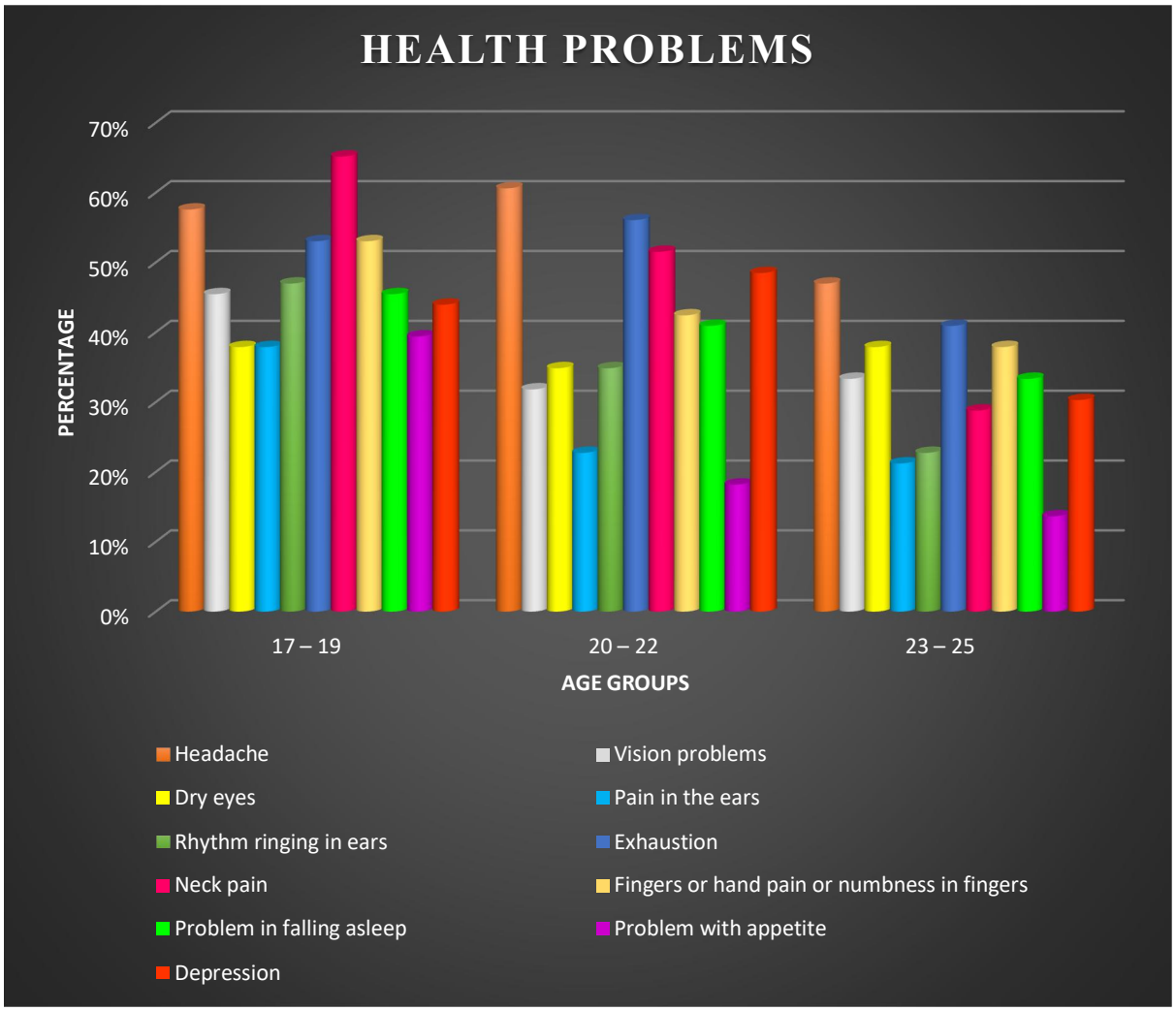


Table 7: Summary statistics for health problems

Summary						
Age Groups	***Count***	***Sum***	***Average***	***Variance***		
17 to 19	11	347	31.54545	32.47273		
20 to 22	11	292	26.54545	77.87273		
23 to 25	11	229	20.81818	40.36364		
Result details						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	633.8788	2	316.9394	6.308964*	0.005163	3.31583*
Within Groups	1507.091	30	50.23636			
Total	2140.97	32				

*$p \leq 0.05$ statistically significant

$F_{stat} > F_{crit}$

5.5 Analysis of syndromes in students

By finding the maximum rate of perceiving syndromes like De Quervain tenosynovitis (37.37%), Cubital tunnel syndrome or cell phone elbow (37.88%), text neck syndrome (46.97%), nomophobia (39.90%) and phantom vibration syndrome (46.97%) had significant association with mobile phone usage. Adolescence (17 to 19 years) was highly affected by long time mobile usage by having De Quervain tenosynovitis (45.45%), Cubital tunnel syndrome or cell phone elbow (46.97%) and phantom vibration syndrome (65.15%). The age category 20 to 22 was mostly affected by Text neck syndrome (50%) and NOMOPHOBIA (46.97%) (Table.8 and Figure.4).

From the results of ANOVA (Table.9) it was found that variance between the age groups and syndromes was found to be statistically significant ($p \leq 0.05$). In this case, p value is equal to 0.021618. F statistical value has two degrees of freedom (2, 12). F statistical value (5.367978) is greater than F critical value (3.885294). It shows that there is statistically significant difference between age groups and syndromes.

Table 8: Distribution of students suffering from common syndromes of excessive mobile phone usage

S.No.	Syndromes	Age groups			Overall
		17 - 19	**20 - 22**	**23 - 25**	
1.	De Quervain tenosynovitis	30 (45.45%)	25 (37.88%)	19 (28.79%)	74 (37.37%)
2.	Cubital tunnel syndrome or cell phone elbow	31 (46.97%)	24 (36.36%)	20 (30.30%)	75 (37.88%)
3.	Text neck syndrome	31 (46.97%)	33 (50%)	29 (43.94%)	93 (46.97%)
4.	NOMOPHOBIA or NO MObile PHone PhoBIA	26 (39.39%)	31 (46.97%)	22 (33.33%)	79 (39.90%)
5.	Phantom Vibration syndrome (PVS)	43 (65.15%)	29 (43.94%)	21 (31.82%)	93 (46.97%)

Figure 4: Syndromes

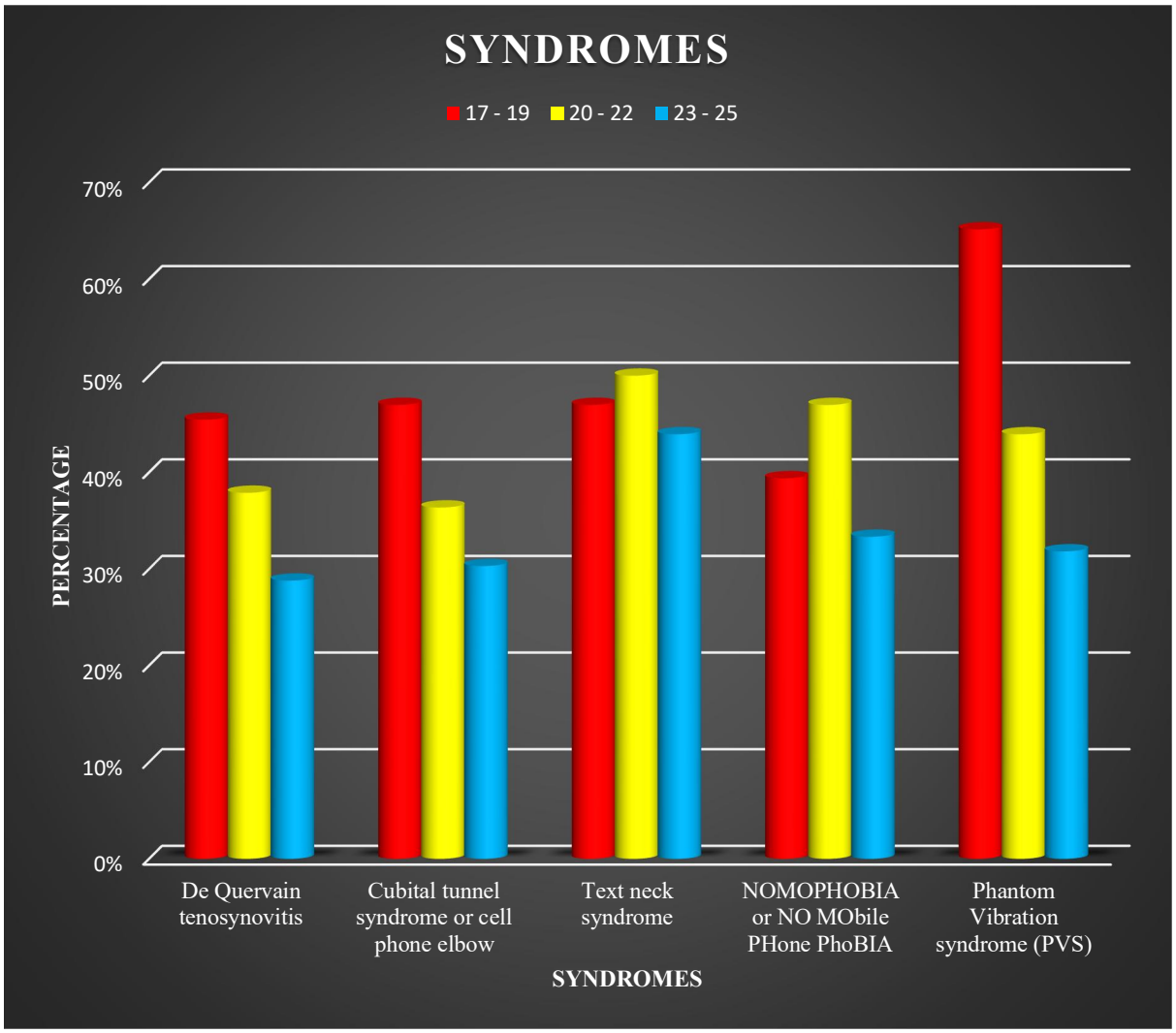


Table 9: Summary statistics for syndromes

Summary						
Age Groups	***Count***	***Sum***	***Average***		***Variance***	
17 to 19	5	161	32.2		40.7	
20 to 22	5	142	28.4		14.8	
23 to 25	5	111	22.2		15.7	
Result details						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	254.8	2	127.4	5.367978*	0.021618	3.885294*
Within Groups	284.8	12	23.73333			
Total	539.6	14				

*$p \leq 0.05$ statistically significant

$F_{stat} > F_{crit}$

5.6 Analysis of smartphone compulsion status among students

Students from the age category 17 to 19 felt that they spent more time on smartphones (87.89%) than they realize compared to other age groups. Most of the students (62.12%) also mindlessly use smartphones rather than doing something productive. Approximately more than half of the student participants felt that they lost track of time while using the phone (54.55%). Amount of time spent on mobile phones per day is increasing for 53.03% participants. Study subjects also use mobile phones while driving or doing other similar activities that require focused attention and concentration (65.15%). More than half of the students use mobile phones while eating (52.53%). It was found in the survey that many students have the habit of keeping their phones on or under their pillow or next to their bed while sleeping (47.98%). It is noted that students also wish to be a little less wired (54.04%) though many seem to have an intense urge to check their mobile phone whenever it buzzes (59.60%). Students also feel reluctant without having a mobile phone even for a short time (51.52%) showing symptoms of NOMOPHOBIA. Even though many of them feel that smartphone usage decreases their productivity (45.96%), they can't limit their time for mobile phone usage which reveals their addiction level. Addiction level is comparatively higher for adolescents (age 17 to 19) than early adulthood (Table.10). Table.11 shows the distribution of students based on their smartphone dependency levels.

From the results of ANOVA (Table.12) it was found that variance between the age groups and smartphone compulsion status was found to be statistically significant ($p \leq 0.05$). In this case, p value is equal to 0.008025. F statistical value has two degrees of freedom (2, 42). F statistical value (5.424537) is greater than F critical value (3.219942). It shows that there is statistically significant difference between age groups and smartphone addiction.

Table 10: Smartphone compulsion status

S.No.	Particulars	Age groups			Overall
		17-19	20-22	23-25	
1.	Smartphone consumes more time than you realize.	58 87.89%	49 (74.24%)	44 (66.67%)	158 (76.26%)
2.	Watching phone mindlessly when you could be doing something more productive.	40 (60.61%)	40 (60.61%)	43 (65.15%)	123 (62.12%)
3.	Lose track of time when using phone.	38 (57.58%)	36 (54.55%)	34 (51.52%)	108 (54.55%)
4.	Spending more time texting, tweeting, or emailing than talking to real-time people.	50 (75.76%)	33 (50.00%)	38 (57.58%)	121 (61.11%)
5.	Amount of time spent on cell phone has been increasing.	31 (46.97%)	39 (59.10%)	35 (53.03%)	105 (53.03%)

Table.10 continued on next page

S.No.	Particulars	Age groups			Overall
		17-19	20-22	23-25	
6.	Secretly wishing to be little less connected to your cell phone.	39 (59.10%)	33 (50.00%)	35 (53.03%)	107 (54.04%)
7.	Sleeping with your phone next to your bed regularly	39 (59.10%)	26 (39.40%)	30 (45.45%)	95 (47.98%)
8.	Engaging in your phone even when it interrupts your other activities.	36 (54.55%)	23 (34.85%)	24 (36.36%)	83 (41.92%)
9.	Using phone while driving or doing other similar activities that require your focused attention.	44 (66.67%)	45 (68.18%)	40 (60.61%)	129 (65.15%)
10.	Use of phone decreases your productivity.	36 (54.55%)	31 (46.97%)	24 (36.36%)	91 (45.96%)

Table.10 continued on next page

S.No.	Particulars	Age groups			Overall
		17-19	20-22	23-25	
11.	Feeling reluctant to be without your smartphone, even for a short time.	35 (53.03%)	29 (43.94%)	38 (57.58%)	102 (51.52%)
12.	Feeling ill-at-ease when you leave your smartphone somewhere or you have no service, or it is broken.	43 (65.15%)	21 (31.82%)	26 (39.40%)	90 (45.45%)
13.	Using smartphone while dining.	43 (65.15%)	32 (48.48%)	29 (43.94%)	104 (52.53%)
14.	Feeling an intense urge to check your phone everytime it buzzes.	43 (65.15%)	36 (54.55%)	39 (59.10%)	118 (59.60%)
15.	Mindlessly checking phone even when there is nothing new or important to see.	49 (74.24%)	33 (50.00%)	43 (65.15%)	125 (63.13%)

Source: Dr. David Greenfield (2017), The Center for Internet and Technology Addiction.

Table 11: Distribution of students based on their smartphone dependency levels

S.No.	Level of dependence	Age groups			Overall
		17 - 19	**20 - 22**	**23 - 25**	
1.	Weak	15	19	25	59 (29.80%)
2.	Moderate	14	29	20	63 (31.82%)
3.	Severe	37	18	21	76 (38.38%)
				Total	198

Table 12: Summary statistics for smartphone compulsion status

Summary						
Age Groups	***Count***	***Sum***	***Average***	***Variance***		
17 to 19	15	624	41.6	46.68571		
20 to 22	15	506	33.73333	57.78095		
23 to 25	15	522	34.8	46.6		
Result details						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	546.3111	2	273.1556	5.424537*	0.008025	3.219942*
Within Groups	2114.933	42	50.35556			
Total	2661.244	44				

*p≤0.05 statistically significant

$F_{stat} > F_{crit}$

5.7 Consulting Dr. Anselm Sophia Leon, Medical Officer, St. Mary's Health Centre

As per the response of Dr. Anselm Sophia Leon, Thoothukudi, there are some health risks associated with excessive usage of mobile phones including headaches, dry eyes, hearing damage, skin pigmentation due to IR effects, memory loss, sleep disturbances, effects on sympathetic nerves and parasympathetic nerves that may lead to seizure and brain function. IR radiation from heat leads to water loss from the body making the person dehydrated. Infant to old age people are affected depending on the time of usage and all are having equal chance for addiction, so only 1 hour per day of usage may be safe. If it is necessary to use more than an hour then one should have a break for at least 30 mins before continuing mobile usage and also advised to avoid using phones beyond 9.30 pm. Introvert having more chances in mobile phone addiction because introvert persons may not be comfortable in socializing so they tend to spend more time on mobile phones and may have a risk of being addicted. For students they are sometimes compelled to use phones while doing research or homework. While there may be advantages of mobile phones, students may not restrict their usage for studies only. They may get distracted and use phones for a long duration. Other people who are gaming or gambling have strong impulse to stay connected to their phones. Addicted individuals may suffer from lack of sleep, stress, digestion problem, obsessed condition.

Mobile usage during meal can cause indigestion and obesity. The person is not taking into account how much of food they are taking in while using mobile phones. Hence the person does not get the satiated feeling and eats more food. People who are addicted to phones tend to have their phones with them on all occassion and may get irritable if their mobile phones are not with them. So, the time wasted on mobile phones can be used to develop some good habits like book reading, household working, gardening, etc. Extremely addicted individuals should undergo psychotherapy, addiction counseling, take medications like Beta blockers and Benzodiazepines to help with severe symptoms of NOMOPHOBIA and digital detox that is weekly at least one full day must be spent without using mobile phones. Everyday people should abstain mobile usage before 2 hours of sleep. Doing neck rotation, Pranayama and hand grip exercise will be helpful. 20-20-20 rule can be followed while using mobile phones that is for every 20 minutes a person looks at a screen, they should look at something 20 feet away for 20 seconds which will reduce the health effects.

5.8 Analysis of the hygienic condition

To analyse the hygienic condition of using mobile phones, samples are swabbed from different mobile phones. There is growth of enteric bacteria that may be potential pathogens like lactose fermenting bacteria, non-lactose fermenting bacteria from seven mobile swabs. Three mobile swabs did not show any bacterial growth (Plate-A). Enteric pathogens were identified as per standard microbiological procedures. MacConkey agar is used for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non- fermenting gram-negative bacteria. Production of bright pink colour colonies on MacConkey agar indicates the presence of enteric pathogens on the mobile phones.

Plate-A

Bacterial cultures from mobile swabs

Growth of lactose fermenting bacteria

Growth of non-lactose fermenting bacteria

No growth

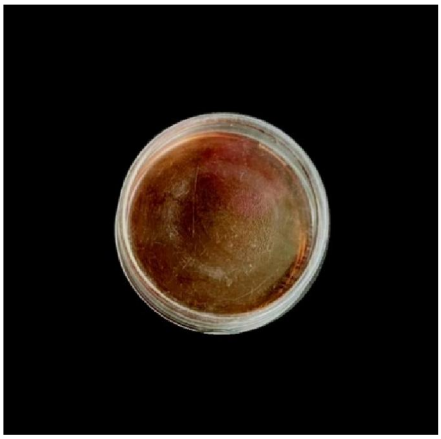


Plate-B

Consulting Dr. Anselm Sophia Leon,

Medical Officer, St. Mary's Health Centre

DISCUSSION

6. DISCUSSION

The smart phone or a mobile phone has become a full day attachment with most of the users all over the globe. It is now very difficult to say who are the people not using mobile/smart phones. The mobile phone users can not even think that the mobile phones are not working for an hour. The main advantage of using a smartphone or mobile phone is that it keeps users online. It is important to point out that smartphones have changed the way we access the internet and benefit from the microcomputers that they are. The people are empowered as they access information and interact with everyone all the time, on the go. Apart from all the above mobile phone or smartphone may be used for entertainment or playing games. However, there are many negative effects of mobile phone or smartphone. Quite a number of researchers have made systematic study on evil effects of mobile phone or smartphone on human health (Nath, 2018)

In this study we specified the relationship between smartphone usage, dependence and health problems among three different age categories 17 -19, 20 – 22 and 23 – 25. At present, smartphones are ubiquitous and used in many cases, from information access to shopping. Usage of smartphones that have easy-access, features and applications increases daily. The excessive and problematic usage due to the facilities that smartphone usage provides has brought smartphone addiction to the forefront (Demirci *et al.,* 2015).

The study was carried out among the college students of Thoothukudi, Tamilnadu, India, where 184 students were single (92.93%) and 14 were married (7.07%). This indicates that the majority of students were single (Table. 1). Tavakolizadeh (2014) made his observation among the students of Gonabad University of Medical Sciences, Iran, where 78.4% of the students were single, 18.9 % were married and 2.7% were divorced.

In our study the majority (89.40%) of the student participants used smartphones only while only a small fraction of students used both smartphone and normal phones (10.10%). At least one in ten people used more than one phone. About three quarters of the study participants used mobile phones for less than 5 years. Most of the study participants (42.42%) used their phones for a duration of about 5 – 8 hours (Table.2). Smartphone use duration averaged 7.8 hours in an Arabian study aged 18–30 years (Osailan, 2021). Another Arabian study showed 6.7 hours of smartphone use duration in 2367 university students (Alosaimi *et al.,* 2016); in a Canadian study of 104 university students, smartphone use duration was 5.1 hours (Berolo *et al.,* 2011). Nearly 72% of South Korean children aged 11–12 years spend 5.4 hours a day on mobile phones, 25% of those children were considered addicts to smartphones (Jeong *et al.,* 2016).

It is reported in our study that 44.44% of the students checked their mobile phone in-between sleep. Majority of the study participants (66.16%)

used their mobile phone in darkness. Approximately 37.37% use their smartphones 15 minutes before they go to bed (Table.2). An experiment conducted by Wood *et al*. (2006) documented that electromagnetic radiation emitted by mobile phones 30 minutes before sleeping was found to delay the onset of melatonin production which in turn might affect sleep. The screen light emitted by mobile phones suppresses the production of melatonin, a hormone that prepares the body for sleep. Short wavelength light, emitted by mobile phones, appears most detrimental to the secretion of melatonin. Sadagopan *et al.* (2017) reported that reading in bed can affect sleep cycle due to the blue light radiating from the screen and lead to decreased levels of melatonin and produce sleep disturbance. Moreover, the effects are immediate and tend to last for hours after light exposure (Exelmans *et al.,* 2016). Additionally, some studies have looked into the effects of exposure to radiofrequency electromagnetic fields emitted by mobile phones on sleep, and found that they increased sleep onset latency and sleep difficulties and lowered melatonin output prior to bedtime (Exelmans *et al.,* 2016).

There is no statistical difference between smartphone activities and age categories ($p>0.05$) (Table.5). It is seen that all the volunteers use their phones in one way or another. Most of the adolescent group use their smartphones for entertainment purposes while the rest of the students use it mainly for study purposes. Responses from students using smartphones for activities like

engaging in social media, communication and meetings, listening to music or videos, studying, playing games and banking were collected. Student volunteers from age group 17 to 18 showed maximum participation in activities like engaging in social media (92.42%), communication (95.45%) and listening to music (100%) and playing games (78.78%). Those from age group 20 to 22 showed maximum smartphone activity in categories like studying (93.93%) and banking (92.42%) (Table.4 and Figure.2). A study revealed an important fact that people are not actually addicted to their smartphones per se; however, it is to the entertainment, information, and personal connections that majority of the respondents were addicted to (Richard *et al.,* 2015). It has been noticed that the use of telephone is a way of coping with stress, anxiety, distraction, or boredom (De-Sola *et al.,* 2019).

Health problems showed statistical difference in correlation with the age category among the volunteers ($p \leq 0.05$) (Table.7). In this study headaches are the most common health problem and the most affected age group is 20 to 22 (60.61%) (Table.6). A study on MP users in Sweden and Norway, reported 8.4-13% of MP users with HAMP (Headache associated with Mobile Phone) (Sandstrom *et al.,* 2001). In Saudi Arabia, HAMP was observed in 22.4% of MP users (Al-Khlaiwi *et al.,* 2004). Santini *et al.* (2001) reported that 10-20% of MP users complained of HAMP in a questionnaire study conducted in France. Discrepancies in the proportion of MP users who experience HAMP may be due

to differences in MP types, demographic features of users, social level of concerns about MP use or media reporting about them (Mortazavi *et al.,* 2007). Headache during or after MP use could be induced by altering conditions during MP use including radiofrequency fields (RFs), psychological factor, temperature change, noise and various combinations thereof (Chu *et al.,* 2011). Exposure to RFs during MP use has been suggested to trigger a variety of symptoms such as headache, fatigue, concentration difficulties, and nausea (Hillert *et al.,* 2008).

In our analysis 36.87% of the students were showing vision syndrome problems (Table.6). It is conversely less when compared to the study conducted by Sadagopan *et al*. (2017) who reported that the prevalence of cell phone vision syndrome in the study population consisting of college students was found to be 83%. Mobile devices that are used for noticing and replying to emails, looking for the climate, reading news, and posting status updates on Facebook may be causing vision problems (Palm *et al.,* 2007). The abrupt change in graphics, brightness and details while students are gaming is one of the main causes of chronic dry eye syndrome. The eyes bear a tremendous amount of reflexes, stress and dryness (Nath, 2018). Visualizing the smaller screens can accelerate a pattern of ophthalmic problems such as headaches, blurred vision, sore eyes, dry eye and muscle strain (Palm *et al.,* 2007). Routine ophthalmic examinations and appropriate vision habits can help to prevent or

decrease the progress of the symptoms associated with cell phone vision syndrome. Normal blinking rate is about 15 times per minute, but this rate was reduced in the person who was staring at the smartphone. The person is quint to read the smaller screens, facial, neck and shoulder muscles are contracted, eyes become fatigued and vision can be blurred or strained. The iPhone's latest update is likely to disturb equilibrium with the new icons zooming in and out. Main complications of using this type of phone include is dizziness.

The age group 17 to 19 were the ones most affected and showed pain in the ears (37.87%) and rhythm ringing in the ears (46.97%). Student participants also had problems with appetite (23.74%), falling asleep (39.90%). A large group of students also reported suffering from depression (40.91%). The age group 20 to 22 showed the maximum rate of depression (48.48%). As analysed among the student participants, longtime smartphone usage causes exhaustion. The study shows that 50% of the students experienced exhaustion after prolonged usage of the phone (Table.6). It is common to see teenagers and even adults exhausted of the long hours spent on smartphones, be it games or surfing the net. It impacts the digestion, breathing rate and heart beat rate (Nath, 2018). A statistical report from the British Chiropractic Association, in 2015, concluded that 45% of young people aged 16–24 years suffered with back pain. Long-term usage of smartphones may also cause incurable occipital neuralgia,

anxiety and depression, nomophobia, stress, eyesight problem, hearing problems, and many other health issues (Leonard, 2019).

There is a statistical difference between syndromes and age groups ($p<0.05$) (Table.9). Phantom Vibration Syndrome (PVS) is one of the most common syndromes suffered by the study subjects (46.97%). Maximum affected category of PVS belongs to the age group 17 to 19 (65.15%) which is followed by the age group 20 to 22 (43.94%) and then by the group 23 to 25 (31.82%) (Table.8 and Figure.4). It is similar to the study conducted by Sunitha *et al*. (2020) where 71% of the UG students (less than 20 years old) experienced PVS and among the PG students (21 -25 years old) 50% reported as experienced with PVS.

The other most common syndrome experienced by the study participants is Text Neck Syndrome (46.97%) (Table.8). Kamaraj *et al.* (2022) also reported that 16.7 % of the students had text neck syndrome and l% of them had severe neck disability. His study group mainly included the students from the age group between 18 to 22 years. Most smart phone tasks users require to stare sharply downwards or to hold their arms out in front of them to read the screen which makes their head move forward and cause an excessive anterior curve in the lower cervical vertebrae and an excessive posterior curve in the upper thoracic vertebrae to maintain balance, placing stresses on the cervical spine and the neck muscles (Vijayakumar *et al.,* 2018). Gazing into the phone for a long

time with one's neck bent and arms in a fixed position pose a serious health risk. Pain, muscle spasms and restlessness are just short-term effects. In the longer run permanent or chronic diseases may occur. Cervical spondylitis, golfer elbow, chronic dry eye syndrome, stiffness in thumbs, neck and back are a few diseases occurring from habituated wrong postures of using smart phones. The typical head down and neck bent position while one is engrossed in their favorite games or chat should be consciously avoided (Nath, 2018).

Our study reported that the age category 17 to 19 also showed maximum levels in De Quervian tenosynovitis (45.45%) and Cubital Tunnel Syndrome (46.97%). Students from age group 20 to 22 were affected by Text Neck Syndrome (50%) and NOMOPHOBIA (46.97%) (Table.8). A recent survey found that 84 percent of the world's population said they could not go one go about in their day without their smartphones, and current research shows that nearly two-thirds of teens and young adults check their phones every 15 minutes or less. The anxiety and stress over missing out on a text or Facebook update can take such a toll on peoples' health that Morningside Recovery Center in California recently founded the first rehab group for NOMOPHOBIA (Nath, 2018).

Addiction to remain online, compulsion to be active on social sites leads to low productivity and impacts the emotional health of the person. Constantly looking for something interesting on the web, social sites and games inhibits

one's emotional ability to focus on one topic for long. It has been already proved that long hours of gaming make anyone impatient, addicted and unproductive. Long time effects may be worse, permanent and affecting the more subconscious layer of behavior (Nath, 2018)

It is investigated from the study that the correlation between age groups and addiction level is statistically significant ($p<0.05$) (Table.12). Addiction is the loss of control, the establishment of a dependent relationship, tolerance, the need for progressively more time and dedication, and severe interference with daily life (Echeburua *et al*., 2009). Students seem to spend more time on their phones than intended. The smartphone compulsion status revealed that 29.80% of students (n=59) exhibited weak dependency, 31.82% (n=63) displayed moderate dependency, and 38.38% (n=76) exhibited severe dependency (Table.11). Jafari *et al*. (2019) also documented that 17.8% (n=78) students had moderate dependency, 10.9% (n=48) had sever dependency, and 71.3% (n=313) were identified as mobile addicts. It is also seen reported in our findings that smartphone dependency decreases with aging (Table.11). Aged populations are very rarely developing addictive mobile phone behavior because of greater self-regulation (Deursen *et al.,* 2015).

The present study reveals that 63.13% students mindlessly check their phone even when there is nothing new or important to see (Table.10). Researchers in Finland found that most people obsessively check their menu

screen, news, e-mail, and apps, even though the likelihood of seeing new and interesting information keeps decreasing (Nath, 2018).

We also found that 65.15% of the students use their mobile phones while driving or doing other similar activities that require focused attention and concentration (Table.10). This may likely increase the incidence of accidents. In November 2009, the Pew Internet and American Life Project reported that one-quarter of American teenagers of driving age admitted to having texted while driving (Madden and Lenhart, 2009). Using telephones during eating, meetings with others, lessons, cycling, skateboarding, or driving a car, and in the toilet may indicate excessive attachment to the telephone. The problem is not the use of the phone as a device itself, but the incorrect use of the applications and tools of that device (De-Sola *et al.,* 2019).

Our findings showed that 7 out of 10 MPs (70%) showed bacterial contamination mostly potential enteric pathogens. Both lactose fermenting and non-lactose fermenting bacteria were isolated from mobile swabs. A study on MPs among college students and staff of Birendra Multiple Campus conducted by Adhikari *et al.* (2018) in Nepal showed a lower prevalence of bacterial contamination (56%). MPs can act as a vehicle for transmitting pathogenic bacteria and other microorganisms (Brady *et al.,* 2007). Contaminated surfaces and equipment, including cell phones, are a source of pathogenic microorganisms, often antibiotic-resistant bacteria, causing diseases in the

social environment and in healthcare facilities (Maksymowicz *et al.,* 2020). Due to the advancement and benefits of MPs, their hazard to human health is often overlooked. It has been reported that a MP can harbour more microorganism than a man's lavatory seat, the sole of a shoe or the door handle (Davis *et al.,* 1996). The combination of constant handling and the heat generated by phone as well as sweat from hands creates an optimum breeding environment for all kinds of microorganisms that are normally found on the skin (Rana *et al.,* 2013). Decontamination of the phone surface with 70% ethyl or isopropyl alcohol should be systematic, because it reduces the number of pathogens on the surface of the phone (Maksymowicz *et al.,* 2020).

Nowadays, mobile phones are a helpful and convenient tool for study, work, organization, or entertainment. However, its excessive use may have a detrimental effect on sleep, while in situations requiring concentration such as learning or driving, it can result in distraction. Careful use of the mobile phone including limiting the time spent on phone applications is essential to prevent inappropriate habits and adolescents' addiction to phones.

SUMMARY

7. Summary

❖ The smartphone has become an essential part of a student's life. For an extended period of time, an individual has to look at their phone's small screen and perform repetitive movements in an uncomfortable posture. In parallel, mobile phones are increasingly used for entertainment as well. Through online media, entertainment gradually becomes a part of their habituation, resulting in an increased rate of social media communication between individuals than live communication.

❖ Students aged 17 to 25 participated in a questionnaire survey to obtain information for the present study.

❖ Health problems and syndromes suffered by students were the focus of the questionnaire. In addition, a smartphone compulsive test was conducted to determine the level of dependence on mobile phones among students.

❖ In Excel, the collected data was examined for statistical significance using ANOVA. From the data we observed statistical difference between age groups and health problems, syndromes and smartphone compulsion.

❖ Long time mobile users were affected with ailments including hand pain, neck pain, ear pain, head ache, vision problems and numbness and psychologically affected by experiencing stress, depression, problem in

sleeping, loss of appetite, exhaustion, NOMOPHOBIA and phantom vibration syndrome.

- ❖ We also performed swab test to find bacterial contamination of mobile phones. Seven out of ten mobiles were contaminated attributing to the poor hygienic and sanitary practices. The enteric bacteria that were potential pathogens, identified on the MacConkey agar plate.

CONCLUSION AND SUGGESTIONS

8. CONCLUSION AND SUGGESTIONS

Present study on the “Mobile phone dependency and health related lifestyle disorders among the college students of Thoothukudi”, reveals that the problematic use of mobile phone adversely affects the quality of life, sleep, vision, appetite, concentration and psychological health. Vision problems such as farsightedness/ myopia, dry eyes, pain in ear, neck, fingers and hand, loss of appetite were common among the students who use their mobile phones frequently. Feeling of exhaustion, anxiety, and depression were also noticed.

In addition, the hours spent on mobile phone use, duration of use, mobile phone usage in darkness, checking the mobile phones in between sleep and taking meal, time interval between mobile phone usage and sleep were analysed. Such analysis provides the addiction level of mobile phone users.

Through experimental analysis, it was proved that mobile phones carry enteric bacteria on it due to user’s unhygienic conditions and may be potential threat to infection.

Suggestions:

- There is a need to intensify awareness programs to address health issues of people caused by excessive dependency on mobile phones.
- Academic calendars for students may be designed to keep the health effects of the overuse of smartphones and laptops while attending online sessions.
- Academic Institutes should issue strict guidelines to avoid overcrowding of online courses.
- Mobile phone users should be advised not to keep the mobile phones near the bed and on the body in order to prevent the continuous exposure of body to the infrared rays emitted from the mobile phones.
- Practice 20-20-20 rule, “When using your screens give your eyes a break so every 20 minutes, take a 20 seconds break and focus your eyes on something at least 20 feet away”.
- Using mobile phone after washing of hands is necessary to prevent the enteric bacterial contamination on the mobile phones. Cleaning the phone with gadget cleanser is also recommended.

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9. BIBLIOGRAPHY

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A STUDY ON HANSEN'S DISEASE IN TWO LEPROSARIA

A field work submitted to

ST. MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

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in partial fulfilment for the award of the degree of

MASTER OF SCIENCE IN ZOOLOGY

by

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

April 2023

CERTIFICATE

This is to certify that this field work entitled, **"A STUDY ON HANSEN'S DISEASE IN TWO LEPROSARIA"** submitted to **St Mary's College (Autonomous), Thoothukudi**in partial fulfilment for the award of the degree of**Master of Science in Zoology** is done under my supervision. It is further certified that this field work report or any part of this has not been submitted elsewhere for any other degree by the following students.

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INTRODUCTION

1. INTRODUCTION

Hansen's disease (HD) also known as leprosy is a chronic infectious disease which is caused by the bacteria *Mycobacterium leprae*. Neglected tropical disease (NTDs) infect over 2.7 billion people worldwide, a disproportionate number of whom reside in low –and middle- income countries (Deribe *et al*., 1756). Hansen's disease is one such NTD that contributes to significant morbidity and disability in certain areas of the world. In 2018 alone, over 208,619 incident cases of leprosy were reported globally, and an estimated 2- 3 million people were living with disease related disabilities (Aseffa *et al*., 2021).

The disease is dreaded because of the damage that occurs in weak and anesthetic hands and feet, as well as in blindness and facial disfigurement. HD produces a chronic infection in humans that affects mainly peripheral nerves and skin but may also affect sites such as the eyes, mucous membranes, bones, mucosa of the upper respiratory tract, and the eyes. In peripheral nerves, *M.leprae* can be found in Schwann cells **(**Graham *et al*.,2010). In the skin, *M.leprae* has an affinity for keratinocytes, macrophages, and histiocytes. Keratinocytes seem to play a key role in the release of the antimicrobial defensing in response to *M. leprae* antigens **(**Cogen *et al*., 2012).

Leprosy is curable and treatment in the early stages can prevent disability. India continues to account for 60% new cases reported globally each year.

Leprosy is spread between people, although extensive contact is necessary. Leprosy has a low pathogenicity and does not spread during pregnancy to the unborn child or through sexual contact. Leprosy occurs more commonly among people living in poverty. Since leprosy is not contagious, people with leprosycan live with their families and go to school and work. The disease is classified into five types according to immunological status. They are tuberculoid, borderline tuberculoid, mid-borderline, borderline lepromatous, and lepromatous leprosy (Sanker *et al*., 2020).

Throughout history, leprosy has been feared and misunderstood. The origin of leprosy is unknown and the disease was first described around 600 BC. There was a debate in 1960s and 1970s about the choice of appropriate name for this disease- leprosy, lepra, Hansen's disease or Hanseniasis (Gramberg and Lendrum, 1952). A strong movement developed in some countries to substitute the name Hansen's disease for leprosy (Rotberg *et al*., 1969). The disease is an important cause of crippling deformities (Landhani *et al*., 1997) and the affected people have high psychosocial problems such as divorce, unemployment, and displacement from their native place of residence (Kaur *et al*., 2002). Psychiatric disorders are highly prevalent in people with Hansen's disease and these preoccupy the healthcare resources. Comorbidity among these patients is observed in both clinical and epidemiological studies (Erinfolami *et al*., 2009).

Stigma attached to these patients has more impact on educated women belonging to a higher socioeconomic group and in joint families (Vyas *et al*., 1982). Because of the fear of infecting family members, women suffer a lot by keeping themselves aloof and are constantly worried about divorce. The psychosocial issues that are commonly related to stigma are people's dignity, social status, employment opportunities, job security, family relationship, and frienships. People have left their families and even spouses and children, fearing the repercussions of the fact that they had Hansen's disease. Eldaron *et al*. (1993) in their study on divorce among Saudi female patients, concluded that average rate of divorce is mainly because of Hansen's disease (14.4% in Saudi women). In a study on the effects of the stigma of Hansen's disease showed negative effect on income generation among the affected people in the Terai area south east Nepal. The negative physical effects of the disease were the main reason for loss of income and employment status (Calcraft *et al*., 2004).

In 1903, the incidence of psychiatric disorders among these patients was found to be less than 6% (Sand *et al*., 1903) whereas in 1927 the incidence of psychosis among these patients was 3% (Cazenavette and Muri, 1931). 70% of these patients felt psychological problems mainly due to the association between physical deformity caused by Hansen's disease and the accompanying disease associated stigma (Price *et al*., 1983). Ramanathan *et al*.(1984) reported a high frequency of psychiatric disorders (55%) in these patients. Yazici *et al*.

(1994) found that 20% of patients with Hansen's disease utilizing outpatient services were found to have comorbid psychiatric disorders. The psychiatric morbidity was found to be positively correlated with physical disability and material status but not age, sex, education, type of Hansen's disease or duration of the illness. The patients of Hansen's disease were more likely to manifest with psychiatric disorders than those suffering from other skin conditions (Erinflomai *et al*., 2009).

The disease presents a broad clinical and histopathological spectrum that is correlated with immunological response of the patient (Ridley *et al*., 1996). At one end of spectrum, in the tuberculoid form, a specific cell mediated immune response to *Mycobacterium leprae* is observed, with lesions characterized by epithelioid granulomas, participation of lymphocytes mainly of Th1 type, and few alcohol-acid-resistant bacilli (Yamamura and Weinberg, 1991). In contrast in the most severe, or lepromatous form, specific cell immune against *M. leprae* is absent, with diffuse dermal lesions characterized by poorly differentiated young macrophages with heavy load of bacilli and small number of Tcells predominately of the Th2 type (Modlin *et al*., 1991). In spectrum of borderline leprosy there are varying degrees of cell-mediated immune response characterized patients with low response to the bacillus (Ridley *et al*., 1985).However, the disease manifestations and complications are determined by the immune response of the host. Therefore proper classification of leprosy is

one of the fundamental issues for treatment and prognosis. Many patients experience nerve damage before, during or after treatment (Pfaltgraff and Bryceson, 1987).

However, in places where bacilloscopic examination is available, patients whose skin-smear exam tested positive are multibacillary regardless of the number of lesions. For better operational classification, some studies have used the *M.leprae* serum lateral flow test (ML-Flow), which correlates the concentration of anti-PGL1 (specific antibody against *M. leprae*) in the patient's peripheral blood with bacillary load (Cambau *et al*., 1997).Serum –positive patients are classified as multibacillary (MB) and serum-negative ones as paucibacillary (PB) (Smits *et al*., 2003).The simplification of the operational classification may mask the true relationship of the immunological response, and other intrinsic genetic factors, limiting the information and preventing further molecular findings that could support epidemiological data collection, treatment and control strategies (Scollard *et al*., 2004).

Multidrug theraphy (MDT) for leprosy is highly effective in curing the mycobacterial infection, but treating the nerve damage is much more difficult. Clinical presentation is unfamiliar to most patients, and the associated immune response may be similar to other more common diseases, such as systematic lupus erythromatosus (Horta Bass *et al*., 2015). It has led to misdiagnosis, delayed treatment, and irreversible neurological damage (Chokkakula *et al*.,

2019). Leprosy is the leading cause of peripheral neuropathy (Jardim and Fernandes, 2004). There are no leprosy patients without peripheral nerve damages, and the mechanism of how it happens is uncertain (Wilder-Smith and Egger, 1997).

The pure neural leprosy (PNL) is characterized by signs and neural symptoms marked with sensitive alterations, like paresthesia, or sensorial deficit equivalent to area of the nerve enlargement, associated or not to the motor or trophic deficits, or autonomic, with skin lesions (Nordeen *et al*.,2007). This form of manifestations of the disease is a well- recognized clinical entity, accounting for 4-16% of patients with leprosy in India (Gridhar *et al*., 1996). A recent study of PNL shows an approximate 9.0% incidence in the southeastern Brazil (Santos and Pereira, 2006). The most commonly affected nerves in the PNL are the ulnar and common fibular nerves (Mahajan *et al*., 2004). The electro neuromyography (ENMG) is indispensable to the studies of peripheral neuropathies (Brasil –Neto *et al*., 1992).Approximately 98% of patients in whom leprosy is confirmed by the traditional methods present electro neuromyographic alterations (Marques *et al*., 2003).

The purpose of controlling leprosy and its management are the early diagnosis and treatment, followed by an early recognition of nerve damage and effective intervention. Due to the high complexity of leprosy, the development of a vaccine and the use of a unique marker for diagnosis are questioned

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

World health organization reported that Leprosy patient recognized to have physical disability result in prejudice and stigma and in 1982 classified the infections into multibacillary and paucibacillary. Duncan *et al*. (1983) suggested that leprosy cases seen in the infant period also have a possible infection from mother through blood or with breast milk.

Sehgal *et al*. (1989) reported that the nerve involvement in Hansen's disease which is detected by enlargement of peripheral nerves. Desikan *et al.* (1995) showed that bacillus can survive for 46 days in most environment and 60 days in water even in backwater they can survive for long time.

BCG vaccination at repeated doses provides protection against leprosy (Pem *et al*., 1996). Abraham *et al*. (1998) documented that individuals with lepromatous infection usually contain many bacilli.

Ji (1998) identified that multidrug therapy decreases the chance of drug resistance. Van Beers *et al*. (1999) defined that direct contact with a patient with leprosy considerably increases the chances of obtaining the disease compared to the rest of the population. Freerksenet *et al*. (2001) reported that medication used for leprosy treatment include dapsone, rifampin, and for the lepromatous disease is clofazimine.

Meima *et al*. (2001) assessed that using sensitive methods including monofilaments depicts the impairment of nerves that occurs a lot earlier in lepromatous disease as compared to tuberculoid disease. Tay *et al*. (2003) concluded that if type II reactions are not treated immediately that may lead to death.

Cavaliere *et al*. (2004) havestated that multidrug therapy was used for the treatment of leprosy and in 1930 the first drug developed were dapsone and sulfane. Britton *et al*. (2004) asserted that the bacteria *M. leprae* affinity for peripheral nerve cells which attacks Schwann cells causes nerve demyelination and also the loss of axonal conductance which presents as numbness.

Eidt *et al*. (2004) have reported that Mycobacterium complex comprises *Mycobacterium leprae and Mycobacterium lepromatosis.* Rea *et al*. (2005) observed that patient affected by type II have alopecia of eyebrows and eyelashes, nasal septal perforation.

Nicholos *et al*. (2005) reported that edema in existing lesions and spontaneous nerve pains are clinical signs of type I reaction. Laskaris *et al*. (2005) stated that it affects the skin, peripheral nerves, the mucosa of the upper airways and other tissues as bone and some viscera and also that the disease are diagnosed by bacteriological and histopathological analysis.

Truman (2005) focused on the damage caused in nerves are lifelong problem although the spreading of disease occurs within the body, starts at

upper respiratory tract and the infection occurs mainly through the skin. Moet *et al*. (2006) argued that old members have risk in the acquisition of leprosy and in some cases the disease leprosy show a bimodal relationship with age. He also stated that men are at greater risk for diseases than women.

Lane *et al*. (2006) showed that patients affected by *M. leprae* progress with focal lesions in several organs and in some patients it is present in liver or marrow. Lahiri *et al*. (2008) reported that the spreading of the disease is mainly by *M. leprae* and environmental conditions.

Kahawita *et al*. (2008) observed that type II reaction results in cellular dysfunction as well the antigen antibody complexes being deposited directly into tissues. Saunderson *et al*. (2008) investigated that the neuropathy is coupled to loss of sensory perception but in some cases pain arises later in path of disease.

Patients develop new subcutaneous nodules which are painful (Kahawita, 2008). Britton *et al*. (2009) found that the nodules may be accompanied by fever and malaise as well as inflammation of nerves.

The National Leprosy Control Programme which was launched in India in 1955 aimed for controlling leprosy using survey, education and through dapsone monotherapy. The programme successfully reduced the National prevelance of leprosy from 57.6/10,000 in March 1981 to 2.44 per 10,000 in March 2004. The goal of The National Leprosy Control Programme is to

consider leprosy as a public health problem and to reduce it as less than 1 case per 10,000 population (Siddiqui *et al*., 2009).

Jaworska (2009) studied that leprosy case detection campaign was conducted to ensure early diagnosis, disability management and stigma alleviation. Gillis *et al*. (2009) reported that the lab technique polymerase chain reaction is readily used for detecting *M. leprae and M. lepromatous* DNA in tissue.

Parkash (2009) documented that leprosy patients were classified into two groups namely paucibacillary and multibacillary and the classification isbased on the number of skin lesions less than or equal to five for paucibacillary and greater than five for the multibacillary form. He also stated that leprosy is a polymorphic infectious disease, which is determined by immune system of the host.

Thompson *et al*. (2009) reported that the bacteria causing leprosy by different species due to their DNA sequences are obligate intracellular organisms. Graham *et al*. (2010) analysed that corticosteroids are generally used for treating leprosy.

Latency period between the exposure to *Mycobacterium leprae* and clinical signs range from 5 to 10 years depending on the type of leprosy(James *et al*., 2011).The bacteria grows best in the temperature of 30^0C or less (Hussein *et al*., 2011).

Barreto *et al*. (2011) documented that more than half of the people affected by Hansen'sdisease shared a small house. Rodrigues *et al*. (2011) reported that the inflammation effect on the peripheral nerve causes dysfunction and sensory loss.

Trindade *et al*. (2011) worked on leprosy that develops after solid organ transplantation and chemotherapy. Bhat *et al*. (2012) reported that transmission mainly occurs through respiratory tract.

Saunderson *et al*. (2012) documented the use of non-steroidal anti inflammatory medications for the patient with mild symptoms. The shift from confinement to outpatient treatment of Hansen disease is better for patient families and their social lives (Scollard, 2012).

Mycobacterium leprae interacts with the host cell lipid metabolism to foster the bacterial intracellular survival (Degang *et al*., 2012). Lockwood *et al*.(2012) stated that the Hanson disease caused acute nerve and skin inflammation.

Nery *et al*. (2013) stated that 95% of type I reaction can occur in the first 2 years after starting Multi Drug therapy treatment. World Health Organization(2014) suggested that ROM treatment including rifampin, ofloxacine and minocylline can be used for treatment and the side effects caused by multidrug therapy are mild.

According to Kumar *et al*. (2014), leprosy presented as an insignificant skin lesion to extensive disease causing profound disability and deformities. White *et al*.(2015) discussed that leprosy may further associated with the immune response to the leprosy bacillus ranging from tuberculoid to lepromatous.

Singh *et al*. (2015) stated that *M. leprae* and *M. lepromatosis* diverged from a common ancestor more than 13million years ago. Fonsea *et al*. (2017) reported that *Mycobacterium leprae* invades both sensory and autonomic nerves causing a reduction in cutaneous sensation and absence of sweating.

Naafs *et al*. (2019) obseved that type II reaction was a type III humoral hypersensitivity reaction. Belachew *et al*. (2019) reported that lack of awareness was the main reasons for missing the diagnosis even at a specialist level.

Thadtbiam (2019) stated that tuberculoid leprosy resulted from infection of an individual with high cell mediated immunity also typifies by less than 5 skin lesions. Menghani *et al*. (2021) did their research on the confirmatory diagnosis and regression of disease in patients under treatment.

Though plenty of research were carried out on diagnosis, types and treatment of Hansen's disease, there is paucity of information regarding the prevalence of the disease and sociodemographic survey.

OBJECTIVES

3. OBJECTIVES

Hansen's disease is one of the oldest diseases known to man. Despite advances in all spheres of medical science, leprosy continues to be a public health challenge in countries like India. The survey has been carried out with the following objectives.

- To find out the severity of leprosy.
- To study the association of leprosy status with the types.
- To find out the socio demographic characteristics of the study population.
- To know the magnitude of stigma and discrimination prevalent in the society.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

We have visited the Holy Family Hansenorium, Tiruchirapalli and St. Joseph Leprosy Hospital, Thoothukudi and interviewed the patients there as a part of our field work. A study was performed using information that have been recorded in two hospitals from the year2016 to 2022. The data was collected from the year 2016 to 2022. In the present study, Holy Family Hansenorium, Tiruchirapalli was considered as Station I and St. Joseph Leprosy Hospital, Thoothukudi as Station II.A questionnaire survey was also undertaken to assess the knowledge, attitude and behaviour of the persons under treatment. Simple questions were asked to the leprosy patients. A sample questionnaire is attached for further references.

4.1 DESCRIPTION OF THE STUDY AREA

STUDY AREA 1:

STATION I

The Holy Family Hansenorium is located in Fathima Nagar, Tiruchirapalli. The Holy Family Hansenorium was founded by (Late) Rt. Rev. Fr. James Mendonza, then Bishop of Tiruchirappalli in the year 1955. The land was allotted by the Government of Tamilnadu for urgent and imminent service to the people in this area. Since 1981 the Congregation of Mother of Sorrows Servants of Mary assumed charges of this institution from the diocese.

STUDY AREA 2

STATION II

St Joseph’s Leprosy Hospital is located in Arockiapuram, Thoothukudi. St Joseph’s Leprosy Hospital began as a single hut in 1949. During the past 70 years, it has treated over 20,000 patients. There are now 47 residents living at the hospital who caught leprosy when they were children. The sisters and staff at St Joseph’s have cared for them throughout their whole lives. As well as treating leprosy, St Joseph's focus is on the physical and social rehabilitation of the residents.

4.2 QUESTIONNAIRE

- Name:
- Age :
- Gender:
- In which city and in which state do you live?
- Current employment status
- Occupation
- Marital Status
- How old were you when you received a diagnosis of leprosy? In what year was this?

- Does anybody else in your family have leprosy?
- Apart from leprosy, do you have other illness?
- What were the first symptoms of leprosy you noticed?

  a) Pale patches on your skin

  b) Pale patches on your skin with no sensation

  c) Lumpy or thickened skin

  d) Runny nose or nose bleed

  e) Difficulty seeing
- Did you have pain or tingling in your arms, legs, hands, feet or around eyes?
- Have you suffered from muscle weakness in your hands, feet, arms or legs and difficulty in moving them?
- Whom did you seek for help after the initial symptoms?
- Do you feel numbness in your hands or in your feet?
- Have you noticed nodules on your skin?
- Have you suffered from loss of eyebrows and eyelashes?
- How often your family visited you?
- How was your life before leprosy?

- What are the changes happen in your life after leprosy?
- Have you faced discrimination in the society after leprosy?

4.3 STATISTICAL ANALYSIS OF THE COLLECTED DATA

ANALYSIS OF VARIANCE (ANOVA):

ANOVA was computed to find out the variation in the data and the significance was tested. The ANOVA was calculated as follows. The calculated F_{stat} was compared with the critical value of $F_{0.05}$ for the two degrees of freedom to draw conclusion about variance components.

Source of variation	***Df***	***SS***	***MS***	***F***
Between groups	k-1	SSB	MSB= SSB/k-1	MSB/MSW
Within groups	n-k	SSW	MSW= SSW/k-1	
Total	n-1			

Where,

F= ANOVA coefficient

SSB = Sum of Squares between groups

SSW= Sum of Squares within groups

MSB= Mean Squares between groups

MSW= Mean Squares within groups

k = Total number of populations

n = Total number of Samples in a populations.

RESULTS

5. RESULTS

A study on Hansen's disease was conducted by visiting the Holy Family Hansenorium, Tiruchirapalli and St. Joseph's Leprosy Hospital, Thoothukudi. The number of cases and types from 2016 – 2022 were statistically analysed. Questionnaire was distributed and the patients were interviewed.

5.1 Statistical Analysis:

5.1.1 Comparison of Total Leprosy Cases of Two Stations

Table.1 represents the total cases of leprosy cases observed in two different Stations between the year 2016 – 2022. The number of leprosy cases observed in Station I is 455 in the year 2016 which is followed by 404 in 2017, 398 in 2018, 382 in 2019, 283 in 2020, 514 in 2021, 673 in 2022 respectively. As same as Station I the number of leprosy cases observed in Station II is 112 in the year 2018 followed by 106 in 2019 , 80 in 2020, 74 in 2021, 66 in 2022 respectively. No cases were detected during the year 2016 and 2017 in Station II.

Figure.1 represents the comparison of leprosy cases between the two different Stations which clearly shows that the prevalence rate of leprosy cases is high in Station I than the Station II between the year 2016 to 2022. An Analysis of Variance (ANOVA) was performed to compare the two Stations on

total leprosy cases from the year 2016 to 2022. In this case, p value < 0.05 shows the results are statistically significant. The statistical F value has two degrees of freedom (1,12). The statistical F value is calculated as 59.05503 which is greater than the critical F value. $F_{stat} > F_{crit}$ clearly shows the results are significantly different. So the data is considered as statistically significant.

5.1.2 Comparison of Multibacillary and Paucibacillary Cases of Two Stations:

The number of multibacillary and paucibacillary cases observed in Station I between the year 2016 – 2022 is shown in Table. 3. The number of multibacillary cases in the year 2016 is 20 which is followed by 23 cases in 2017, 18 cases in 2018, 20 cases in 2019, 8 cases in 2020, 18 cases in 2021 and 17 cases in 2022 as recorded in Station I respectively. The number of Paucibacillary cases in the year 2016 is 4 which is followed by 5 in 2017, 3 in 2018, 6 in 2019, 1 in 2020, 4 in 2021 and 5 in 2022.

Table. 4. explains the multibacillary and paucibacillary cases observed in Station II between the year 2016 – 2022. The number of Multibacillary cases in the year 2018 is 2 which is followed by 1 case in 2019, 1 case in 2020, 1case in 2021 and 1 case in 2022 . No multibacillary cases were observed in the year 2016 and 2017. The percentage of Paucibacillary cases in the year 2018 is 2

which is followed by 1 case in 2019, 1 case in 2022 respectively. No paucibacillary cases were observed in the year 2016, 2017, 2020 and 2021.

A comparison of both multibacillary and paucibacillary cases of the Station I is shown in the Figure. 2. It shows that more multibacillary cases than paucibacillary cases in the year 2016, 2017, 2018, 2019, 2020 and 2021. In 2022 the prevelance of paucibacillary cases is high than multibacillary cases. Figure. 3 shows the comparison of both multibacillary and paucibacillary cases of the Station II. It shows that more paucibacillary cases than multibacillary cases were detected in the year 2018, 2019 and 2022.

An ANOVA (Analysis of Variance) was performed to compare the two Stations on multibacillary cases of leprosy from the year 2016 to 2022. In this case, p value < 0.05 shows that the results are statistically significant. The statistical F value has two degrees of freedom (1, 12). The statistical F value is calculated as 87.57233 which is greater than the critical F value. $F_{stat} > F_{crit}$ clearly shows the results are significantly different. So the data is considered as statistically significant. An ANOVA was also performed to compare the two Stations on paucibacillary cases of leprosy from the year 2016 to 2022. In this case, p value < 0.05 shows the results are statistically significant. The statistical F value has two degrees of freedom (1, 12). The statistical F value is calculated as 25.04348 which is greater than the critical F value. $F_{stat} > F_{crit}$ clearly shows

the results are significantly different. So the data is considered as statistically significant.

5.1.3 Comparison of Ulcer Cases of Two Stations:

The number of leprosy cases affected by ulcer were observed in two different Stations between the year 2016 - 2022 . The number of ulcer cases observed in Station I is 369 in the year 2016 which is followed by 334 in 2017, 335 in 2018, 314 in 2019, 210 in 2020, 404 in 2021 and 566 in 2022. As same as Station I the number of ulcer cases observed in Station II is 48 in the year 2018 followed by 45 in 2019 , 32 in 2020, 28 in 2021, 21 in 2022 respectively. No cases were detected during the year 2016 and 2017 in Station II.

Figure.4 shows the comparison of leprosy cases affected by ulcer cases in both the Station I and Station II. It represents that more ulcer cases were detected in the year 2018, 2019, 2020, 2021 in Station II than Station I. No ulcer cases were detected during the year 2016 and 2017 in Station I. In 2022, Station II has more ulcer cases than Station I.

An ANOVA (Analysis of Variance) was performed to compare the two Stations on ulcer cases of leprosy from the year 2016 to 2022. In this case, p value < 0.05 shows the results are statistically significant. The statistical F value has two degrees of freedom (1, 12). The statistical F value is calculated as 65.20481 which is greater than the critical F value. $F_{stat} > F_{crit}$ clearly shows the

results are significantly different. So the data is considered as statistically significant.

The questionnaire survey clearly depicted that the patients were affected by pain or tingling sensation, muscle weakness, numbeness, loss of eyebrows and eyelashes. Although the first symptoms administered by them were pale patches on skin with or without sensation,lumpy or thickened skin and difficulty in vision.

Table 1: Comparison of total leprosy cases for Two Stations

YEAR	STATION I	STATION II
2016	455	-
2017	404	-
2018	398	112
2019	382	106
2020	283	80
2021	514	74
2022	673	66
TOTAL	3109	438

Figure 1: Comparison of total leprosy cases for Two Stations

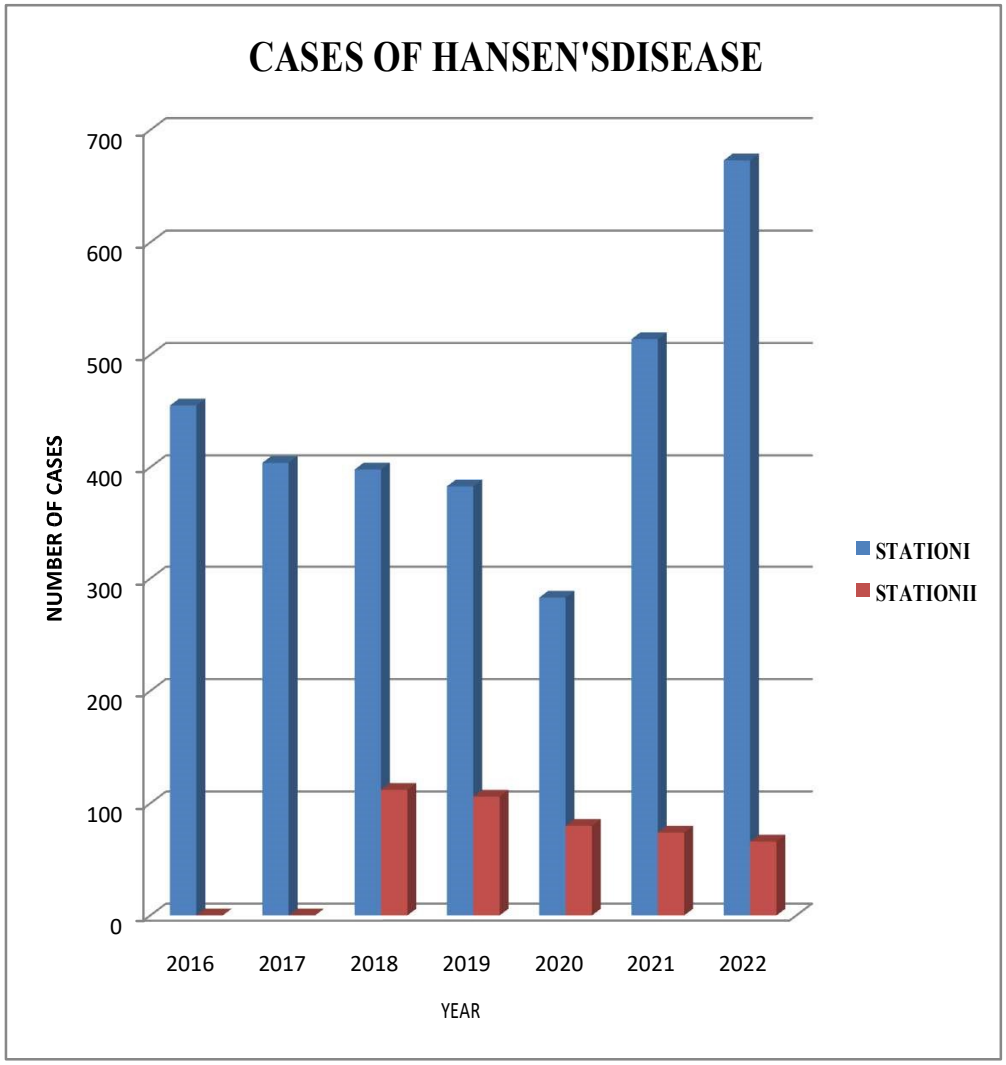

Table 2: Analysis of variance for Total Leprosy Cases in Two Stations

Groups	*Count*	*Sum*	*Average*	*Variance*
STATION I	7	3109	444.1429	15157.14
STATION II	7	438	62.57143	2100.952

Source of Variation	*SS*	*df*	*MS*	*F stat*	*F crit*
Between Groups	509588.6	1	509588.6	59.05503*	4.747225*
Within Groups	103548.6	12	8629.048		
Total	613137.2	13			

*p value< 0.05 statistically significant

Table 3: Comparison of Multibacillary and Paucibacillary Cases in Station I

YEAR	MULTIBACILLARY CASES	PAUCIBACILLARY CASES
2016	20	4
2017	23	5
2018	18	3
2019	20	6
2020	8	1
2021	18	4
2022	17	5
TOTAL	124	28

Figure 2: Comparison of Multibacillary and Paucibacillary Cases in Station I

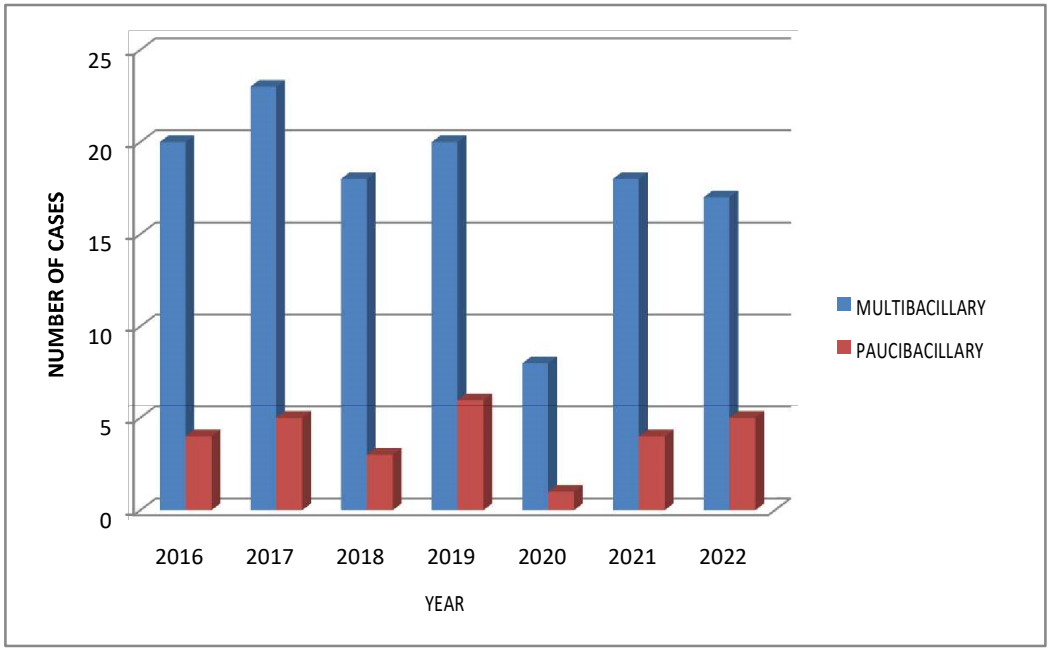

Table 4: Comparison of Multibacillary and Paucibacillary Cases in Station II

YEAR	MULTIBACILLARY CASES	PAUCIBACILLARY CASES
2016	0	0
2017	0	0
2018	2	2
2019	1	1
2020	1	0
2021	1	0
2022	1	1
TOTAL	6	4

Figure 3: Comparison of Multibacillary and Paucibacillary cases in Station II

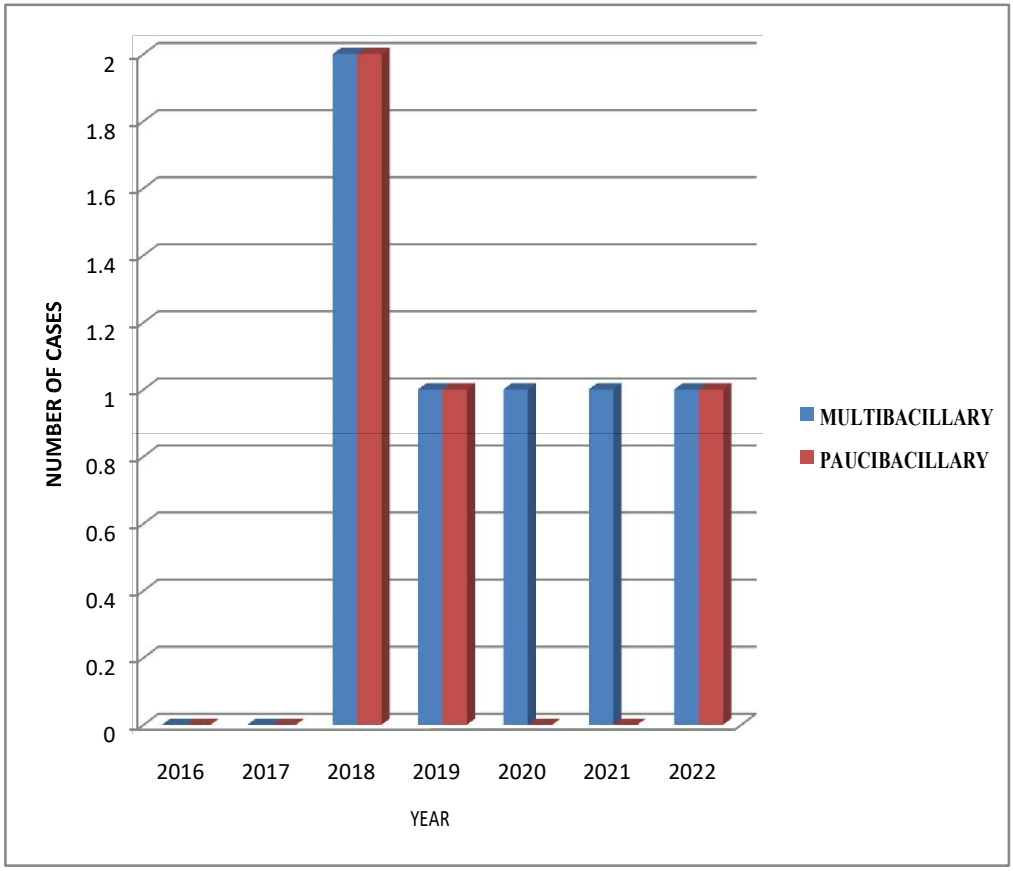

Table 5: Analysis of Variance for Multibacillary cases of leprosy in Two Stations

Groups	*Count*	*Sum*	*Average*	*Variance*
STATION I	7	124	17.71429	22.2381
STATION II	7	6	0.857143	0.47619

Source of Variation	*SS*	*df*	*MS*	*F stat*	*F crit*
Between Groups	994.5714	1	994.5714	87.57233*	4.747225*
Within Groups	136.2857	12	11.35714		
Total	1130.857	13			

*p value< 0.05 statistically significant

Table 6: Analysis of Variance for Paucibacillary cases of leprosy in Two Stations

Groups	*Count*	*Sum*	*Average*	*Variance*
STATION I	7	28	4	2.666667
STATION II	7	4	0.571429	0.619048

Source of Variation	*SS*	*df*	*MS*	*Fstat*	*F crit*
Between Groups	41.14286	1	41.14286	25.04348*	4.747225*
Within Groups	19.71429	12	1.642857		
Total	60.85714	13			

*p value< 0.05 statistically significant

Table 7: Comparison of Ulcer Cases of the Two Stations

YEAR	STATION I	STATION II
2016	361	0
2017	334	0
2018	335	48
2019	314	45
2020	210	32
2021	404	28
2022	566	21
TOTAL	2532	174

Figure 4: Comparison of Ulcer Cases of the Two Stations

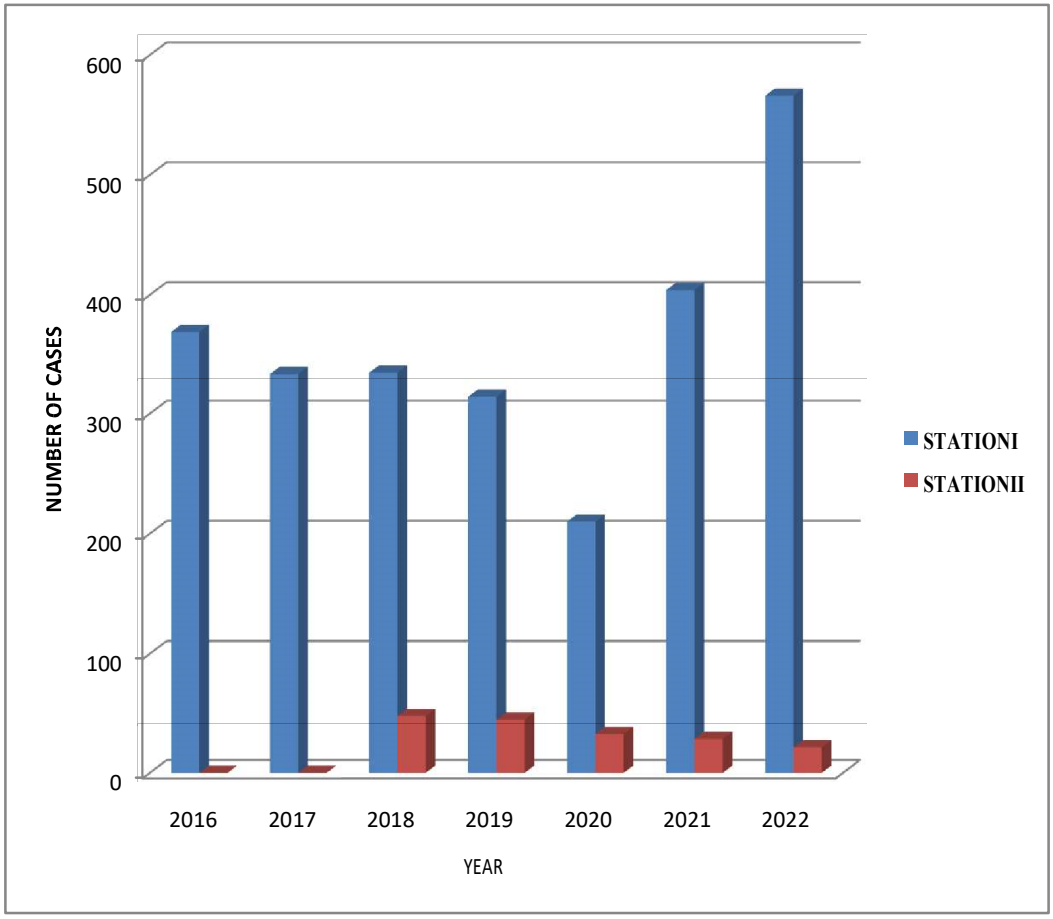

Table 8: ANOVA for Ulcer Cases of leprosy for Two Stations

Groups	*Count*	*Sum*	*Average*	*Variance*
Station I	7	2532	361.7143	11724.9
Station II	7	182	26	374.3333

Source of Variation	*SS*	*df*	*MS*	*Fstat*	*F crit*
Between Groups	394464.3	1	394464.3	65.20481*	4.747225*
Within Groups	72595.43	12	6049.619		
Total	467059.7	13			

*p value< 0.05 statistically significant

PlateA

Leprosy patients in the Rehabilitation Centre

PlateB

Collecting Data from the incharge of Rehabilitation Centre

Plate C

Interviewing the patients in the Rehabilitation Centre

DISCUSSION

6. DISCUSSION

Hansen's disease is one of the most misunderstood diseases. Throughout human history, it is feared; a whole host of myths and misconceptions surround the disease since time immemorial. As far as the mistaken beliefs are concerned, many believe leprosy is a hereditary disease, a curse, or a punishment from God. Even after discovering the germ that causes the disease, leprosy patients are stigmatised and shunned. The popular perception is that leprosy is an ancient disease that has been eradicated many years ago. But the reality is, leprosy is still prevalent, with more than 200,000 people being diagnosed every year worldwide**.** Despite these measures; studies in the last decades have shown that the expected decline in the new case detection rate and the incidence of leprosy has not occurred. Analysis of trends of leprosy in a well defined geographical population over a period provides useful information on how the disease has evolved over the years (Rinaldi *et al*., 2005).

Leprosy is one of the oldest diseases known to mankind. Despite the advancements made byscience and technology, this curable disease remains misunderstood and dreaded. India is home to the largest number of new leprosy cases globally. Tamilnadu is one among state of high level of leprosy cases in India.The country was still seen to have largest number of leprosy patients in 2018(Hariharan, 2020). In the present study, the prevalence rate is high in the

2018 at Thoothukudi and in 2022 in Tiruchirapalli. The prevalence rate of leprosy cases is high in Station I than the Station II from the year 2016 to 2022. The Two Stations were significantly different from each other when comparing the prevalence rate.

When the leprosy patient is with 5 or less number of skin lesions nearly half of them had multiple nerve thickening it is referred as multibacillary. There were more of multibacillary cases in the study group. Increase in multibacillary cases may also be due to late identification of leprosy cases and is an eye opener to strengthen the control measures. More number of multibacillary cases were registered in the Leprosy Centre Government Medical College, Manjeri from October 2014 to September 2019 (Sanker *et al*.,2020). In the two Stations, more number of multi bacillary cases were registered during the year 2017 at Station I and 2018 at Station II. In Station I, about 23 multibacillary cases were recorded in the year 2017 followed by 20 cases in 2016. In Station II, about 2 cases were recorded in the year 2018 followed by 1 case in 2019.

Paucibacillary leprosy comprises cases with 1 to 5 skin lesions, single nerve trunk involvement, and AFB (Acid- Fast Bacillus) negativity.The high proportion of multibacillary cases than paucibacillary cases in our study could be a sign of existence of inaccessible pockets of population harboring undiagnosed leprosy patients for a long time(Chhabra N*et al*.,2015). In the

current study, more number of paucibacillary cases were registered during the year 2019 at Station I and 2018 at Station II. In Station I, about 6 paucibacillary cases were observed in the year 2019 followed by 5 in 2017. In Station II, about 2 paucibacillary cases were observed in the year 2018 followed by 1 case in 2019. The results show there were less number of paucibacillary cases than multibacillary cases. According to Oliveira *et al*. (2017)leprosy patients with and without plantar ulcers, were found to be either overweight or obese.The multipronged approach through medical intervention for ulcer care, preventive screening for associated risk factors and patient counselling for healthy lifestyle help in reducing the morbidity associated with leprosy plantar ulcers (Upputuri *et al*., 2020). In our present study, The comparison of ulcer cases of two different Stations clearly shows there were less ulcer cases recorded in Station II than Station I.

In the cross-sectional study by Xiaohua Chen *et al*.(2021), leprosy patients with 5000 symptoms, were analyzed. The symptoms related to disability,clinical feature and facial features were predominantly presented in delayed diagnostic group. Numbness, erythema, Painless nor pruritic skin lesions, eyebrow hair loss, and tubercles were common symptoms of leprosy. The symptoms related to skin and leprosy reaction were mainly existed in multibacillary group. Pale patches on skin with or without sensation, lumpy or thickened skin, pain or tingling of arms, hands, feet or around eyes, muscle

weakness, numbness, nodules, loss of eyebrow and eyelashes were the common symptoms observed in our current study.

Many patients are affected mentally, not because of the disease, but because of society's rejection of them. One third of black patients in studied in South Africa were found to have contemplated suicide after their diagnosis of leprosy (Bekri W et al., 1998). Negative attitudes towards people with leprosy act to destroy the patient's psychological and social health, but also can affect them physically. The shame associated with this disease can prevent people from seeking treatment until significant disability has occurred, while those who have been treated may never be cured in a truly holistic way nor be accepted back into society (Rafferty J, 2005). In the present study, the patients from the rehabilitation centres said there were no discrimination regarding leprosy even though people in many areas with leprosy are often ostracized by their communities, reporting insults, rejection and hate till today.

Though the prevalence and incidence rates for leprosy have been significantly reduced as a result of the control strategies of the World Health Organization (WHO), new cases still occur (Kundakci *et al*., 2019). In spite of the established fact that leprosy is least infectious and completely curable, the social stigma still lingers and remains a major obstacle to self-reporting and early treatment. Early detection depends almost completely on voluntary reporting which implies awareness of the disease and its treatment facilities. In India, the country with largest case load, Multi Drug Therapy (MDT) has

brought down the prevalence of disease in 2012 (Chhabra *et al*., 2015). In the current study, the prevalence rate of leprosy in present situation is low than the previous mentioned years in Station I than Station II. This positively indicates the effective implementation of Multi Drug Therapy (MDT). The possible reasons for this could be voluntary reporting to health facility. Voluntary reporting is based on increased awareness using health education activities about the disease. Eventhough there is a decline in leprosy cases, the transmission is still active. Early diagnosing and voluntary reporting and early treatment will help to eliminate leprosy.

SUMMARY

7. SUMMARY

- In the present study, the cases of Hansen's disease administered in two different stations were observed for assuming the prevalence rates.
- The prevalence rate of Hansen's disease was high in Station I than in the Station II from the year 2016 to 2022. The prevalence ratein the two stations were statistically significant.
- More number of multibacillary cases were registered in the year 2017 at station I and in the year 2018 at station II.
- More number of paucibacillary cases were registered in the year 2019 at station I and in the year 2018 at station II.
- The comparison of ulcer cases of the disease in two different stations clearly show that the recorded ulcer cases were more in station I than in station II.
- Pale patches on skin with or without sensation, lumpy or thickened skin, pain or tingling of arms, hands, feet or around eyes, muscle weakness, numbness, nodules, loss of eyebrows and eyelashes were the common symptoms observed in the patients.
- The present study shows that transmission of Hansen's diseaseis still active even though there is overall decline of the disease in the country. Early diagnosing, voluntary reporting and early treatment will help to eliminate leprosy.

CONCLUSIONS AND SUGGESTIONS

8. CONCLUSIONS AND SUGGESTIONS

Hansen's disease also called as leprosy, is a major health concern worldwide. Leprosy is caused by a slow growing bacteria *Mycobacterium leprae.* The patients are identified with hypopigmented patches or thickened tender nerves. Leprosy though considered to be eliminated from India, it is still prevalent in many areas.

In the present study, leprosy cases administered in the two stations namely St. Joseph's Leprosy Hospital, Thoothukudi and Holy Family Hansenorium, Tiruchirapalli were analysed. The prevalence rate washigh in Holy Family Hansenorium, Tiruchirapalli and the reason includes voluntary reporting of the patients as well as the available facilities such as surgery in the hospital.

The multibacillary cases are more when compared with paucibacillary cases.Identification of the correct clinical types facilitates proper treatment and avoid the risk of relapse.

The best way to prevent the spread of Hansen's disease is the early diagnosis and treatment of people who are infected. Leprosy is curable by the treatment provided in the early stages.Leprosy is treated with the use of antibiotics such as Rifampicin, Dapsone and Clofazimine called MultiDrug Therapy. Thus, in attempting to eradicate the disease there is still the necessity

to study and research about this disease for better understanding the pattern of the disease occurrence, prevalence and transmission.

The presence of leprosy cases highlighted the need for continuation of targeted leprosy control activities and active case detection. The National Leprosy Eradication Programme has taken massive steps for the reduction of the leprosy cases. The Government needs to conduct more awareness programme about misbelief and misconception of leprosy disease to the public. The Non-Governmental organization also should take the responsibility to create awareness through the already cured people. To eradicate the disease it becomes inevitable to educate the people about the cause, symptoms, diagnosis, treatment and prevention of communicable diseases including leprosy.

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EVALUATION OF AIR POLLUTION STATUS BASED ON APPORTIONMENT AND VEHICULAR TRAFFIC MAPPING INVENTORY AND HEAVY METALS IN THE THOOTHUKUDI CITY

A field work submitted to

ST. MARY'S COLLEGE (Autonomous), Thoothukudi

affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY, TIRUNELVELI

in partial fulfilment for the award of the degree of

MASTER OF SCIENCE IN ZOOLOGY

by

M. JENOVIN	22APZO04
R. MARIA SAHAYA CHRISTIKA	22APZO06
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DEPARTMENT OF ZOOLOGY

ST.MARY'S COLLEGE (AUTONOMOUS), THOOTHUKUDI

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

April 2023

CERTIFICATE

This is to certify that the field work entitled," **EVALUATION OF AIR POLLUTION STATUS BASED ON APPORTIONMENT AND VEHICULAR TRAFFIC MAPPING INVENTORY AND HEAVY METALS IN THE THOOTHUKUDI CITY** " submitted to **St. Mary's College (Autonomous), Thoothukudi** in partial fulfilment for the award of the degree of **Master of Science in Zoology** is done under my supervision. It is further certified that this field work report or any part of this has not been submitted elsewhere for any other degree by the the following students .

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DECLARATION

We do hereby declare that this field work entitled, " **EVALUATION OF AIR POLLUTION STATUS BASED ON APPORTIONMENT AND VEHICULAR TRAFFIC MAPPING INVENTORY AND HEAVY METALS IN THE THOOTHUKUDI CITY** " submitted by us for the award of the degree of Master of Science in Zoology is the result of our original independent research work carried out under the guidance of **Dr. Mrs. N. Arokiya Mary. M.Sc.. M.Phil., Ph.D.,** Associate Professor, Department of Zoology, St. Mary's College (Autonomous). Thoothukudi, and it has not been submitted elsewhere for the award of any other degree.

Place : Thoothukudi **Signature of the candidate**

Date :

M. Jenovin

M. JENOVIN

R. Maria Sahaya Christika

R. MARIA SAHAYA CHRISTIKA

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1.0 INTRODUCTION

Environmental pollution harms earth's ecosystems due to toxic substances and energy release into air, land and water. Air pollution is the most noxious among all of the pollution types (Rehman *et al.*, 2021; Khan *et al.*, 2021) Vehicular emission is a significant source of air pollution.

Air pollution mainly affects those living in large urban areas, where road emissions contribute the most to the degradation of air quality. There is also a danger of industrial accidents, where the spread of a toxic fog can be fatal to the populations of the surrounding areas. The dispersion of pollutants is determined by many parameters, most notably atmospheric stability and wind (Kelishadi *et al.*, 2010)

Clearly, urbanization and industrialization are reaching unprecedented and upsetting proportions worldwide in our era. Anthropogenic air pollution is one of the biggest public health hazards worldwide, given that it accounts for about 9 million deaths per year (WHO, 2019).

The United Nations estimated that over 600 million people in urban areas worldwide were exposed to dangerous levels of traffic generated air pollutant (Cacciola *et al.*, 2002). Primary pollution from motor vehicles is pollution that is emitted directly into the atmosphere, whereas secondary pollution results from chemical reactions between pollutants after they have been released into air (Caserini *et al.*, 2013).

Harmful substances emitted by exhausts and automobile emissions are deposited and accumulated daily in the urban dust on road pavement together with primary and secondary particles from other anthropogenic and natural sources (Amato *et al*., 2009).

This century has witnessed air pollution to be one of the major environmental concerns with the impacts becoming prominent with time. It severely affects human health, quality of life and is rated as the greatest environmental risks to human health and is the most dangerous form of pollution (World Bank, 2016).

Extreme air pollution is recorded in India, where the air quality reaches hazardous levels. New Delhi is one of the more polluted cities in India. Flights in and out of New Delhi International Airport are often cancelled due to the reduced visibility associated with air pollution. Pollution is occurring both in urban and rural areas in India due to the fast industrialization, urbanization, and rise in use of motorcycle transportation. Nevertheless, biomass combustion associated with heating and cooking needs and practices is a major source of household air pollution in India and in Nepal (Parajuli *et al*., 2016).

Air pollution has various health effects. The health of susceptible and sensitive individuals can be impacted even on low air pollution days. Short-term exposure to air pollutants is closely related to COPD (Chronic Obstructive Pulmonary Disease), cough, shortness of breath, wheezing, asthma, respiratory disease, and high rates of hospitalization (a measurement of morbidity). The

long-term effects associated with air pollution are chronic asthma, pulmonary insufficiency, cardiovascular diseases, and cardiovascular mortality. According to a Swedish cohort study, diabetes seems to be induced after long-term air pollution exposure (Eze *et al*., 2014).

Air pollution not only affects respiratory and cardiovascular systems; it also has been shown to have significant effects on the central nervous system (Costa *et al*., 2019; Guxens and Sunyer, 2012). Magnetic resonance imaging (MRI) data demonstrate that air pollution is associated with damage to the prefrontal cortex and altered neurodevelopment in a variety of areas in children (Herting *et al*., 2019).Neurological effects of air pollution provide a biologically plausible route to disruption of cognitive function, and research into the effects of air pollution on neuropsychological and cognitive outcomes throughout the life course is accumulating (Clifford *et al*., 2016).

Outdoor air pollution alone causes 2.1 to 4.21 million deaths annually (Silva *et al*., 2013., Leleiveld *et al*.,2019).Overall ,air pollution causes the death of around 7 million people worldwide each year, and is the largest single environmental health risk (McCauley,2016)

Recently in developing countries, enormous environmental problems due to inadequate environmental planning and monitoring have emanated. In such places, environmental problems like air pollution in urban centers due to increased volume of traffic on ill-treated roads, are aggravating the already

serious problems caused by poor, absent or inadequate sanitary facilities (Alo *et al*.,2007).

Vehicle exhausts as well as industrial emission contribute to air pollution due to incomplete combustion of carbon containing fuels, which cause formation of various gases, liquids and solid particles. In most developing countries of the world vehicular growth has not been checked properly by environmental regulating authorities loading to increased level of pollution (Han and Naeher, 2006).

Vehicular emissions cause heavy metal pollution and exert negative impacts on environment and roadside vegetation. Heavy metals like iron (Fe), cadmium(Cd), lead (Pb), copper (Cu), chromium (Cr), nickel (Ni), zinc (Zn) and manganese (Mn) are released from several wires, alloy, tires and pipes of vehicles into roadside surroundings (Ubsa *et al*.,2013; Nazzal *et al*.,2013;Authman *et al*., 2015; Adamiec *et al*.,2016).

Motor vehicles are regarded as the main source of air pollution globally. Motor vehicles release carbon monoxide, the major part of nitrogen oxides (NOx), volatile organic compounds (VOCs), toxic chemicals and some fine particles (Talbi *et al*., 2018; Franca *et al*., 2017; Chauhan, 2010; Nawaria and Kush 2012). The sources of heavy metals include leather tanning, lead-acid batteries, fluorescent, fuel, battery industry and thermal power plants (Verma and Dwivedi, 2013).

Heavy metals such as Fe, Cu, Ni, Cd, Cu, Pb and Zn are released through different parts of vehicles (Eteh *et al*., 2021; Shuaib *et al*., 2021). Heavy metals emitted from various sources are accumulated on the soil surface (Weber *et al*., 2021; Xu *et al*., 2021; Yang *et al*., 2021). Human activities are the main cause of heavy metal pollution (Komijani *et al*., 2021; Aguilera *et al*., 2021). Every year, millions of tons of heavy metals are released into the air, which destroy environmental ecology and ecosystem and negatively affect human health (Bafana *et al*., 2018; Thijs *et al*., 2020).

Heavy metals have harmful effects on human health, and exposure to these metals has been increased by industrial and anthropogenic activities and modern industrialization. Heavy metals are generally referred to as those metals which possess a specific gravity of more than $5g/cm^3$ and adversely affect environment and living organisms (Jarup, 2013).

The most commonly found heavy metals include arsenic, cadmium, chromium, copper, lead, nickel and zinc all of which cause risks for human health and environment (Morais *et al*., 2012). Carcinogenic metals such as arsenic, cadmium and chromium can disrupt DNA synthesis and repair (Clancy *et al*., 2012; Koedrith *et al*., 2013).Heavy metals are significant environmental pollutants and their toxicity is a problem of increasing significance for ecological, evolutionary, nutritional and environmental reasons (Nagajyoti *et al*., 2010; Jaishankar *et al*., 2013).

Thoothukudi is one of the most polluted cities in Tamil Nadu due to its unrestricted growth. The presence of raw materials markets, transportation networks, capital and labour makes Thoothukudi a major industrial centre in Tamil Nadu. All these factors resulted in a rapid population growth especially through migration (Malumfashi *et al.*, 2011).Urban transport, cargo freight services, manufacturing industries and thermal power plants are the major sources of anthropogenic pollution. As a consequence the assimilative capacity of atmosphere is being stressed (Joyce, 2014).

The pearl city, Thoothukudi , the energy and economic hub of South India is a vibrant city providing numerous job oppurtunities and hence have a large vehicles population .It imposes a cost on society as it increases both morbidity and mortality.

The environmental information on roadside contamination with heavy metals due to vehicular pollutions in Thoothukudi is limited. Hence present study was taken up to document the effects of vehicular exhausts with special reference to heavy metal accumulations in the areas with heavy traffic.

2.0 AIMS AND OBJECTIVES

The problem of air pollution has been brought on by the rapid population growth in urban areas, changing consumption habits, and unplanned industrial urban development. In many urban areas, vehicles account for a large portion of the total air pollution load. Today, anthropogenic air pollution is a fact of life.

The industrial revolution made amazing technological advances, but it also started the manufacturing of massive amounts of pollutants that were released into the air without any thought as to how they would effect health.

This inquiry was done in an effort to gather various statistics and information. The major objectives are

- To choose areas with lots of traffic.
- To study the sources of air pollution.
- To analyse for a week how many vehicles pass in a day per hour.
- To determine the pH and electrical conductivity of the soil.
- To research the presence of lead, and cadmium in the soil samples.
- To offer a framework for pollution mitigation measures.

3.0 REVIEW OF LITERATUTE

Chemical compounds released into the atmosphere as a result of human activities or those that are the consequences of the interaction of chemical emission have harmful effects on health. Medina *et al.*, and Ehrlich (2010) both covered these consequences (2010). The nature of the molecule in question, its quantity in the air, and the duration of human exposure are the three main factors that determine whether air pollution has harmful health impacts.

Acute exposure to air contaminants can have a wide range of toxic effects, according to Cropper (2011). High levels of pollutants are linked to an increase in various cardiac and respiratory disorders, as well as death. These are typical in densely inhabited or highly industrialised areas.

Numerous studies document an increase in mortality brought on by respiratory issues. Evidently, the mechanism was connected to exposure to air pollution by Schwartz *et al.,* (2012). Numerous publications assert an increase in cardiovascular disease related fatalities, which would point to a mechanism with an indirect connection to air pollution. The main causes of death are exposure to sulphates, ozone, and particulate matter. People with cardiac or respiratory conditions are more likely to die as a result of exposure to air pollution.

The consequences of population are more likely to affect some population groups than others. This has drawn the interest of numerous researchers (Peterson *et al.,* 2013, Pope *et al.*, 2013). The elderly and neonates, who are at the ends of the life cycle, exhibit higher mortality linked to exposure primarily to particulate matter and sulphates. Compared to the rest of the population, these groups' biological defence mechanisms are less effective. Smoking practises may also be linked to an increase in mortality brought on by exposure to air pollution. This phenomena is probably brought on by smokers' 30% worse lung function than non-smokers of the same age.

According to Cooper *et al.*, (2014), Gamble and Lewis (2014), and Pooley *et al.,* (2014), exposure to air pollution is linked to a wide range of acute illnesses. These include upper and lower respiratory tract illnesses, pneumonia, chronic obstructive pulmonary disease, and coughs with phlegm.

In a similar vein, Anderson *et al.*, (2016) assessed the impact of pollutants on ma Macrophages, one of cellular lineages involved in the respiratory system's defence mechanisms.

In 2017, Gupta and Chakarbartty investigated traffic related air pollution in Indian cities. They asserted that resisting in a large city in India is equivalent to smoking 10 to 20 cigarettes each day.

Wenling *et al.*, (2018) conducted research on the topic and found that good air quality was crucial for enhancing public health. Future measures

should extend the time when the air is clean while managing pollution by reducing or controlling severe pollution.

Chan-Na-Zhao *et al*., (2019), studies on air pollution revealed that air pollution causes major autoimmune disease including systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis and type 1 diabetes mellitus.

Effects of vechicular emission of two plants *Amaranthus spinosus Linn.* and *Croton bonplandianum Bail*. were studied by Smita Ray and Mala Neogy (2022). The study showed that both of the experimental plant's epidermal cells and stomatal size were affected by vehicular emissions. Data showed that *C .bonplandianum* was more tolerant to vehicular pollution than *A.spinosis*, which was more susceptible.

Heavy metal accumulation by road side vegetation and implication for pollution control studied by Rubina *et al*., (2021). Results revealed that heavy metal accumulation is significant in plants growing along road sides. In order to stop vehicular pollution along road side and other contaminated places, these species control should be applied.

Long term exposure to PM is reported by Jie chen *et al.*,(2020). The study showed clear evidence that both $PM_{2.5}$ and PM_{10} were associated with increased mortality from all causes, cardiovascular disease, respiratory disease and lung cancer.

Indoor air pollution is analysed by Ke-cheng chan *et al.,* (2022). One of the main sources of indoor air pollution is cooking and incense burning. Lung cancer risk was raised by routine cooking and indoor incense use.

Studies of air pollution on reproductive and endocrine systems have reported associations of TRAP, secondhand smoke, organic solvents and biomass fuelled cooking with adverse birth outcomes studied by Eva L Siegel *et al.*,(2023) reported that air pollution contributes to infertility.

The effects of exposure to high levels of air pollution on people have been shown through pollution episodes that have taken place in many places around the world.

4.0 MATERIALS AND METHODS

4.1 Description of the study area – Thoothukudi (Plate- A)

Thoothukudi (formerly Tuticorin) is a port city, a municipal corporation and an industrial city in Thoothukudi district in the Indian state of Tamil Nadu. The city lies in the Coromandel Coast of Bay of Bengal.

Thoothukudi is known as "Pearl City" due to the 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. The current estimate population of Thoothukkudi city in 2023 is 327,000.Thoothukudi is one of the fastest growing Major Ports in India, Thoothukudi, is also an "Emerging Energy and Industrial hub of South India" as a large number of Power plants are located in the coastal city of Thoothukudi. Roadways are the major mode of transport to Thoothukudi, while the city also has rail, air, and sea transport.

Study area:

During December 2022 March 2023 the data on vehicles passing by is gathered in a few key locations around Thoothukudi. In Thoothukudi places like College Entrance, Market signal, Harbour highway Road, Thermal power plant road were selected as study area.

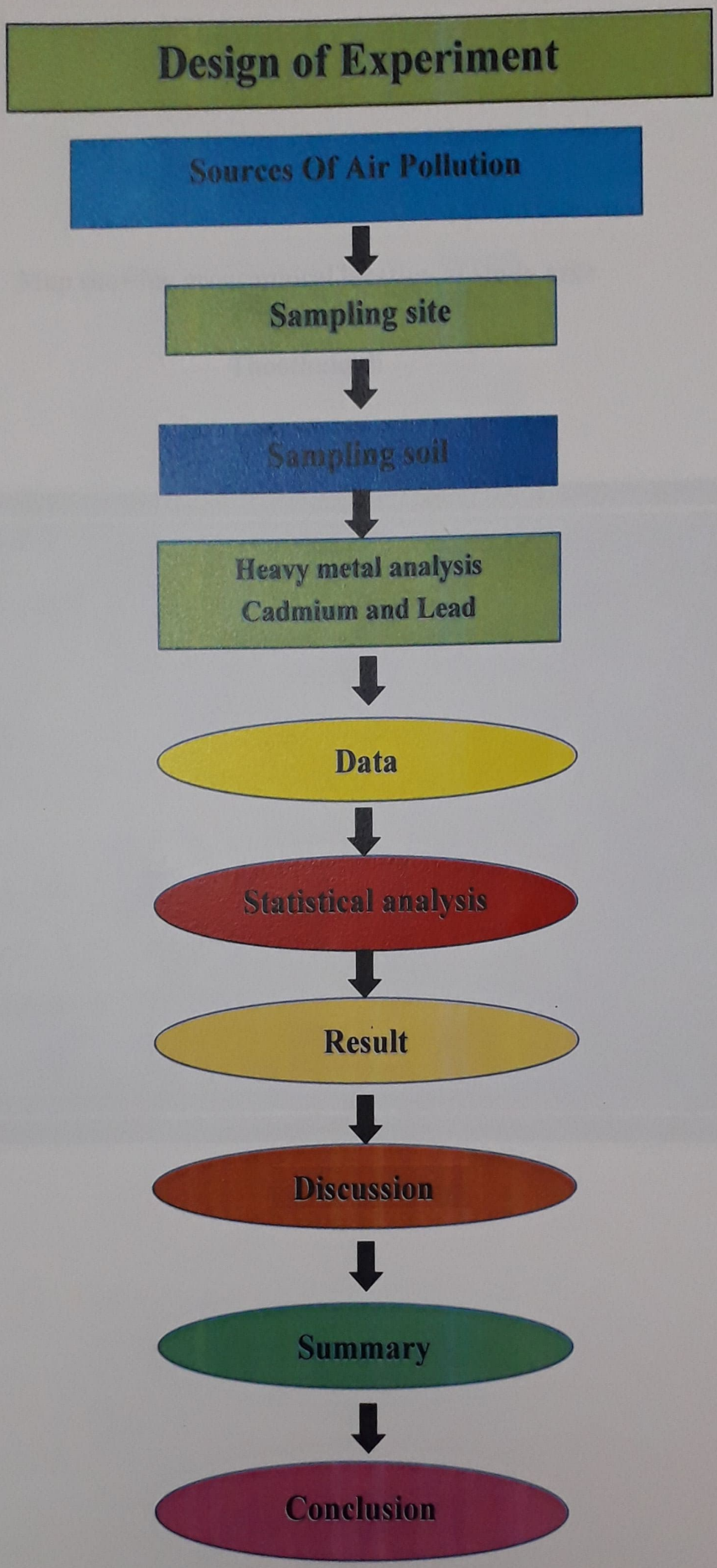

Design of Experiment
Sources Of Air Pollution
Sampling site
Sampling soil
Heavy metal analysis
Cadmium and Lead
Data
Statistical analysis
Result
Discussion
Summary
Conclusion

Plate – A

Map showing geographical location of study area

Thoothukudi

Plate – B

Sampling sites

College Entrance

Market Signal

Harbour Highway

Thermal power plant road

Sampling Sites (Plate B)

Sampling Site 1 - College Entrance (Residential Area)

St. Mary's College, Thoothukudi, is a women's general degree college located in Thoothukudi, Tamil Nadu. It was established in the year 1948. The college is affiliated with Manonmaniam Sundaranar University. This college offers different courses in arts, commerce and science.

Sampling Site 2 – Market Signal (Commercial Area)

Thamizh Salai (known previously as Palayamkottai road) arterial road witnesses traffic round the clock, and three traffic signals help prevent snarl-ups to some extent.

The private vegetable market spans over 1.9 acres of land near a major roundabout, the old bus stand, corporation office, and hospitals. The market houses over 50 stores, in addition to the vegetable shandies and auction area, and is frequented by traders and the public.

Sampling Site 3 – Harbour Highway (Industrial Area)

Harbour highway located in Tuticorin Harbour Estate, Tuticorin. Harbour is one of the oldest fishery ports in the east coast of india. Due to its commercial and economic importance from the marine fisheries point of view, many vehicles and container trucks passing by the harbour highway. Now a

days it is considered as one of the major fishing harbours on the east coast of India. Due to this the harbour highway is always a busy and rush way by the transport of vechicles.

Sampling Site 4 – Thermal Power plant Road (Industrial Area)

Thoothukudi Thermal Power Station is a power plant situated near newport of Thoothukudi in Tamil Nadu, India, on the sea shore of Bay of Bengal. It has 5 units with a total installed capacity of 1,050 MW and spread over 160 hectares (400 acres). All the unit are coal based. Coal is transported by sea through ship from Haldia, Paradeep, Vizag Port to TTPS. Coal transported by ship is given to crushers which crush the coal particles to 10-20mm in diameter. The crushed coal is fed to coal grinding mills with bowl roller via coal bunkers. The powdered coal is given to pulverisers and to furnace through forced draft fans. There are four mills around the furnace as well as oil injecting nozzles from oil storage for tangential firing.

4.2 Data collection:

Apportionment and Vehicular Traffic Mapping Inventory

The data of the vehicles travelling through the study region was estimated by calculating the number of vehicles passing by. The vehicles were tallied for one hour for a week. Peak traffic hour (8 a.m to 9 a.m) was selected.

Average was calculated day-wise and vehicle type wise. The rate of passage of vehicles per minute was calculated.

Collection of Soil samples:

The soil samples were collected from the selected study areas. 250 gram of soil were taken and weighed. The soils were collected in the plastic bags.

Soil testing:

- pH
- Electrical Conductivity

Heavy metals

- Lead
- Cadmium

pH (EPA method – 9045 D Rev 4 : 2004)

pH of the soil samples was determined by pH meter. To 20 g of soil in a 50-mL beaker, added 20 mL of reagent water, covered, and continuously stirred the suspension for 5 min. The soil suspension was allowed to stand for about 1 hr to allow most of the suspended clay to settle out from the suspension or filter or centrifuge off the aqueous phase for pH measurement. Adjusted the electrodes in the clamps of the electrode holder so that, upon lowering the electrodes into the beaker, the glass electrode will be immersed just deep enough into the clear supernatant solution to establish a good electrical contact

through the ground-glass joint or the fiber-capillary hole. Inserted the electrodes into the sample solution in this manner. pH was measured.

Electrical Conductivity (IS 14767 : 2000 (RA 2016))

It was measured by electrical conductivity meter by measuring the electrical resistance of a 1:5 soil : water suspension. This indicates the amount of soluble (salt) ions in soil.

Assay of heavy metals

(Lead – EPA 3050B – 1996 (Rev – 2) / EPA 7420 – 1986)

(Cadmium – EPA 3050B – 1996 (Rev – 2) / EPA 7130 – 1986)

A soil sample was dried for 5 to 6 hours at 105℃ in a hot air oven before being ground into a fine powder. In order to completely digest the sample until residue was formed, 0.5 g of it was treated with concentrated nitric acid and perchloric acid in a ratio of 1: 3 v/v. using 0.1N HCL, the residue was dissolved and diluted to 25 ml. By using an atomic absorption spectrometer, the sample's metal concentration was estimated. The amount was calculated from standard values.

Statistical analysis :

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

1. **Mean :**

The average ($\bar{x}$) is calculated as follows.

$$\bar{X} = \Sigma x/n$$

where

x = data obtained

Σx = sum of value of sample

n = total number of sample

n = number of observation

2. Correlation

The Karl Pearson's correlation coefficient was computed to assess the association between the variables.

Correlation coefficient r was calculated by the following formula

$$\mathbf{r} = \frac{\mathbf{n}(\sum \mathbf{xy}) - (\sum \mathbf{x})(\sum \mathbf{y})}{\sqrt{[\mathbf{n}\sum \mathbf{x}^2 - (\sum \mathbf{x})^2][\mathbf{n}\sum \mathbf{y}^2 - (\sum \mathbf{y})^2]}}$$

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

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

= absolute value of the correlation coefficient

= number of paired observations that were made

Critical values of 't' are compared with the calculated t values and decision about the acceptance or rejection of H_o or H_A was made.

3. Analysis of Variance (ANOVA)

ANOVA was carried out to find out the variation the data and in the significance was tested.

Steps in computation

1. Grand Total $= \Sigma^a \Sigma_n X$

2. Sum of squared observation $= \Sigma^a \Sigma_n X$

3. Sum of squared group totals divided by $n=1/n \Sigma^a [\Sigma_n X]^2$

4. Grand total squared and divided by total sample size = Correction term (CT)

   $= CT\ 1/an \Sigma^a [\Sigma_n X]^2$

5. SS total $= \Sigma^a \Sigma_n X^2 - CT$

6. SS between groups $= 1/n \Sigma^a [\Sigma_n X]^2 - CT$

7. SS within group $= SS_{total} - SS_{between}$

8. The ANOVA table was constructed as follows

Source of variation	Df	SS	MS	Fs
Between groups	a-1	$SS_{\text{between groups}}$	$\frac{SS_{\text{between}}}{a-1}$	$\frac{MS_{\text{between}}}{MS_{\text{with in groups}}}$
Within groups	a (n-1)	$SS_{\text{with in groups}}$	$\frac{SS_{\text{with in groups}}}{a-1}$	
Total	an(-1)			

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.

5.0 RESULTS

Air pollution is a mix of hazardous substances from both human-made and natural sources. Air pollution is a familiar environmental health hazard. It is a major threat to global health and prosperity. Traffic emissions are responsible for a large proportion of ambient air pollution. Vehicular pollution can influence the quality of soil, pH and the chemistry of the soil can be amended due to pollution. Vehicular pollution, a serious man made pollution causes detrimental changes and upsets nature's dynamic ecological balance.

The present investigation was designed to elucidate the vehicular emission patterns and heavy metals status of selected samples of soil. The estimation which was used in retrospective assessment revealed a distinct pattern of variation.

5.1. Pollution hotspots:

The details of the sources of pollution hotspots is depicted in Table I. The areas of pollution hotspots are seen in

RESIDENTIAL: College Entrance

COMMERCIAL: Market signal

INDUSTRIAL: Harbour Highway

Thermal Power plant Road.

5.2. Apportionment and vehicular traffic mapping inventory:

College entrance:

The data of vehicular traffic mapping inventory at this site was shown in Table II. From the survey, the total number of vehicles crossed per day for a duration of one week is 4084, the number of vehicles crossed per day for a minute is 68. The type of vehicle which crossed maximum at this site is observed as Bike with a percentage of 79%, and minimum is observed as Lorry with a percentage of 0.2%. More number of vehicles was observed on Day IV, least number of vehicles was observed on Day VII.

Market signal:

The data of vehicular traffic mapping inventory at this site was shown in Table III. From the survey, the total number of vehicles crossed per day for a duration of one week is 15,438 and the number of vehicles crossed per day for a minute is 257. The type of vehicle which crossed maximum at this site is observed as Bike with a percentage of 70.5%, and Minimum is observed as Lorry with a percentage of 1.35%. More number of vehicles was observed on Day VII, least number of vehicles was observed on Day II.

Harbour highway:

The data of vehicular traffic mapping inventory at this site was shown in Table IV. From the survey, the total number of vehicles crossed per day for a duration of one week is 14,475 and the number of vehicles crossed per day for a minute is 241. The type of vehicle which crossed maximum at this site is observed as Bike with a percentage of 56 %, and Minimum is observed as Auto with a percentage of 0.2%. More number of vehicles was observed on Day VII, least number of vehicles was observed on Day II.

Thermal power plant road:

The data of vehicular traffic mapping inventory at this site was predicted in Table V. From the survey, the total number of vehicles crossed per day for a duration of one week is 12,524 and the number of vehicles crossed per day for a minute is 209. The type of vehicle which crossed maximum at this site is observed as Lorry with a percentage of 26 %, and Minimum is observed as Auto with a percentage of 1.44%. More number of vehicles was observed on Day V, least number of vehicles was observed on Day VII.

5.3 Statistical analysis

The correlation coefficients showing the direction and degree of relationship between the variables is shown in the Table VI. The correlation coefficients are organized in the form of correlation matrix. Both positive and negative relationship

was observed. The strength of relationship was less. The degree varied from low degree to fairly high degree.

The results of ANOVA (Days vs Vehicles) are given in Table VII. The variance between days was statistically significant. Variance between sampling sites was statistically non – significant (Table VIII).

5.3. Characterization of soil samples:

pH:

The results depicting the pH of sampling sites of our study areas are shown in Table IX. The value of pH ranged from 7.8 to 8.7.Maximum value was registed at College Entrance with a pH of 8.7 .Minimum value was registed at Thermal Powerplant Road with a pH of 7.8

Electrical conductivity:

The results of Electrical Conductivity of sampling sites of our study areas are given in Table IX. The value ranged from 258 µs/cm to 3600 µs/cm. High level of conductivity is observed at Harbour Highway with a value of 3600µs/cm and the low level of conductivity is observed at College Entrance with a value of 258 µs/cm.

Heavy metal analysis:

The results depicting the status of the heavy metals Lead and Cadmium are shown in Table IX. The value of lead was between BDL to 84.26 mg/kg. Maximum

amount of lead was observed at Harbour Highway with a value of 84.26mg/kg and BDL at College Entrance. The Cadmium value was at BDL in all the sampling sites.

The results of the present study clearly established the pollution from vehicular emissions and the sources of air pollutions and the presence of heavy metal (Lead) in the sampling sites.

Table – I

Identification of hotspots of pollution in Thoothukudi city

Description of the area	Sources
Residential	College • Mosquito repellents • Cleaning supplies • Scented candles • Perfumes • Deodorants • Burning dhoop , smoke stick , incense sticks , oil – lit lamps , light candles • Cooking • Tobacco smoking
Commercial	Market signal • Night time shops • Waste burning • Construction site • Vehicular emissions
Industrial	• Harbour • Thermal power plant

Plate – c

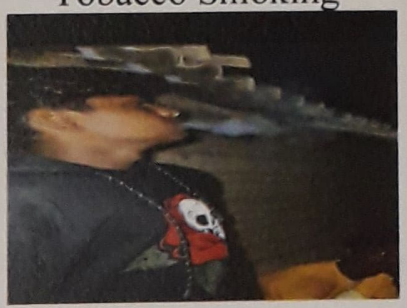
This plate shows different sources of pollution in Thoothukudi city

Residential

Mosquito repellents

Cooking

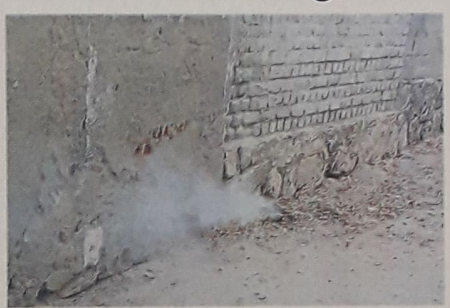
Tobacco Smoking



Commercial

Night time shop

Waste burning



Construction site

Construction site

Vehicular emission

Industrial

Thermal and Harbour road

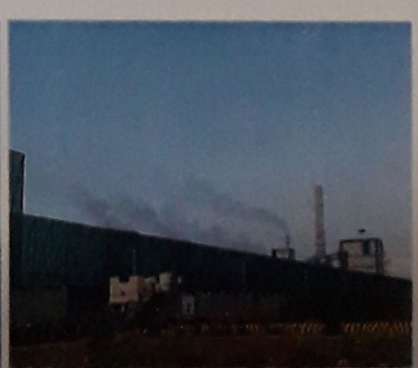


TABLE - II

COLLEGE ENTRANCE

DAYS	TYPE OF VEHICLES													
	BIKE	%	AUTO	%	CAR	%	VAN	%	BUS	%	LORRY	%	TOTAL NUMBER OF VEHICLES	RATE OF PASSAGE OF VEHICLES
DAY I	520	16%	52	12%	34	14%	15	13%	3	12%	1	10%	625	10 Per/min
DAY II	407	13%	60	13%	36	14%	12	10%	4	16%	1	10%	520	9 Per/min
DAY III	476	15%	63	14%	40	16%	19	16%	6	24%	2	20%	606	10 Per/min
DAY IV	548	17%	74	16%	32	13%	16	14%	3	12%	1	10%	674	11 Per/min
DAY V	453	14%	68	15%	42	17%	23	20%	4	16%	1	10%	591	10 Per/min
DAY VI	438	13%	71	16%	35	14%	17	14%	3	12%	2	20%	566	9 Per/min
DAY VII	387	12%	65	14%	31	12%	15	13%	2	8%	2	20%	502	8 Per/min
TOTAL	**3229**	**100%**	**453**	**100%**	**250**	**100%**	**117**	**100%**	**25**	**100%**	**10**	**100%**	**4084**	-
AVERAGE	**461**		**65**		**36**		**17**		**4**		**1**		-	-

Figure 1: College Entrance

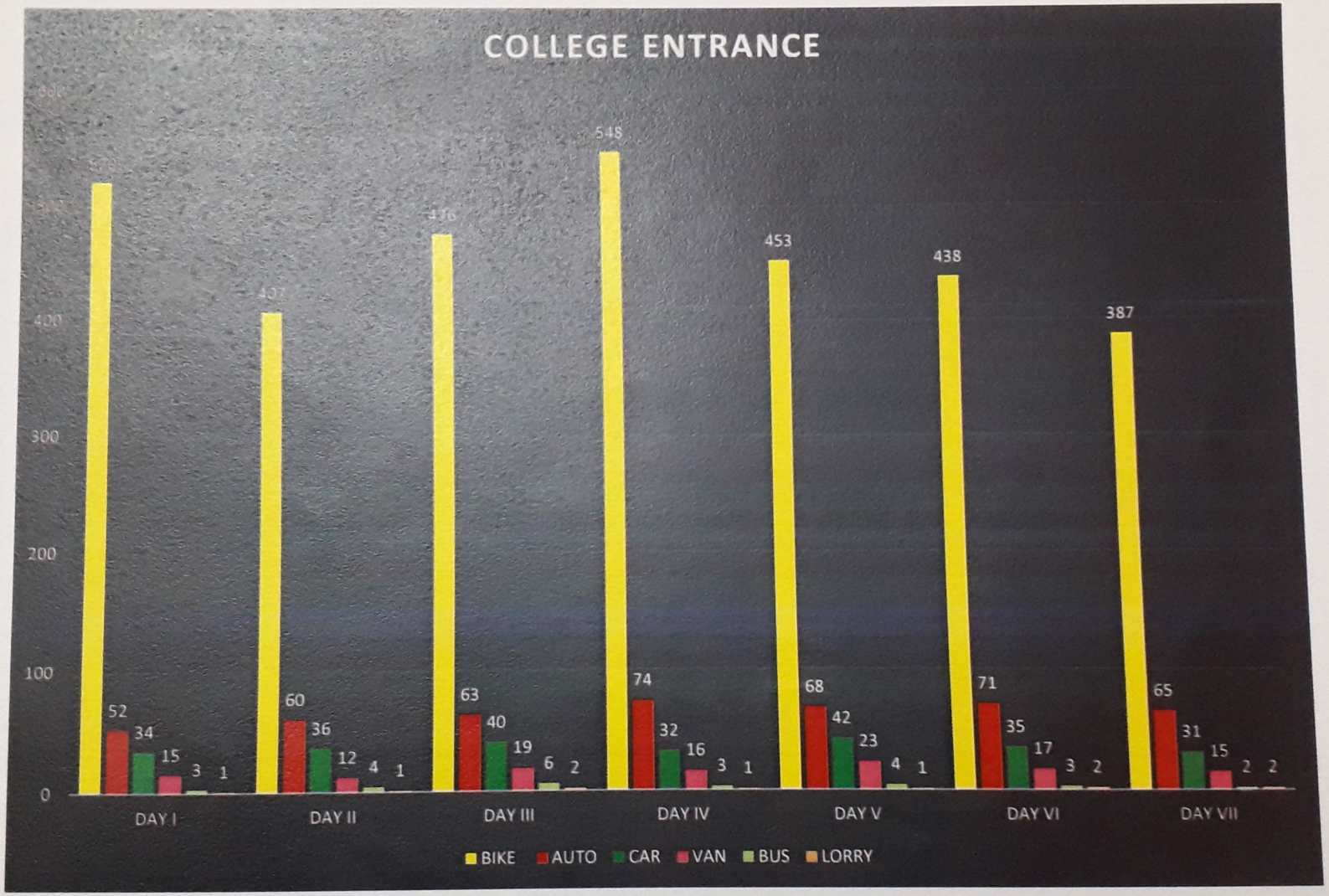


TABLE – III

MARKET SIGNAL

DAYS	TYPE OF VEHICLES													
	BIKE	%	AUTO	%	CAR	%	VAN	%	BUS	%	LORRY	%	TOTAL NUMBER OF VEHICLES	RATE OF PASSAGE OF VEHICLES
DAY I	1708	16%	150	12%	320	15%	34	11%	80	13%	26	12%	2318	39 Per/min
DAY II	1026	9%	183	15%	222	11%	50	15%	96	15%	36	17%	1613	26 Per/min
DAY III	1580	14%	176	14%	284	13%	42	13%	98	15%	32	15%	2212	38 Per/min
DAY IV	1496	14%	166	13%	302	14%	52	16%	82	13%	37	18%	2135	36 Per/min
DAY V	1622	15%	192	16%	312	15%	46	14%	86	14%	28	14%	2286	38 Per/min
DAY VI	1732	16%	186	15%	323	15%	43	13%	97	15%	30	14%	2411	40 Per/min
DAY VII	1745	16%	194	15%	352	17%	58	18%	94	15%	20	10%	2463	41 Per/min
TOTAL	**10909**		**1247**		**2115**		**325**		**633**		**209**		**15438**	-
AVERAGE	**1558**		**178**		**302**		**46**		**90**		**30**		-	-

Figure 2 : Market Signal

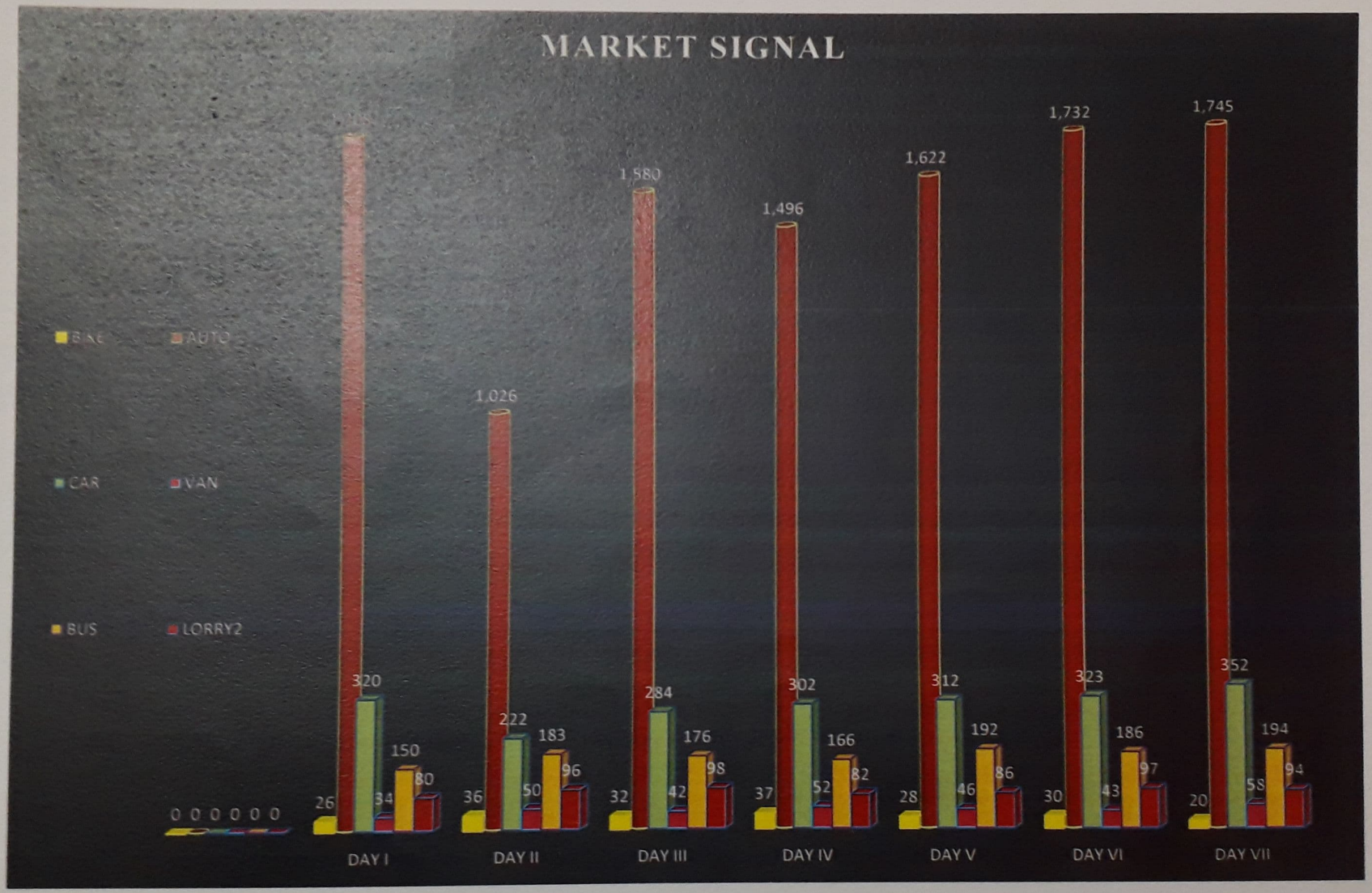


TABLE – IV

HARBOUR HIGHWAY

DAYS	TYPE OF VEHICLES												TOTAL NUMBER OF VEHICLES	RATE OF PASSAGE OF VEHICLES
	BIKE	%	AUTO	%	CAR	%	VAN	%	BUS	%	LORRY	%		
DAY I	1253	15	8	19	324	12	34	17	24	15	543	17	2186	36 Per/min
DAY II	1023	13	5	12	288	10	22	12	31	20	472	15	1841	31 Per/min
DAY III	984	12	6	14	351	13	27	14	21	14	508	16	1897	32 Per/min
DAY IV	1164	14	3	7	429	16	33	17	28	18	342	10	1999	33 Per/min
DAY V	1108	14	7	17	507	19	21	11	12	8	503	16	2158	36 Per/min
DAY VI	1084	13	9	21	435	16	26	14	16	10	497	15	2067	34 Per/min
DAY VII	1532	19	4	10	383	14	29	15	23	15	356	11	2327	39 Per/min
TOTAL	**8148**		**42**		**2717**		**192**		**155**		**3221**		**14475**	-
AVERAGE	**1164**		**6**		**388**		**27**		**22**		**460**		-	-

Figure 3 : Harbour Highway

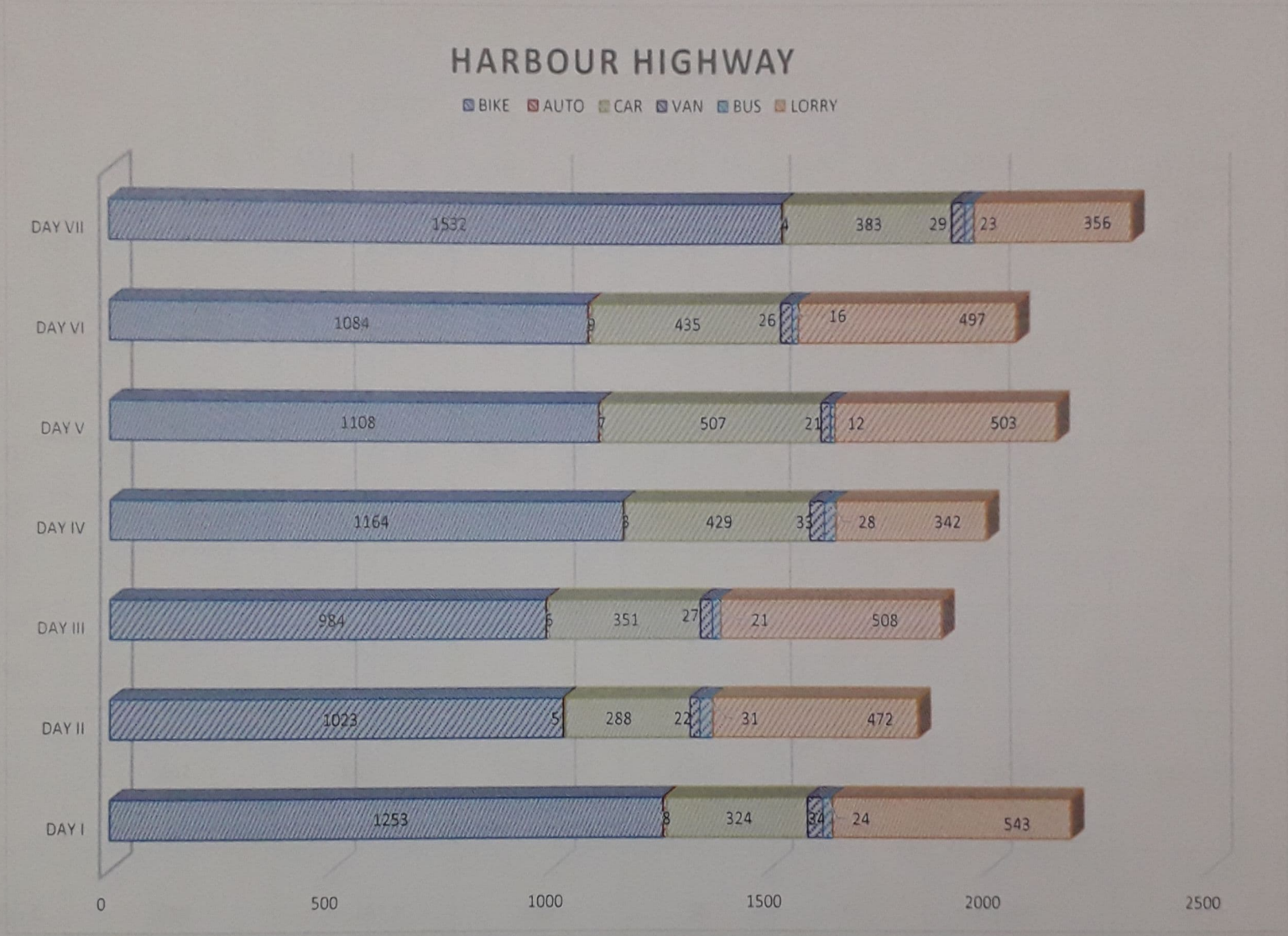


TABLE – V

THERMAL POWER PLANT ROAD

DAYS	TYPE OF VEHICLES													
	BIKE	%	AUTO	%	CAR	%	VAN	%	BUS	%	LORRY	%	TOTAL NUMBER OF VEHICLES	RATE OF PASSAGE OF VEHICLES
DAY I	1084	14%	28	15	144	14	35	13	30	15	480	15	1801	30 Per/min
DAY II	1070	14	24	13	151	15	42	16	30	15	472	14	1789	30 Per/min
DAY III	1092	15	32	18	170	17	38	14	34	17	487	15	1853	31 Per/min
DAY IV	1089	14	20	11	130	13	40	15	25	12	460	14	1764	29 Per/min
DAY V	1105	15	34	19	157	15	41	15	35	17	491	15	1863	31 Per/min
DAY VI	1069	14%	23	13	138	13	33	12	27	13	452	14	1742	29 Per/min
DAY VII	1052	14%	20	11	135	13%	39	15%	23	11%	443	13%	1712	29 Per/min
TOTAL	7561		181		1025		268		204		3285		125241	-
AVERAGE	1080		26		146		38		29		469		-	-

Figure 4 : Thermal Power Plant Road

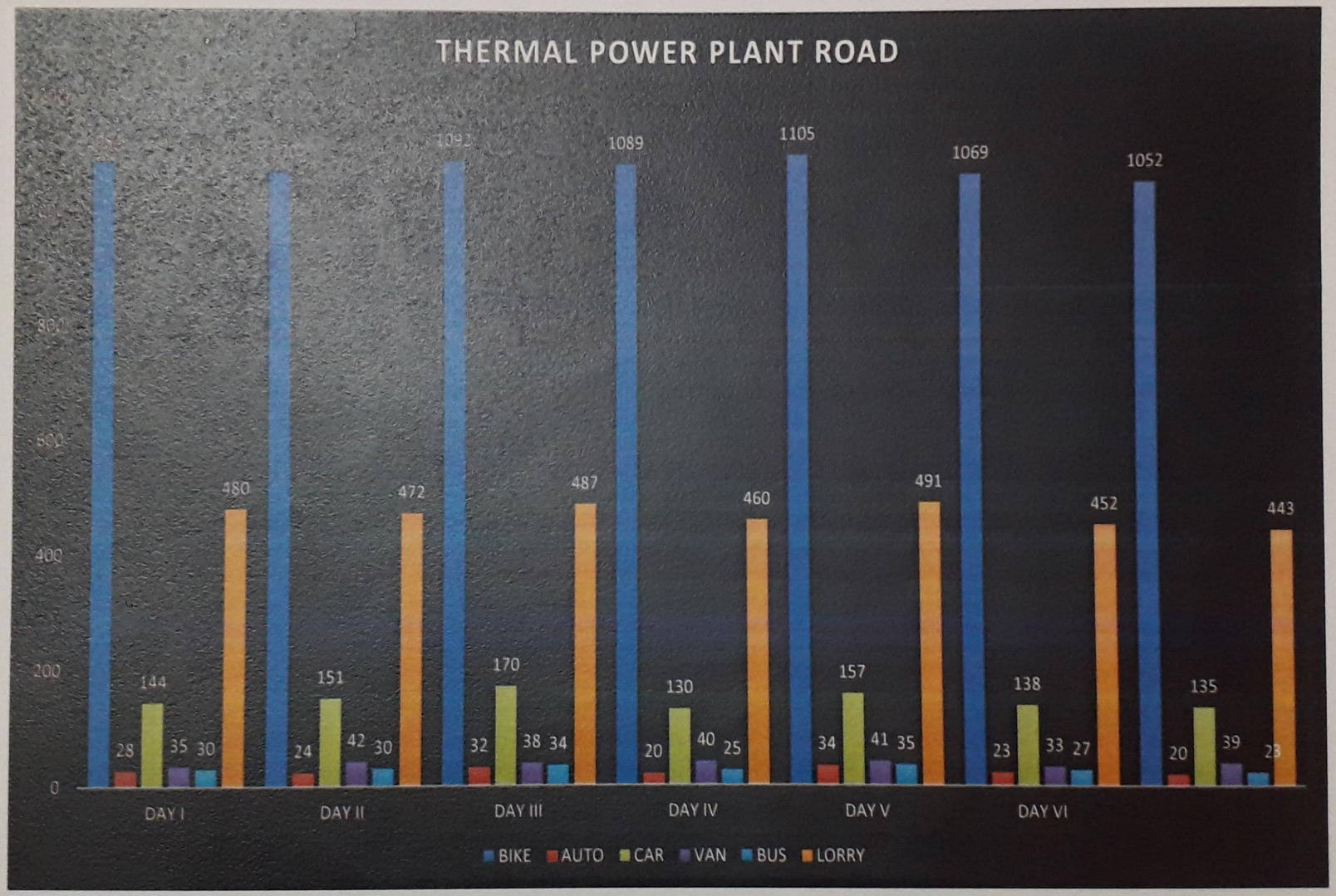


TABLE – VI

Correlation coefficient matrix between the sampling sites

	BIKE	AUTO	CAR	VAN	BUS	LORRY
BIKE	1	-	-	-	-	-
AUTO	0.036945	1	-	-	-	-
CAR	-0.04301	-0.03702	1	-	-	-
VAN	0.160346	0.359676	0.726264*	1	-	-
BUS	0.12591	-0.12233	0.784766*	0.379771	1	-
LORRY	-0.44461	0.206277	-0.08843	0.076397	0.070014	1

***p<0.05 statistically significant**

TABLE – VII

ANOVA for sampling sites – Days vs vehicles

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Days	1.55E+08	5	30910487	14.6995*	2.52E-05	2.901295
vehicles	13389373	3	4463124	2.122442	0.140193	3.287382
Error	31542383	15	2102826			
Total	1.99E+08	23				

***p<0.05 statistically significant**

TABLE – VIII

ANOVA for sampling sites

ANOVA						
Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	13389373	3	4463124	0.479661	0.700058	3.098391
Within Groups	1.86E+08	20	9304741			
Total	1.99E+08	23				

p>0.05 statistically non significant

Table – IX

Characterization of soil samples

Sampling sites	pH	Electrical Conductivity (µs/cm)	Heavy metals (mg/kg)	
			Lead	Cadmium
College Entrance	8.7	258	BDL	BDL
Harbour Highway	8.5	3600	84.26	BDL
Market Signal	8.5	803	9.70	BDL
Thermal Power plant Road	7.8	1091	46.58	BDL

6.0 DISCUSSION

Air pollution is one of the serious environmental concerns of the urban cities where majority of the population is exposed to poor air quality. Several factors cause air pollution in Thoothukudi. Based on the study areas the factors are classified as residential, commercial and industrial. The sample site 1 college entrance comes under residential and the main source of air pollution is due to mosquito repellents, cleaning supplies, scented candles, perfumes, deodorants, burning of dhoop, smoke stick, incense sticks, oil lit lamps, light candles and cooking. The sample site 2 Market signal comes under commercial and the sources are night time shops, tobacco smoking, waste burning, building materials, vehicular emission. The sampling site 3 comes under industrial. As Thoothukudi is industrialized city, the emission by the industries are main source. The study includes the harbour and thermal power plant.

Polluion Due To Bakery And Night Time Shops :

Large amounts of energy are used during the cooking process all across the world, but especially in developing nations (Bansal *et al.,* 2013;Poltorak *et al., 2015*). Natural gas, charcoal, wood, kerosene, liquefied petroleum gas, electricity, biogas, and biomass are among the many fuel types typically used for cooking (Isler *et al.,* 2008). The rapid expansion of the restaurant industry has increased the severity of cooking odour emissions (Afrane and Ntiamoah, 2012). In several parts of the world, complaints about restaurant throughout the

cooking procedures, significant quantities of damaging air pollutants and green house gases are released every day. The most significant location for cooking is a restaurant, where both locals and visitors frequently spend a cooking fume and odour emissions have been rise (Pang and Wong, 2002). The heating and cooking processes employed in restaurants, where a variety of foods are cooked using a variety of fuels, produce pollutants (Alves *et al.,* 2015).

Charcoal is widely used for barbecuing in the majority of restaurants worldwide since it has a high heating value, is inexpensive when compared to other fuels, can be conveniently stored, and imparts a distinct flavour and texture to the meal (Lahiff *et al.,*2013; Vicente *et al.,*2018). In addition to several trace elements, charcoal contains a variety of organic and inorganic molecules, including hydrocarbons, sulphur, water, and oxygen (Tanser *et al.,*2013; Rahman and Kim 2012). As a result, burning charcoal releases a significant number of harmful substances into the air, both in the solid and gaseous stages (Lewtar,2007).

Burning charcoal is thought to be a source of volatile organic compounds (VOCs) and polycyclic aromatic hydrocarbons (PAHs) (Pandey *et al.,* 2009; Kabir *et al.,* 2010), both of which contribute to the formation of photochemical ground level ozone and have a number of harmful health effects, including carcinogenicity (Hsieh *et al.,* 2012; Huang *et al.,* 2011). When sulphur, a fuel ingredient found largely in coal, gasoline, kerosene, and diesel, is burned during

cooking or any other high temperature combustion, sulphur dioxide gas (SO_2) can be produced. Chronic bronchitis and irritations of the eyes and mucous membranes are brought on by the presence of SO_2 in the air (Usinger 1999). Other cooking methods used in restaurants, such as charbroiling, frying, and baking, are also taken into consideration (Kleeman *et al.*,1999; Nolte *et al.*, 1999).

Household Air Pollution :

LPG is predominantly propane or a blend of propane and butane, whereas natural gas is primarily methane. Natural gas burns with less air than other fuels (an air-togas ratio of 10:1). LPG, on the other hand, releases nearly three times as much energy when burned (93.2 MJ/m3 through LPG versus 38.7 MJ/m3 through natural gas), despite requiring more air for combustion (an air-to-gas ratio of 25:1). Natural gas is more dense than natural gas, which is more dense than LPG (1.52:1:0.55). nitrogen dioxide. In contrast to natural gas, which rises towards the roof and has fewer adverse effects on health, gas leakage when using LPG tends to settle in the home's air at human levels.

Natural gas burning results in the creation of a number of gases, including sulphur oxides, mercury compounds, and particulate matter, as well as nitrogen oxides, principally nitrogen dioxide. Wood, crop waste, animal dung cakes, and wood charcoal are all examples of biomass fuel (Apte and Salvi,2016). Around 3 billion people, or half of the world's population, use biomass every day for heating or cooking, burning around 2 million kg of it.

Particulate matter and gaseous air pollutants, including phenols and free radicals, carbon monoxide (CO), nitrogen dioxide, sulphur dioxide, formaldehyde, hydrocarbon complexes, and other inorganic and organic substances like polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds, and chlorinated dioxins, are present in extremely high concentrations in these homes. High volumes of CO are also created, particularly when burning charcoal (Clark *et al* ., 2013) . However, among biomass fuels, burning wood results in the least amount of PM 2.5 and CO emissions (Chen *et al.*,2017).

Smoking :

One of the main causes of indoor air pollution in a home is smoking tobacco in any form. Around 1.1 billion people smoke worldwide, and that number is rapidly growing. Smoke from cigarettes contains 7,357 different chemical substances, including nicotine, heavy metals, benzene, CO, heterocyclic amines, PAHs, cyanide, and formaldehyde. Additionally, burning tobacco releases a lot of $PM_{2.5}$. (burning one cigarette emits 7 to 23 mg of $PM_{2.5}$) (Rodgman 2009).

Insecticides And Pest Control :

The developing and rural world continues to struggle with infectious and communicable diseases, which are typically spread by vectors like mosquitoes, ticks, and other insects. Malaria and dengue are two mosquito-borne diseases that are a major threat. Mosquito management is urgently needed in order to

reduce the rising mortality and morbidity rates of various diseases transmitted by mosquitoes. Chemical repellents are used to achieve this. The mosquito coil is the most popular and often used repellent. Around 2 billion people use mosquito coils to protect themselves from the risks brought on by illnesses spread by mosquitoes.

A typical mosquito coil includes 0.1% pyrethroids, the active ingredient, while the remaining 99.9% is made up of binders, resins, and combustible materials like coal dust coconut husks and dust. These coils are sold worldwide each year in quantities of 12,000,000. After being lit, the coil is left to smoulder for six to seven hours. According to earlier research, burning one of these coils releases the same amount of PAH and particulate matter as burning 50 cigarettes. The pollution levels only reach the safe levels when the windows and doors are left open (Salvi *et al.*, 2016). Other mosquito deterrents, such as vaporizers, sprays, ointments, and medicated sheets, produce gaseous air pollutants that irritate the mucosa of the airways but less particle matter.

Perfumes, Deodorants And Cleaning Agents :

Kitchen odours and indoor air pollution are more concentrated in homes with poor ventilation. This justifies the use of smells and perfumes to raise the standard of hygiene in the home. Improved hygiene calls for the use of cleaning supplies, scented candles, perfumes and deodorants, and other items to make the house cosier and more pleasant. Steinemann (2015) investigated the volatile organic chemicals emitted by 37 frequently used consumer goods, such as air

fresheners, laundry detergents, cosmetics, and cleaning agents (Dimitroulopulouo *et al.,* 2015). Almost all religions use perfumes in some capacity throughout their regular religious exercises. During their daily prayers, Hindus and Buddhists burn dhoop, or smoke sticks, incense sticks, scents, and oil-lit lamps. Hindu wedding ceremonies frequently entail blazing a sacred fire made of wood and cakes of animal excrement for at least two to three hours each. In India alone, there are 3 million places of religious worship, and 10 million marriages take place there annually. Christians also light candles during prayer, especially at special occasions like Easter and Christmas. Bakhoor and Oudh, which release scents when placed over hot charcoal, are commonly used as fragrances in Islamic households. According to a number of studies, burning these scents releases dangerous quantities of air pollutants.

Construction site :

Air pollutants occur not only in the transport, industry or coal combustion sectors (Bourdin *et al*., 2013; Muleski *et al*., 2005) but also in the construction sector (Wu *et al*., 2016; Yang *et al*., 2020). Thereby pollutants have different sources of origin within the construction sector or the built environment. Outdoor sources include construction machinery at sites (Challoner *et al*., 2014; Ruan *et al*., 2019), production of building materials (Bogush *et al*., 2020 ;Ekinci *et al*., 2020) or pollutant emergence at other different life cycle stages of buildinds such as the end-of-life stage. Numerous people suffer from

diseases such as acute respiratory diseases, chronic obstructive pulmonary disease, lung cancer, heart disease and strokes due to air pollution.

Building Material :

Household paints and varnishes generate large volumes of volatile organic compounds, adding to the load of indoor air pollution. Because they are kept together by adhesives that also produce volatile organic compounds, furniture made of particle board is also a source of emissions similar to those of VOCs (Banlokke *et al.,* 2015). Electronics and furniture made of foam include fire retardants called polybrominated diphenyl ethers (PDBEs). These barely detectable emissions of PentaPDBEs and DecaPDBEs contribute to house hold air pollution (Webster *et al.,*2015).

An increased occurrence of indoor wall moisture has been linked to faulty plumbing, either alone or in combination with environmental factors (Wang *et al.,* 2014) . For the growth of fungi like *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium*, these walls create the perfect environment (Alves *et al.,* 2016). Several kinds of Gram-positive and Gram-negative bacteria, such as *Streptococcus*, *Micrococcus*, *Staphylococcus*, *Mycobacterium*, *Nocardia*, and *Streptomyces*, as well as fungi thrive on moist, mouldy walls (Kilpelainen *et al.,* 2001). Household air pollution is largely caused by these fungi's budding spores, microbial particles, volatile organic compounds, mycotoxins, and bacteria's endotoxins (Thrasher, 2016).

Vehicular pollutants :

Carbon monoxide (CO), nitrogen oxides (NO_x), photochemical oxidants, air toxics such as (C_6H_6), aldehydes, 1,3 butadiene (C_4H_6), lead (Pb), particulate matter (PM), hydrocarbons (HC), sulphur dioxide (SO_2), and polycyclic aromatic hydrocarbons are themain pollutants released as vehicle/fuel emissions (PAHs). While hydrocarbons and carbon monoxide are the main pollutants from gasoline and diesel-powered vehicles, particulates and oxides of nitrogen are the main pollutants from diesel-powered vehicles. Both the environment and human health are negatively impacted by vehicle emissions. It is thought that these contaminants have an immediate impact on the cardiovascular and respiratory systems. Particularly high levels of suspended particulate matter and sulphur dioxide are linked to increased mortality, morbidity, and compromised pulmonary function.

Industrial Emissions :

Industrial processes release large volumes of chemicals, hydrocarbons, and organic compounds into the air. The air's greenhouse effect is caused by an excessive amount of carbon dioxide. Because greenhouse gases absorb infrared light from the planet's surface, their existence benefits the environment. Inadequate levels of these gases and particulate matter (PM) in the atmosphere are to blame for the recent climate change (Beuchemin *et al.*,2007; Heede

2006). Climate change has been accompanied by a rise in greenhouse gases, which is the result of greenhouse gas emissions from many sources (Ahrens 2007). The global problem of greenhouse gas emissions, which alters the climate, has a negative influence on human and natural resource development as well as economic progress (Abbasi and Abbasi 2014 ; Bilgen 2008). The principal greenhouse gases (GHGs) and their relative concentrations are methane (4–9%), nitrous oxide (3–7%), water vapour (H_2O), and carbon dioxide (9–26%, respectively) (Russell 2007). CO_2 and CH_4 are the two greenhouse gases that have the greatest impact on rising global surface temperatures (Hansel *et al.*,2007) .

Among them the major source of air pollution is due to transportation, where the abundance of poorly-maintained vehicles, use of petrol fuel, and poor controlling are making transportation the major air polluting sector and it is the leading contributor to air pollution (Mukherjee , Mukherjee , 2013)

The rapid urbanization in India has resulted in a tremendous increase in the number of motor vehicles. As the number of vehicles continues to grow and the consequent congestion increases, vehicles are now becoming the main source of air pollution in urban India.

Vehicular pollution is the introduction of harmful material into the environment by motor vehicles. These materials, known as pollutants, have several bad effects on human health and the ecosystem. Transportation is a

major source of air pollution in many countries around the world due to the high number of vehicles that are available on the roads today. Vehicular pollution has grown at an alarming rate due to growing urbanisation in India. The air pollution from vehicles in urban areas, particularly in big cities, has become a serious problem.

When compared to other vehicle platforms, market signal found to be stronger in terms of the overall vehicle count. The number of vehicles passed by the market signal for one hour for a week is 15,438. Because there are bus stand, Hospitals, Medicals and Wholesale shops etc., near to market signal. All the sampling locations have a large number of two-wheelers. Particularly in Thermal power plant Road and harbour highway there is an increased number of lorry due to export and import for transporting industrial needs. In college entrance the number of vehicle is at peak during weekdays and the number is reduced during weekend.

Soil Characteriation :

The ph of the soil samples was alkaline, which varied among sites. Soil electrical conductivity measures the ablity of soil water to carry electric current. From the current study by comparing the other sample the electrical conductivity is high in harbour highway road (3600 µs/cm) and very low in college entrence (258 µs/cm) .

Heavy metal accumulation in soil :

Soils from different backgrounds have shown different contamination origins depending on the potential heavy metals sources in the surrounding. Several investigations have revealed the main source of the cadmium/lead contamination in their study area.

Heavy metals such as Cu, Zn, Pb, and Cd contained in contaminated soils have a high potential bioavailability to soil biota, plants and humans.

A dangerous environmental contaminant called lead has highly damaging effects on a number of human organs. From the present study high amount of lead accumulation seen in harbour highway road. High level is due to heavy load lorry. Below detectable level observed in college entrence. Pb can be absorbed through the skin, although it is primarily absorbed through the stomach and respiratory systems. Due to pathways including immune-modulation, oxidative stress, and inflammation, exposure to Pb can result in neurological, respiratory, urogenital, and cardiovascular diseases. Additionally, Pb may cause inflammatory reactions in many organs and upset the equilibrium of the oxidant-antioxidant system. Pb exposure has a number of disorders related with it and can change how the body functions physiologically (Joseph *et al.*, 2005; Jacobs *et al.*, 2009; Kianoush *et al.*, 2012).

Although, Cadmium (Cd) presence is uncommon, it is naturally exists in water, soil and minerals like sulphate, chloride, carbonate and hydroxide salts.

During industrial activity, high amounts of Cd in water, air and soil might arise, which may result in significant human exposure to Cd. Moreover, eating contaminated food will expose to a lot of Cd. Smoking is another way to be exposed to Cd, and it can raise Cd levels in the blood and urine. The presence of Cd in contaminated water may interfere with vital bodily functions and cause short-or-term illness (Jiang *et al.,* 2015; Richter *et al.,* 2017; Cao *et al*., 2018). From the present study all the sampling site contains below detectable level of cadmium.

7.0 SUMMARY

Living in the modern century where amelioration in science and technology has made life both pleasant and challenging. It cannot be denied that one of the perils of this modernization, urbanization and globalization is pollution. The roadways and automobiles are emerging as a major contributors of air pollution. Air pollution is the cause of alarming numbers of premature deaths, as well as serious impacts on human health and the environment.

In the present investigation, evaluation of air pollution status based on apportionment and vehicular mapping inventory and heavy metals was made. The findings of the present study are

- Analysis of vehicular pollution of four sampling sites for one week was studied, it shows that the bike was travelled majorly through college entrance, market signal and harbour highway.
- Minimum vehicle travelled was predicted as lorry in college entrance, market signal and harbour highway.
- More number of lorry was recorded in Thermal powerplant road as it is industrial area.
- pH values lied in the range of 7.8 to 8.7 and more pH was seen in the college entrance with a value of 8.7 and less pH was seen in Thermal powerplant road with a value of 7.8.

- Electrical conductivity at the sampling sites were observed. High amount of conductivity was observed at Harbour highway with a value of 3600µs/cm and low amount of conductivity was observed at college entrance with a value of 258µs/cm.
- High electrical conductivity was in harbour highway as it is industrial area and low electrical conductivity was in college entrance as it belongs to residential areas.
- Among the two heavy metals analysed lead content was highest (84.26mg/kg) in soil sample of Thermal power plant road.
- Cadmium content was below detectable limit in all sampling sites.
- The level of lead was higher than cadmium because of the presence of lead in the fuel and in the vehicular exhaust.

The challenge in addressing vehicular pollution and climate change over the coming decades will be striving to maximize synergestic efforts and to minimize the conflicts to protect our environment and future.

8.0 SUGGESTIONS AND CONCLUSION

In India, automobile pollution has increased at a startling rate as a result ofrising urbanisation. The unfortunate truth about vehicle pollution has an effect on demand and economic growth.

Implementable recommendations must be followed to reduce traffic pollution.

- Reducing fuel consumption by improving the efficiency of automobiles and trucks.
- Zero-emission automobiles to reduce dangerous air pollutants.
- Switch to alternative fuels such as natural gas, methanol, ethanol, and hydrogen instead of conventional fuels.
- Renewable energy sources such as hydro, wind, and solar energy can provide environmental advantages.
- Lessening our driving can reduce motor pollution, protect the environment, and improve public health. This includes offering alternatives to the use of automobiles, such as mass transit, bicycle, and pedestrian paths.
- Human efforts can also help to lessen the damaging environmental impacts. Car pooling, mass transit, biking, taking public transportation, and other methods to reduce mile we cover while driving.

- Achieving success requires civic education. To make the world a far better environment to live in, it is crucial to raise everyone's knowledge of these issues and to instill in them a feeling of responsibility. Innovative legislation and good policies to lessen the effects of pollution.
- Optimal performance and reduced air pollution can both be achieved with regular vehicle maintenance.
- A double win would result from getting rid of old cars.
- Enhancing green space (belts, barriers, etc.) and wise plant species choice.
- Using protective clothing, such as a pollution mask, to save our health damaging contaminants.
- The odd/even number system in Delhi.

There is only one Earth, so we should make every effort to preserve it. Individual responsibility for a cleaner planet is where it all starts. The amount of vehicular pollution can be decreased and managed when people adopt a more proactive approach. India will be cleaner and greener.

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