Bioprospecting the Marine Invertebrates of Goa for Pharmaceutical Potential

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, **"Bioprospecting the Marine Invertebrates of Goa for Pharmaceutical Potential"** is based on the results of investigations carried out by me in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Samantha Fernandes D'Mello and the same has not been submitted elsewhere for the award of a degree

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Date: 08 April 2024

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report "**Bioprospecting the Marine Invertebrates of Goa for Pharmaceutical Potential**" is a bonafide work carried out by Ms. Samradni Rohit Paigankar under my supervision in partial fulfilment of the requirements for the award of the degree of Masters of Science in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

Dr. Samantha Fernandes D'Mello

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PREFACE

In recent years, the exploration of marine ecosystems for pharmaceutical potential has been of great interest to the researchers worldwide. This preface serves as an introduction to the idea of bioprospecting from marine invertebrates for their potential pharmaceutical activities. As we move deeper into the ocean, we find a vast diversity of marine invertebrates, each harbouring different types of bioactive compound. The marine invertebrates like the sponge, molluscs and sea anemones being filter feeders and due to their defence mechanisms tend to produce bioactive compounds. These compounds can be checked for their pharmaceutical potential to treat some of the most challenging modern lifestyle diseases

The process of bioprospecting pharmaceutical compounds from marine invertebrates is accompanied by a lot of challenges. It requires a balance between scientific exploration and environmental conservation, as we seek to harness the potential of these organisms while preserving the ecosystems.

In this thesis, we discuss about the marine invertebrates like sponge, mollusc and sea anemones as a potential source that produces bioactive compounds. The bioactive compounds produced by them are checked for various pharmaceutical properties. Besides bioprospecting these marine invertebrates for pharmaceutical potential, their conservation is also equally important. Therefore, for the purpose of environmental conservation concept like in-vitro propagation is also discussed in this thesis.

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ABBREVIATIONS

- ℃ - Degree Celsius
- µg - Micro gram
- µL - Micro liter
- mL – Milli liter
- mg – Milli gram
- g- Gram
- % - Percentage
- RT – Room temperature
- ZMA – Zobell Marine agar
- ZMB – Zobell Marine broth
- OD – Optical density
- PBS – Phosphate buffer saline
- DPPH – 2,2-diphenyl-1-picrylhydrazyl

ABSTRACT

The modern lifestyle has led to a lot of health issues, necessitating the exploration of new pharmaceutical compounds. In addition to terrestrial ecosystems, marine environments offer promising opportunities for bioprospecting. Marine invertebrates are particularly noteworthy for their production of a wide array of bioactive compounds, often attributed to their filter feeding behaviour and defensive mechanisms. This study focused on analyzing three marine organisms namely, the golf ball sponge, horn shell, and nine species of sea anemones for their pharmaceutical potential. The methanol extract of sea anemone sp. 1 was recorded to show maximum DPPH scavenging of 93.27 % indicating significant antioxidant activity. On the other hand, reducing power assay of methanol extract of *B. goanensis* reported highest antioxidant content of 14.38 µg/mL. The crude extract sea anemone sp.5 and sp.4 was observed to have anti-diabetic and anti-inflammatory property respectively with 25.81 % inhibition of amylase and 46.19 % inhibition of albumin denaturation. The coagulation and anti-coagulation assay revealed the coagulation property of the crude extracts of sp.4 and sp.6 and anti-coagulation property of sp.2 and *A. anjunae*.

Furthermore, to investigate the potential contribution of associated microbes, the gut microbiomes of two sea anemone species were isolated and analyzed for pharmaceutical properties. To ensure the conservation of marine ecosystems, in vitro propagation techniques were employed for sea anemones.

CHAPTER - I

1. INTRODUCTION

1. INTRODUCTION

1.1 BACKGROUND

In order to combat diseases emerging due to modern lifestyle and to improve human health, pharmaceutical research continually seeks novel sources of therapeutic compounds. Despite significant progress, the discovery of new drugs remains a pressing need, particularly in light of emerging drug resistance and unmet medical needs. The term “pharmaceutical” refers to substances or products used in the diagnosis, treatment, prevention or mitigation of disease or medical condition in humans or animals. This substance could include drugs, medications, vaccines, biologics and medical devices (WHO EMRO | Pharmaceutical Products | Health Topics, 2014). Pharmaceuticals derived from plants, animals, and marine organisms offer an invaluable array of natural compounds with therapeutic potential. The exploration of marine organisms, including algae, bacteria, sponges, and mollusks, represents a promising opportunity for drug discovery. These organisms produce a wide range of bioactive compounds with diverse pharmacological properties, ranging from antiviral peptides to anticancer agents. The coastal areas, abundant with marine life, are especially rich in such compounds, offering immense potential for treating various diseases (Sheikh et al., 2018).

Goa forms a small coastal state of the Western Ghats and has a coastline of about 120 kms and has sandy beaches and rocky shoreline. Qualitative and quantitative distribution of marine biota along the Goa coast varies during pre-monsoon, and post monsoon. The marine ecosystem comprises a vast diversity ranging from microorganisms, algae, sponges, sea anemones, molluscs, fish and many more

(Sonak, 2017). These organisms produce bioactive compounds for their survival and defence and are mainly supported by associated microorganisms that aid them in defence mechanisms. The variety of bioactive compounds produced consists of polysaccharides, proteins, fatty acids, phenolic compounds, terpenoids, enzymes and minerals. These compounds are of human benefit as they possess activities like antioxidant and antitumor effects. The marine invertebrates discussed in this study belong to the phylum cnidaria, porifera and mollusca respectively (Shirsath et al., 2023).

Cnidarians are a phylum of aquatic invertebrates that include animals such as jellyfish, corals, and sea anemones. They are characterized by the presence of specialized cells called cnidocytes, which contain stinging structures called nematocysts used for defense and prey capture. **Porifera** is the phylum of sponges, which are multicellular aquatic organisms known for their porous bodies. Sponges filter feed by pumping water through their bodies, trapping food particles in specialized cells called choanocytes. **Mollusks** are a diverse phylum of invertebrate animals that include familiar organisms such as snails, clams, octopuses, and squids. They are characterized by a soft body typically enclosed in a calcareous shell (Comparing and Contrasting Invertebrates, 2016).

Sea anemones (phylum cnidaria) produce venom that contain peptides that modulate ion channels and are used for pain relief (Prentis et al., 2018). **Sponges** (phylum porifera) are immobile multicellular organisms consisting of a sticky substance called mesophyll and have pitcher-like bodies supported by spicules and spongia. These organisms produce chemical compounds, such as alkaloids,

terpenoids, polyphenolic compounds, peptides, and sterols, which have the potential to treat various ailments (Mayer et al., 2019). **Horn shell** (phylum Molluscs) is consumed as marine foods and bioactive peptides from blue mussel extracts effectively reduce hypertension. Ground abalone and its shells are utilized for treating eye conditions (Shirsath et al., 2023).

It is important to uphold sustainable practices to safeguard marine ecosystems. The marine resources like sea anemone can be harnessed for its pharmaceutical properties sustainable by carrying out in-vitro propagation. They possess remarkable regenerative abilities that enable them to repair and regenerate damaged or lost body parts. Sea anemones can regenerate various structures, including tentacles, oral discs and even entire body (Minasian, 1976).

The present study focuses on bioprospecting the marine invertebrates of Goa for their pharmaceutical potential which can aid in combating certain disease or improving human health. We explore three phyla of invertebrates that is cnidarians (nine species of Sea anemones), porifera (Golf Ball sponge) and mollusc (Horn shell) for their pharmaceutical potential. The crude extracts and the solvent extracts of the marine invertebrates are checked for antioxidant, anti-diabetic, anti-inflammatory, anticoagulant and antimicrobial pharmaceutical properties. Since it is necessary to ensure the preservation of marine ecosystems while harnessing their therapeutic benefits in-vitro propagation of the sea anemone was also carried out.

1.2 AIM AND OBJECTIVE

Aim:

To bioprospect marine invertebrates from the costal ecosystem of Goa for pharmaceutical potential.

Objectives:

1. Collection and identification of invertebrates from the marine ecosystem of Goa.
2. Screening the marine invertebrates for pharmaceutical potential.
3. Partial characterisation of selective compound.
4. Screening of the gut microbiota and in vitro propagation of sea anemone.

1.3 RESEARCH HYPOTHESIS

The biodiversity of marine invertebrates holds untapped potential for the discovery and development of novel pharmaceutical compounds with therapeutic applications ("From Monsoons to Microbes: Understanding the Ocean's Role in Human Health," 2000).

This hypothesis suggests that the diverse range of marine invertebrates, including sponges, sea anemone, and molluscs, harbour bioactive compounds that have the potential to be developed into pharmaceutical drugs. The hypothesis proposes that through systematic bioprospecting efforts, researchers can uncover previously unknown compounds with therapeutic properties, thereby expanding the pharmaceutical repertoire and addressing medical needs. This hypothesis serves as the foundation for research endeavours aimed at exploring the pharmaceutical potential of marine invertebrates and could guide investigations into specific organisms, habitats, and biochemical pathways to validate its assertions.

1.4 SCOPE

Bioprospecting, particularly in marine invertebrates, holds immense promise for discovering novel pharmaceutical compounds. Marine environments, especially invertebrate-rich habitats like coral reefs and rock pools, are vast and largely unexplored reservoirs of biological diversity. The unique adaptations of marine invertebrates to extreme conditions offer a rich source of bioactive compounds with potential therapeutic applications.

One of the key advantages of bioprospecting from marine invertebrates is the vast biodiversity present in these ecosystems. With millions of species, each with its own distinct biochemical makeup, the potential for discovering new pharmaceutical compounds is staggering. Moreover, many marine invertebrates possess defensive mechanisms against predators or pathogens, which often involve the production of potent chemical compounds. These compounds have evolved over millions of years and may exhibit remarkable efficacy and specificity against human diseases.

However, the full scope of bioprospecting from marine invertebrates has yet to be realized. Challenges such as limited access to remote marine habitats, the complexity of sample collection and extraction, and the need for sustainable harvesting practices must be addressed. Additionally, the identification and characterization of bioactive compounds from marine organisms require interdisciplinary collaboration between biologists, chemists, pharmacologists, and bioinformaticians.

CHAPTER - II

2. LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 Impact of modern lifestyle on health

Lifestyle encompasses the behaviours, habits, and choices adopted by individuals, groups, and societies, shaped by various factors including geography, economics, politics, culture, and religion. It reflects the unique characteristics of inhabitants in a particular time and place, encompassing their daily activities, work routines, leisure pursuits, and dietary patterns. In recent years, researchers have increasingly recognized lifestyle as a critical determinant of health. According to the World Health Organization (WHO), a significant portion of individual health and quality of life is influenced by lifestyle factors. Unfortunately, a substantial number of people lead unhealthy lifestyles, resulting in illness, disability, and premature death. Common health problems associated with unhealthy lifestyles include metabolic disorders, musculoskeletal issues, cardiovascular diseases, hypertension, obesity, and violence (Farhud., 2015).

Today, societal changes and technological advancements have introduced new challenges to people's lives. Malnutrition, unhealthy eating habits, tobacco use, excessive alcohol consumption, substance abuse, and stress have become prevalent features of modern lifestyles. Research indicates that lifestyle choices profoundly impact both physical and mental well-being (Gardner, C. et al., 2007). For example, modern lifestyles, characterized by hectic schedules, sedentary habits, and unhealthy dietary choices, significantly impact health, particularly in terms of stress and diabetes. Chronic stress from high-pressure work environments and social demands, combined with unhealthy lifestyles,

contributes to the rising prevalence of stress-related health issues like diabetes. (Mozaffarian, D. et al., 2011).

Bioprospecting diverse bioresources for potential pharmaceuticals is essential in tackling health issues arising from modern lifestyles. Pharmaceuticals such as antioxidants, anti-inflammatories, antidiabetics, and antimicrobials play a crucial role in today's world. Antioxidants combat free radicals, reducing cancer and cardiovascular disease risks. Anti-inflammatory agents alleviate conditions like arthritis and asthma. Antidiabetic compounds regulate blood sugar levels, preventing diabetes complications. Antimicrobial agents fight infectious diseases, vital for addressing antibiotic resistance. Their varied benefits highlight the significance of continuous research and development in this area for overall well-being and longevity (Sarian et al., 2017).

2.2 Pharmaceutical

In the effort to address diseases arising from modern lifestyles and enhance human health, pharmaceutical research constantly seeks innovative sources of therapeutic substances. Despite notable advancements, the need for discovering new drugs remains urgent, especially with the emergence of drug resistance and unmet medical requirements. The term "pharmaceutical" encompasses substances or products utilized in diagnosing, treating, preventing, or alleviating diseases in humans or animals. This category includes drugs, vaccines, biologics, and medical devices (WHO EMRO | Pharmaceutical Products | Health Topics, 2014). Pharmaceuticals sourced from plants, animals, and marine organisms provide a diverse array of natural compounds with therapeutic properties, expanding the

pharmaceutical field significantly. While traditional medicine has long acknowledged the medicinal benefits of botanical and zoological sources, contemporary pharmacology continues to uncover novel applications and advantages of these natural reservoirs. Exploring marine organisms, such as algae, bacteria, sponges, and mollusks, presents a promising avenue for drug discovery. These organisms produce various bioactive compounds with diverse pharmacological effects, ranging from antiviral peptides to anticancer substances. Coastal regions, teeming with marine life, offer particularly abundant sources of such compounds, presenting vast potential for treating a multitude of ailments (Shirsath et al., 2023).

The tricyclic alkaloid ascidiathiazone, extracted from the ascidian *Aplidium* species, was observed to affect the production of superoxide by human neutrophils in vitro and ex vivo. Research suggests that these two compounds hold promise as potential leads for anti-inflammatory pharmaceuticals (Kalimuthu & Kim, 2013). Eryloside F, sourced from the sponge *Erylus formosus,* has demonstrated potent activity as a thrombin receptor antagonist (Stead, et al., 2000). Callyspongynic acid, a polyacetylenic acid derived from the sponge *Callyspongia truncata*, exhibits inhibitory effects on α-glucosidase (Lebovitz, 1992). Norzoanthamine, a marine alkaloid belonging to the zoanthamine class, is isolated from a colonial zoanthid, *Zoanthus* sp. This compound has shown potential in protecting skeletal proteins like collagen and elastin from external stresses. It is considered a promising candidate for the treatment and prevention of osteoporosis (Kinugawa, et al., 2009).

2.3 Marine Invertebrates

Marine invertebrates encompass a vast array of organisms inhabiting the world's oceans, ranging from microscopic plankton to large mollusks and echinoderms. These diverse creatures play crucial roles in marine ecosystems, contributing to nutrient cycling, habitat formation, and biodiversity. Marine invertebrates exhibit a remarkable array of adaptations to their underwater environment, including specialized feeding mechanisms, locomotion strategies, and defence mechanisms. From colorful corals to elusive nudibranchs, marine invertebrates captivate scientists and enthusiasts alike with their intricate forms and behaviours. They have also captured the attention of researchers in fields such as pharmacology, biotechnology, and ecology due to their potential applications in drug discovery, biomaterials, and environmental monitoring (Marine Invertebrates – Geological Oceanography Lab, 2016).

2.3.1 Sea Anemone

Sea anemones produce bioactive compounds that could have applications in medicine. These compounds often have properties like anti-inflammatory, analgesic, and anti-cancer effects (Thangaraj et al., 2018) For instance, some sea anemone venoms contain peptides that can modulate ion channels and potentially be used for pain relief. One example of a compound derived from sea anemones is "ShK toxin," which stands for *Stichodactyla helianthus* K+ extracted from the venom of the sea anemone *Stichodactyla helianthus*. ShK toxin has the ability to block specific potassium channels in cells, which has shown potential as an immunosuppressant and has been investigated for its use in treating autoimmune diseases like rheumatoid arthritis (Prentis et al., 2018).

The extracts from sea anemones *Anemonia sulcata* and *Actinia equinan* shows anti-inflammatory and cytotoxic effects. The alkaloid homarine in extracts showed effectiveness in reducing inflammation and inhibiting phospholipase A2 responsible to break fatty acids that are responsible for inflammation. The evaluation on human gastric cells revealed a non-classical mechanism of apoptosis(Silva et al., 2017). The mucus of sea anemones comprises water, minerals, proteins, carbohydrates, and lipids. Various methods are employed for extraction and isolation of bioactive compounds including aqueous, methanol and ethanol extraction, filtration, evaporation, and lyophilization (Flórez-Fernández et al., 2020). Purification methods include C18 column fractionation, semi preparative chromatography on selected fractions from gel filtration, and membrane separation for mucus extract components before chromatography (Flórez-Fernández et al., 2020). The extract of sea anemone *Heteractis aurora* from the Southeast coast of India demonstrated significant inhibition against bacterial(*Klebsiella oxytoca*, *Escherichia coli*) and fungal pathogens(*Botrytis cinerea, Trichoderma koning*). The extract exhibited antioxidant activity and a hemolytic activity (Thangaraj et al., 2018).

Sea anemones have diverse microbial communities associated with them, which play important roles in their health and ecology. These microbial communities consist of bacteria, archaea, fungi, and other microorganisms. The bacteria from the microbiota of sea anemones (*Anemonia sulcata* and *Actinia equina*) for their antimicrobial activity against human, agricultural, and aquaculture pathogens. Among the 27 strains with antibacterial activity, 12 also exhibited antifungal activity (León-Palmero et al.,2018).

2.3.2 Marine Sponge

Algae possess diverse colour pigments aiding in photosynthesis and producing various bioactive compounds. Similarly, sponges harbor anti-inflammatory and anti-tumor compounds. The review highlighted 106 compounds from algae and sponges like amines, brominated phenols, kainic acid from algae and spongistain-I and 8-hydroxy manzamine from sponges showcasing medicinal potential. Compounds, like protein kinase C inhibitors from sponges, were associated with tumor development (Naqvi et al.,2022). The modified nucleotides in *Cryptotethya crypta* led to discovery of the leukemia drug cytarabine. Sponges yield mainly terpenes, alkaloids, and lipids, exhibiting anti-tumors and antimicrobial properties (Romano et al.,2022).Eribulin derived from the sponge *Halichondria okadai* is a synthetic derivative used as a chemotherapy drug for metastatic breast cancer. It disrupts microtubule dynamics, leading to cell cycle arrest and apoptosis (Crews et al., 2003). Bryostatin obtained from the bryozoan *Bugula neritina* has shown potential in the treatment of Alzheimer's disease and certain types of cancer. It modulates protein kinase C and affects cellular signaling pathways(Shen et al., 2012). Discodermolide isolated from the Caribbean sponge *Discodermia dissoluta*, has demonstrated anti-cancer properties by stabilizing microtubules and inhibiting cell division (Crews et al., 2003). Spongistatin extracted from the sponge *Spirastrella cunctatrix*, show potent anticancer activity by inhibiting microtubule assembly and inducing apoptosis (Perdicaris et al.,2013).

Scopularide is a bioactive compound found in the marine sponge *Tethya aurantium.* It exhibits various pharmacological properties, including antimicrobial and anticancer activities (Mayer et al., 2019)Research on

scopularide and other compounds from marine organisms continues to explore their potential in medicine and Biotechnology. The genera *Haliclona*, *Xestospongia* and *Neopetrosia* are known to produce renieramycin and quinones. The renieramycin E found in extracts of *Haliclona* sponges are actually produced by the symbionts *Endohaliclona renieramycinifaciens*. Due to genome reduction, the formation of chemo bacteriocytes is seen in marine sponges. These symbiotic bacteria live within sponges, receive nutrients and protect them from other bacteria and in turn produce defence molecules that benefit the marine sponge (Romano et al., 2022).

2.3.3 Horn shell

Horn shell extracts, derived from *Conus sp*., offer promising applications in pharmaceutical formulations. Rich in bioactive compounds like peptides, proteins, and enzymes, these extracts exhibit antioxidant, antimicrobial, and anti-inflammatory properties. Potential applications include developing supplements to mitigate oxidative stress, combat microbial infections, alleviate inflammation-related conditions, support cognitive function, and enhance immune system modulation (Shirsath et al., 2023). Such formulations hold significant therapeutic potential for promoting overall health and longevity, addressing neurodegenerative diseases, and bolstering immunity against infections. Continued research into the bioactive constituents and mechanisms of action of horn shell extracts underscores their potential as valuable ingredients in pharmaceutical products.Mollusk extracts offer health benefits such as antioxidant, anticancer and anti-infectious, extensively reviewed in this study. The review provides insights into the efficacy of isolation techniques and

subsequent bioactivity analysis, with discussions on future developments in extraction-bioactivity (Odeleye et al., 2019).

Novel biphasic bioceramic nanopowders comprising hydroxyapatite and β-tricalcium phosphate were synthesized from *Cerithium vulgatum* shells using an innovative chemical method. Analyzed through FT-IR, X-ray diffraction, and SEM, these biologically sourced powders exhibit characteristics suitable for potential use in nanoceramic biomaterial fabrication (Gündüz et al., 2014).

2.4 Why bio-prospect marine invertebrates of Goa

Situated on India's western coast, Goa spans approximately 120 kilometers along its shoreline. According to Hartog (1993), Goa is home to two sea anemone species, *Bunodosoma goanensis* and *Anthopleura anjunae*, which are endemic to the region, alongside numerous other sea anemone species. Apart from sea anemones, other marine invertebrates like sponges and mollusks, which are filter feeders, offer opportunities for investigating their production of bioactive compounds. During low tide, the gathering of these marine invertebrates and exploration of their potential could enhance their utilization effectively.

2.5 In-vitro propagation of Sea Anemone

Adhering to sustainable practices is crucial to safeguard marine ecosystems and ensure the continual availability of vital resources for pharmaceutical research and development. Sea anemones, notable members of the Cnidaria phylum, boast remarkable regenerative capabilities, allowing them to heal and regenerate damaged body parts. This process, known as regeneration, is essential for their

survival and reproduction (Van der Burg & Prentis, 2021). Through in-vitro propagation methods, sea anemones can be sustainably harnessed for their pharmaceutical properties, enabling the preservation of marine ecosystems while benefiting from their valuable therapeutic potential.

Nematostella vectensis, a common burrowing sea anemone, was bred under controlled conditions, reproducing both sexually and asexually. They were fed *Artemia* sp. *nauplii* every other day and *Mytilus* sp. tissue every eight days, resulting in synchronized spawning every eight days. Spawning involves the release of eggs or sperm. In an experiment, isolated female and male clonemates spawned predominantly after mussel feeding and water changes. Fertilization occurred in vitro, with embryos gastrulating within 12-15 hours and developing into juvenile anemones within 2-3 weeks. This species shows potential as a model for cnidarian developmental research (Hand et al., 1992).

Longitudinal fission and pedal laceration are the most common asexual reproductive modes employed by sea anemones. In longitudinal fission, the sea anemone stretches transversely to generate two fragments of similar size. Tissue tears along a longitudinal plane parallel, and usually close to the oral-aboral axis. In pedal laceration, many small tissue fragments separate from the pedal disk and regenerate into new individuals (Minasian, 1976). In controlled laboratory settings, in-vitro propagation of sea anemones can be achieved through longitudinal fission, wherein the organisms are bisected into two equal halves, each containing essential anatomical components for regeneration. Optimal conditions such as salinity, food availability, and temperature are critical factors

influencing the success of this process. By maintaining these conditions within prescribed ranges, researchers can foster the regeneration and growth of sea anemones, facilitating their propagation for experimental and conservation purposes (Rabinowitz et al., 2016).

CHAPTER - III

3. MATERIALS AND METHODOLOGY

3. MATERIALS REQUIRED

Chemicals Used

- Methylene blue
- Ethanol
- Methanol
- Hexane
- ZMA
- ZMB
- Ascorbic acid
- Gallic acid
- Quercetin
- Dichlofenac sodium
- DPPH
- Potassium hexaferricyanide
- Trichloro acetic acid
- Ferric chloride
- PBS
- Albumin
- Alpha-amylase
- Starch
- Aluminium chloride
- Sodium hydroxide
- DNSA
- Acarbose
- Sodium nitrate

Apparatus

- Autoclave
- Laminar Air Flow
- Biosafety cabinet
- SORVALL ST 8R refrigerated bench top centrifuge
- Borosil separating funnel
- UV mini 1240 UV-Vis spectrophotometer
- Microscope
- Refractometer
- pH meter (pH 700, Eutech Instruments, Thermo Fisher Scientific, India)
- Hot air oven
- Refrigerator
- Waterbath

3. METHODOLOGY

3.1 Collection and Identification of Samples (Sea anemones, Horn shell, and Golf Ball sponge)

Nine species of sea anemone, one species of mollusc and sponge each was collected from Anjuna (Lat 15.584591º, Long 73.736502º) and Vainginim (Lat 15.454703º, Long 73.814643º) beach as seen in the Fig.3.1 (A), (B) and (C) along the coast of Goa during the low tide post monsoon season. The collected samples were identified based on their morphology and confirmed by Dr. Preeti Pereira from Zoology Discipline, School of Biological Sciences and Biotechnology, Goa University. A study on the types of nematocysts cells present in sea anemone tentacle along with its histology was also carried out in order to identify sea anemones

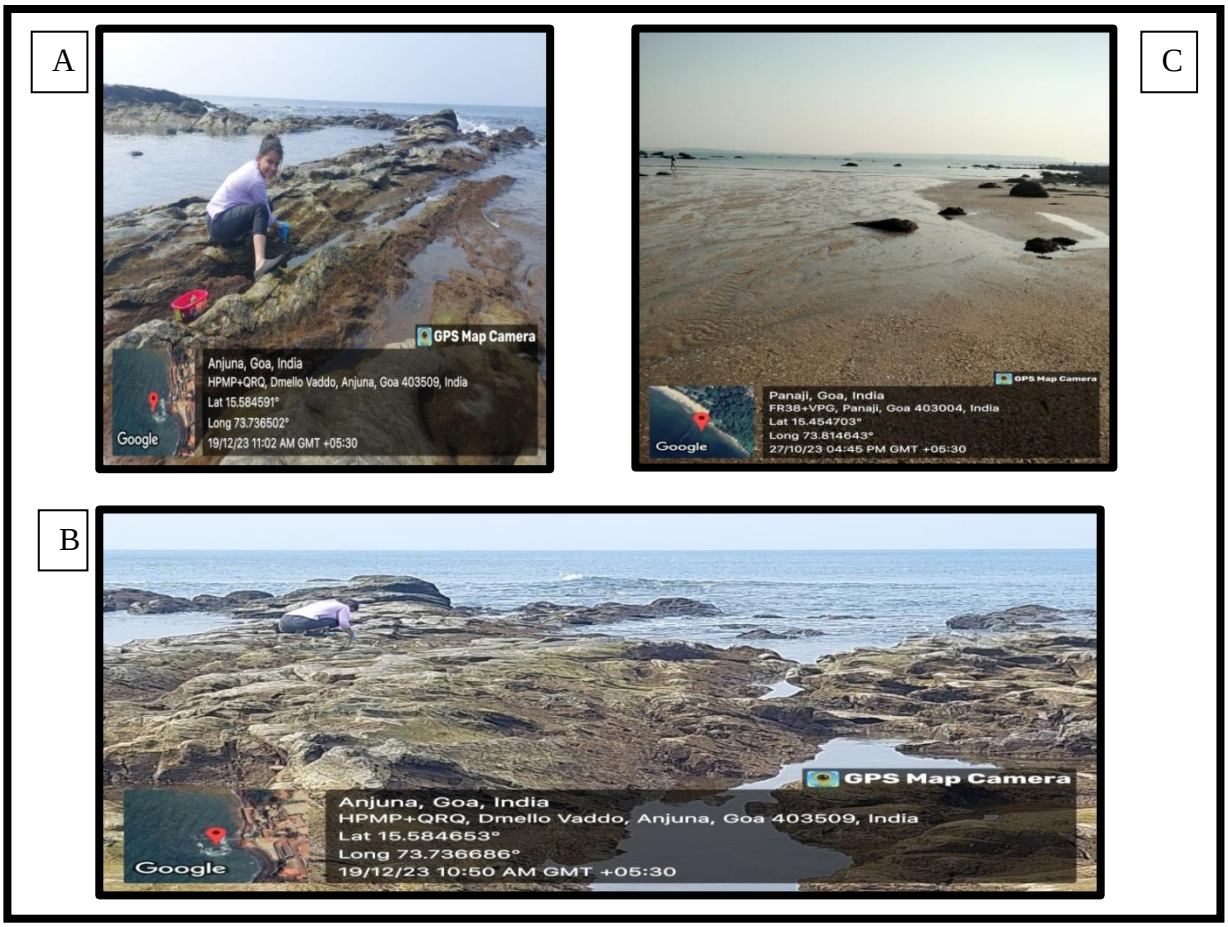


Figure 3.1: (A) and (B) Sample collection at Anjuna beach. (C) Sample collection at Vainginim beach.

3.1.1 Identification based on morphology of sea anemones, Horn shell, and Golf Ball sponge

Three species of sea anemones namely, *Anthopleura anjunae, Anthopleura nigrescens* and *Bunodosoma goanensis* along with Golf Ball sponge and Horn shell was identified based on their morphological characteristics and confirmed by Dr. Preeti Pereira from Zoology Discipline, School of Biological Sciences and Biotechnology, Goa University. The morphological characters like column colour and oral disc pattern for sea anemone identification for considered. Whereas, for the Horn shell and sponge the characteristic shell pattern and structure and colour of the sponge body was observed respectively.

3.1.2 Identification of nematocysts cells in sea anemone

Identification of the sea anemones was done by checking for the type of nematocysts present in their respective tentacles. As per the protocol given by the Hartog (1993), the tentacles of sea anemone were cut and washed with distilled water followed by staining with 0.5% methylene blue. It was then washed again and transferred on to a clean slide and smashed using a cover slip, followed by observation under the microscope (100X objective).

3.1.3 Histological study of sea anemones

Confirmation of identified species of sea anemones was done by histological studies. According to Hartog (1993), the sea anemones were anesthetised using 6% magnesium chloride followed by dissecting it with sterile scalpel and blade to obtain cross sections of the column. The section obtained was preserved in 10% formaldehyde and sent for 8µm thick microtome sectioning to Dr. Ashwini's

Pathology laboratory, Panjim. The sections were stained using Masson trichrome staining protocol. The sections were first deparaffinised followed by staining with eosin and methylene blue (Hartog, 1993). The stained sections were observed for internal structures under the microscope under 40X, 100X and 400X magnifications.

3.2 Maintenance of sea anemones

A glass tank for maintenance of Sea anemone and Horn shell was set up keeping in mind all the natural parameters like salinity, temperature, food and aeration as seen in the Fig.3.2 (A), (B) and (C). The tanks were fitted with aerators in order to maintain a continuous oxygen supply. Hatched *Artemia salina* was fed to the sea anemones once a day. The salinity of the water was kept under check by using a refractometer. The tank was cleaned once a week with addition of only 25% new sea water.

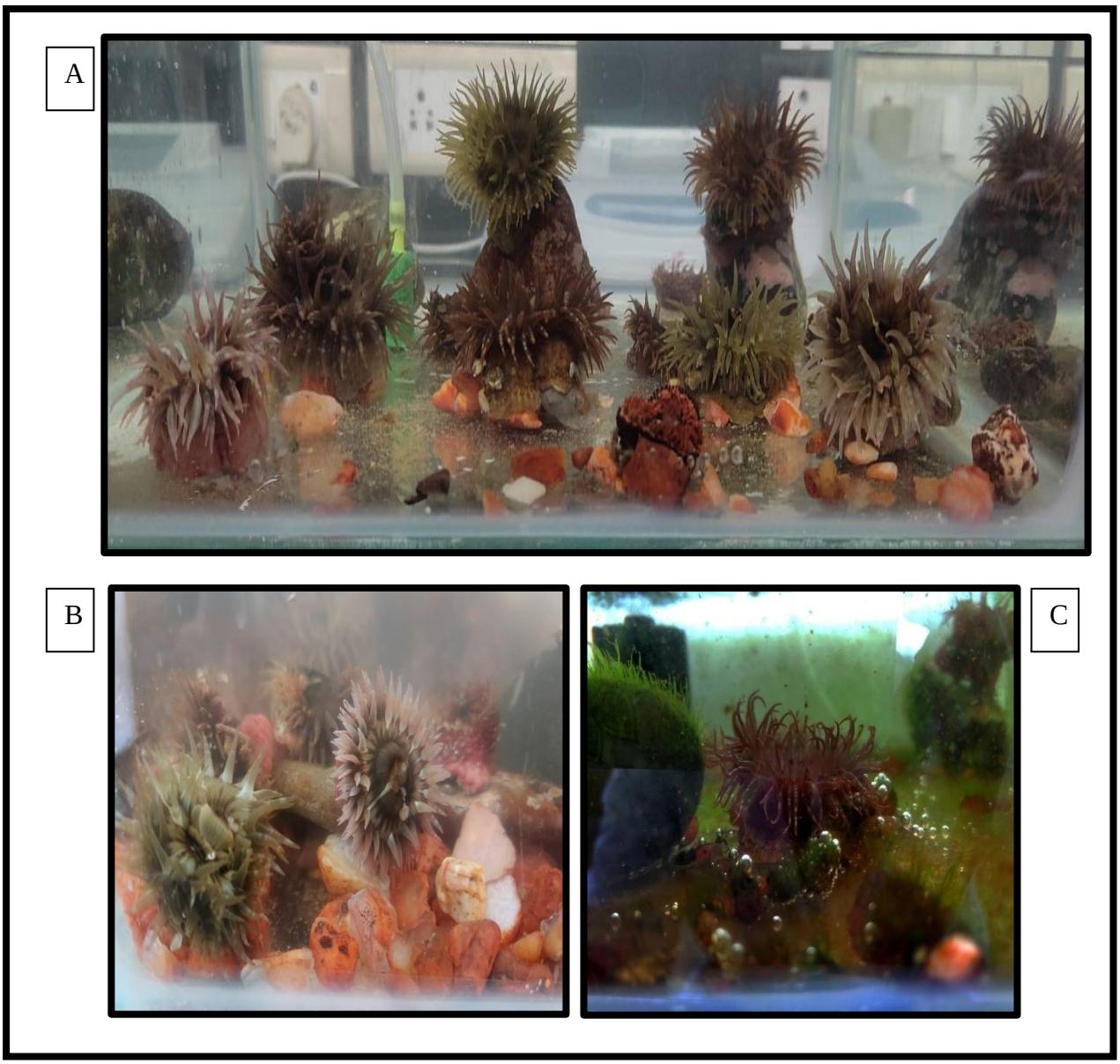


Figure 3.2: (A), (B) and (C) Tank set up for maintenance of sea anemones

3.3 In-vitro propagation of sea anemones

In-vitro propagation of sea anemone was carried out by first anesthetising the sea anemone in 6% magnesium chloride followed by dissecting it into equal halves using a sterile blade and scalpel as seen in the Fig. 3.3 (A) and (B). The dissected anemone was immediately washed with fresh sea water and kept in well aerated tanks with *Artemia salina* for regeneration.

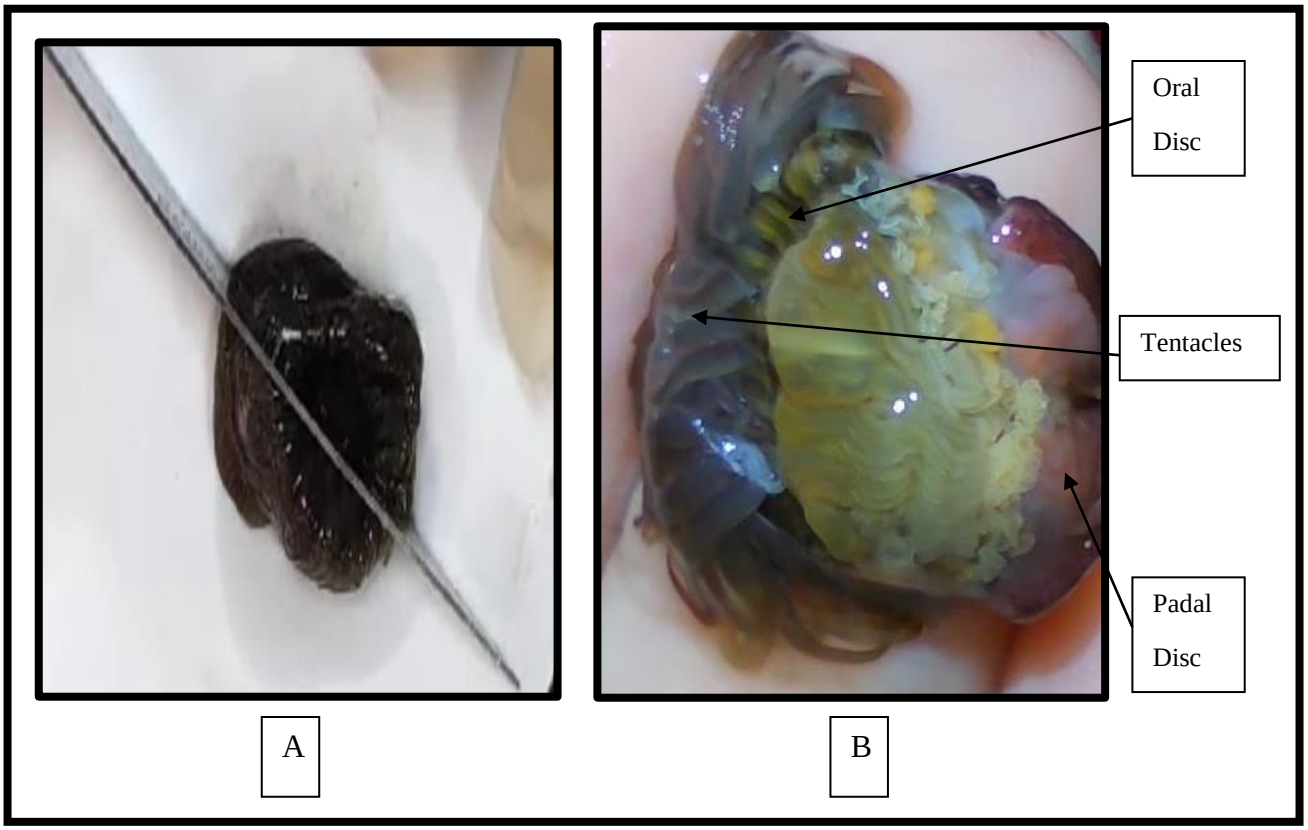


Figure 3.3: (A) Sea anemone being dissected with sterile blade (B) Sea anemone washed immediately after dissection

3.4 Preparation of crude extract

Crude extract preparation was carried out by the method mentioned in Hamayeli et al., (2019). The samples were first washed with distilled water to remove all the impurities followed by cutting it into small pieces using sterile blade. The pieces were then macerated in 96% ethanol to make it into a fine paste as depicted in the Fig.3.4 (A), (B) and (C). The ethanol was then allowed to evaporate at room temperature to leave behind crude extract powder which was used for further analysis. The weight and other dimensions of the respected samples were recorded prior to crude extract preparation.

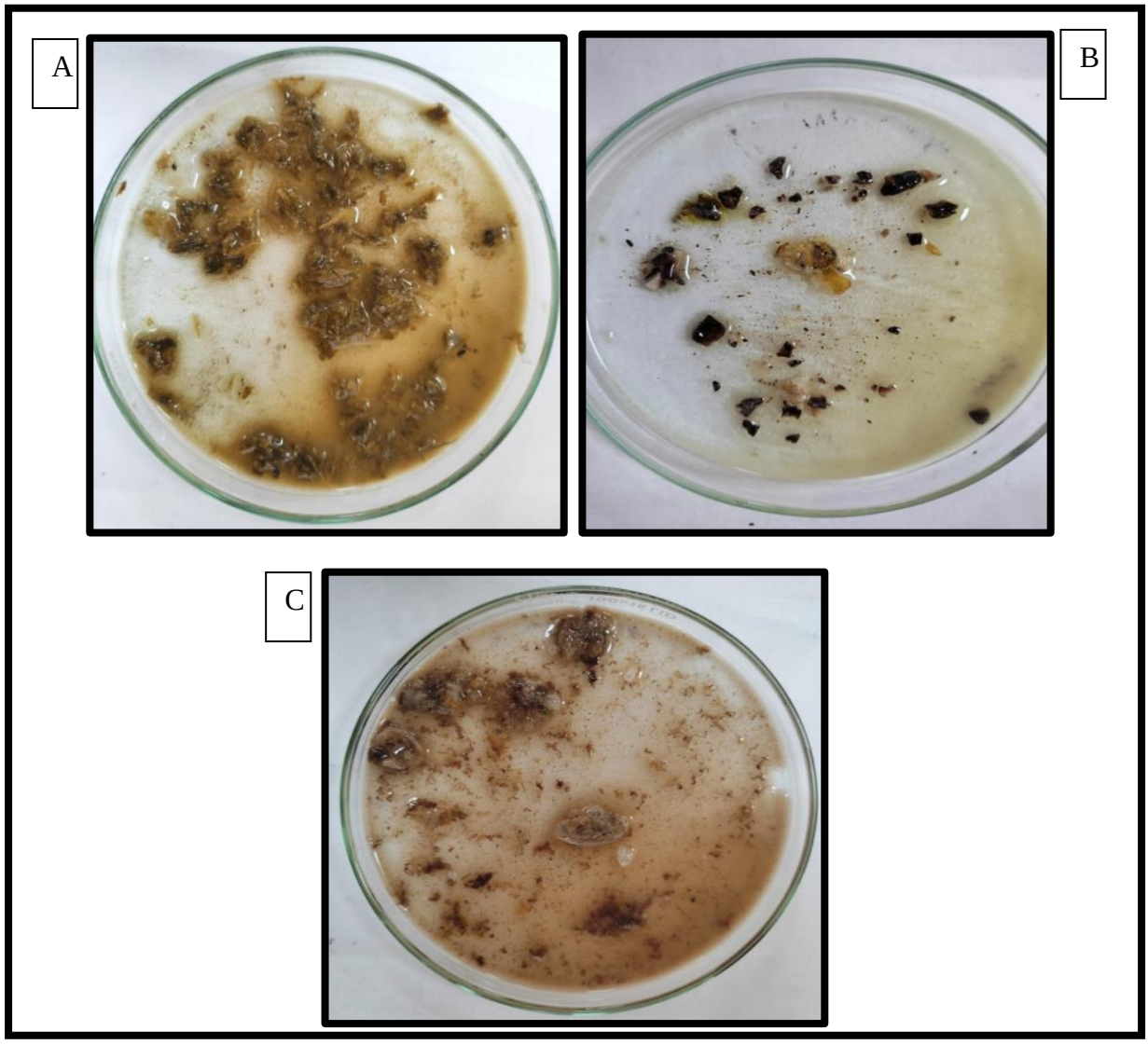


Figure 3.4: Crude extract of (A) Golf Ball sponge, (B) Horn shell and (C) Sea anemone

3.5 Partial purification of crude extract by solvent extraction method

Solvent extraction was performed for the crude extract by using one polar and one non polar solvent. The polar solvent used was methanol whereas the non-polar solvent was hexane. According to Babbar et al., (2012) for solvent extraction 7.5mg of crude extract was partitioned in 25mL methanol and 25mL hexane using a Borosil separating funnel as illustrated in the Fig.3.5.

Figure 3.5: Partitioning crude extract in methanol and hexane using Borosil separating funnel

3.6 Isolation of gut microbiota of sea anemones

3.6.1 Isolation and colony characterisation of bacteria

The gut microbiota of two sea anemone species namely, *A. anjunae* and *A. nigrescens* was isolated on ZMA before and after food. Sterile cotton swab was inserted into the gut of sea anemone via oral disc and then was mixed in 3% saline followed by serial dilution. The dilution 10^{0}, 10^{-2} and 10^{-4} was plated on ZMA and incubated at RT for 24 hours. The isolated colonies were checked for their colony characteristics like colour, shape, elevation, opacity, consistency and Gram character by performing standard Gram staining protocol (Coico, 2005).

3.6.2 Biochemical analysis of isolates

KB001 HiMVIC Biochemical strips were utilised for the biochemical analysis of the isolates.

3.7 Screening crude, methanol, hexane extract and bacterial isolates for pharmaceutical potential

The crude extract was used in the powered form after dissolving in ethanol while the methanol and hexane extracts were utilised directly. For the bacterial isolates, eight unique isolates were taken and allowed to grow in ZMB. The broth was then centrifuged by using SORVALL ST 8R refrigerated bench top centrifugeat 5,000 rpm for 10 minutes at 4°C. The pellet was discarded and the supernatant was analysed for pharmacological potential (Jan et al., 2022)

.

3.7.1 Antioxidant activity – DPPH assay

DPPH assay is performed to determine the antioxidant activity of a substance. The reduction of DPPH reagent by antioxidant in the sample can be determined spectrophotometrically. This is important because antioxidants protect the body from free radicals that can cause diseases like cancer. The antioxidant activity of the crude, methanol, hexane extract and bacterial isolate was analysed using DPPH radical scavenging activity (Athavale & Jirankalgikar, 2023). The standards were made using ascorbic acid as stock (0.1 mg/mL) and different dilution were made with different concentrations by using ethanol as the diluent. For the crude extract, 3 mg of the crude extract was dissolved in 3mL ethanol; while for solvent extracts and isolates 3 mL of solvent extract / broth was mixed with 1 mL of 0.3mM DPPH reagent followed by 30 minutes incubation in the dark at RT. The absorbance was recorded using UV mini 1240 UV-Vis spectrophotometer at 517 nm and compared with the standard.

$$\% \text{ Scavenging} = \frac{\text{O.D.of Blank} - \text{O.D of Sample}}{\text{O.D.of Blank}} \times 100.$$

3.7.2 Reducing power assay

The reducing power assay is conducted to analyse the ability of substance to donate electrons and act as antioxidant. The substance with strong reducing power can neutralise harmful radicals in the body, which are linked to diseases like cancer and aging. This test helps to determine the potential antioxidant activity of the substance. The protocol mentioned by (Ci, 2016) was used to assess the reducing power of the samples. The standards were made using

ascorbic acid as stock (0.1mg/mL) and different dilutions were made with diverse concentrations by using ethanol as the diluent. For crude extract, 2.5 mg of the extract was dissolved in 2.5 mL ethanol; for solvent extract and isolate, 2.5 mL of solvent / broth was used as the sample. The sample was mixed with 1 % potassium hexaferricyanide and incubated at 50℃ for 20 minutes. To this mixture, 2.5 mL of 10 % trichloro acetic acid was added followed by centrifugation at 3000 rpm for 10 minutes using a SORVALL ST 8R refrigerated bench top centrifuge. After centrifugation, 2.5 mL of the supernatant was taken in another tube with 2.5 mL of distilled water, to which 0.5 mL 0.1 % ferric chloride was added. The absorbance was recorded at 700 nm and compared with the standard.

3.7.3 Anti-diabetic assay – alpha-amylase inhibition activity

The alpha amylase inhibition test for antidiabetic assay is conducted to screen the substance for presence of antidiabetic compound. In the presence of an anti-diabetic compound, the activity of alpha amylase is inhibited thereby preventing breakdown of starch to sugar. This proves to be beneficial for the people suffering from diabetes as it helps to regulate blood glucose level. The protocol mentioned by Jan et al. (2022) was used to test samples for anti-diabetic activity. The standards were made using acarbose as stock (12 mg/ mL) and dilutions were made with different concentrations by using ethanol as the diluent. For crude extract, 1 mg of the extract dissolved in 1 mL ethanol was used; while for the solvent extract and isolate, 1 mL of solvent / broth was mixed with 0.032 mg/mL alpha-amylase. This mixture was incubated for 25 minutes at 37 °C. To this, 1 mL of 1 mg/mL starch was added and incubated at 37℃ for 45 minutes. The

sugar break down was then estimated by DNSA method. The absorbance was recorded at 540 nm and compared with the standard.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of Blank} - \text{Absorbance of Sample}}{\text{Absorbance of Blank}} \times 100$$

3.7.4 Anti-inflammatory assay – Inhibition of albumin denaturation

The albumin denaturation inhibition assay was performed to screen the sample for anti-inflammatory activity. This assay determines presence of anti-inflammatory compound as a degree of albumin denaturation inhibition. Under conditions like high temperature albumin denatures, but the presence of anti-inflammatory compound inhibits denaturation of the albumin which can be estimated spectrophotometrically. The anti-inflammatory assay was performed according to the protocol mentioned by Bhutiya, (2020). The standards were made using dichlofenac sodium as stock (10 mg/mL) and dilutions were made with different concentrations using ethanol as the diluent. For crude extract, 2 mg of the extract dissolved in 2 mL ethanol was used while for solvent extract and isolate, 2 mL of solvent / broth was mixed with 200 µl of 10 mg/mL albumin. To this, 2.8 mL PBS buffer pH 6.4 was added followed by incubation at 37℃ for 15 minutes and 70℃ for 5minutes. The absorbance was recorded at 660 nm and compared with the standard.

$$\% \text{ Inhibition} = \left(\frac{\text{Absorbsnce of Sample}}{\text{Absorbance of Blank}} - 1\right) \times 100$$

3.7.5 Anti-microbial assay – Agar well diffusion method

The anti-microbial assay was performed by agar well diffusion method to screen the samples for production of anti-microbial compounds that would inhibit the growth of other micro-organisms. The Muller Hinton agar was utilised for this test due to its ability to provide reproducible results Anti-microbial assay was performed for methanol extract, hexane extract and bacterial isolates. The samples were tested for anti-microbial activity against three pathogens namely, *Pseudomonas aeruginosa*, *Escherichia coli*, *and Shigella* sp. by agar well diffusion method. The pathogenic cultures were spread plated on the Muller Hinton agar plates followed by piercing wells using a sterile cock borer. 100 µL of the test samples were then loaded in the well and the plates were incubated at 37°C for 24 hours. The plates were then observed for clearance zone after the incubation period (Mayer et al., 2019).

3.7.6 Coagulation and Anti-coagulant Assay

The Coagulation and anti-coagulant assay was performed to check for the presence of any anticoagulant compound or clotting factor in the crude extract. If the anti coagulant compound is present then it can be used as a drug to prevent blood from clotting or as a blood thinning agent. The presence of a clotting factor could be used in medicine to prevent excessive bleeding during surgery. It also could be used for the people suffering with haemophilia and diabetes as these diseases affect the blood clotting ability. The protocol given by Xiong et al., (2009) was followed to perform this test. Three blood groups namely, A+, B+ and O- were used to check for anticoagulant property of the compound. The sample was prepared by dissolving 1mg of crude extract in 1 mL of sterile saline. 0.1 M

EDTA was used as the positive control for anticoagulant and 1 % $FeCl_3$ was used as the clotting factor. The detailed protocol for the coagulation and anti-coagulation assay is mentioned in the Table 3.2 and 3.2.

Table 3.1: Protocol for Coagulation assay

	Blood group A+	**Blood group B+**	**Blood group O-**
Positive control	EDTA + Blood + $FeCl_3$	EDTA + Blood + $FeCl_3$	EDTA + Blood + $FeCl_3$
Negative control	EDTA + Blood	EDTA + Blood	EDTA + Blood
Sample	EDTA + Blood + Sample	EDTA + Blood + Sample	EDTA + Blood + Sample

Table 3.2: Protocol for Anti-coagulation Assay

	Blood group A+	**Blood group B+**	**Blood group O+**	**Blood group AB-**
Positive control	EDTA + Blood	EDTA + Blood	EDTA + Blood	EDTA + Blood
Negative control	Blood	Blood	Blood	Blood
Sample	Blood + Sample	Blood + Sample	Blood + Sample	Blood + Sample

3.8 Partial characterisation of selective compound

3.8.1 Flavonoid analysis

The flavonoid assay is performed to quantify the total flavonoid content in the substance. Flavonoids are known for reducing inflammation and lowering risk of chronic disease like cancer. Significantly higher amount of flavoinods signify better antioxidant activity and health benefits. The protocol mentioned by Ci (2016) was used to estimate flavonoid content in the samples. The standards were made using quercetin as stock (0.1 mg/mL) and different dilutions were made

with different concentrations by using ethanol as diluent. For crude extract, 1 mg of the extract dissolved in 1 mL ethanol was used For the solvent extract and isolate, 1 mL of solvent / broth was mixed with 4 mL distilled water, followed by addition of 0.3 mL of 5 % sodium nitrate. The reagents were mixed well and incubated for 5 minutes at RT. To these tubes, 0.3 mL of 10 % aluminium chloride was added followed by 6 minutes incubation at RT. After the incubation, 2 mL of 1 M sodium hydroxide and 3.3 mL distilled water was added and the absorbance was recorded at 510 nm and compared with the standard.

3.8.2 Phenol analysis

The phenol estimation test is carried out to determine the total phenolic content of a substance. Phenolic compounds are reported to have antioxidant properties and hence play major role in reducing oxidative stress. The protocol mentioned by Siddiqui et al., (2017) was followed for the phenol estimation test. The standards were made using gallic acid as stock (0.1 mg/mL) and different dilutions were made with different concentrations by using ethanol as diluent. For crude extract, 1mg of the extract was dissolved in 1mL ethanol; while for the solvent extract and isolate, 1mL of solvent / broth was used as the sample. It was mixed with 5mL Folin reagent followed by addition of 4 mL of 7.5% sodium nitrate. The mixture was incubated for 30 minutes in dark at RT. The absorbance was recorded at 765 nm and compared with standard.

3.8.3 Thin Layer Chromatograpgy (TLC)

Thin layer chromatography was performed to separate the compounds present in the methanol extract of the samples based on the retention factor. The Rf value of

the separated compounds was calculated using the mentioned formula. The TLC was performed according to the protocol mentioned by Borbon et al., (2016). The silica coated aluminium plates were used to separate the compounds in the sample. The TLC chamber was saturated for 24 hours with the solvent system, methanol: chloroform (3 : 2). The next day, silica plates were marked for spotting samples using a pencil. The samples were spotted thrice using a capillary tube. Once the sample was dried, the silica plate was kept in the saturated TLC chamber and allowed to run three forth. The plate was then removed and solvent front was immediately marked followed by drying. Once the plate was completely dried, it was placed in the chamber saturated with iodine fumes for visualisation of separated compound. The visible spots were marked using a pencil and the Rf of the marked spots were calculated..

$$\text{Rf} = \text{Distance travelled by solute} / \text{distance travelled by the solvent}$$

3.8.4 Fourier – transform infrared spectroscopy (FT-IR)

Fourier – transform infrared spectroscopy (FT-IT) is performed to analyse the chemical composition of a substance based on its infrared absorption spectrum. The FT-IR anlyses gives an idea about the functional groups present in the sample which could be contributing to various pharmaceutical activities. The methanol extracts of the samples with best results were directly given in glass vials for FT-IR analysis in the Chemistry Discipline, School of Chemical Sciences, Goa University. The samples were analysed by using Bruker, alpha-2 FT-IR machine (Thangaraj et al., 2018).

CHAPTER - IV

4. RESULTS AND DISCUSSION

4.1 Identification of collected samples

Among the 11 collected samples, three species of sea anemones, one species of sponge and one species of mollusc was identified.

4.1.1 Golf Ball Sponge

The golf ball sponge was observed to be spherical in shape and resembled appearance of a golf ball as seen in Fig.4.1 (A). The size of the sponge was noted to be 4.2 cm. Its surface had tiny pores and the exterior surface was observed to have slightly rough texture. Internally, the sponge had soft, gelatinous consistency and complex network of interconnected chambers. These chambers are known to facilitate water circulation and nutrient absorption. The sponges were found attached to rocky substrates and had characteristic orange yellow hue (Sonak, 2017).

4.1.2 Horn shell

Horn shell size was recorded to be 6-7 cm long with large, thick brown coloured shell, with body highly inflated and short spire as seen in Fig.4. (B). Most of the shell was occupied by the body whorl. The body whorl had broad brown and white spiral ribs separated by narrow grooves. Widely spaced nodules were observed. Outer lip was turned outward. Noumbilicus. Columella and aperture was white. Posterior canal was observed to be deep (Sonak, 2017)

The Golf Ball sponge and Horn shell isolated from the coasts of Goa were morphologically on par with the characteristics described by Sonak (2017).

Figure 4.1 : (A) Golf Ball sponge. (B) Horn shell

4.1.3 SEA ANEMONE SPECIES 7

Species 7 was characterised by a dark greenish brown column with plae verrucae with a reddish central area. The acrorhagi was creamy in colour. The base was observed to be pale. The tentacles were semi- translucent, creamy to greyish with opaque creamy spots. The oral disc had various creamy to yellow and some dark radii as depicted in Fig.4.2 (A). The tentacles show presence of basitrich and spirocysts nematocysts cells as seen in Fig.4.2 (B). For the visualisation of the microtome sections certain features like mesenterial filament and mesentery margin arrangement are clearly visible as illustrated in Fig.4.2 (C). As per literature the characteristics of this species signifies *Anthopleura anjunae.* The morphological description, types of nematocysts cells in the tentacles and the histology lies in accordance with (Hartog., 1993).

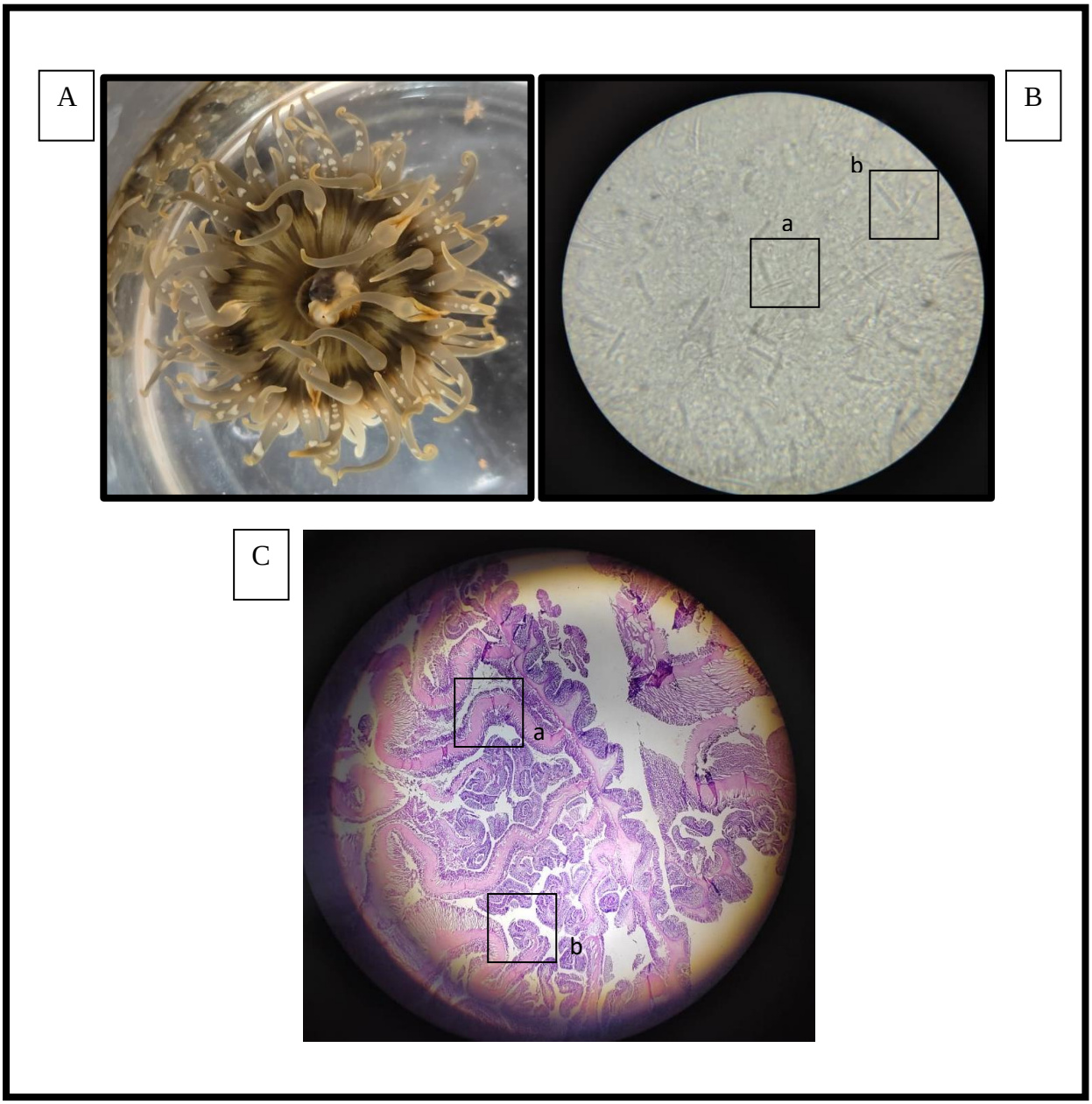


Figure 4.2: (A) *Anthopleura anjunae* expanded (B) nematocysts cells in the tentacle, a: basitrich; b: siprocyst (C) microtome section under 4X objective, a: mesentery margin; b: mesenterial filament

4.1.4 SEA ANEMONE SPECIES 8

The species 8 was observed for column to have dark reddish cast. The column also had strongly adhesive verrucae usually holding bits of gravel. The verrucae, were arranged in longitudinal. They were paler than the rest of the column. White-tipped The achrorhagi were white tipped, The oral disc was occasionally

extending into a cone. The area immediately surrounding the mouth was observed to be black. The colour of the ground of oral disc is lighter than the column. The mesenteric insertions were observed as dark lines as seen in Fig.4.3 (A). The tentacles show presence of basitrich and spirocysts nematocysts cells as depicted in Fig.4.3 (B). For the visualisation of the microtome sections certain features like gonads were clearly visible as illustrated in Fig.4.3 (C). As per literature the characteristics of this species signifies *Anthopleura nigrescens*

The morphological description, types of nematocysts cells in the tentacles and the histology lies in accordance with (Acuna et al., 2022).

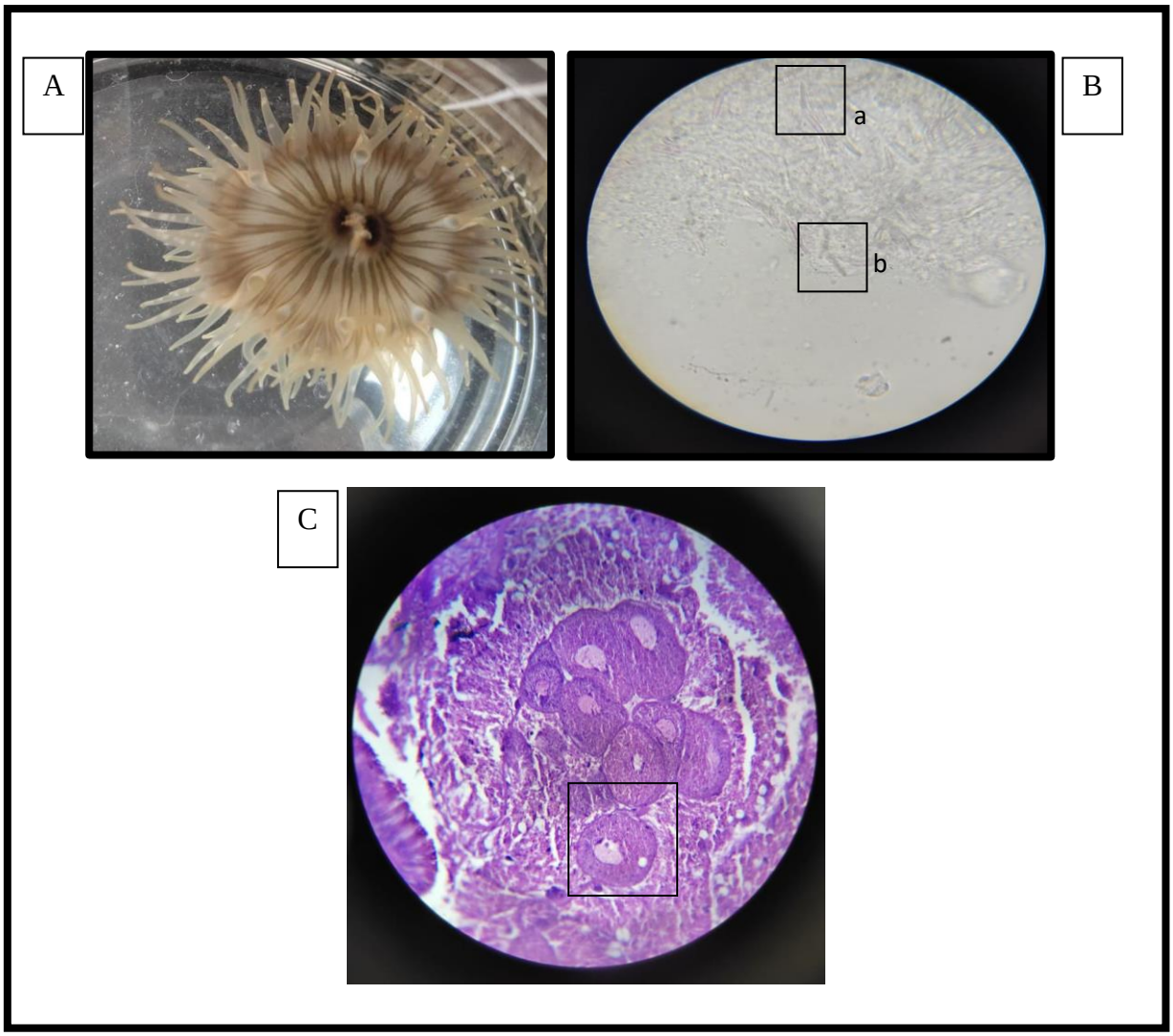


Figure 4.3: (A) *Anthopleura nigrescens* expanded (B) nematocysts cells in the tentacle, a: basitrich; b: siprocyst (C) microtome section under 10X objective, Gonads

4.1.5 SEA ANEMONE SPECIES 9

Species 9 of sea anemone was noted to have densely covered column with non adhesive verrucea. The oral disc had no characteristic pattern. The tentacles were short and contracted. The colour of the column, tentacles and oral disc was observed to be brownish crimson to brick red as depicted in the Fig.4.4 (A). The tentacles show presence of basitrich and spirocysts nematocysts cells as seen in Fig.4.4 (B). For the visualisation of the microtome sections certain features like sphincter were clearly visible as illustrated in Fig.4.4 (C). As per literature the characteristics of this species signifies *Bunodosoma goanensis*

The morphological description, types of nematocysts cells in the tentacles and the histology findings lies in par with Hartog., (1993).

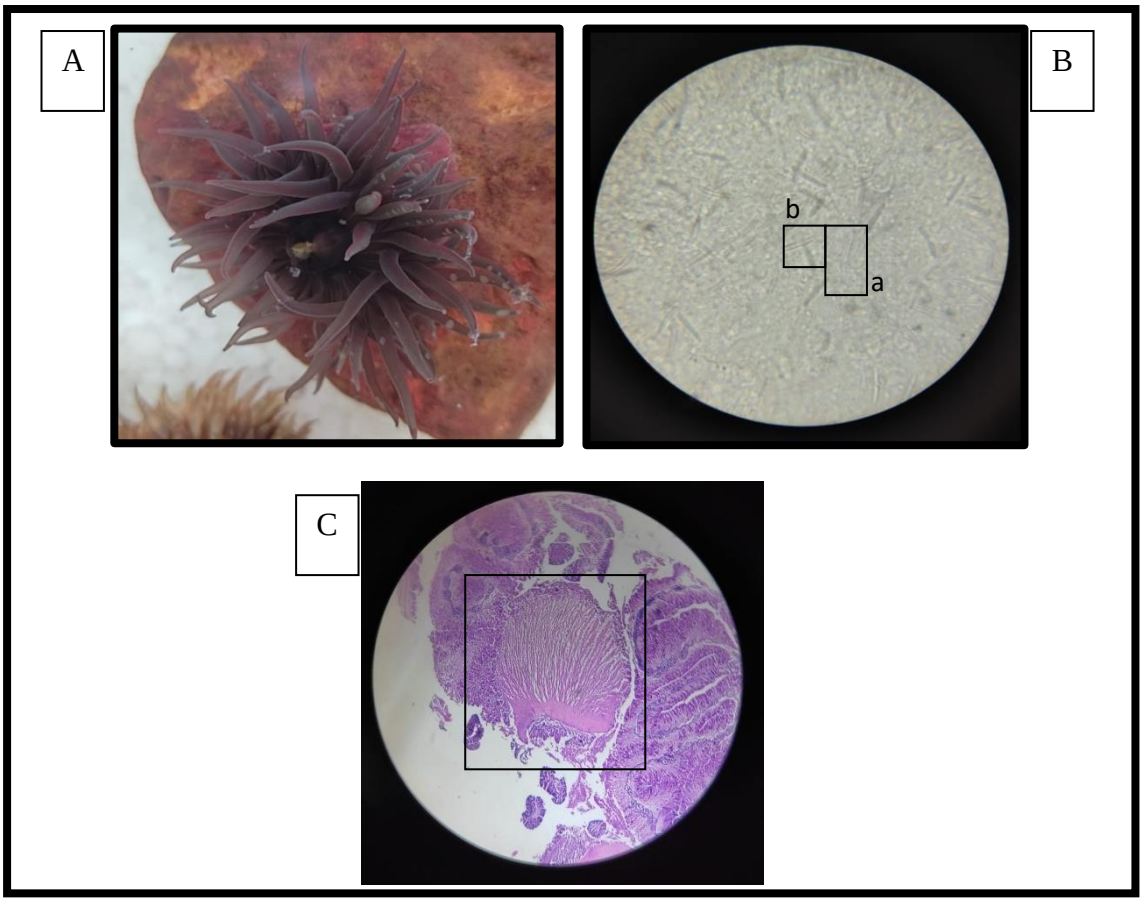


Figure 4.4: (A) *Bunodosoma goanensis* expanded (B) nematocysts cells in the tentacle, a: basitrich; b: siprocyst (C) microtome section under 10X objective, Sphincter

4.2 In-vitro propagation of sea anemones

Through in-vitro propagation methods, sea anemones can be sustainably harnessed for their pharmaceutical properties, enabling the preservation of marine ecosystems while benefiting from their valuable therapeutic potential. In controlled laboratory settings, in-vitro propagation of sea anemones was carried out, wherein the organisms were bisected into two equal halves, each containing essential anatomical components for regeneration. With optimal conditions such as salinity, food availability, and temperature the dissected sea anemones were observed to grow into full individual within 40 days. Two such cycles of in-vitro propagations were carried out.

According to MinasianJr, (1976), in longitudinal fission, the sea anemone stretches transversely to generate two fragments of similar size. Tissue tears along a longitudinal plane parallel, and usually close to the oral-aboral axis. This supports the concept of in-vitro propagation of sea anemone.

4.3 Isolation and Colony characterisation of gut microbiota of sea anemone

Total seven unique isolates were obtained from the gut of *Anthopleura anjunae*.. Four unique isolates were obtained on ZMA plates (before feeding) while three unique isolates were obtained from samples procured on ZMA plates after feeding as seen in Fig. 4.5 (A) and (B). A total of seven unique isolates were obtained from the gut of *A. nigrescens.* Four unique isolates were obtained on ZMA plates (before feeding) while four isolates were obtained after feeding as seen in Fig.4.5 (C) and (D). The colonies were observed for their morphological characteristics as reported in table 4.1.

The 14 isolates obtained showed Gram negative character which is in accordance with the research by Leach & colleagues that discuss about the bacterial community associated with sea anemones. As per literature, these bacteria could belong to seven bacterial classes that is, Gammaproteobacteria, Mollicutes, Betaproteobacteria, Flavobacteria, Alphaproteobacteria, Deltaproteobacteria and Phycisphaerae where all the bacteria has Gram negative character except Millicutes (Leach et al., 2019).

Table 4.1: Colony Characteristics of bacterial isolates before and after feeding from *A. anjunae* and *A. nigrescens*

A. anjunae							
	Before food				**After food**		
Isolate	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 1	Isolate 2	Isolate 3
Colour	Pale Yellow	Yellow	White	White	White	Pale Yellow	White
Margin	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Elevation	Elevated	Elevated	Elevated	Elevated	Elevated	Elevated	Elevated
Opacity	Opaque	Translucent	Opaque	Translucent	Opaque	Opaque	Translucent
Consistency	Lubricious	Lubricious	Lubricious	Lubricious	Lubricious	Lubricious	Lubricious
Gram character	Gram negative rod	Gram negative rod	Gram negative rod	Gram negative rod	Gram negative rod	Gram negative rod	Gram negative rod
A. nigrescens							
	Before food			**After food**			
Isolate	Isolate 1	Isolate 2	Isolate 3	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Colour	Yellow	White	Pale Yellow	Yellow	White	White	Pale Yellow
Margin	Circular	Circular	Circular	Circular	Circular	Irregular	Circular
Elevation	Elevated	Elevated	Elevated	Elevated	Elevated	Elevated	Elevated
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Translucent	Opaque
Consistency	Lubricious	Lubricious	Lubricious	Lubricious	Lubricious	Lubricious	Lubricious
Gram character	Gram negative rod	Gram negative rod	Gram negative rod	Gram negative rod	Gram negative rod	Gram negative rod	Gram negative rod

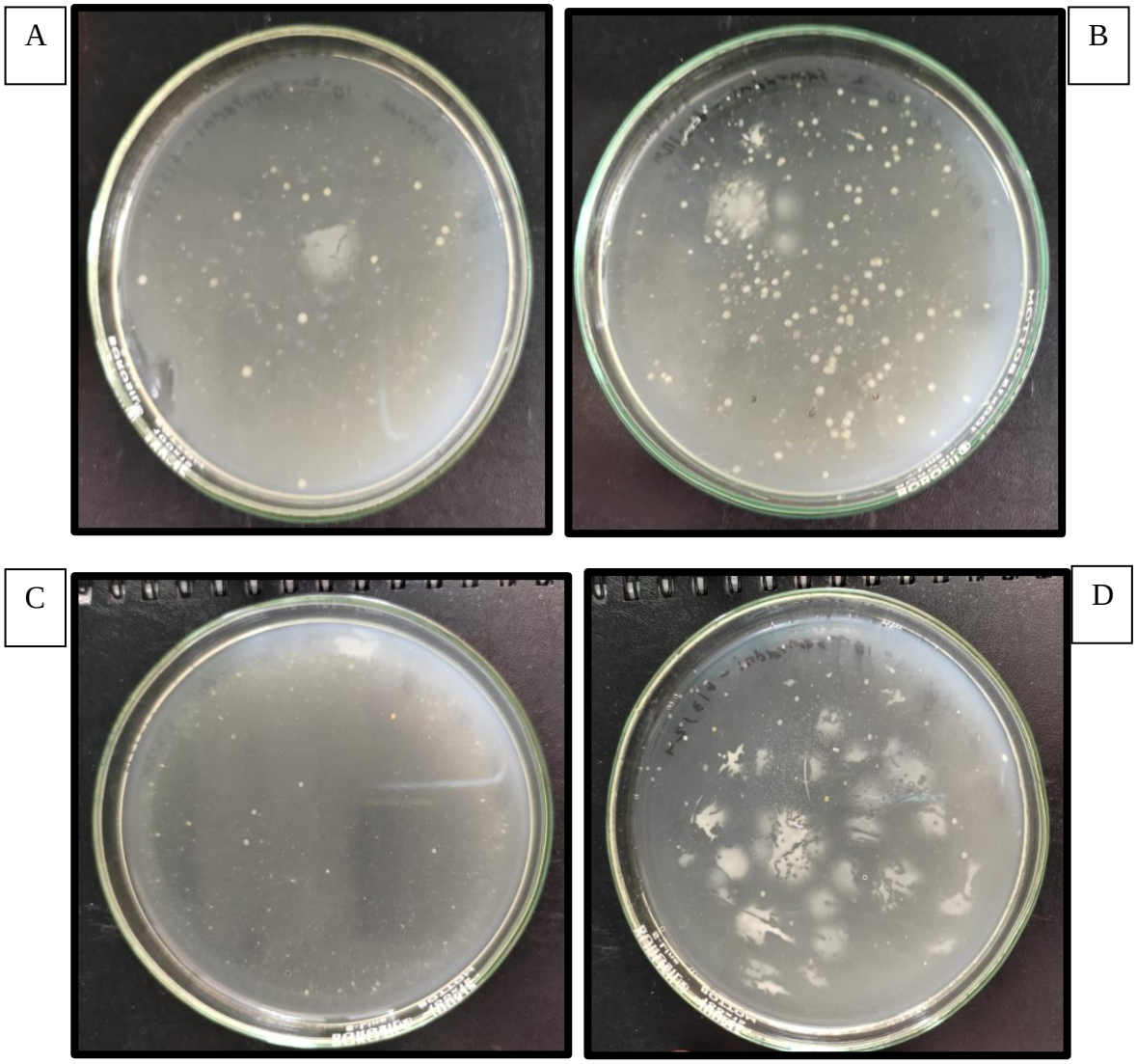


Figure 4.5: Bacterial isolates obtained from the gut of *Anthopleura anjunae:* (A) Before feeding, (B) After feeding

Bacterial isolates obtained from the gut of *Anthopleura nigrescens:* (C) Before feeding, (D) After feeding

4.4 Biochemical analysis of gut microbiota of sea anemone

The results obtained for biochemical test performed using KB001 HiMVIC Biochemical strips are recorded in the table 4.5.

Table 4.2: Biochemical Analysis of the bacterial isolates from *A. anjunae* and *A. nigrescens*

A. anjunae							
Before food					**After food**		
	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 1	Isolate 2	Isolate 3
Indole	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-
Voges Proskauer's	-	-	-	-	-	-	-
Citrate	-	-	-	-	+	-	-
Glucose	+	-	+	-	+	+	-
Adonitol	-	-	+	-	-	-	-
Arbinose	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-
Sorbitol	-	-	+	-	+	-	-
Mannitol	-	-	+	-	+	-	-
Rhamnose	-	-	-	-	+	-	-
Sucrose	+	-	+	-	+	+	-
A. nigrescens							
Before food				**After food**			
	Isolate 1	Isolate 2	Isolate 3	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Indole	-	+	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-
Voges Proskauer's	-	-	-	-	-	-	-
Citrate	-	+	-	+	-	-	-
Glucose	+	+	+	+	+	+	-
Adonitol	-	-	+	-	-	+	-
Arbinose	-	-	+	+	-	+	-
Lactose	-	-	-	+	-	+	-
Sorbitol	+	-	+	-	+	+	-
Mannitol	+	+	-	+	+	+	-
Rhamnose	-	-	-	+	-	+	-
Sucrose	+	+	+	+	+	+	-

4.5 Screening of the crude, methanol and hexane extract and bacterial isolates for pharmaceutical potential

4.5.1 Antioxidant Activity – DPPH Assay

4.5.1.a Crude extract, Methanol extract and Hexane extract

The % scavenging for the crude, methanol and hexane extract of all samples are documented in Table 4.3. As seen in the Fig.4.6 the sea anemone sp. 3 showed significantly more *($p < 0.01$)* DPPH scavenging activity of 53.11 % than sp.2 for the crude extract. For the methanol extract and hexane extract sea anemone sp. 1 showed significantly *($p < 0.01$)* higher scavenging activity of 93.27 % and 23.83 % than sponge respectively. In the study conducted by Choudhury & Raghunathan, (2019), the aqueous extract of sea anemone species *H. magnifica* showed DPPH scavenging activity of 52.6 %. This shows that the crude extract of sea anemone sp. 3 and methanol extract of sea anemone sp. 1 has more scavenging activity compared to *H. magnifica.*

Table 4.3: Antioxidant assay of crude, methanol and hexane extract

Sample	Crude Extract % Scavenging	Methanolic Extract % Scavenging	Hexane Extract % Scavenging
Sponge	$5.88 \pm 0.83^{c,d}$	8.19 ± 0.88^{a}	4.36 ± 0.06^{a}
Horn shell	$5.43 \pm 0.69^{c,d}$	12.02 ± 0.88^{b}	6.67 ± 0.19^{c}
A. anjunae	8.02 ± 0.8^{d}	14.56 ± 0.67^{c}	7.79 ± 0.30^{d}
A. nigrescene	$2.3 \pm 0.97^{a,b}$	13.99 ± 0.88^{c}	6.04 ± 0.22^{c}
B. goanensis	0.81 ± 0.71^{a}	$29.1 \pm .49^{f}$	5.27 ± 0.12^{b}
Sp. 1	$4.35 \pm 1.89^{b,c}$	$\mathbf{93.27 \pm 0.4^{g}}$	$\mathbf{23.83 \pm 0.36^{h}}$
Sp. 2	1.17 ± 0.96^{a}	18.38 ± 0.68^{d}	11.81 ± 0.08^{f}
Sp. 3	$\mathbf{53.11 \pm 0.74^{g}}$	20.43 ± 0.70^{e}	9.97 ± 0.50^{e}
Sp. 4	19.18 ± 1.81^{f}	14.56 ± 0.51^{c}	17.46 ± 0.9^{g}
Sp. 5	8.15 ± 0.78^{d}	21.21 ± 0.80^{e}	$6.01 \pm 0.34^{b,c}$
Sp. 6	12.38 ± 2.02^{e}	14.42 ± 0.73^{c}	8.14 ± 0.26^{d}

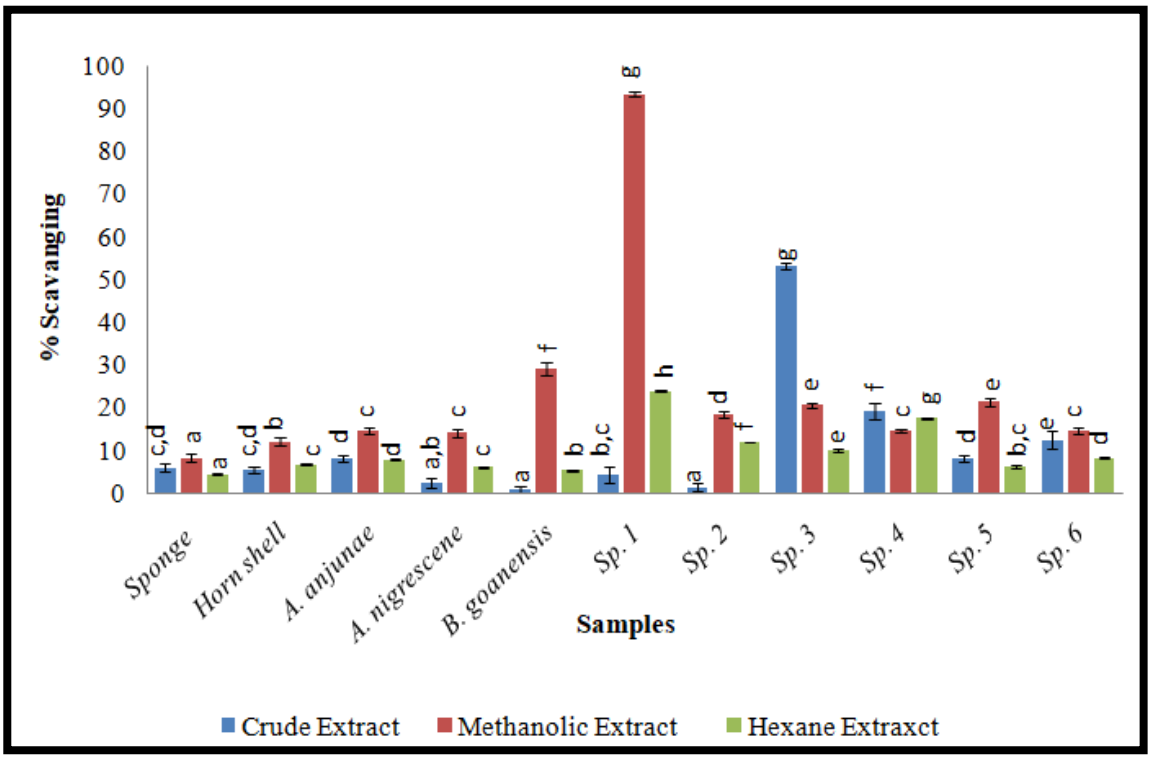


Figure 4.6: Graph depicting antioxidant activity of the crude, methanol and hexane extract

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means are statistically significant (ANOVA; $P < 0.01$ and subsequent post hoc multiple comparison with Duncan's test)

4.5.1.b Bacterial Isolate

The % scavenging for the bacterial isolates are documented in Table 4.4. The bacterial isolate 7 depicted significantly higher DPPH scavenging activity of 46.45 % *($p < 0.01$)* than isolate 4 as seen in the Fig.4.7. According to the study done by Balakrishnan et al., (2014) on associated bacteria with the sponge, *Tedania anhelans* the isolates showed 55 % of radical scavenging. This intends that the isolate 7 obtained from the gut of *A. anjunae* before food has comparatively lesser scavenging activity.

Table 4.4: Antioxidant assay of gut microbiota

Sample	% Scavenging
Isolate 1	$31.88 \pm 1.27^{b,c}$
Isolate 2	$39.83 \pm 1.99^{c,d}$
Isolate 3	$19.25 \pm 1.82^{a,b}$
Isolate 4	17.56 ± 13.06^{a}
Isolate 5	$25.39 \pm 1.11^{a,b}$
Isolate 6	$21.90 \pm 3.41^{a,b}$
Isolate 7	$\mathbf{46.45 \pm 2.35^{d}}$
Isolate 8	$23.22 \pm 2.18^{a,b}$

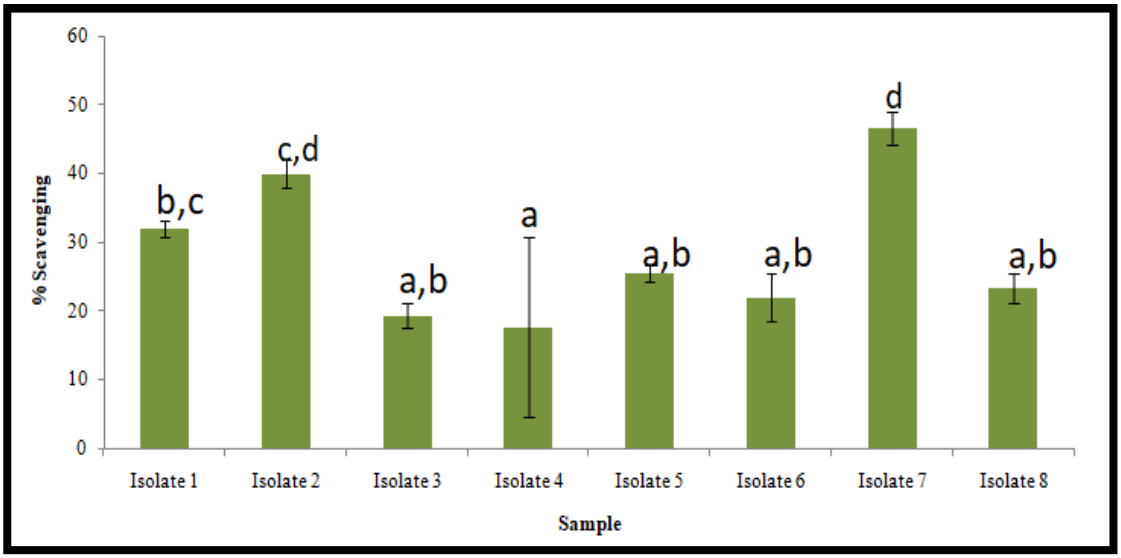


Figure 4.7: Graph depicting antioxidant activity of gut microbiota of *A. nigrescens* and *A. anjunae*

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means are statistically significant (ANOVA; $P < 0.01$ and subsequent post hoc multiple comparison with Duncan's test)

4.5.2 Reducing Power Assay for the Crude extract, Methanol and Hexane extract

The concentration of antioxidant present in the crude, methanol and hexane extract of all samples are documented in Table 4.5. As depicted in the Fig.4.8, Golf Ball sponge showed significantly higher reducing power *($p < 0.01$)* of 9.3 µg/mL than sea anemone sp. 5 for the crude extract. For the methanol extract and hexane extract *Bunodosoma goanensis* and sea anemone sp. 6 showed significantly higher *($p < 0.01$)* reducing power of 14.38 µg/mL and 0.40 µg/mL than sea anemone sp.3 respectively.

The study by Thangaraj et al., (2018) reported reducing power of 0.27 µg/mL for 1 mg of crude extract of sea anemone .*H. aurora*. This shows that the reducing power of the Golf Ball sponge crude extract, methanol extract of *Bunodosoma goanensis* and hexane extract of sea anemone sp. 6 is significantly high when compared with sea anemone *H. aurora.*

Table 4.5: Reducing Power of the Crude extract, Methanol extract and Hexane extract

Sample	Crude Extract (µg/mL)	Methanolic Extract (µg/mL)	Hexane Extract (µg/mL)
Sponge	**9.13 ± 0.04**[a]	5.49 ± 0.05[a]	0.01 ± 0.03[a]
Horn shell	1.77 ± 0.06[a]	4.39 ± 0.04[b]	0.00 ± 0.01[a]
A. anjunae	0.84 ± 0.05[a]	8.82 ± 0.04[c]	0.05 ± 0.05[a]
A. nigrescene	0.83 ± 0.04[b]	7.19 ± 0.07[d]	0.05 ± 0.04[a]
B. goanensis	0.62 ± 0.03[b]	**14.38 ± 0.03**[e]	0.00 ± 0.01[a]
Sp. 1	0.96 ± 0.05[c]	11.45 ± 0.04[f]	0.01 ± 0.03[a]
Sp. 2	0.74 ± 0.06[c]	11.03 ± 0.05[g]	0.06 ± 0.03[a]
Sp. 3	0.35 ± 0.03[c]	1.26 ± 0.05[h]	0.01 ± 0.04[a]
Sp. 4	0.57 ± 0.03[d]	9.32 ± 0.07[i]	0.04 ± 0.03[a]
Sp. 5	0.34 ± 0.04[e]	14.19 ± 0.05[i,j]	0.14 ± 0.03[b]
Sp. 6	0.24 ± 0.06[f]	14.30 ± 0.04[j]	**0.40 ± 0.05**[c]

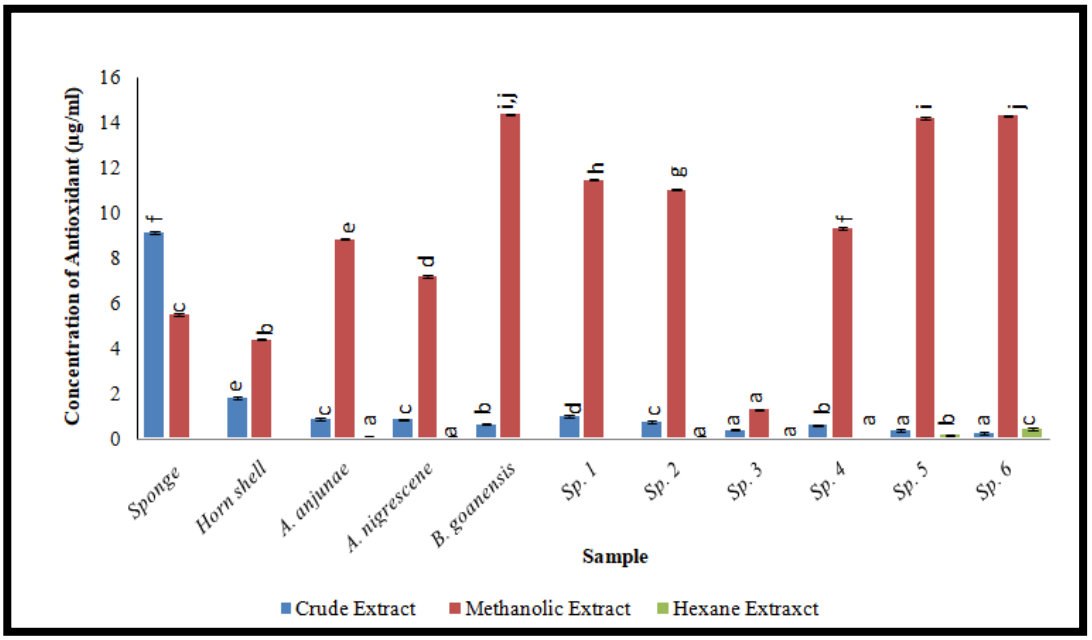


Figure 4.8: Graph depicting Reducing Power of the Crude, Methanol and Hexane extract

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means are statistically significant (ANOVA; $P < 0.01$ and subsequent post hoc multiple comparison with Duncan's test)

4.5.3 Anti-diabetic Assay – (alpha- amylase inhibition assay)

4.5.3.a Crude extract, Methanol and Hexane extract

The % inhibition of alpha amylase by the crude, methanol and hexane extract of all samples is documented in Table 4.6. The Fig.4.9 indicates the sea anemone sp. 5 cause significant *($p < 0.01$)* inhibition of alpha amylase of 25.18 % than the Golf Ball sponge for the crude extract. For the methanol extract sea anemone sp. 2 showed significant *($p < 0.01$)* inhibition of alpha amylase of 22.40 %. There was no anti-diabetic property seen in hexane extract indicating no separation of compound with anti-diabetic property. In the study conducted by Tamrakar et al., (2007), the methanol extract of soft coral *Sinularia firma and Sinularia erecta* reported blood glucose lowering effect of 14.5 % and 16.1% respectively. This shows that the crude extract of sea anemone sp. 5 and methanol extract of sea anemone sp. 2 has more alpha amylase inhibition activity compared to *Sinularia firma*

Table 4.6: Anti-diabetic assay of Crude extract and Methanol extract

Sample	Crude Extract (% Inhibition of amylase)	Methanolic Extract (% Inhibition of amylase)
Sponge	0.24 ± 0.0^{a}	7.97 ± 0.08^{a}
Horn shell	0.76 ± 0.07^{a}	0.08 ± 0.14^{a}
A. anjunae	$1.72 \pm 0.72^{a,b}$	0.08 ± 0.07^{a}
4 *A. nigrescene*	0.28 ± 0.8^{b}	20.16 ± 0.30^{a}
. *B. goanensis*	5.06 ± 0.24^{b}	0.08 ± 0.14^{a}
Sp. 1	6.71 ± 0.35^{c}	0.04 ± 0.07^{a}
5 **Sp. 2**	3.01 ± 0.67^{c}	**22.40 ± 0.30^{b}**
. Sp. 3	2.89 ± 0.4^{d}	0.04 ± 0.07^{c}
Sp. 4	1.72 ± 0.69^{e}	0.04 ± 0.07^{d}
3 **Sp. 5**	**25.81 ± 0.61^{f}**	0.52 ± 0.14^{e}
. Sp. 6	21.10 ± 0.48^{g}	4.97 ± 0.07^{f}

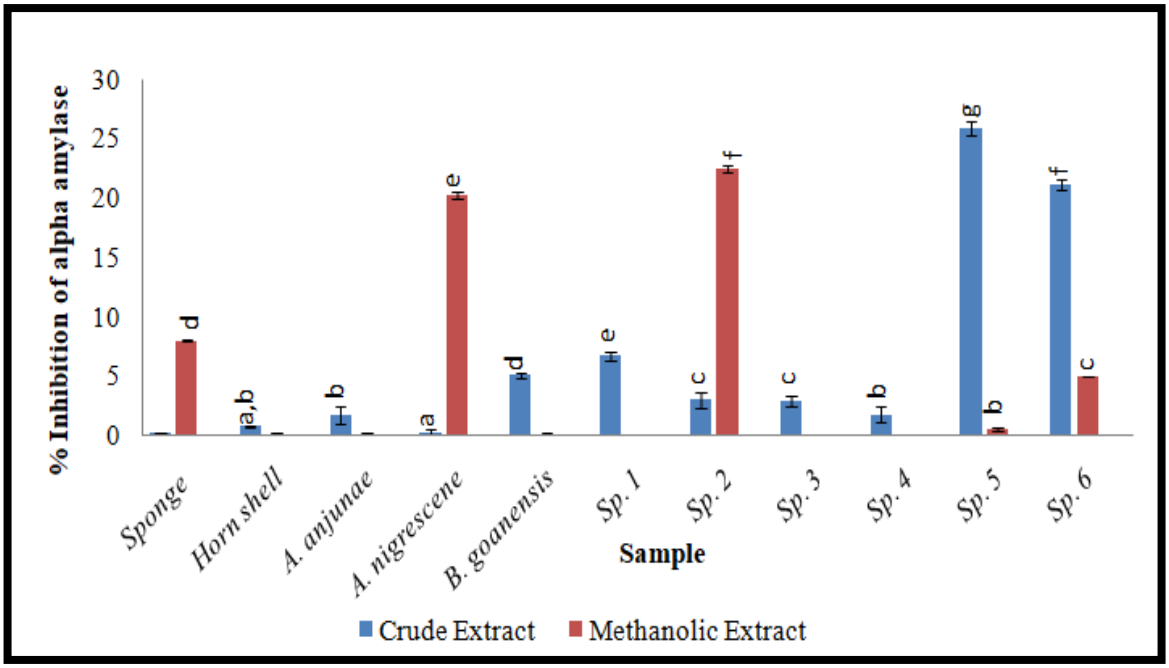


Figure 4.9: Graph depicting Anti-diabetic activity of the Crude and Methanol extract

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means are statistically significant (ANOVA; $P < 0.01$ and subsequent post hoc multiple comparison with Duncan's test)

4.5.3.b Bacterial Isolate

No significant alpha amylase inhibition was seen in the bacterial isolates from the gut of *A. anjunae* and *A. nigrescens* indicating no potential as anti-diabetic pharmaceutical. According to the Ansarizadeh A. et al., (2022), the metabolites isolated from the associated bacteria with the sponge *Haliclona* sp. and *Niphates* sp showed 3.46 % and 3.84 % alpha amylase inhibition.

4.5.4 Anti-inflammatory Assay- (Inhibition of albumin denaturation)

4.5.4.a Crude extract, Methanol and Hexane extract

The % inhibition of denaturation of albumin by the crude, methanol and hexane extract of all samples is documented in Table 4.7. The Fig.410 indicates sea anemone sp. 4 exhibit significantly higher *($p < 0.01$)* inhibition of albumin denaturation of 46.19 % than sp. 5 for the crude extract. For the methanol extract sea anemone sp. 4 was reported for significant *($p < 0.01$)* inhibition of albumin denaturation of 23.88 %. There was no anti-inflammatory property seen in hexane extract indicating no separation of compounds with anti-inflammatory property. In the study conducted by Fristiohady et al., (2019), crude extract of marine sponge *Callyspongia* sp. reported inhibition of albumin denaturation by 55 % . This shows that the crude extract of sea anemone sp. 4 and methanol extract of sea anemone sp. 4 has lesser Anti-inflammatory activity compared to marine sponge *Callyspongia* sp.

Table 4.7: Anti-inflammatory assay of Crude extract and Methanol extract

Sample	Crude Extract (% Inhibition)	Methanolic Extract (% Inhibition)
Sponge	N.D	N.D
Horn shell	N.D	N.D
A. anjunae	$17.37 \pm .16^{a}$	9.26 ± 0.0^{a}
A. nigrescene	23.10 ± 0.58^{a}	11.84 ± 0.0^{a}
B. goanensis	19.95 ± 2.27^{b}	11.11 ± 1.07^{a}
Sp. 1	21.87 ± 3.05^{c}	12.46 ± 0.0^{a}
Sp. 2	N.D	N.D
Sp. 3	N.D	N.D
Sp. 4	$\mathbf{46.19 \pm 1.57^{d}}$	$\mathbf{23.88 \pm 0.75^{d}}$
Sp. 5	7.08 ± 0.74^{e}	3.91 ± 0.87^{e}
Sp. 6	37.53 ± 1.18^{f}	19.36 ± 0.74^{f}

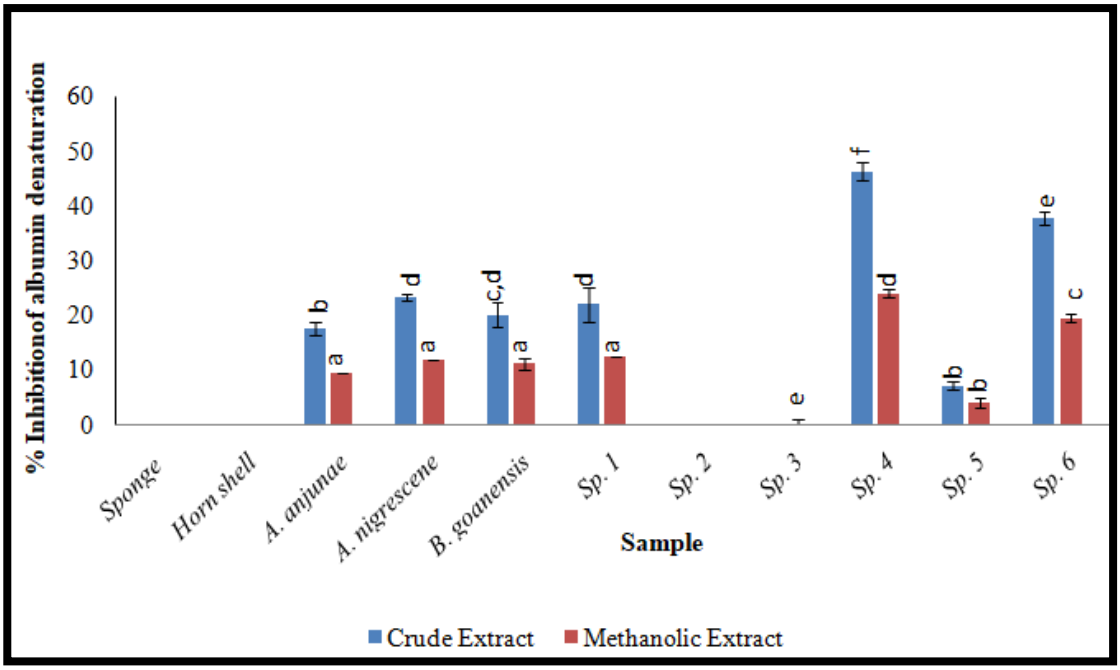


Figure 4.10: Graph depicting Anti-inflammatory activity of Crude and Methanol extract

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means are statistically significant (ANOVA; $P < 0.01$ and subsequent post hoc multiple comparison with Duncan's test)

4.5.4.b Bacterial Isolate

No significant inhibition of albumin denaturation was seen in the bacterial isolates from the gut of *A. anjunae* and *A. nigrescens* indicating no potential as anti-inflammatory pharmaceutical. According to (Lee et al., 2024) two compounds isolated from the sea anemone-derived fungus *Arthrinium arundinis* MA30 showed 71 % and 81 % anti-inflammatory activity.

4.5.5 Anti-microbial Assay – by Agar Well Diffusion Method

4.5.5.a Methanol and Hexane extract

There was no formation of clearance zone for methanol and hexane extract indicating absence of anti-microbial activities. The reason for this could be failure of separation of anti-microbial compound from the crude in both the phases. The study by Borbón et al., (2016) has reported that the acetone and diethyl ether extracts of *A. nigrescens* has significant anti microbial activity against *Proteus vulgaris* and *Pseudomonas aerugenosa* then the methanol extract.

4.5.5.b Bacterial Isolate

The zone of clearance formed were negligible hence, there is need to screen the isolates from the gut of sea anemone against more pathogens.

4.5.6 Coagulation Assay

The crude extract of species 6 and species 4 of sea anemones were observed to show agglutination in all the three blood groups used within 1 minute of adding the extract. This indicates that the crude extract of sp.6 and sp.4 has a compound that acts like clotting factor. According to the study by Quintal et al., (2023), high flavonoid concentrations contribute to anticoagulant properties of compound. The crude extract of sp.6 and sp.4 is reported to have fewer flavonoid compound as seen in Table 4.8. The lesser concentration of these antithrombotic compounds could be one of the reasons for blood clotting, especially when combined with other factors that promote blood clotting. As per the study by Reis et al., (2023), diabetes has marked effect of the transport of phenolic compound by VLDL resulting in decreased concentration of phenolic compound. This consequently

decreases the antioxidant capacity. In such cases, clotting factors that function better in the absence of phenolic compounds become promising as wound healing drug for the diabetic patients. The crude extract of other samples did not show any blood clotting properties. A study by Wang et al., (2021) reported sponging material, SFM from marine sponge *Spongiaofficinalis* for its hemostatic property. It was observed to have hemostatic time of 3 minutes for common carotid artery injury in rabbit model.

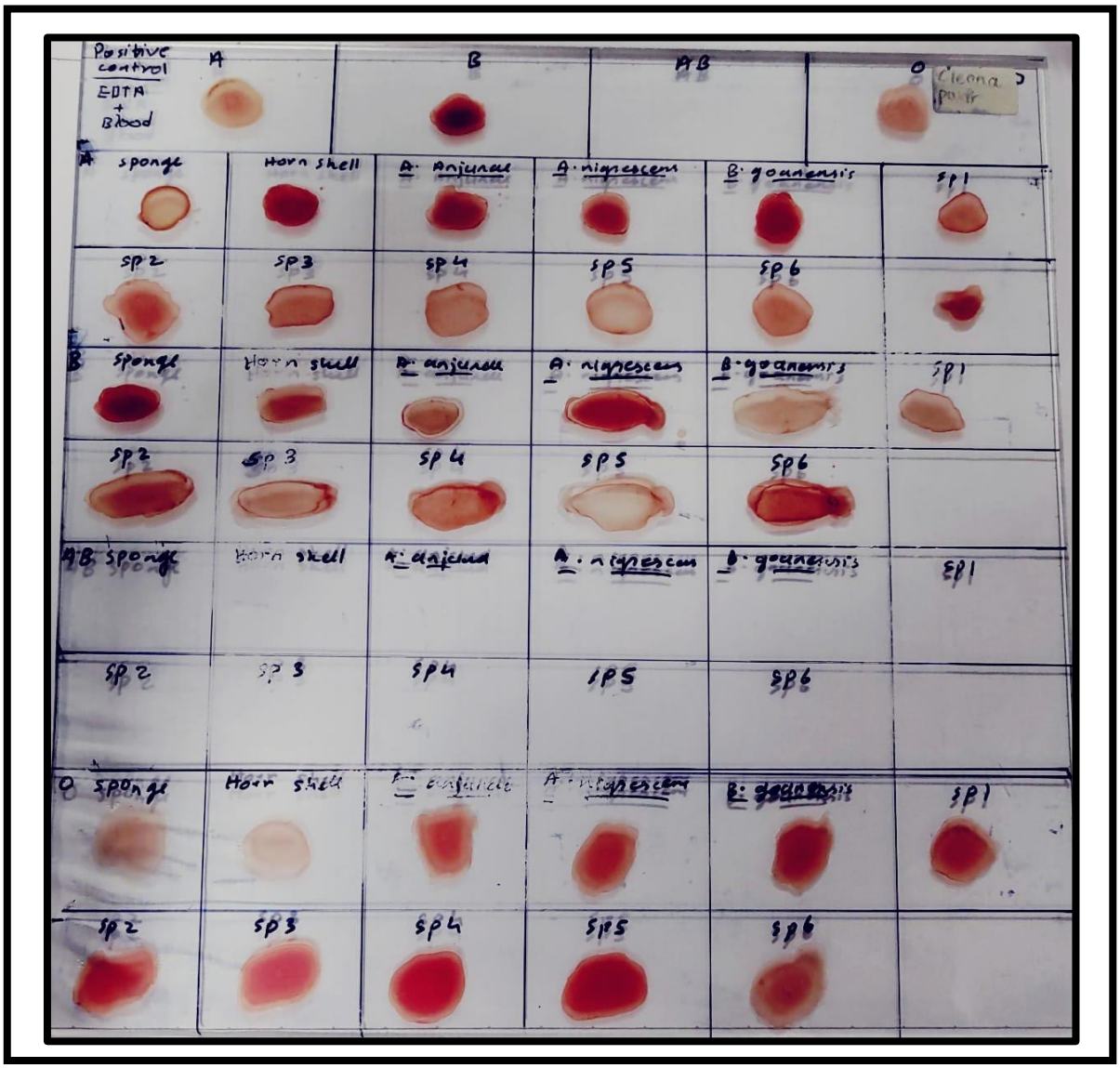


Figure 4.11: Coagulation assay of crude extracts performed for blood group A+, B+ and O-

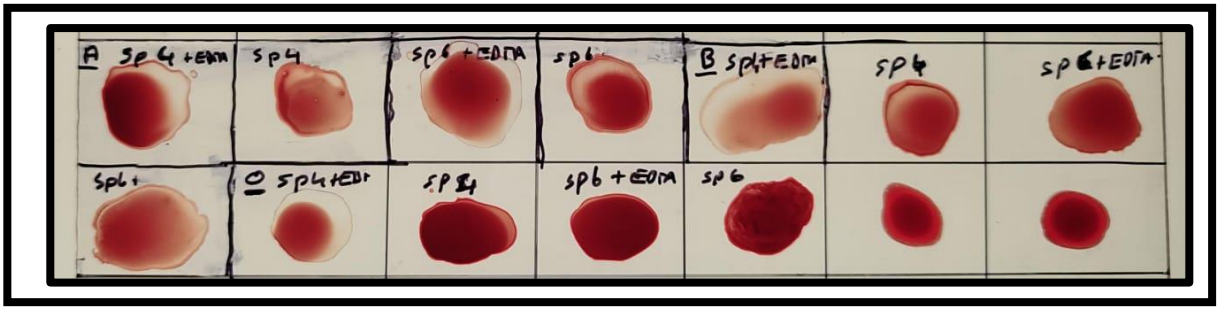


Figure 4.12: Confirmatory test for presence of clotting factor in crude extract of sp.4 and sp. 6 performed for blood group A+, B+ and O-

4.5.7 Anti-coagulation Assay

The crude extract of *A. anjunae* and species 2 were observed to show anti-coagulant activity in all the four blood groups used. This indicates that the crude extract of sp.2 and *A. anjunae* has a compound that acts like an anti-coagulant. A study by Gomez-Guzman et al., (2018) has reported that polyphenolic compounds like flavonoids have antithrombotic properties along with antioxidant properties. During oxidative stress there is generation of free radicals that damage the endothelial cells lining blood vessels. The damage promotes activation of clotting factors leading to thrombosis. Antioxidants counteract oxidative stress thereby reducing endothelial damage and inhibiting thrombosis. The methanolic extract of sp.2 is noted to have highest phenol content of 2.32 µg/mL and *A. anjunae* is seen to have high flavonoid content of 380.12 µg/mL as depicted in Table 4.8 and 4.9. This indicates that the sp.2 and *A. anjunae* possess anti-coagulant properties along with antioxidant activity conferred mainly by the phenol and flavonoid content. A study by Martin, (1966), has reported presence of anticoagulant in the sea anemone species, *Rhodactishowesii*, that cause 10 times more prolongation of coagulation time compared with heparin. The crude extract of other samples did not show any anticoagulant properties.

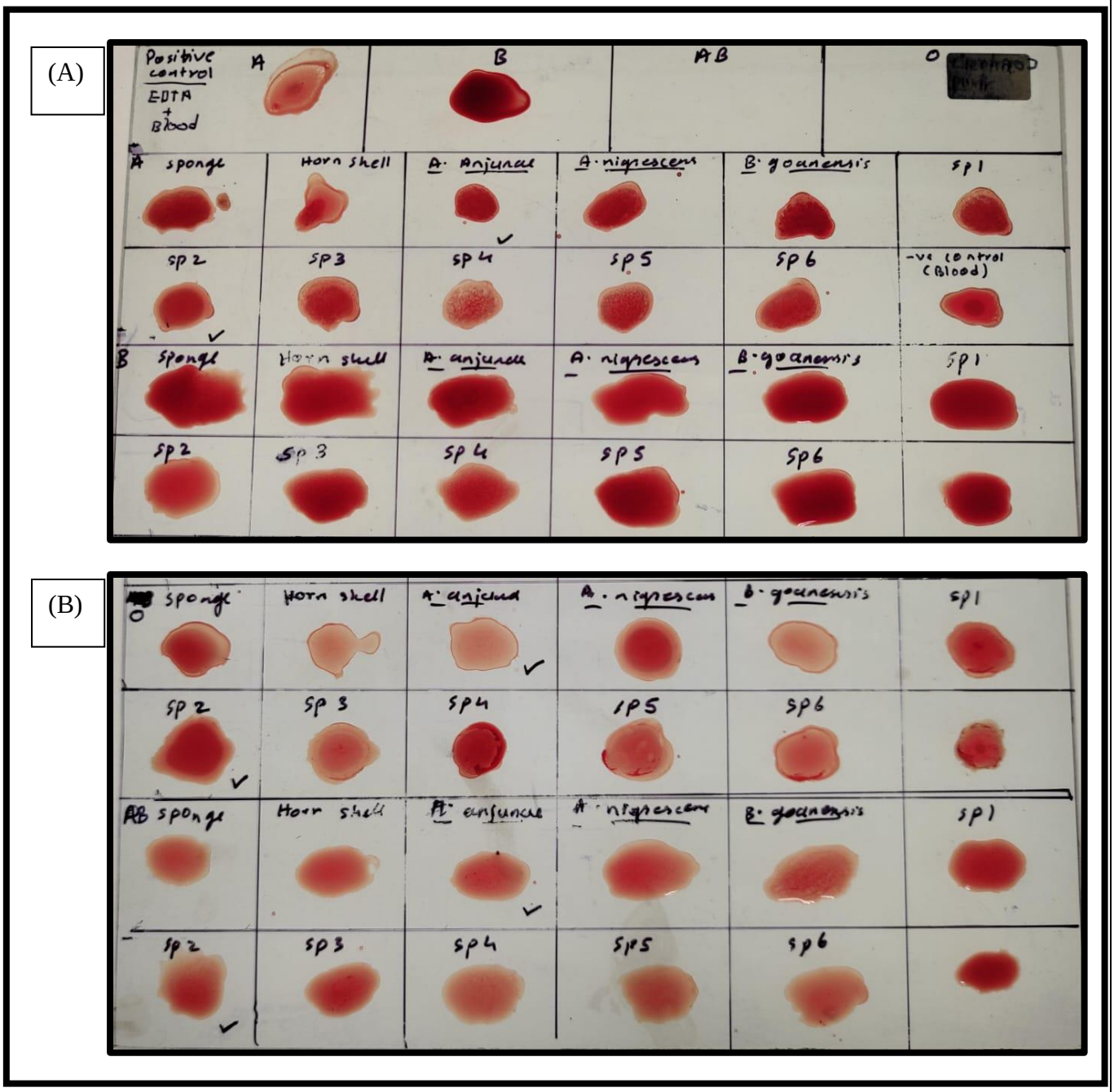


Figure 4.13: Anticoagulant assay of crude extracts performed for blood group (A) A+, B+ and (B) O+ and AB-

4.6 Partial characterisation of selective compound

4.6.1 Flavonoid Assay

4.6.1.a Crude extract, Methanol extract and Hexane extract

The concentration of flavonoids present in the crude, methanol and hexane extract of all samples are documented in Table 4.8. Sea anemone sp. 4 showed significantly higher flavonoid content *($p < 0.01$)* of 21.65 µg/mL than *A. nigrescens* for the crude extract as seen in Fig. 4.14. For the methanol extract and hexane extract sea anemone species 6 and species 4 showed significantly higher flavonoid content of 396.79 µg/mL and 86.46 µg/mL than sp. 2 and sp.5 respectively.

The study by (Rhandour et al., 2016) showed flavonoid content of 5.48 µg/mL for 1mg of crude extract of marine sponge *Iricinia spinulosa*. This shows that the flavonoid content in methanol extract of sea anemone sp.6 is significantly higher when compared to marine sponge *Iricinia spinulosa*. The hexane extract showed significantly lesser concentrations of flavonoid the reason being ineffeciant separation of compounds into non polar phase like hexne.

Table 4.8: Flavonoid content in Crude, Methanol and Hexane extract

Sample	Crude Extract (µg/mL)	Methanolic Extract (µg/mL)	Hexane Extraxct (µg/mL)
Sponge	8.10 ± 1.12^{c,d}	252.88 ±1.12^{f}	N.D
Horn shell	5.86 ±0.75^{b,c}	222.16 ±1.51^{d}	62.95 ±0.75^{g}
A. anjunae	15.68 ±1.31^{e}	380.12 ±9.87^{i}	4.24 ±0.94^{c}
A. nigrescene	1.50 ±0.57^{a}	122.28 ±1.12^{b}	6.48 ±0.57^{d}
B. goanensis	8.97 ±1.31^{d}	325.15 ±0.94^{h}	6.85 ±0.94^{d}
Sp. 1	6.48 ±0.94^{b,c}	234.72 ±0.57^{e}	37.82 ±0.94^{f}
Sp. 2	17.18 ±1.31^{e,f}	65.19 ±1.12^{a}	22.28 ±0.75^{e}
Sp. 3	5.11 ±0.99^{b}	214.20 ±0.94^{c}	2.13 ±0.75^{b}
Sp. 4	**21.65 ±1.31^{g}**	208.35 ±1.14^{c}	**86.46 ±1.12^{h}**
Sp. 5	N.D	305.62 ±0.94^{g}	0.51 ±0.94^{a,b}
Sp. 6	18.42 ±0.78^{f}	**396.79 ±3.34^{j}**	4.49 ±1.31^{c}

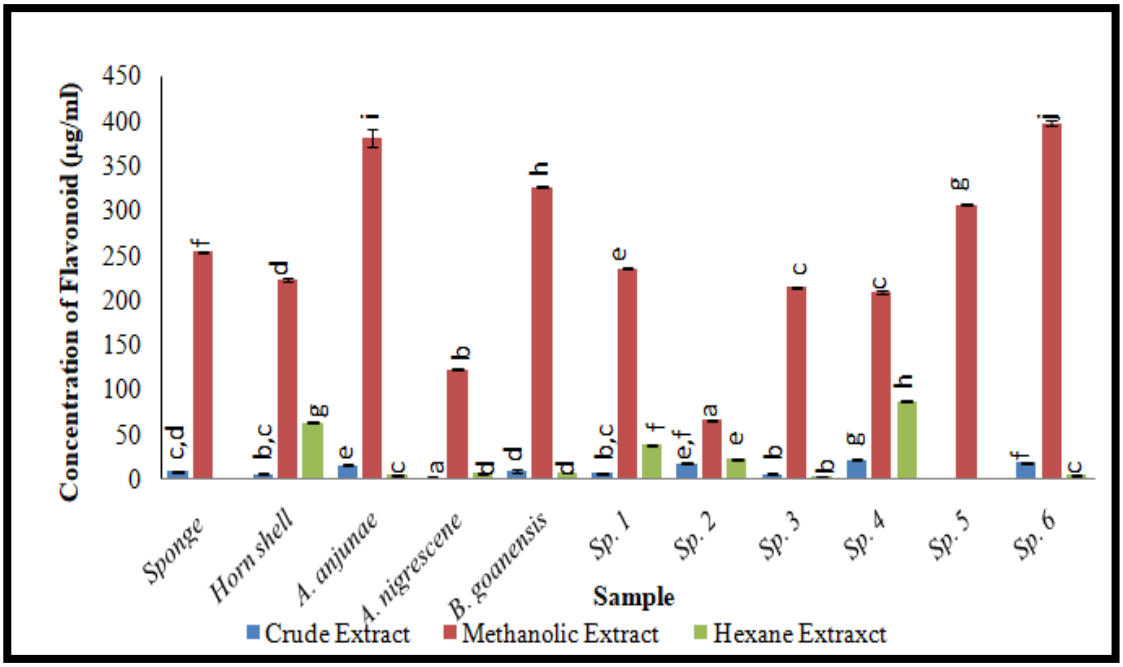


Figure 4.14: Graph depicting Flavonoid content in the Crude, Methanol and Hexane extract

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means are statistically significant (ANOVA; $P < 0.01$ and subsequent post hoc multiple comparison with Duncan's test)

4.6.2 Phenol Assay

4.6.2.a Crude extract, Methanol extract and Hexane extract

The concentration of phenol present in the crude, methanol and hexane extract of all samples are documented in Table 4.9. Golf Ball sponge showed significantly higher phenol content *(p < 0.01)* of 16.87 µg/mL than *A. anjunae* for the crude extract as seen in Fig. 4.15. For the methanol extract and hexane extract sea anemone sp. 2 showed significantly higher phenol.content *(p < 0.01)* of 2.32 µg/mL and 1.19 µg/mL respectively. The study by Rosyantari et al., (2021) reported phenol content of 15.26 mg/mL for 1 mg of crude extract of marine sponge *Stylissa flabelliformis*. This shows that the phenol content in the Golf Ball sponge crude extract is significantly less when compared with marine sponge *Stylissa flabelliformis*

Table 4.9: Phenol content in Crude extract, Methanol extract and Hexane extract

Sample	Crude Extract (µg/mL)	Methanolic Extract (µg/mL)	Hexane Extraxct (µg/mL)
Sponge	**16.87 ± 0.04**^{d}	N.D	N.D
Horn shell	4.68 ± 0.09^{b}	N.D	N.D
A. anjunae	0.03 ± 0.10^{a}	0.44 ± 0.07^{b}	N.D
A. nigrescene	0.10 ± 0.06^{a}	N.D	N.D
B. goanensis	0.01 ± 0.04^{a}	N.D	N.D
Sp. 1	N.D	0.91 ± 0.07^{c}	N.D
Sp. 2	0.06 ± 0.07^{a}	**2.32 ± 0.07**^{d}	**1.19 ± 1.12**^{b}
Sp. 3	7.86 ± 0.10^{c}	N.D	N.D
Sp. 4	0.16 ± 0.08^{a}	N.D	N.D
Sp. 5	0.12 ± 0.11^{a}	N.D	N.D
Sp. 6	N.D	N.D	N.D

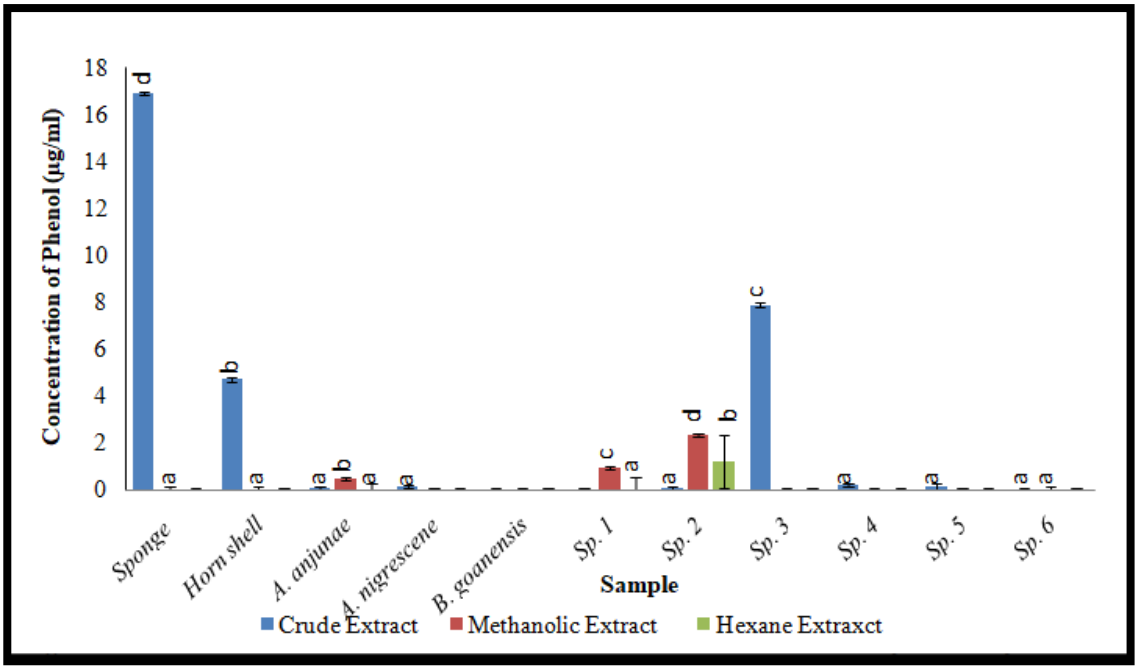


Figure 4.15: Graph depicting Phenol content in the Crude, Methanol and Hexane extract

Each value is the mean ± SD of three replicates (n = 3). Within each bar, means are statistically significant (ANOVA; $P < 0.01$ and subsequent post hoc multiple comparison with Duncan's test)

4.6.3 Thin Layer Chromatography (TLC)

The TLC plate was observed to have two distinct spots for the methanol extract of sea anemone species, *B. goanensis* as depicted in Fig. 4.16. The Rf value of spot 1 and spot 2 were calculated to be 0.62 and 0.72 respectively. The other spots observed for other methanol extracts on the silica plate were not well defined. Various solvent system were tried by using solvents like methanol, chloroform, hexane, butanol, ethanol, ethyl acetate, ethanol and water in different ratios. This suggests that experimenting with different solvent system is necessary to obtain better results to pool the sample together for NMR. In a study conducted by Borbon et al., (2016), the crude extract of *A. nigrescens* is reported to show

presence of amino acids visualised as distinct spots by ninhydrin reaction in thin layer chromatography.

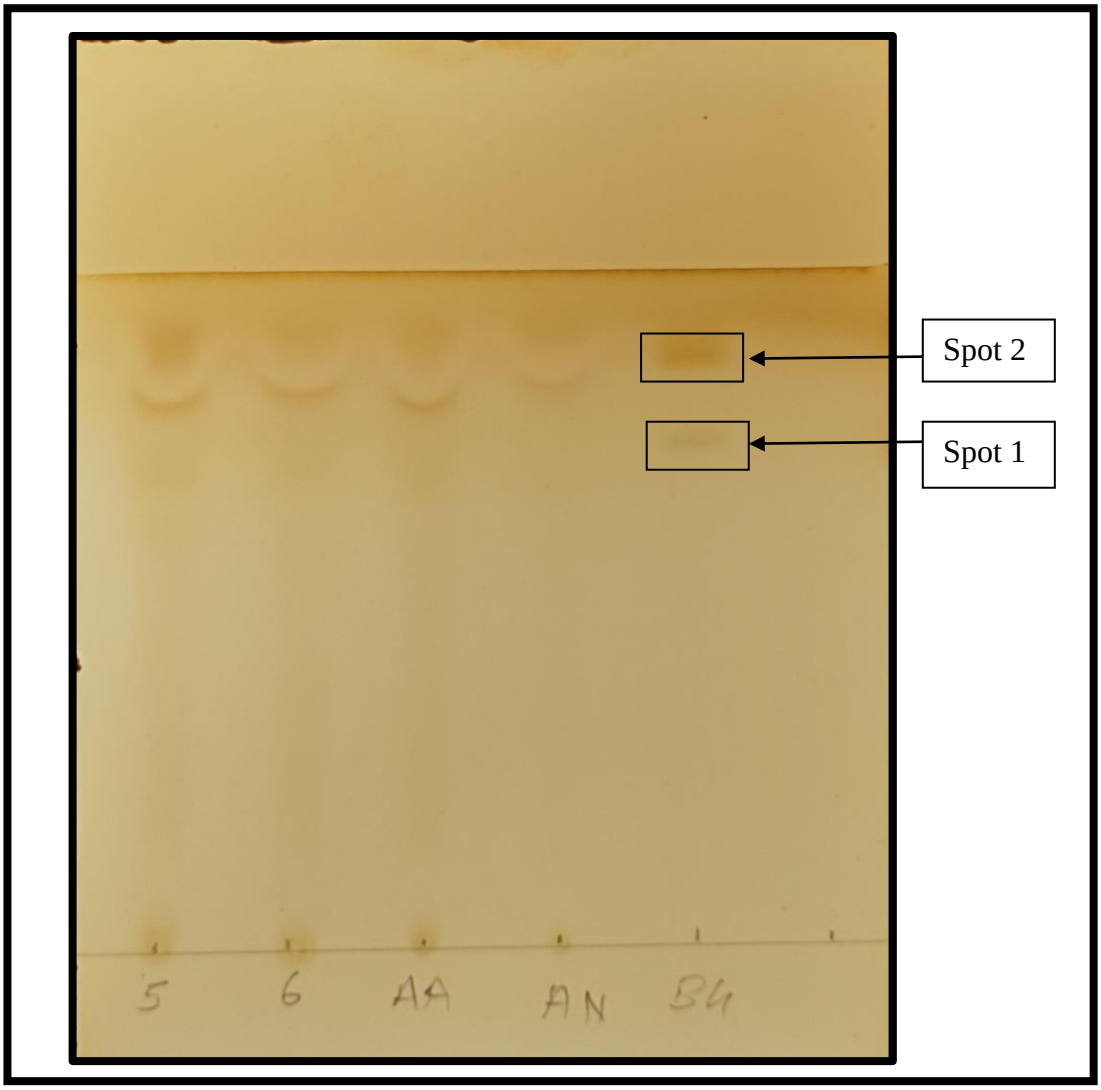


Figure 4.16: Silica plate observed for two distinct spots for methanol extract of *B. goanensis*

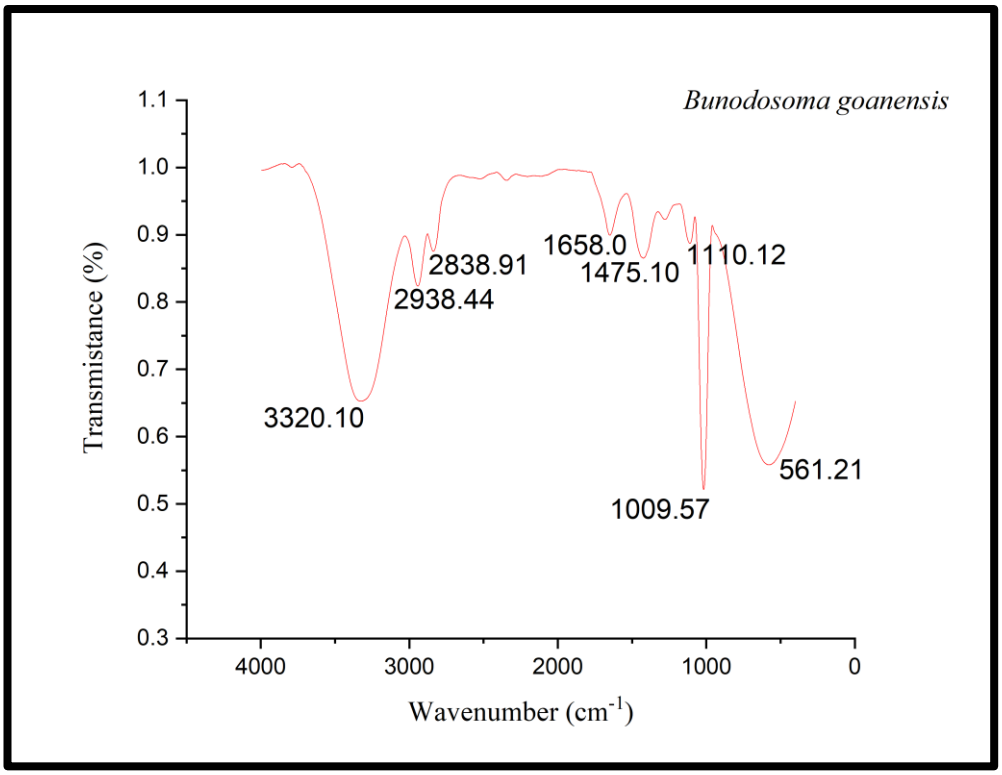
4.6.4 FT-IR

The FTIR data was analysed using OriginPro 2024 software as seen in Fig. 4.17. The wave number values estimated from graph were compared with FT-IR database, (https://instanano.com/all/characterization/ftir/ftir-functional-group-search/) to obtain information about the functional group present in the samples.

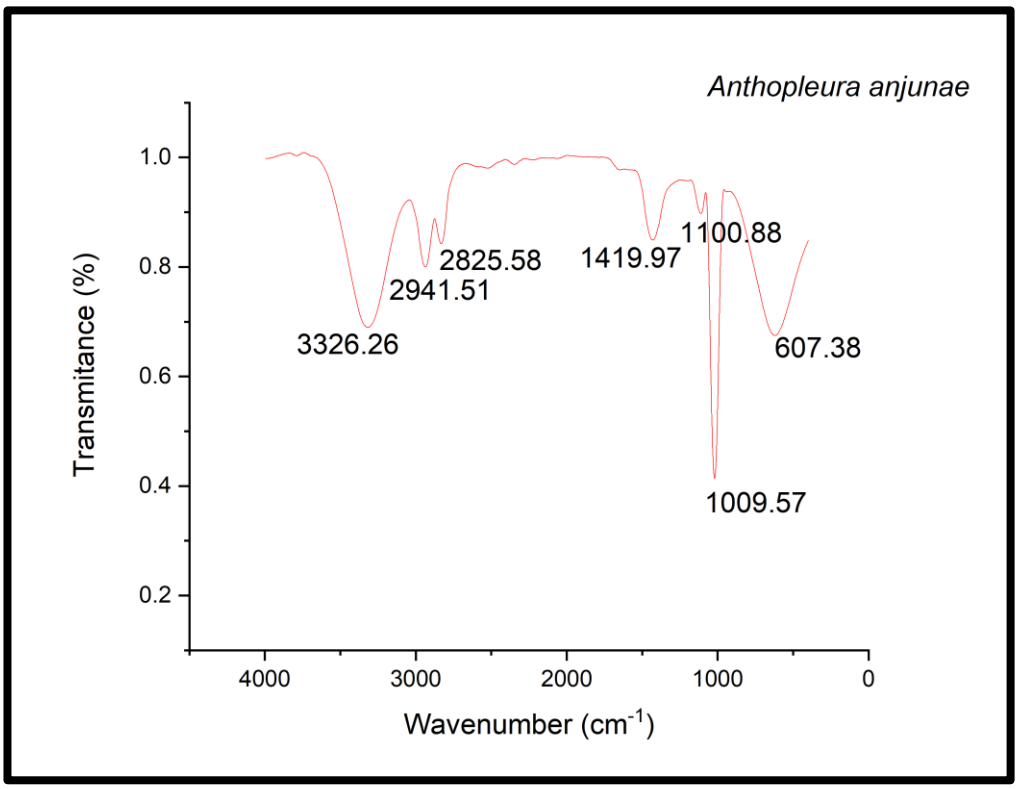
The study by Thangaraj et al., (2018) reported the presence of functional groups like secondary amine, alkane, halo compound and aliphatic primary amine in the crude extract of sea anemone, *H. aurora*. The FT-IR data analysis for methanol extract of *B. goanensis, A. anjunae*, sp.1, sp.2 and sp.4 showed presence of similar functional groups like N-H, C-H, C=C, C-O, C-F, C-Br, C-I and O-H as resported in the Table 4.10. The nature of these functional groups were found to be secondary amine, alkane, amine salt, alkene, secondary alcohol, fluoro compound, halo compound, aliphatic primary amine and carboxylic acid.

Table 4.10: FT-IR analysis of methanol extract of *Bunodosoma goanensis*, *Anthopleura anjunae*, sp. 1, sp. 2 and sp. 4

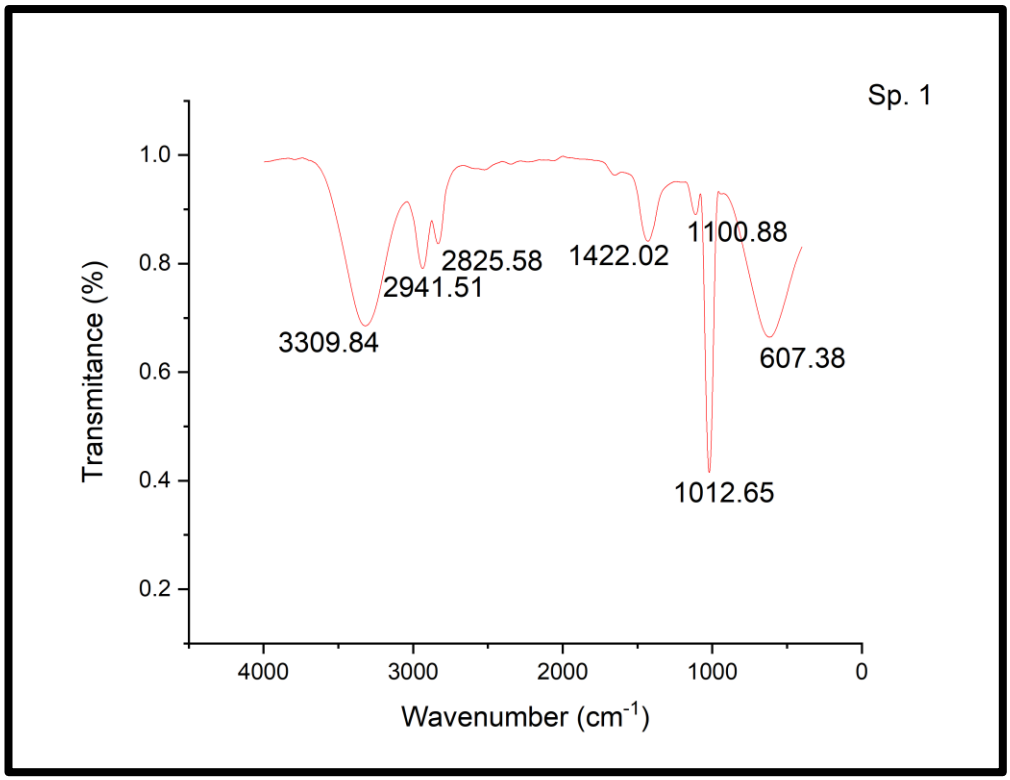
Sample	Wavenumber (cm^{-1})	Functional group	Nature
Bunodosoma goanensis	3320.10	N-H	Secondary amine
	2938.44	C-H	Alkane
	2838.91	N-H	Amine salt
	1658.0	C=C	Alkene
	1475.10	C-H	Alkane
	1110.12	C-O	Secondary alcohol
	1009.57	C-F	Fluoro compound
	561.21	C-I	Halo compound
Anthopleura anjunae	3326.26	N-H	Secondary amine
	2941.51	C-H	Alkane
	2825.58	N-H	Amine salt
	1419.97	O-H	Alcohol
	1100.88	C-O	Secondary alcohol
	1009.57	C-F	Fluoro compound
	607.38	C-Br	Halo compound
Sp. 1	3309.84	N-H	Aliphatic primary amine
	2941.51	C-H	Alkane
	2825.58	N-H	Amine salt
	1422.02	O-H	Carboxylic acid
	1100.88	C-O	Secondary alcohol
	1012.65	C-F	Fluoro compound
	607.38	C-Br	Halo compound
Sp. 2	3317.03	N-H	Secondary amine
	2941.51	C-H	Alkane
	2825.58	N-H	Amine salt
	1422.02	O-H	Carboxylic acid
	1100.88	C-O	Secondary alcohol
	1012.65	C-F	Fluoro compound
	607.38	C-Br	Halo compound
Sp. 4	3320.10	N-H	Secondary amine
	2941.51	C-H	Alkane
	2825.58	N-H	Amine salt
	1422.02	O-H	Carboxylic acid
	1100.88	C-O	Secondary alcohol
	1012.65	C-F	Fluoro compound
	624.82	C-Br	Halo compound


Bunodosoma goanensis
Transmistance (%)
Wavenumber (cm-1)
3320.10
2938.44
2838.91
1658.0
1475.10
1110.12
1009.57
561.21

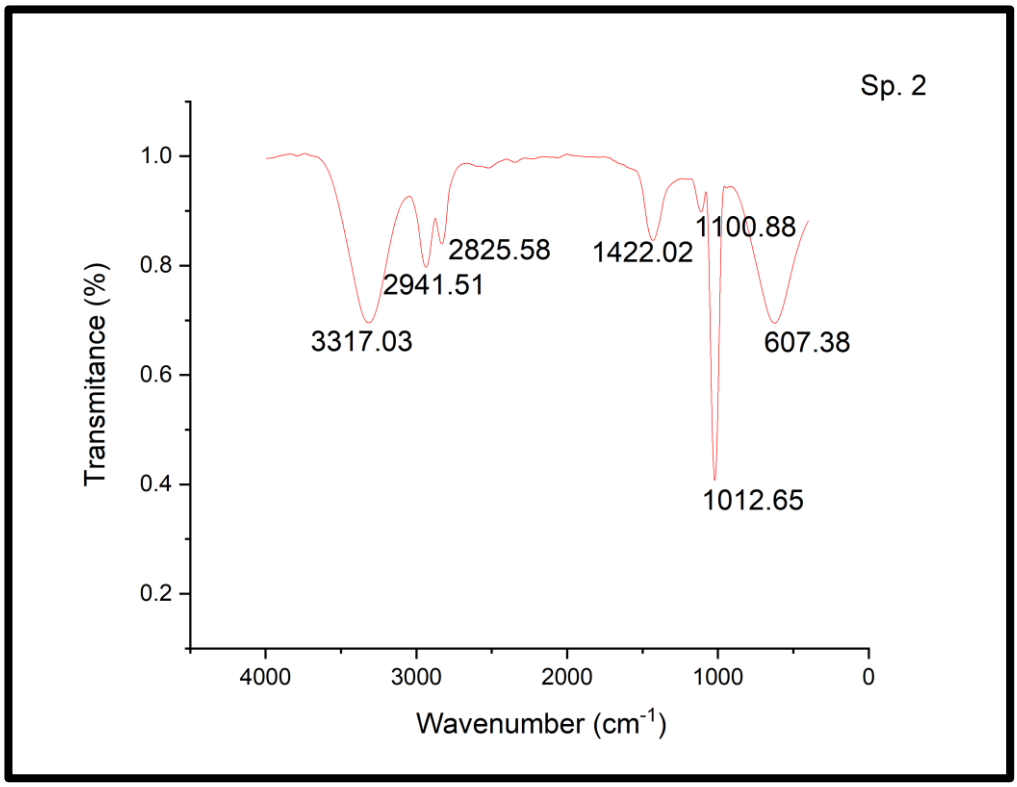
(A)


Anthopleura anjunae
Transmitance (%)
Wavenumber (cm-1)
3326.26
2941.51
2825.58
1419.97
1100.88
1009.57
607.38

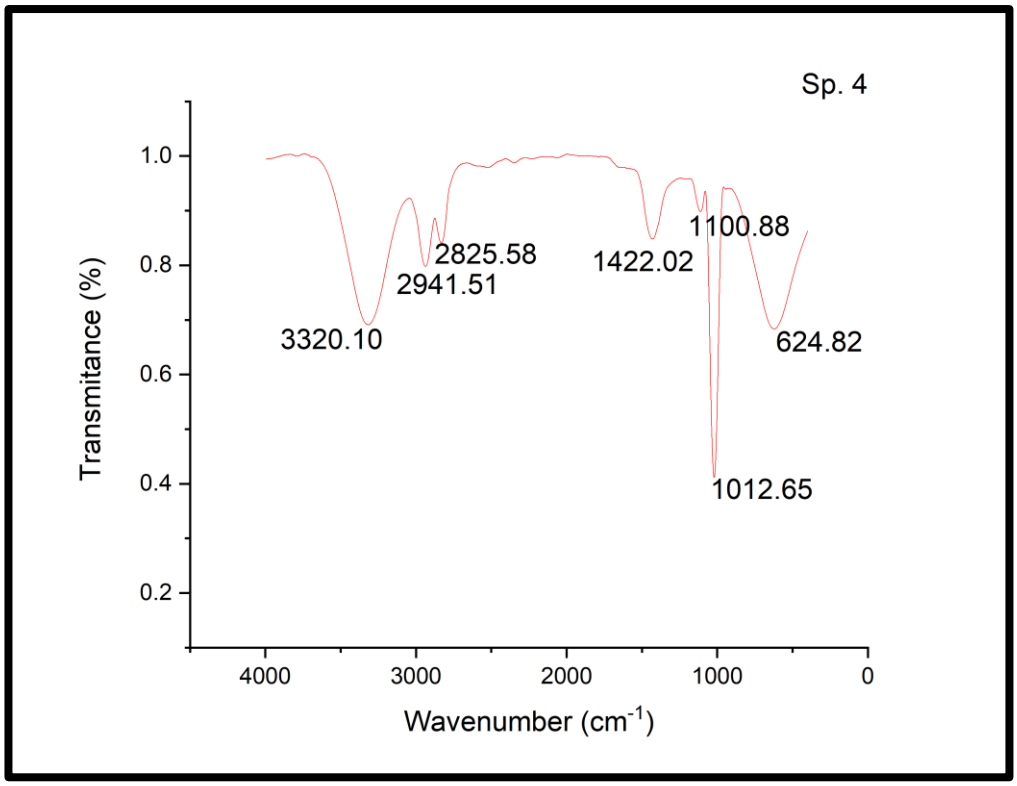
(B)


Sp. 1
Transmitance (%)
1.0
0.8
0.6
0.4
0.2
3309.84
2941.51
2825.58
1422.02
1100.88
607.38
1012.65
4000
3000
2000
1000
0
Wavenumber (cm-1)

(C)


Sp. 2
Transmitance (%)
1.0
0.8
0.6
0.4
0.2
3317.03
2941.51
2825.58
1422.02
1100.88
607.38
1012.65
4000
3000
2000
1000
0
Wavenumber (cm-1)

(D)



(E)

Figure 4.17: Graph depicting FT-IR data of methanol extract of (A) *Bunodosoma goanensis* , (B) *Anthopleutra anjunae*, (C) Sp. 1 , (D) Sp. 2 and (E) Sp. 4

SUMMARY

Similar to terrestrial ecosystems, marine ecosystems are also valuable sources of new pharmaceutical compounds. This study aimed to explore the pharmaceutical potential of marine invertebrates, such as the Golf Ball sponge, Horn shell, and nine species of sea anemone. The crude, methanol and hexane extract of the organisms were screened for antioxidant, antimicrobial, anti-inflammatory, anti-diabetic, and anticoagulant properties. Due to their filter feeding and defensive behaviour, marine invertebrates produce numerous bioactive compounds beneficial to humans. Methanol extract of sea anemone sp. 1 and *B. goanensis* was identified for their antioxidant potential. The crude extract of sea anemone sp. 5 and sp. 4 showed anti-diabetic and anti-inflammatory property respectively. The crude extracts of sp. 4 and sp. 6 that could be potentially used for blood clotting in diabetic patients. While, *A. anjunae* and sp. 2 demonstrated anticoagulation activity. However, since these organisms associate with many microbes, the contribution of gut microbes to pharmaceutical compounds was also investigated. Extracts showing pharmaceutical potential underwent partial characterization using methods like TLC and FTIR to identify functional groups. To preserve natural ecosystems while screening natural resources for pharmaceuticals, techniques like in vitro propagation were employed.

Table 4.11: Screening marine invertebrates for various

Sample	Extract	Antioxidant Assay (% Scavenging)	Reducing Power Assay(µg/mL)	Anti-diabetic Assay(% Inhibition)	Anti-inflammatory Assay (% Inhibition)	Coagulation Assay	Anti-coagulation Assay	Flavonoid Assay (µg/mL)	Phenol Assay (µg/mL)
Sponge	Crude	5.88	9.13	0.24	0			16.87	8.10
	Methanol	8.19	5.49	7.97	0			252.88	0
	Hexane	4.36	0.01	0	0			0	0
Horn shell	Crude	5.43	1.77	0.76	0			4.68	5.86
	Methanol	12.02	4.39	0.08	0			222.16	0
	Hexane	6.67	0	0	0			62.95	0
A. anjunae	Crude	8.02	0.84	1.72	17.37		**Positive**	0.03	15.68
	Methanol	14.56	8.82	0.08	9.26			380.12	0.44
	Hexane	7.79	0.05	0	0			4.24	0
A. nigrescene	Crude	2.3	0.83	0.28	23.10			0.10	1.50
	Methanol	13.99	7.19	20.16	11.84			122.28	0
	Hexane	6.04	0.05	0	0			6.48	0
B. goanensis	Crude	0.81	0.62	5.06	19.95			0.01	8.97
	Methanol	29.1	**14.38**	0.08				325.15	0
	Hexane	5.27	0.00	0	0			6.85	0
Sp. 1	Crude	4.35	0.96	6.71	21.87			0	6.48
	Methanol	**93.27**	11.45	0.04	12.46			234.72	0
	Hexane	23.83	0.01	0	0			37.82	0
Sp. 2	Crude	1.17	0.74	3.01	0		**Positive**	6.00	17.18
	Methanol	18.38	11.03	22.40	0			65.19	2.32
	Hexane	11.81	0.06	0	0			22.28	1.19
Sp. 3	Crude	53.11	0.35	2.89	0			7.86	5.11
	Methanol	20.43	1.26	0.04	0			214.20	0
	Hexane	9.97	0.01	0	0			2.13	0
Sp. 4	**Crude**	19.18	0.57	1.72	**46.19**	**Positive**		0.16	**21.65**
	Methanol	14.56	9.32	0.04	23.88			208.35	0
	Hexane	17.46	0.04	0	0			86.46	0
Sp. 5	**Crude**	8.15	0.34	**25.81**	7.08			0.12	0
	Methanol	21.21	14.19	0.52	3.91			305.62	0
	Hexane	6.01	0.14	0	0			0.51	0
Sp. 6	Crude	12.38	0.24	21.10	37.53	**Positive**		0	18.42
	Methanol	14.42	14.30	4.97	19.36			**396.79**	0
	Hexane	8.14	0.40	0	0			4.49	0

CONCLUSION

This study revealed the significance of marine invertebrates as sources of bioactive compounds for novel pharmaceuticals.

Antioxidant activity: The methanol extract of sea anemone sp. 1 exhibited significant DPPH scavenging activity of 93.27 %, concluding the presence of potential antioxidant compounds.

The methanol extract of *B. goanensis* also demonstrated high antioxidant content of 14.38 µg/mL, indicating its ability to reduce free radicals effectively.

Anti-diabetic activity: The crude extract of sea anemone sp. 5 displayed promising inhibition of amylase of 25.81 %, concluding its potential as a drug for diabetes treatment.

Anti-inflammatory activity: The crude extract of sp. 4 exhibited anti-inflammatory properties by inhibiting albumin denaturation by 46.19 %, indicating its ability to prevent inflammation-induced protein denaturation.

Clotting activity: Revealed the presence of clotting factors in the crude extracts of sp. 4 and sp. 6 that could be potentially used for blood clotting in diabetic patients.

Anti-coagulation activity: *A. anjunae* and sp. 2 demonstrated anticoagulation / anti-thrombotic activity which can be used to prevent blood clotting.

These findings reveals the variety of bioactive compounds present in these marine invertebrates, suggesting their potential therapeutic applications in various health conditions.

FUTURE PROSPECT

- The crude extract can be purified for the bioactive compounds and the same can be used for the further analysis.
- The extracts and pure compounds can be characterised by various techniques like GC-MS and NMR.
- Investigate cultivation of sea anemone in synthetic seawater and in association with marine fish.

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APPENDIX

1. **DPPH Reagent**

   Dissolve 1mg of DPPH powder in 13 mL Ethanol

2. **Folin Reagent**

   Mix 1mL Folin reagent in 9mL distilled water

3. **DNSA Reagent**

   Mix 3 g of sodium potassium tartrate in 50 mL of distilled water.

   Mix 1 g of DNSA powder in 20 mL of 2 M NaOH.

   Mix both solutions together

REFERENCE

1. Acuña, F. H., González-Muñoz, R., Garese, A., & Díaz-Ferguson, E. (2022). First records of Anthopleura nigrescens (Verrill, 1928) and Telmatactis panamensis (Verrill, 1869) (Cnidaria, Anthozoa, Actiniaria) from Parque Nacional Coiba, Pacific coast of Panama. Check List, 18(5), 1141–1146. https://doi.org/10.15560/18.5.1141
2. Ansarizadeh A, Kafilzadeh F, Tamadoni Jahromi S, Kargar M, Gozari M. (2022). Isolation, identification and evaluation of the anti-diabetic activity of secondary metabolites extracted from bacteria associated with the Persian Gulf sponges (Haliclona sp. and Niphates sp.). http://jifro.ir/article-1-5036-en.htmL
3. Babbar, N., Oberoi, H. S., Sandhu, S. K., & Bhargav, V. K. (2012). Influence of different solvents in extraction of phenolic compounds from vegetable residues and their evaluation as natural sources of antioxidants. Journal of Food Science and Technology, 51(10), 2568–2575. https://doi.org/10.1007/s13197-012-0754-4
4. Balakrishnan, D., Bibiana, A. S., Vijayakumar, A., Santhosh, R. S., Dhevendaran, K., & Nithyanand, P. (2014). Antioxidant Activity of Bacteria Associated with the Marine Sponge Tedania anhelans. Indian Journal of Microbiology, 55(1), 13–18. https://doi.org/10.1007/s12088-014-0490-8
5. Bhutia, S. (2020). EVALUATION OF IN VITRO ANTI-INFLAMMATORY ACTIVITY OF CITRUS MACROPTERA MONTR. Asian Journal of Pharmaceutical and Clinical Research, 101–103. https://doi.org/10.22159/ajpcr.2020.v13i8.
6. Borbón, H., Váldes, S., Alvarado-Mesén, J., Soto, R., & Vega, I. (2016). Antimicrobial properties of sea anemone Anthopleura nigrescens from Pacific coast of Costa Rica. Asian Pacific Journal of Tropical Biomedicine. https://doi.org/10.1016/j.apjtb.2016.01.014
7. Choudhury, S., & Raghunathan, C. (2019). The Cytotoxic Effect and Antioxidant Properties of Actiniarian Sea Anemones. Asian Fisheries Science, 32(1), 42–47. https://doi.org/10.33997/j.afs.2019.32.01.006
8. Comparing and Contrasting Invertebrates. (2016). https://www.msnucleus.org/membership/htmL/k-6/lc/organ/4/lco4_5a.htmL
9. Coico, R. (2005). Gram Staining. Current Protocols in Microbiology, 00(1). https://doi.org/10.1002/9780471729259.mca03cs00

10. Crews P, Gewick WH, Schmitz FJ, France D, Bair KW, et al. (2003) Molecular approaches to discover marine natural products anticancer leads-An update from a drug discovery group collaboration. Pharmac Biol 41: 39-52.
11. De Zoysa, M. (2012). Medicinal Benefits of Marine Invertebrates. Advances in Food and Nutrition Research. https://doi.org/10.1016/b978-0-12-416003-3.00009-3
12. Farhud D. D. (2015). Impact of Lifestyle on Health. Iranian journal of public health, 44(11), 1442–1444.
13. Flórez-Fernández, N., Torres, M. D., Braz, L., Grenha, A., Loret, E., & Domínguez, H. (2020). Seaweed and Sea Anemones Proteins as a Source of New Pharmaceutical Active Principles. Springer EBooks, 203–219. https://doi.org/10.1007/978-981-15-5017-1_11
14. Fristiohady, A., Wahyuni, W., Malik, F., Purnama, L. O. M. J., Sadarun, B., & Sahidin, I. (2019). ANTI-INFLAMMATORY ACTIVITY OF MARINE SPONGE CALLYSPONGIA SP. AND ITS ACUTE TOXICITY. Asian Journal of Pharmaceutical and Clinical Research. https://doi.org/10.22159/ajpcr.2019.v12i12.34737
15. Gardner, C. D., Kiazand, A., Alhassan, S., Kim, S., Stafford, R. S., Balise, R. R., ... & King, A. C. (2007). Comparison of the Atkins, Zone, Ornish, and LEARN diets for change in weight and related risk factors among overweight premenopausal women: the A TO Z Weight Loss Study: a randomized trial. Jama, 297(9), 969-977.
16. Gündüz, O., Şahin, Y. M., Agathopoulos, S., Ben-Nissan, B., & Oktar, F. N. (2014). A New Method for Fabrication of Nanohydroxyapatite and TCP from the Sea SnailCerithium vulgatum. Journal of Nanomaterials, 1–6. https://doi.org/10.1155/2014/382861
17. Hand, C., & Uhlinger, K. R. (1992). The Culture, Sexual and Asexual Reproduction, and Growth of the Sea Anemone Nematostella vectensis. The Biological Bulletin, 169–176(2). https://doi.org/10.2307/1542110
18. Hartog, J.C. den & J. Vennam (1993). Some Actiniaria (Cnidaria: Anthozoa) from the west coast of India. Zool. Med. Leiden 67 (42), 24.xii.1993:601-637, figs. 1-47, tabs. 1-6.— ISSN 0024-0672., available online at http://www.repository.naturalis.nl/document/149281
19. Jan, B., Zahiruddin, S., Basist, P., Irfan, M., Abass, S., & Ahmad, S. (2022). Metabolomic Profiling and Identification of Antioxidant and Antidiabetic Compounds from Leaves of Different Varieties of Morus alba Linn Grown in

Kashmir. ACS Omega, 7(28), 24317–24328. https://doi.org/10.1021/acsomega.2c01623

20. Kalimuthu, S., & Kim, S. (2013). Marine Invertebrate Natural Products for Anti-Inflammatory and Chronic Diseases. Evidence-based Complementary and Alternative Medicine. https://doi.org/10.1155/2013/572859
21. Kinugawa, M., Fukuzawa, S., & Tachibana, K. (2009). Skeletal protein protection: the mode of action of an anti-osteoporotic marine alkaloid, norzoanthamine. Journal of bone and mineral metabolism, 27, 303-314.
22. Leach, W. B., Carrier, T. J., & Reitzel, A. M. (2019). Diel patterning in the bacterial community associated with the sea anemone Nematostella vectensis. Ecology and Evolution, 9(17), 9935–9947. https://doi.org/10.1002/ece3.5534Leach, W. B., Carrier, T. J., & Reitzel, A. M. (2019, August 13). Diel patterning in the bacterial community associated with the sea anemone Nematostella vectensis. Ecology and Evolution, 9(17), 9935–9947. https://doi.org/10.1002/ece3.5534
23. Lebovitz, H. E. (1992). Oral antidiabetic agents: the emergence of α-glucosidase inhibitors. Drugs, 44(Suppl 3), 21-28.
24. Lee, Y. S., Wu, H. C., Huang, S. J., Hsiao, G., Chi, W. C., & Lee, T. H. (2024). Anti-inflammatory constituents from a sea anemone-derived fungus Arthrinium arundinis MA30. Phytochemistry, 219, 113998. https://doi.org/10.1016/j.phytochem.2024.113998
25. León-Palmero, E., Joglar, V., Álvarez, P. A., Martín-Platero, A. M., Llamas, I., & Reche, I. (2018). Diversity and antimicrobial potential in sea anemone and holothurian microbiomes. PLOS ONE, e0196178(5). https://doi.org/10.1371/journal.pone.0196178
26. Mayer, A. M. S., Guerrero, A. J., Rodríguez, A. D., Taglialatela-Scafati, O., Nakamura, F., & Fusetani, N. (2019). Marine Pharmacology in 2014–2015: Marine Compounds with Antibacterial, Antidiabetic, Antifungal, Anti-Inflammatory, Antiprotozoal, Antituberculosis, Antiviral, and Anthelmintic Activities; Affecting the Immune and Nervous Systems, and Other Miscellaneous Mechanisms of Action. Marine Drugs, 5(1). https://doi.org/10.3390/md18010005
27. Marine Invertebrates – Geological Oceanography Lab. (2016). https://mLmL.sjsu.edu/geooce/research/microcosms/marine-invertebrates/#:~:text=Within%20the%2035%20phyla%20mentioned,(shellfish%2C%20snails%2C%20squid%2C

28. Martin, E. J. (1966). Anticoagulant from the Sea Anemone Rhodactis howesii. Experimental Biology and Medicine, 121(4), 1063–1065. https://doi.org/10.3181/00379727-121-30966
29. McClatchey, W., & Stevens, J. (2001). An Overview of Recent Developents in Bioprospecting and Pharmaceutical Development. Springer eBooks. https://doi.org/10.1007/978-94-015-9779-1_2
30. Minasian, L. L. (1976). Characteristics of Asexual Reproduction in the Sea Anemone, Haliplanella Luciae (Verrill), Reared in the Laboratory. Springer eBooks. https://doi.org/10.1007/978-1-4757-9724-4_
31. Mozaffarian, D., Hao, T., Rimm, E. B., Willett, W. C., & Hu, F. B. (2011). Changes in diet and lifestyle and long-term weight gain in women and men. New England journal of medicine, 364(25), 2392-2404.
32. Naqvi, S. A. R., Sherazi, T. A., Hassan, S. U., Shahzad, S. A., & Faheem, Z. (2022). Anti-inflammatory, Anti-infectious and Anti-cancer Potential of Marine Algae and Sponge: A Review. European Journal of Inflammation. Retrieved December 3, 2023, from https://doi.org/10.1177/20587392221075514
33. Odeleye, T., White, W. L., & Lu, J. (2019). Extraction techniques and potential health benefits of bioactive compounds from marine molluscs: a review. Food & Function, 2278–2289(5). https://doi.org/10.1039/c9fo00172g
34. Ovchinnikova, T. V. (2019). Structure, Function, and Therapeutic Potential of Marine Bioactive Peptides. Marine Drugs, 505(9). https://doi.org/10.3390/md17090505
35. Perdicaris, S., Vlachogianni, T., & Valavanidis, A. (2013). Bioactive Natural Substances from Marine Sponges: New Developments and Prospects for Future Pharmaceuticals. Natural Products Chemistry & Research, 01(03). https://doi.org/10.4172/2329-6836.1000114
36. Prentis, P. J., Pavasovic, A., & Norton, R. S. (2018). Sea Anemones: Quiet Achievers in the Field of Peptide Toxins. Toxins. Retrieved December 3, 2023, from https://doi.org/10.3390/toxins10010036
37. Rabinowitz, C., Moiseeva, E., & Rinkevich, B. (2016). In vitro cultures of ectodermal monolayers from the model sea anemone Nematostella vectensis. Cell and Tissue Research, 693–705(3). https://doi.org/10.1007/s00441-016-2495-6

38. Ren, C., Liu, Z., Wang, X., & Qin, S. (2022). The seaweed holobiont: from microecology to biotechnological applications. *Microbial Biotechnology*, *15*(3), 738–754. https://doi.org/10.1111/1751-7915.14014
39. Rhandour, Z., Tarbaoui, M., Oumam, M., Elamraoui, B., Bennamara, A., & Abourriche, A. (2016). Determination of polyphenols, tannins, flavonoids and antioxidant activity in extracts of two genus Ircinia marine sponges of Atlantic Morrocan Coast. Frontiers in Marine Science, 3. https://doi.org/10.3389/conf.fmars.2016.04.00129
40. Romano, G., Almeida, M., Varela Coelho, A., Cutignano, A., Gonçalves, L. G., Hansen, E., Khnykin, D., Mass, T., Ramšak, A., Rocha, M. S., Silva, T. H., Sugni, M., Ballarin, L., & Genevière, A. M. (2022). Biomaterials and Bioactive Natural Products from Marine Invertebrates: From Basic Research to Innovative Applications. Marine Drugs, 20(4), 219. https://doi.org/10.3390/md20040219
41. Rosyantari, A., Prasedya, E., Ilhami, B., Martyasari, N., Padmi, H., Abidin, A., Ambana, Y., Kirana, I., & Sunarwidhi, A. (2021). Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Antioxidants Activity of Marine Sponge Stylissa flabelliformis Ethanol Extract. IOP Conference Series: Earth and Environmental Science, 913(1), 012109. https://doi.org/10.1088/1755-1315/913/1/012109
42. Sarian, M. N., Ahmed, Q. U., Mat So'ad, S. Z., Alhassan, A. M., Murugesu, S., Perumal, V., Syed Mohamad, S. N. A., Khatib, A., & Latip, J. (2017). Antioxidant and Antidiabetic Effects of Flavonoids: A Structure-Activity Relationship Based Study. BioMed Research International, 2017, 1–14. https://doi.org/10.1155/2017/8386065
43. Sheikh, M., Rathore, D. S., Gohel, S. D., & Singh, S. P. (2018). MARINE ACTINOBACTERIAASSOCIATED WITH THE INVERTEBRATE HOSTS: A RICH SOURCE OF BIOACTIVE COMPOUNDS: A REVIEW. ResearchGate. https://www.researchgate.net/publication/337413829_MARINE_ACTINOBACTERIA_ASSOCIATED_WITH_THE_INVERTEBRATE_HOSTS_A_RICH_SOURCE_OF_BIOACTIVE_COMPOUNDS_A_REVIEW
44. Shen S, Liu D, Wei C, Proksch P, Lin W (2012) Purpuroines A-J, halogenated alkaloids from the sponge Iotrochota purpurea with antibiotic activity and regulation of tyrosine kinases. Bioorg Med Chem 20: 6924-692
45. Siddiqui, N., Rauf, A., Latif, A., & Mahmood, Z. (2017). Spectrophotometric determination of the total phenolic content, spectral and fluorescence study of the

herbal Unani drug Gul-e-Zoofa (Nepeta bracteata Benth). Journal of Taibah University Medical Sciences, 360–363(4). https://doi.org/10.1016/j.jtumed.2016.11.006

46. Silva, T. C., Andrade, P. B., Paiva-Martins, F., & Valentão, P. (2017). In Vitro Anti-Inflammatory and Cytotoxic Effects of Aqueous Extracts from the Edible Sea Anemones Anemonia sulcata and Actinia equina. International Journal of Molecular Sciences, 653(3). https://doi.org/10.3390/ijms18030653
47. Sonak, S. M. (2017). Marine Shells of Goa. Springer. http://books.google.ie/books?id=Xh4xDwAAQBAJ&pg=PA185&dq=Marine+Shells++of+Goa&hl=&cd=1&source=gbs_api
48. Stead, P., Hiscox, S., Robinson, P. S., Pike, N. B., Sidebottom, P. J., Roberts, A. D., ... & Langley, D. (2000). Eryloside F, a novel penasterol disaccharide possessing potent thrombin receptor antagonist activity. Bioorganic & Medicinal Chemistry Letters, 10(7), 661-664.
49. Tamrakar, A. K., Tiwari, P., Ahmad, R., Kumar, R., Lakshmi, V., Srivastava, M. N., & Srivastava, A. K. (2007). Antihyperglycaemic activity of Sinularia firma and Sinularia erecta in streptozotocin-induced diabetic rats. Medicinal Chemistry Research, 17(2–7), 62–73. https://doi.org/10.1007/s00044-007-9037-4
50. Thangaraj, S., Bragadeeswaran, S., & Gokula, V. (2018). Sea Anemones as Potential Source for Bioactive Metabolites. International Journal of Peptide Research and Therapeutics, 591–604(2). https://doi.org/10.1007/s10989-018-9705-x
51. Velho-Pereira, S. (2014). Retrieval of euryhaline eubacterial and haloarchaeal bionts from nine different benthic sponges: reflection of the bacteriological health of waters of Mandapam, India. https://nopr.niscpr.res.in/handle/123456789/28763
52. Velho-Pereira, S. (2014). Retrieval of euryhaline eubacterial and haloarchaeal bionts from nine different benthic sponges: reflection of the bacteriological health of waters of Mandapam, India. https://nopr.niscpr.res.in/handle/123456789/28763
53. Van der Burg, C. A., & Prentis, P. J. (2021). The Tentacular Spectacular: Evolution of Regeneration in Sea Anemones. Genes, 12(7), 1072. https://doi.org/10.3390/genes12071072
54. WHO EMRO | Pharmaceutical products | Health topics. (2014). World Health Organization - Regional Office for the Eastern Mediterranean. https://www.emro.who.int/health-topics/pharmaceutical-products/index.htmL

55. Xiong, J., Fang, W., Fang, W., Li, B., Huo, J. L., Kong, Y., & Li, Y. (2009). Anticoagulant and antithrombotic activity of a new peptide pENW (pGlu-Asn-Trp). Journal of Pharmacy and Pharmacology. https://doi.org/10.1211/jpp/61.01.0012