Chemically mediated ecological interactions of Sea anemone *Bunodosoma goanense* (den Hartog & Vennam, 1993)

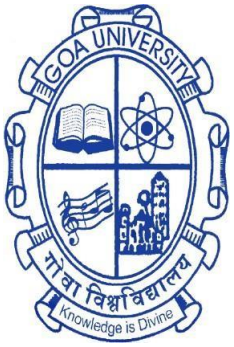
A Thesis submitted in partial fulfillment for the Degree of

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GOA UNIVERSITY



By

Cecelia Perpetua Celsa Menezes

CSIR-National Institute of Oceanography
Dona Paula, Goa-India

May 2025

DECLARATION

I, Ms. Cecelia Perpetua Celsa Menezes hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or Institution for the award of any research degree.

Place: Dona Paula
Date: Cecelia P.C. Menezes

CERTIFICATE

I hereby certify that the above Declaration of the candidate, Ms. Cecelia Perpetua Celsa Menezes, is true and the work was carried out under my supervision.

Dr. Narsinh L. Thakur
Chief Scientist
Chemical Oceanography Division
CSIR-National Institute of Oceanography, Goa

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"There's always going to be another mountain,
I'm always going to want to make it move."

~ Miley Cyrus *'The Climb'*

This thesis is dedicated in honor of all the animals that have been sacrificed and tested on in the pursuit of scientific knowledge.

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Chapter 1

Introduction and Literature Review

1.1 Ecological interactions of marine invertebrates

The marine ecosystem is a complex web of interactions among organisms, shaped by their environments and evolutionary adaptations. Among these, ecological interactions of marine invertebrates play an important role in maintaining biodiversity and ecosystem stability, particularly in dynamic habitats such as the intertidal zone. The intertidal zone, located between the high and low tide marks, presents one of the most dynamic and challenging environments for marine organisms. Here, marine invertebrates experience fluctuating conditions such as desiccation, temperature shifts, salinity changes, and intense competition for space and resources (Little, 2000). To survive, intertidal organisms, particularly sessile or sedentary ones, have evolved adaptive strategies, both morphological and behavioral, to maintain their ecological niche. They survive by interacting with other organisms in their habitat, engaging in ecological interactions ranging from predation and competition to mutualism and parasitism.

1.2 Chemically mediated interactions

Among the many strategies employed by marine invertebrates, chemically mediated interactions play a crucial role. These involve the production and detection of bioactive compounds that serve as signals, helping organisms communicate, deter predators, inhibit competitors, or attract symbionts (Hay, 2009). Chemically mediated interactions are particularly significant in intertidal zones, where physical barriers and resource limitations increases the reliance on chemical signaling for survival and reproductive success (Pawlik, 2011). Sessile organisms like sponges, corals, and sea anemones often rely heavily on such chemical means to interact with their environment due to limited mobility. Within this context, the chemically mediated interactions of marine invertebrates offer a rich field for exploring ecological dynamics and their potential applications. For instance, chemical

defenses in sponges and corals have been shown to deter herbivores, while allelopathic interactions among seaweeds influence community structure (Rasher & Hay, 2010). These interactions not only shape species distributions but also hold promise for biotechnological advancements, such as the development of novel pharmaceuticals derived from marine natural products. To gain deeper understanding into these interactions, it is important to study organisms that show such strategies clearly. Sea anemones, for instance, are cnidarian invertebrates well-known for their ability to use bioactive molecules in various ecological interactions.

1.3 Sea Anemones

Sea anemones are often described as "flowers of the sea" due to their beautiful and colourful floral-like appearance, but they are marine predatory benthic invertebrate animals belonging to the invertebrate Phylum Cnidaria (order Actiniaria, Class Anthozoa) (Figure 1.1). Sea anemones are supposedly one of the most primitive animals, along with corals, jellyfish, and hydras, that constitute the oldest eumetazoan phylum, the Cnidaria (Putnam *et al*., 2007) and ever since have been widely distributed around the world, inhabiting the various marine environment from the tropics to the poles, from the coasts to the deep seas. They are usually attached to rocks or suitable substrata and are mostly sessile animals, but if required they can move short distances by gliding along the seafloor. The adult polyp consists of a cylindrical column attached to the substratum via the pedal disc at one end. At the opposite end lies the oral disc which consists of the centrally located actinopharynx and a crown of tentacles (Figure 1. 2).



Figure 1.1: Sea anemones found on the Rocky shores of Goa, India.

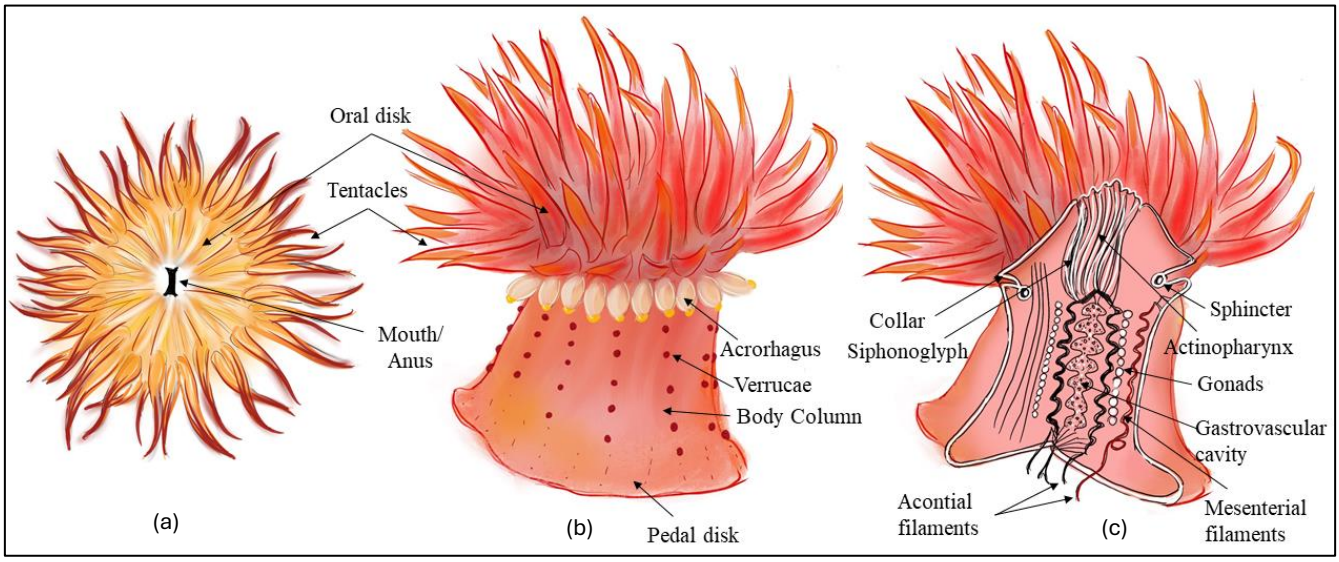


Figure 1.2: Anatomy of Sea anemone (a) External anatomy (Top view), (b) External anatomy (Side view) and (c) Internal anatomy.

1.3.1 Ecological importance of Sea anemones

Sea anemones have an important role in the marine ecosystem. They are predatory animals that prey on free-living organisms like crustaceans, molluscs, and fish, thus acting as a link between the pelagic and benthic ecosystems (Brusca & Brusca, 2003). They play key

biogeochemical roles in benthic ecosystems. Mostly, they are solitary animals, but some are also known to show symbiosis, especially in the coral reef ecosystem where they offer refuge and protection to other reef animals and in exchange receive leftover food and fish metabolic waste as a source of nitrogen (Cleveland *et al.,* (2011)).

1.3.2 Chemical Ecology of Sea Anemones

In the natural environment, there exist various kinds of interactions between living organisms, essentially mediated by chemical cues. All organisms produce, emit, detect and respond to these chemical signals. The study of the role of these chemical interactions between living organisms and their environment is known as Chemical ecology (Dyer *et al*., 2018). These interactions at the molecular level play an important role in the fine tuning of processes which increase the survival of a species in its habitat, such as, in feeding or prey capture, reproduction, optimizing the survival conditions for the offspring or avoiding potential predators (Klaschka, 2009). Sea anemones play a vital role in marine ecosystems, engaging in complex ecological interactions mediated by chemical compounds. As sedentary organisms, they rely heavily on their venom to interact with their environment. Their venom, composed of peptides, proteins, and small molecules, helps in prey capture, defense against predators, and competition for space, etc. In addition to venom, sea anemones release chemical cues that facilitate symbiotic relationships with other marine organisms, such as crustaceans and gastropods. These chemical mediators regulate behaviours like host recognition, settlement, and competition. Beyond their ecological roles, sea anemones also hold potential for human applications, as explored through bioprospecting.

1.3.3 Bioprospecting of Sea Anemone Venom

Bioprospecting of marine organisms has gained significant attention in recent years due to the discovery of bioactive compounds with therapeutic potential. Sea anemones are a promising source of venom peptides that have evolved to target ion channels and cellular membranes. These venom components play crucial roles in prey capture, defense, and other ecological interactions. Many sea anemone toxins, such as actinoporins, Kunitz-type peptides, and other neurotoxins, have shown pharmacological relevance in treating neurological disorders, cardiovascular diseases, and even cancer. The specificity of sea anemone peptides in modulating voltage-gated ion channels (Nav and Kv channels) makes them valuable tools in neurobiology and drug discovery (Menezes and Thakur, 2022).

1.4 Literature Review

The sea anemone is one of the most venomous animals in the world and is known to be a rich source of toxic proteins and peptides (Madio *et al*., 2017; Madio *et al*., 2019). These venomous molecules are more than just toxins as they are involved in various ecological interactions of the sea anemone such as in predatory feeding, spatial competition and aggressive encounters, self-defence, and even in larval recruitment (Schendel *et al.,* (2019)). There are numerous reviews of sea anemone toxins concerning their bioactivity and pharmacological applications, but the knowledge of the ecological role of these molecules is limited. O'Hara *et al*., (2021) reviewed literature studying the effect of ecological factors such as environmental temperature, salinity, ontogeny, geographic location, and diet on cnidarian venom and stated that venom ecology research is necessary for pharmacologically important cnidarians to facilitate the advancement of medical treatments. Similarly, Schendel *et al*., (2019) in their review on the importance of behaviour and venom system morphology in understanding its ecology and evolution mentioned that more studies of behaviour, morphology and ecologically relevant toxin pharmacology will boost our understanding of the evolution of venoms toxins, and enable the discovery of their potential as sources of molecular tools and therapeutic and agrochemical lead compounds. Ashwood *et al.,* (2020) reviewed the diversity of venom composition between Meduzoa and Anthozoa. They suggest that the temporal and spatial expression of protein and peptide toxins is a consequence of the functional and ecological factors. In their review, Surm & Moran (2021) provided insights into how the development and life-history dynamics of animals shape the evolution of their venom and its ecological implications. There is significant scope for studies to understand the chemical ecology of these animals and the ecological role of their toxins as it can pave the way for the discovery of novel and unexpected biotechnological applications. In this review, we focus on sea anemone venom to understand its role in the various chemically mediated ecological interactions and the current biotechnological application.

1.4.1 Sea anemone venom tissue

Marine venoms are probably as myriad as marine biodiversity. As a result of the co-evolution of processes between the host, parasite, predator, and prey, these venomous animals have developed various glands or tissues for the production storage, and dispersal of their venom. The most distinct characteristic of their phylum cnidaria is the presence of stinging cells called Cnidocytes or nematocyte s which play a particularly important role in prey capture

and anti-predatory defense of the animal. Cnidocytes contain a stinging capsule known as a Cnida. Among anthozoans there are three categories of cnidae: (1) Nematocysts, which have harpoon-like structures that inject venom into the prey and paralyzes it (atrichs, isorhizousholotrichs, anisorhizousholotrichs, b-mastigophores/basitrichs/ spirullae, microbasic p-mastigophores, microbasic p-mastigophores) (2) Spirocysts, which consists of a string that is fired and entangle the prey and (3) Ptychicysts, these release sticky substances to hold onto prey, also build protectoral tube in tube-dwelling sea anemones (England and Robson, 1991; Zenkert, 2011). Based on their functions, these cnidae sub-types are differentially located of the animal. The number of cnidae and their distribution is influenced by the body size, prey type, and selectivity in prey capture. The quantity of cnidae subtypes present in various locations of the animal is conserved and unique to every species, therefore playing an important role in the taxonomical identification of the anthozoan species (Carlgren, 1949). Among the three cnidae subtypes, only the nematocysts are known to contain venom. The production of venom from other classes of cnidae is uncertain. Nematocysts are present on tentacles, acrorhagi, acontia and mesentrial filaments and their distribution differs (Macrander *et al.,* 2016, Kass-Simon and Scappaticci 2002). The nematocyst has a small vesicle loaded with venom, inner filament, and external sensory hair. A touch to the hair mechanically sets off a cell explosion, that releases a harpoon-like structure that pins to and injects a dose of venom in the predator (Beckmann and Özbek, 2012)). Besides nematocysts, venom is also secreted from the ectodermal cells (Moran *et al.,* 2012b).

1.4.1.1 Evolution of sea anemone venom

The evolution of venom systems in sea anemones is closely linked to the diversification and specialization of cnidocytes, the stinging cells responsible for venom production and delivery. Rather than being produced by a single specialized gland, venom in sea anemones is synthesized and stored in multiple cnidocyte types distributed across different tissues, allowing functional specialization and ecological versatility.

Sunagar et al. (2018) demonstrated that toxin and toxin-like genes exhibit strong cell type specific expression patterns in sea anemones, revealing that venom evolution is driven in part by changes in cnidocyte identity, developmental regulation, and gene expression programs. Their cell-specific transcriptomic analyses showed that distinct cnidocyte

subtypes express different repertoires of venom genes, highlighting the role of regulatory evolution in shaping venom composition. In addition to cell-type specialization, venom evolution in sea anemones involves gene duplication and diversification of major toxin families, including actinoporins, Kunitz-type protease inhibitors, and ion channel-modulating peptides (Macrander & Daly, 2016; Surm & Moran, 2021). Such duplication events, followed by neofunctionalization or differential expression, contribute to lineage-specific venom phenotypes.

Ontogenetic and spatial regulation of venom expression further contribute to venom evolution. Studies have shown that venom composition and toxin gene expression can vary across developmental stages and tissues, reflecting functional requirements associated with prey capture, defense, and competition (Columbus-Shenkar et al., 2018; Schendel et al., 2019). These findings indicate that venom evolution in sea anemones is a dynamic process shaped by both developmental and ecological factors.

Together, these studies suggest that sea anemone venom evolution is best understood as the evolution of an integrated venom system, involving cnidocyte diversification, gene family expansion, and context-dependent expression. Integrating ecological observations with molecular and functional analyses therefore provides critical insight into how venom systems diversify and adapt over evolutionary time.

1.4.2 Role of venom and other chemical cues in the ecological interactions of sea anemone

To understand the role of venom toxins, it is important to know when and for what purpose the venom is employed. Venom toxins of sea anemones are perhaps the most functionally distinct (Schendel *et al*., (2019)). Sea anemones are covered with cnidocytes and toxin-secreting epidermal gland cells and do not have a centralized venom delivery system (Moran *et al*., (2012b)). The venom varies dramatically across the body Macrander *et al.,* (2016) and even tentacle types (Ashwood *et al.,* (2021a)). Therefore, the ecological role of toxins can be understood from the distribution of venom across the body of the sea anemone. Ashwood and co-workers (2021b) reported that the regionalization of toxin production remains consistent with the segregation of the ecological functions of venom across the envenomating structures. For example, venom toxins in the tentacles aid in prey capture and defense, whereas those in the acrorhagi are responsible for intra-specific aggression, in the

mesenterial filaments for food digestion, in acontia for self-defense, and in the gametes for protection of the eggs and zygote (Basulto *et al.,*2006; Beckmann & Özbek (2012); Surm *et al.,* (2019), Madio *et al.*, (2018); Macrander *et al.,* (2015)). Additionally, sea anemones have different venom toxins during different stages of their life cycle (Columbus-Shenkar *et al.* (2018)).

1.4.2.1 Feeding

Sea anemones are typically predators that capture and feed on smaller prey , but some species also show symbiosis with other organisms in their habitat species (Baker, (1931)), while few others are known to be parasitic during their larval stages (Panikkar, (1938)).

1.4.2.1.1 Predation

Sea anemones rely upon chemical and mechanical cues to detect prey (Watson *et al*., (2009); Watson & Mire, (2004); Watson & Hessinger, (1989)). Chemoreceptors and mechano-receptors located on the supporting cells of the tentacles (Watson and Hessinger, 1988) control the nematocyst (Thorington and Hessinger, 1990). Thorington and Hessinger (1988) successfully triggered the discharge of nematocyst by *Diadumene lineata* and *Exaiptasia diaphana* when chemosensitized by N-acetylneuraminic acid (NANA) and proline. Weir and fellow workers (2020) proposed that the Cav2.1 voltage-gated calcium channel orthologue (nCaV) plays a role as molecular filters for sensory discrimination and demonstrated that cnidocytes from *Nematostella vectensis* used this specialized (nCaV) to integrate dynamic voltage signals produced by distinct sensory stimuli. Some deep-sea sea-anemone species use lures to attract their prey. A novel fluorescent protein cjFP510 had been isolated from the tentacles of deep-sea anemone *Cribrinopsis japonica.* The biological role of this protein has been proposed to be the attraction of prey (Tsutsui *et al*., 2016). Frank and Bleakney (1978) reported that exposure to *Nematostella vectensis* Nv1 toxin causes paralysis in arthropods. Macrander, Moran & Reitzel (2017) and others evaluated the effects of *Nematostella vectensis* Nv1, NvePTx1, NEP3 toxins on *Fundulus* larvae, nematode and *Artemia* copepod preys. The extracellular NANA and glycine, originating from wounded prey, cause the release of captured prey to allow ingestion (Thorington and Hessinger, 1998). Sea anemones are rich in neurotoxins and cytolytic toxins that are responsible for the paralysis, immobilization and death of the prey. Granulitoxin and Cangitoxin from *Bunodosoma cangicum* is potent neurotoxin responsible for the paralysis and immobilization of the prey (Santana *et al*., (1998); Zaharenko *et al.,* (2008b)). Crassicorin reported from the pharynx of

Urticina crassicornis is a neurotoxin responsible for the paralysis of prey (Kim *et al*., (2017)). Likewise, Kaliseptine from *Anemonia sulcata (*Schweitz *et al*.,(1995); Ishida *et al*., (1997) are voltage-gated ion channel toxins that are responsible for the paralysis and immobilization of prey. A lethal toxin (AvTX-60A) was discovered in sea anemone *Actineria villosa* which kills its prey by a pore-forming mechanism (Oshiro *et al*., 2004). Alvarado-Mesén *et al.*, (2019) reported the role of a cytotoxin Nigrelysin in the prey capture of *Anthopleura nigrescens.* Similarly, Caissarolysin I (Bcs I), Bandaporin, Tenebrosins, Equinatoxin and Magnificalysin are actinoporins reported in the venom of *Bunodosoma caissarum, Anthopleura asiatica*, *Actinia tenebrosa*, *Actinia equina* and *Heteractis magnifica* respectively that are involved in cytotoxic cell death of the prey (de Oliveira et al., (2006); Kohno *et al*., (2009); Norton *et al.,* (1990); Ferlan & Lebez (1974); Šuput, (2014), Khoo *et al.* (1993)).

Some sea anemone preys have developed a mechanism to escape the predator when captured. Kicklighter and Derby (2006) reported that the ink and other metabolites in *Aplysia californica* secretions were aversive to the predator anemone *Anthopleura sola*. The sea hare escapes from *A. sola* by eliciting tentacle shrivelling and/or retraction, which lead to anemones dropping ensnared sea hares.

1.4.2.1.2 Parasitism

The larval stages of certain species of sea anemones are known to be parasitic to other marine organisms. Spaulding (1972) reported that the tentacles of jellyfish *Clytia gregaria, Clytia hemisphaerica, Aequorea forskalea , itrocoma cellularia ,* and *Mitrocomella polydiodemata* are parasitized by the larvae of burrowing sea anemone *Peachia quinquecapitata,* where it feeds on the gonads and other tissues of its host before liberating as juveniles. Similarly, the planula of *Edwardsiella lineata* is an endoparasite that infects the ctenophore *Mnemiopsis leidyi* (Reitzel *et al*., 2006). To date, there are no reports on the kairomones that induce parasitic behaviour in the cnidarian.

1.4.2.2 Symbiosis

Even though sea anemones produce toxic compounds, many species of these cnidarians have mutualistic relationships with other marine organisms. The most common symbionts are the single-celled algae, either Zooxanthalae and/or Zoochlollerae that live in the gastro-dermal cells of the oral disc and tentacles (Figure 1. 3a) where they receive protection and ensured availability of sunlight and nutrients from the metabolic waste from its host and in turn, the

cnidarians receive oxygen and food such as glycerine, glucose, and alanine (Ruppert *et a*l., (2004). Terminal sugar residues, Mannose-Mannose, Galactose-β-(1-4)-N-acetylglucose amine along with other glycoproteins present on the cell wall of symbiotic zooxanthellae are responsible for the establishment of symbiosis between the single-celled algae and sea anemone *Exaiptasia diaphana* (Lin *et al*., 2000).

Sea anemones also exhibit symbiosis with some species of fish and invertebrates which are immune to their sting (Figure 1. 3c). This immunity can be innate as well as acquired via the process of acclimatization which leads to chemical camouflaging in the fish (Elliott and Richard, 1997). The fish receives protection from the predator. The fish aids in better circulation of oxygen helps in removal of parasites and necrotic tissue from the cnidarian and exchange of nutrients also occurs (Mariscal,1970). Murata *et al*., (1986) studied the species-specific partnership between *Heteractis crispa* (Sea anemone) and *Amphiprum perideraion* (Anemonefish), and *Stichodactyla gigantea* and *A. ocellaris* (Anemonefish). This study revealed the presence of a new pyridinium compound, Amphikueumin from *Heteractis crispa* which induces characteristic attracted swimming in *A. perideraion*. Similarly, the sea anemone *Stichodactyla gigantea* released tyramine and tryptamine that incites the symbiotic fish *A. ocellaris* to swim towards it with a tail-wagging and active searching habit. Another study was conducted by Miyagawa and Hidaka (1980) to understand the chemical attraction of *Amphiprion clarkii* juvenile by symbiotic sea anemones *Exocoelactis actinostoloides , Exocoelactis actinostoloides,* and *Stichodactyla haddoni* . The results of this caging experiment demonstrated that the juvenile *Amphiprion clarkii* is attracted to symbiotic sea anemones by some chemical substances released from them and not by any visual cues and that they can discriminate the symbiotic sea anemone species by some chemical substances released from their symbiotic sea anemone.

Many species of crabs and gastropods harbour sea anemones on their appendages or carapace/shells (Figure 1. 3b) to camouflage in their environment and the sea anemone in turn receives tiny chunks of food while the host is feeding and protecting from predators (Guinot *et al*.,(1995). *Hepatus ephiliticus* initiated the transfer of the sea anemone *Calliactis tricolor* onto its body by rubbing the tentacles of the cnidarian on its carapace (Guinot *et al*., 1995). Brooks and Rittschof (1995) reported that the porcellanid crab *Porcellana sayana* is also strongly attracted chemically to the symbiont sea anemone *Calliactis tricolor*, however, the active molecules that emanate from *C. tricolor* and attract the crabs is still unknown. The capability of the hermit crabs *Dardanus venosus* and *Pagurus pollicaris* to identify their

symbiotic sea anemone *Calliactis tricolor* chemically was reported by Brooks (1991). Izumi and co-authors (2018), reported for the first time a symbiotic relationship between a homoscleromorph sponge (*Oscarella sp*.) and Sea anemone (*Tempuractis rinkai*) (Figure 1. 3d). The sea anemone hides inside the sponge during a potential attack by a predator. In turn, the sea anemone helps to deter the sea slug *Berthella sp.* which is known to feed upon homoscleromorph sponges.

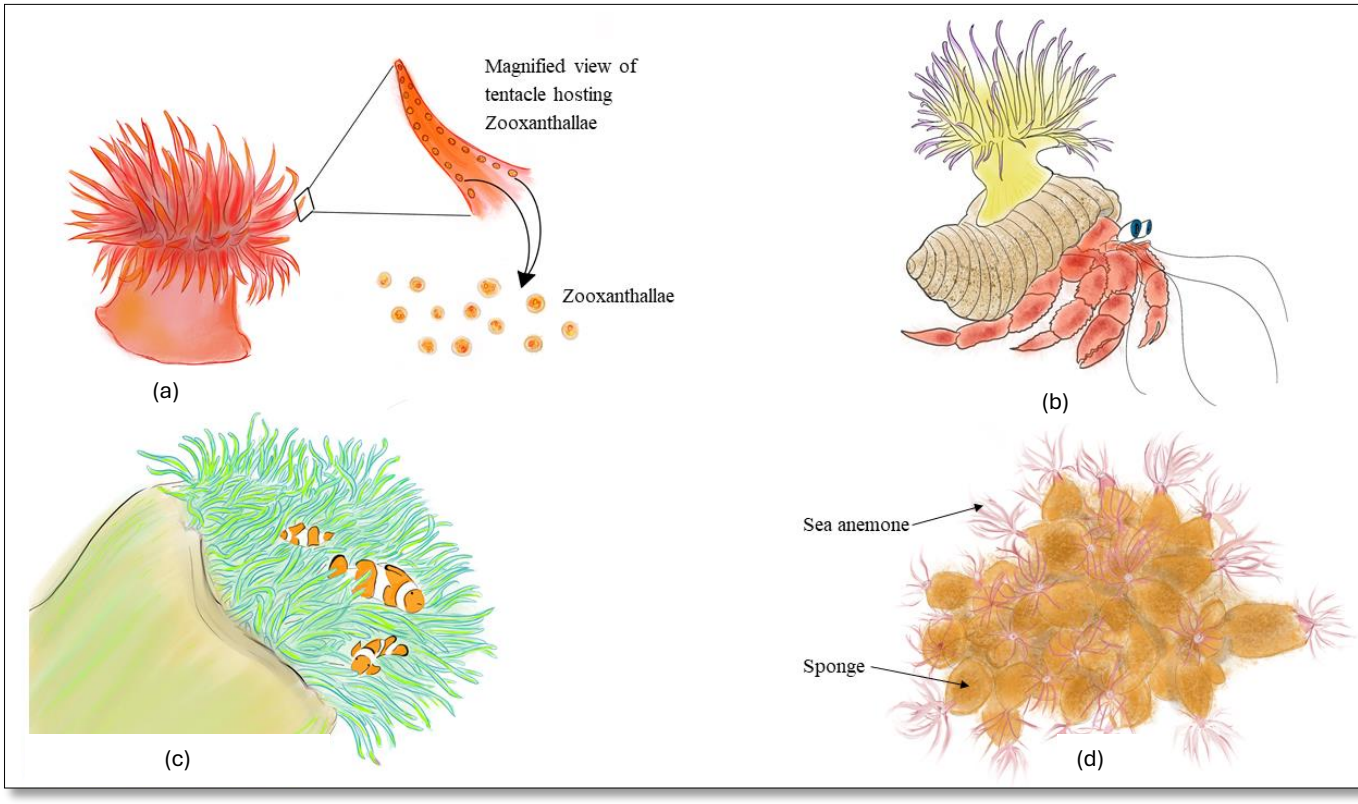


Figure 1.3 a: Symbiosis between Sea anemone and Zooxanthallae; Figure 1. 3b: Symbiosis between Sea anemone and Hermit crab; Figure 1. 3c: Symbiosis between Sea anemone and Pomocentridae (Anemone fish); Figure 1. 3d: Symbiosis between Sea anemone and homoscleromorph Sponge (*Oscarella* sp.)

1.4.2.3 Larval recruitment

Recruitment is the process by which young individuals undergo larval settlement and become part of the adult population. This is achieved through the detection of physical and/or chemical cues. Space for larval recruitment of *Condylactis gigantea* is ensured in a space-restricted environment due to the toxins it produces (Bak and Borsboom, 1984). The toxin NvePtx1of sea anemone *Nematostella vectensis* protects eggs and planulae from fish predators. (Columbus-Shenkar *et al*., 2018). Mussel beds of *Mytilus californianus* serve as

an important refuge site for the settlement of the *Anthopleura xanthogrammica* juvenile population. This indicates that the juveniles' ability to detect the location of mussel beds via chemical cues. The juveniles later migrate from juvenile habitat to adult habitat, dislodge and prey upon the mussels (Sebens, 1981). Weis *et al.* (2005) attempted to induce settlement and metamorphosis of *Anthopleura elegantissima* and *A. artemisia* larvae using various inducers such as Metamorphosin A, 12-O-tetradecanoylphorbol- 13-acetate, Phorbol 1 2,13-dibutyrate, KCl, NH_4CI, γ -aminobutyric acid (GABA) and 3,4--di hydroxyphenylalanine (DOPA). They were not successful in their attempts probably due to other biological and physical parameters such as light intensity, duration, age of the larvae, and combination of chemical cues which were not considered during the study.

1.4.2.4 Spatial competition

Marine organisms compete for space in crowded ecosystems such as tidepools (Figure 1.4) and coral reefs. The organisms may crowd, undercut, crush, overshadow, overgrow, digest, or poison their neighbours (Quinn, 1982; Branch, 1984).

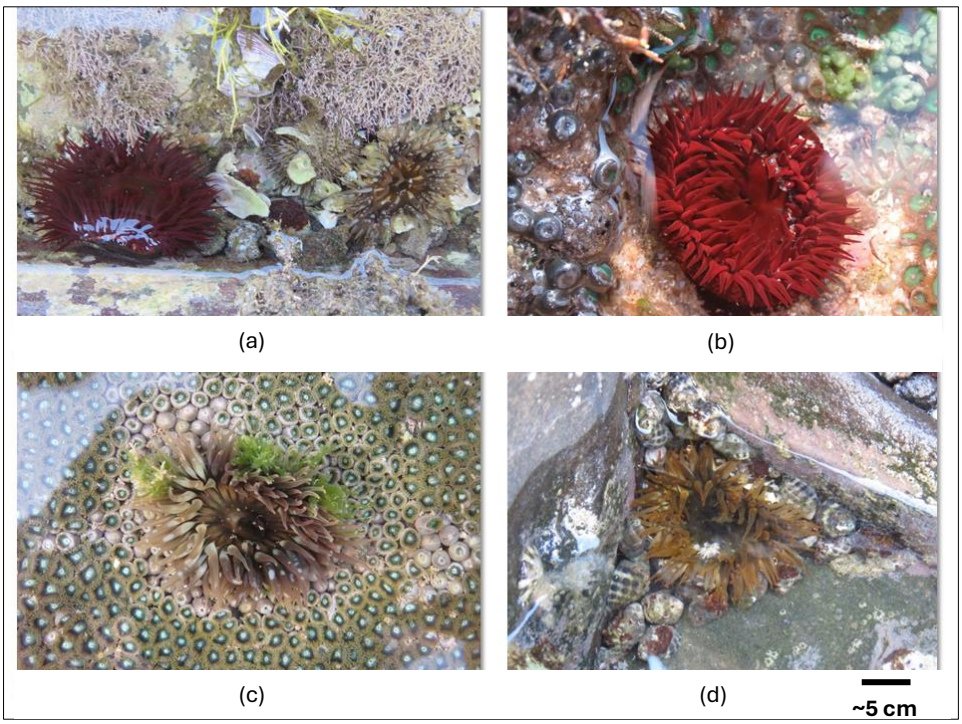


Figure 1.4: Images of Sea anemones from the tidepools of the rocky shores of the West Coast of India. This is highly competitive habitat with regard to space. Sea anemones maintain their niche in this habitat by using their venom arsenal to compete with other intertidal organisms. Figure 1. 4a & 1. 4b: Sea anemone in tidepools along with neighbours- Sea

urchin, Macro-algae, sponge, Zoanthus. Figure 1.4c & 1.4d: Sea anemone seen in space crunch with Zoanthus and Gastropods

Aggression involves very exquisite intra-and interspecific recognition (Bigger, 1982). Most cnidarians use their venom to inhibit growth or kill their spatial competitors. Catch tentacles were used in intraspecific and interspecific aggression among *Cereus pedunculatus, Cylista elegans*, *and Cylista troglodytes* (Williams, (1975)). The acrorhagi are specialized nematocysts filled sacs containing a cocktail of venom that is used to cause damage during spatial competition with non-clonal intraspecific individuals (Jenner & Undheim (2017); Surm *et al*., (2019); Ayre & Grosberg (1995)) (Figure 1. 4). An injured anemone closes, moves away, and sometimes may release its pedal disc from the substratum (Brace, (1990)). In the anemones, *Cereus pedunculatus, Cylista elegans*, *and Cylista troglodytes*, the catch tentacles were used offensively against members of their species from different localities and other species (Purcell, (1977)). Bak and Borsboom (1984) studied the allelopathic interaction between the giant sea anemone *Condylactis gigantea* and benthic algae. Algal biomass and spore germination was inhibited by the cnidarian exudates consisting of terpenes.

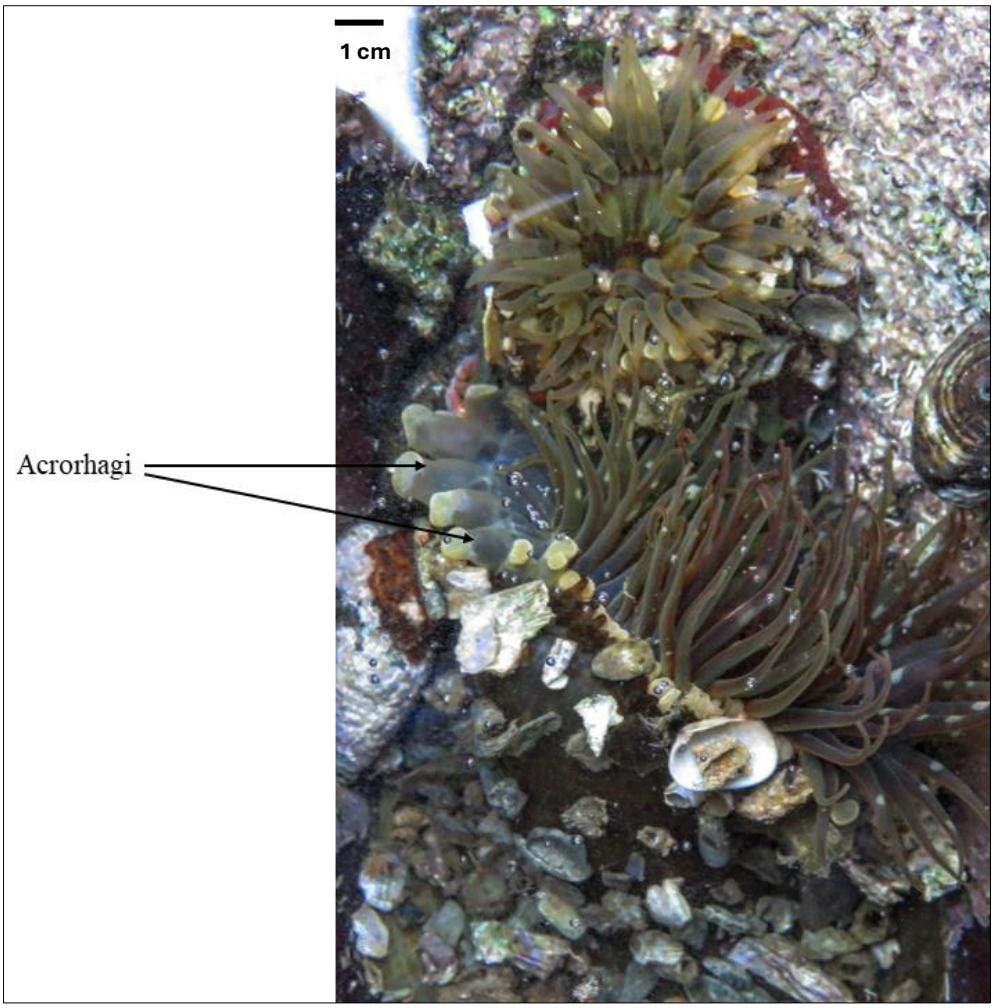


Figure 1.5: *Anthopleura anjunae* displaying its acrorhagi during non-clonal intraspecific aggression

1.4.2.5 Defence against predators

Sea anemones are soft and stationary organisms making them very vulnerable to predation. The anti-predatory mechanism can be defensive as well as offensive. The sea anemones can either actively defend themselves by using their tentacles to sting the predator or it can retract their tentacles and column, exposing the predator to its ectodermal mucus on the body column.

The sea anemone venom contains various compounds of proteinic nature including pore-forming toxins or Cytolysin, acid-sensing ion channel toxins, phospholipases Na^+ channel toxins, K^+ channel inhibitors, other neurotoxins, haemolytic toxins, and proteinase inhibitors (Hessinger and Lenhoff, 1988). The defensive behaviour of sea anemones, when attacked by the nudibranch *Aeolidia papillos*, was studied by Edmunds and co-workers (1976). They reported that *Anemonia sulcata* uses its tentacles to defend itself against the predator,

whereas *Actinia equina* responds by tentacle and column retraction, and inflating its column, exposing its acrorhagi to the eolid. *Anthopleura elegantissima* responds in similar ways. *Anemonia* crawls away but it does not detach. Exposure to the Nv1 toxin of the starlet sea anemone *Nematostella vectensis* leads to contraction paralysis of its predator the grass shrimps (Kneib, 1985). Similarly, Christomanos (1953) isolated purple pigment and protein threads from *Adamsia rondeletii* and suggested that its possible function was in self-defence of the sea anemone. Sea anemones are known to disperse alarm pheromones to alert their conspecifics against predators as a strategy to reduce predation. Howe and Sheikh (1975) reported that anthopleurin acts as a toxin in addition to an alarm pheromone. When attacked by a predator, *Anthopleura elegantissima* immediately withdraws its tentacles and oral disc. Now the predator is exposed only to the body of the sea anemone that contains the highest amount of anthopleurin. After feeding upon the sea-anemone, the predator travels through the water and aids in the spread of anthopleurin, alerting conspecifics to defend themselves (Howe, 1976). *Anthopleura sp*. moves away from injured or crushed conspecifics, indicating the release and detection of alarm pheromones (Howe and Sheik, 1975). Similarly, the attack by *Clinocottus globiceps* leads to the alarm response in nearby *A. xanthogrammica* (Hand, 1996). Acontia, a unique defence tissue consisting of thread-like structures containing numerous stinging cells, located in the gastrovascular cavity of anemone. When threatened, the sea anemone ejects the acontia through its actinopharynx, and toxins are injected into the predator (Lam *et al*., (2017)). Edmunds *et al*., (1976) studied the behaviour of sea anemones when attacked by Aeolidia and reported that *Cylista elegans, Cereus pedunculatus, Aiptasia couchii, Actinothoe sphyrodeta* and *Metridium senile* eject acontia as a defence response.

1.4.3 Types of sea anemone venom

The venom composition is not known in detail, but it mainly consists of proteinaceous substances such as peptides, proteins, enzymes, and proteinase inhibitors and non-proteinaceous molecules like purines, quaternary ammonium compounds, biogenic amines, and betaines (Martins *et al.,* 2009; Jouiaei *et al*., 2015b; Frazão *et al*., 2012; Mariottini *et al*., 2014). This venom cocktail varies from species to species but predominantly consists of molecules that act as neurotoxins, cytotoxins and induce pain and inflammation (Surm *et al.,* 2019; Mariottini & Pane, 2010; Honma & Shiomi, 2006; Madio *et al*., 2019). (Refer Table 1.1 for a list of sea anemone venom peptides reported in literature)

1.4.3.1 Cytotoxins:

Cnidarian venom is known to exhibit cytotoxic activity, which induces necrosis, apoptosis, prevents cell growth. Therefore, the ecological function of cytotoxins is to aid in effective prey capture and defence against predators and competitors (Anderluh &Macek, (2002)).

1.4.3.1.1 Pore-forming toxins (PFT):

These are proteins that disrupt biological membranes by lesion or pore formation. The plasma membrane permeability of the target cells is altered, possibly causing cell death (Los *et al*., 2013).

1.4.3.1.1.1 Actinoporins:

The 'sea anemone pore-forming proteins' are collectively referred to as Actinoporins, a sub-class of Pore-forming toxins, a type of cytolysin.

1.4.3.1.1.2 Membrane attack complex/ Perforins (MACPF):

Perforins are cytolytic pore-forming proteins that bind to cell membranes and oligomerize to form pores up to the size of 20nm in the cell, leading to cytolysis (Liu *et al*., 1995). MACPF is used by sea anemones to kill prey and deter predators (Oshiro *et al.,* 2004)).

1.4.3.1.1.3 Hydralysins-Related Toxin

Hydralysins are non-nematocyte-derived beta-pore-forming toxins in cnidaria, discovered in the digestive endodermal cells of Chlorohydra viridissima (Zhang *et al.,* 2003; Sher et al., 2005). They play a role in the lysis and digestion of prey by binding to specific receptors and forming pores in the tissue (Sher et al., 2005; Sher et al., 2008; Moran et al., 2012b).

1.4.3.1.2 Phospholipases:

Enzymes such as phospholipases constitute a small component of sea anemone venom but are very poorly studied (Madio *et al*., 2019). These are enzymes that catalyse the hydrolysis of 2-acyl ester bonds of 3-sn-phospholipids leading to fatty acids and lysophospholipids production (Yu *et al*., 1993).

1.4.3.1.3 Metalloprotease:

These are metal-dependent proteases that degrade the extra-cellular matrix causing necrosis and haemorrhage and preventing blood clot formation in the prey or predator (Fox & Serrano (2005); Fry *et al*., (2009)).

1.4.3.2 Neurotoxins:

Neurotoxins are low molecular weight peptides that disrupt nerve tissue (Madio *et al*., 2019). Neurotoxicity is caused by blocking neuron cellular processes such as inter-neuron communication and membrane depolarization by the toxin. The major ecological role of neurotoxins is the paralysis and immobilization of the prey (Madio *et al*., 2019).

1.4.3.2.1 Voltage-gated Potassium (Kv) Channel Toxins (KTxs):

These toxins inhibit the potassium current through channels Kv1. and Kv3. subfamilies and intermediate conductance calcium-activated potassium channels (Frazão *et al*., 2012). PHAB is a type of KV toxins and is the shortest sea anemone toxin comprising of 17 residues, κ-actitoxin-Ate1a (Ate1a) peptide. It plays a role in prey immobilization that is achieved through inhibition of Shaker-type KV channels (Madio *et al.,* 2018)

1.4.3.2.2 Voltage-Gated Sodium (Nav) Channel Toxins (NaTxs):

Voltage-gated ion channels are transmembrane proteins that facilitate the electrical signalling of cells. NaTxs bind to the Nav channels resulting in the interference in generation and conduction of action potential and release of neurotransmitters in synapses (Gilchrist *et al*., (2014)).

1.4.3.3 Kunitz peptides:

The Kunitz-type protease inhibitors belong to the serine protease inhibitors family**.** Kunitz peptides reported in sea anemones display Kv blocking activity and protease inhibiting activity (Peigneur *et al*.,2011; Schweitz *et al.,* 1995).

1.4.3.4 Acid Sensing Ion Channel Blockers/Inhibitors:

ASICs are a family of voltage insensitive and proton-sensing channels which are responsible for Na^+ influx and play important roles in mediating pain sensation (Waldmann *et al.,* 1997, Dibas and Dibas, (2019)). Peptides with ASIC inhibition potential have been recently discovered in Cnidarians (Osmakov *et al*.,(2013); Rodríguez *et al*.,(2014)). Their ecological role is to repel potential predators by inducing pain (Jami et al., (2018)).

1.4.3.5. TRPV1 Inhibitor:

TRPV is a highly calcium-selective family of transient receptor potential cation channels (TRP channels) in animals (Winston *et al*; (1988)). TRPVs are molecular triggers of pain

stimulus and TRPV inhibitors attenuate the expression of TRPV1 and thus can be used to develop analgesics (Jouiaei *et al*., 2015b).

1.4.3.6 Acrorhagin:

Acrorhagins are di-sulphide-rich peptides isolated from the Acrorhagi of sea anemone *Actinia equinia* (Krishnarjuna *et al.,* 2021). Acrorhagin I have no homologies with any known toxins, while acrorhagin II is similar to spider neurotoxins hainantoxin-I and Tx 3-2 and cone snail neurotoxin ω-conotoxin MVIIB (Honma *et al*., 2005b). The acrorhagi toxins of *Actinia equina* induce tissues necroses by the intracellular formation of reactive oxygen species while lacking the neurotoxic activity (Bartosz *et al*., 2008). The ecological function of Acrorhagin is to deter encroaching intra-specific spatial competitors by causing paralysis and necrosis of the tissue.

Table 1.1: List of Venom peptides with the source organism, toxin type, and ecological function.

Sr. No.	Peptide	Source organism	Toxin type	Ecological function	Reference
1.	Fra-E	*Actinia fragacea*	Actinoporins	Prey capture or/and defense against predators and competitors	Morante *et al*., (2019)
2.	Nigrelysin	*Anthopleura nigrescens*	Actinoporins	Prey capture or/and defense against predators and competitors	Alvarado-Mesén *et al*., (2019)
3.	CJTOX I and CJTOX II	*Cribrinopsis japonica*	Actinoporins	Prey capture or/and defense against	Tsutsui & Sato (2018)

				predators and competitors	
4.	RTX-A, RTX-S, RTX-G, RTX-S II, Hct-A and Hct-S4	*Heteractis crispa*	Actinoporins	Prey capture or/and defense against predators and competitors	Leichenko *et al*., (2014); Tkacheva *et al*., (2011); Klyshko *et al*., (2004); Monastyrnaia *et al*. (1999); Monastyrnaya *et al*., (2002).
5.	Fragaceatoxin C	*Actinia fragacea*	Actinoporins	Prey capture or/and defense against predators and competitors	Bellomio *et al*., (2009)
6.	Gigantoxin-4	*Stichodactyla gigantea*	Actinoporins	Prey capture or/and defense against predators and competitors	Hu *et al.,* (2011)
7.	Caissarolysin I (Bcs I)	*Bunodosoma caissarum*	Actinoporins	Prey capture or/and defense against predators and competitors	de Oliveira *et al*., (2006)
8.	Avt-I	Globular vesicles of the tentacles of	Actinoporins	Prey capture or/and defense	Uechi *et al*., (2005)

		Actineria villosa		against predators and competitors	
9.	Or-A and Or-G	*Oulactis orientalis*	Actinoporins	Prey capture or/and defense against predators and competitors	Il'ina *et al*., (2005)
10.	Src I	*Sagartia elegans*	Actinoporins	Prey capture or/and defense against predators and competitors	Jiang *et al*., (2003)
11.	PsTX-20A	*Phyllodiscus semoni*	Actinoporins	Prey capture or/and defense against predators and competitors	Nagai *et al*., (2002)
12.	Bandaporin	*Anthopleura asiatica*	Actinoporins	Prey capture or/and defense against predators and competitors	Kohno *et al*., (2009)
13.	Unnamed pore-forming protein	*Anthopleura dowii*	Actinoporins	Prey capture or/and defense against predators and competitors	Ramírez-Carreto *et al*., (2019)

14.	Tenebrosins TN-A, TN-B, TN-C	*Actinia tenebrosa*	Actinoporins	Prey capture or/and defense against predators and competitors	Norton *et al.,* (1990)
15.	Equinatoxin II	*Actinia equina*	Actinoporins	Prey capture or/and defense against predators and competitors	Ferlan & Lebez (1974)
16.	U-AITx-Ate1	*Actinia tenebrosa*	Cytotoxin	Prey capture or/and defense against predators and competitors	Elnahriry *et al*., (2019)
17.	Magnificalysin I, Magnificalysin II, HMg III, Cytolysin HmT	*Heteractis magnifica*	Actinoporins	Prey capture or/and defense against predators and competitors	Khoo *et al.* (1993); Wang *et al.,* (2000); Samejima *et al*., (2000).
18.	Src-1	*Sagartia elegans*	Actinoporins	Prey capture or/and defense against predators and competitors	Jiang *et al.,* (2002)
19.	SmT-2,SmT-2	*Stichodactyla mertensii*	Actinoporins	Prey capture or/and defense	Samejima *et al*., (2000)

				against predators and competitors	
20.	UpI	*Urticina piscivora*	Actinoporins	Prey capture or/and defense against predators and competitors	Cline *et al*., (1995)
21.	AvTX60	Nematocysts of *Actineria villosa*	Membrane attack complex/ Perforins	Prey capture or/and deter predators	Oshiro *et al*., (2004)
22.	PsTX-60B	*Phyllodiscus semoni*	Membrane attack complex/ Perforins	Prey capture or/and deter predators	Satoh *et al*., (2007)
23.	Sticholysin I and II	*Stichodactyla helianthus*	Phospholipase	Prey capture or/and deter predators	del Monte-Martinez *et al.,* (2014); Anderluh &Macek, (2002)
24.	Unnamed	Tentacle Venom of *Condylactis gigantea*	Phospholipase	Prey capture or/and deter predators	Romero *et al*., (2010)
25.	PLA2	*Bunodosoma caissarum*	Phospholipase	Prey capture or/and deter predators	Martins *et al*., (2009)
26.	Aerolysin	*N. vectensis*	Hydralysin-related toxin	Lysis and digestion of prey	Moran *et al.,* 2012a

27.	Zinc dependent metalloproteinase	*Epiactis japonicus*	Metalloproteinase	Prey capture or/and deter predators	Babenko *et al.,* (2017).
28.	Disintegrin/ Zinc dependent metalloproteinase	*Aulactinia verrucose*	Metalloproteinase	Prey capture or/and deter predators	Domínguez-Pérez. (2018).
29.	Granulitoxin	*Bunodosoma cangicum*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Santana *et al*., (1998)
30.	AeK	*Actinia equina*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Minagawa *et al*., (1998)
31.	APETx1, APETx2, APETx3	*Anthopleura elegantissima*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Peigneur *et al*., (2012)
32.	Kappa-actitoxin-Ate1a	*Actinia tenebrosa*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Madio *et al*., (2018)
33.	Cangitoxin II and Cangitoxin III	*Bunodosoma cangicum*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Zaharenko *et al.,* (2008a)
34.	Blood-depressing substance-1 (BDS-1), also known as kappa-actitoxin-Avd4a,	*Anemonia sulcata*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Diochot *et al*., (1998); Liu *et al*., (2012)
35.	Crassicorin-I and Crassicorin-II	The pharynx of *Urticina crassicornis*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Kim *et al*., (2017)

36.	AnerK, AETX I, AETX II, AETX III, AETX-K	*Anemonia erythraea*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Hasegawa *et al*., (2006); Shiomi *et al*., (1997)
37.	Kaliseptine	*Anemonia sulcata*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Schweitz *et al*., (1995).
38.	BcsTx1, BcsTx2 and BcsTx3	*Bunodosoma caissarum*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Orts *et al*., (2013a); Orts *et al*., (2013b)
39.	Bcg III 21.75	*Bunodosoma cangicum*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Osmakov *et al*., (2013)
40.	Bgk, U-AITX-Bg1a-e	*Bunodosoma granuliferum*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Aneiros *et al*., (1993); Rodríguez *et al*. (2012)
41.	Ca I and CaK	*Cryptodendrum adhaesivum*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Maeda *et al*., (2010); Yamaguchi *et al.,* (2010)
42.	Halcurin	*Halcurias carlgreni*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Ishida *et al*., (1997)
43.	HmK	*Heteractis magnifica*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Gendeh *et al*., (1997)
44.	OspTx2a	*Oulactis sp*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Sunanda *et al.,* (2018)
45.	Metridin	*Metridium senile*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Krebs & Habermehl, (1987)

46.	SgK, SHTX I/SHTX II, shtx-k	*Stichodactyla gigantea*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Yamaguchi *et al*. (2010); Honma *et al*., (2008)
47.	SmK	*Stichodactyla mertensii*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Yamaguchi *et al*., (2010)
48.	ShK	*Stichodactyla helianthus*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Castañeda *et al*., (1995)
49.	Gigantoxin-II and Gigantoxin-III	*Stichodactyla gigantea*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Shiomi *et al*., (2003)
50.	Av3	*Anemonia viridis*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Moran *et al*., (2007)
51.	Hk7a and Hk2a	*Anthopleura sp*.	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Xiang *et al*., (2008)
52.	Cangitoxin	*Bunodosoma cangicum*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Cunha *et al*., (2005)
53.	BcIII and BcIV	*Bunodosoma caissarum*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Oliveira *et al*., (2006)
54.	Nv1	Ectodermal gland cells of *Nematostella vectensis*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Moran *et al*., (2012a)
55.	NvePtx1	Eggs and Planula of *Nematostella vectensis*	Voltage-gated Sodium Channel Toxins	Protection of eggs and planula from predator	Columbus-Shenkar *et al*., 2018

56.	CgNa	*Condylactis gigantea*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Billen and co-workers (2010)
57.	ApC	*Anthopleura elegantissima*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Salceda and co-workers (2006)
58.	Av3	*A. elegantissima*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Gur and fellow workers (2014)
59.	RpII	*Heteractis magnifica* mucus	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Sintsova *et al*., 2018
60.	Calitoxin I and Calitoxin II	*Calliactis parasitica*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Cariello *et al*., 1989; Spagnuolo *et al*., 1994
61.	ATX-I, ATX-II and ATX-III	*Anemonia sulcata*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Alsen, (1983)
62.	Peptide toxin Am I, Peptide toxin Am II and Peptide toxin Am III	*Antheopsis maculata*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Honma and others (2005a)
63.	Toxin AFT-I and Toxin AFT-II	*Anthopleura fuscoviridis*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Sunahara *et al*., 1987
64.	Anthopleurin-B, Toxin PCR1, Toxin PCR2, Toxin PCR3,	*Anthopleura xanthogrammica*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey.	Norton, 1981; Kelso *et al*., 1998;

	Toxin PCR4, Toxin PCR5, Toxin PCR6, Toxin PCR7			Alarm pheromone	Howe and Sheikh., (1975)
65.	Ade-1	*Exaiptasia diaphana*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Nesher *et al*., 2013
66.	Bc-III and Bc-IV	*Bunodosoma caissarum*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Oliveira *et al.*, 2004); Oliveira *et al.*, (2006)
67.	Delta-actitoxin-Bca1a	*Bunodosoma capens*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	van Losenoord *et al*., (2019)
68.	Bg II and Bg III	*Bunodosoma granuliferum*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Loret *et al*., (1994)
69.	Toxin AnmTx Cj 1c-1, Toxin CjTL7, Toxin CjTL8	*Epiactis japonica*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Babenko *et al*., (2017)
70.	Re-I, RpII, RpIII, Neurotoxin-I, Neurotoxin-II, Neurotoxin-III, Neurotoxin-IV, and Hcr 1b-3, Hcr 1b-2, Hcr 1b-4.	*Heteractis crispa*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Shiomi *et al.,(*1996); Zykova,(1985); Zykova *et al*., (1988a); Zykova *et al*., (1988b); Zykova *et al*., (1989); Kalina *et al*., (2018);

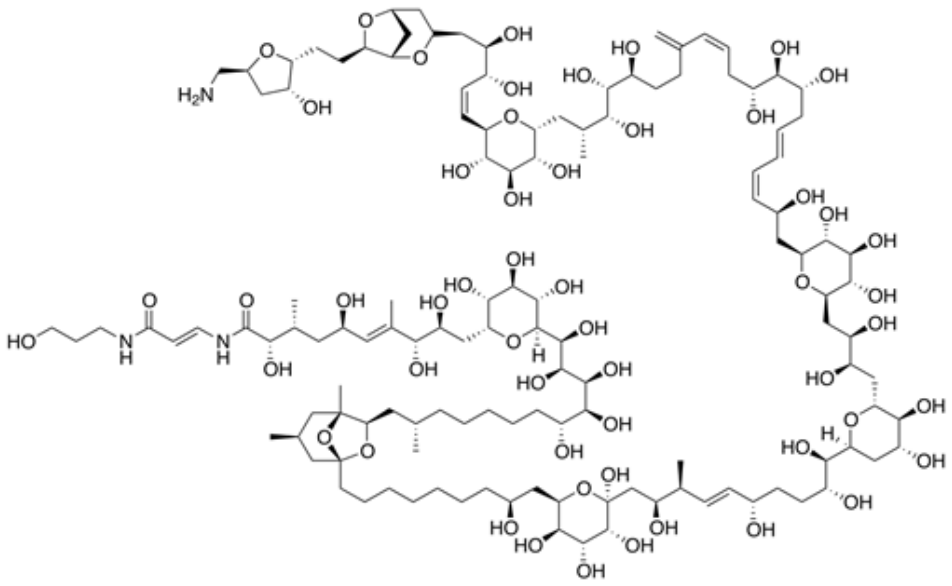
					Kalina *et al*., (2020); Metrione *et al*., (1987)
71.	SHTX IV	*Stichodactyla haddoni*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	*Honma et al., (2008)*
72.	U-SHTX-Sdd1	*Homostichanthus duerdeni*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Cassoli *et al*., (2013)
73.	MS9.1	*Metridium senile*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Logashina *et al*., (2017a).
74.	APEKTx1	*Anthopleura elegantissima*	Kunitz peptides	Paralysis and Death of prey	Peigneur *et al*., (2011)
75.	AXPI-I and -II	*Anthopleura xanthogrammica*	Kunitz peptides	Paralysis and Death of prey	Minagawa *et al*., (1997)
76.	Unammed Kunitz-type peptides	The mucus of *Heteractis magnifica*	Kunitz peptides	Paralysis and Death of prey	Sintsova *et al*., (2018)
77.	HC1, HC2, HC3, HCRG, HCRG1, HCRG2, HCRG21	*Heteractis crispa*	Kunitz peptides	Paralysis and Death of prey	Kozlov et al., 2009; Gladkikh *et al*., 2020; Monastyrnaya *et al.,* 2016; Gladkikh *et al*., 2015; Gladkikh *et al*., 2012

78.	AEAPI,	Acrorhagi venom of *Actinia equina*	Kunitz peptides	Paralysis and Death of prey	Minagawa *et al.,* (2008)
79.	AXAPI	Acrorhagi venom of *Anthopleura xanthogrammica*	Kunitz peptides	Paralysis and Death of prey	Minagawa *et al.,* (2008)
80.	AFAPI-I and AFAPI-III	Acrorhagi venom of *Anthopleura fuscoviridis*	Kunitz peptides	Paralysis and Death of prey	Minagawa *et al.,* (2008)
81.	Iq-peptide	*Heteractis magnifica*	Kunitz peptides	Paralysis and Death of prey	Kvetkina *et al.,* (2018)
82.	ShPI-1, also known as the bovine pancreatic trypsin inhibitor (BPTI)-Kunitz-type protein	*Stichodactyla helianthus*	Kunitz peptides	Paralysis and Death of prey	García-Fernández *et al*., (2016)
83.	Kalicludine-1, Kalicludine-2, and Kalicludine-3	*Anemonia sulcata*	Kunitz peptides	Paralysis and Death of prey	Schweitz *et al*., (1995)
84.	SHTX II	*Stichodactyla haddoni*	Kunitz peptides	Paralysis and Death of prey	Honma *et al*., 2008
85.	Gigantoxin I, SHTX V	*Stichodactyla gigantea*	SCRiP	Bacterial defence	Honma *et al*., 2008; Shiomi *et al.,* 2003).
86.	Ueq 12-1	*Urticina eques*	SCRiP	Bacterial defence	Logashina *et al*., 2017b

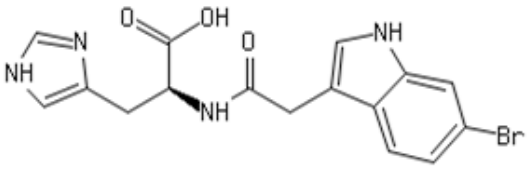
87.	Msen-SCRiP	*Metridium senile*	SCRiP	Bacterial defence	Jouiaei *et al*., 2015a
88.	π-AnmTX Ugr 9a-1	*Urticina grebelnyi*	ASIC inhibitors	Repel potential predators	Osmakov *et al*.,(2013)
89.	PhcrTx1	*Phymanthus crucifer*	ASIC inhibitors	Repel potential predators	Rodríguez *et al*.,(2014)
90.	τ-SHTX-Hcr2b (APHC1), (τ-SHTX-Hcr2c (APHC2) and τ-SHTX-Hcr2d (APHC3)	*Heteractis crispa*	*TRPV1 Inhibitors*	Trigger pain in predator or competitor	Andreev *et al*., (2008); Kozlov *et al*., (2009).
91.	Acrorhagin I, II	*Actinia equina*	*Arorhagin*	Deter encroaching intraspecific competitor	Honma *et al*., (2005b)
92.	κ-Actitoxin-Ate1a	*Actinia tenebrosa*	*PHAB*	Paralysis and immobilization of prey	Madio *et al.,* (2018).

1.2.3.7 Non-proteinaceous molecules

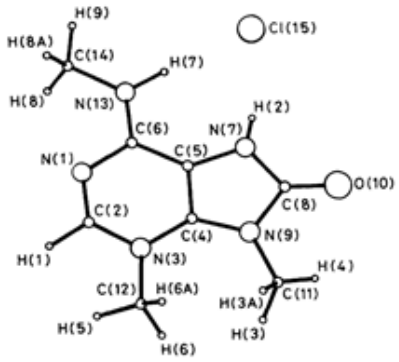
Heteractis crispa has been reported to produce Palytoxin (Figure 1. 5a), a polyether, that is a vasoconstrictor and one of the most poisonous non-proteinaceous toxins known to date (Mahnir *et al.,* 1992). Bunodosine 391 is an acylated amino acid discovered from *Bunodosoma cangicum* (Figure 1. 5b). It is reported to act on serotonin receptors and has analgesic activity (Zaharenko *et al.,* 2011). Caissarone (3,9-dimethyl-6-methylimino-8-oxo-3,6,8,9-tetrahydropurine) is an iminopurine from *Bunodosoma caissarum* which is the first marine compound reported as an adenosine receptor antagonist (Figure 1. 5c). Caissarone is reported to increase motility in isolated mammalian gut preparations (Cooper *et al*., 1995). 1,2-iminomethyl-3-methyl-6-aminomethyl-9H-purine was extracted from *Cylista troglodytes* (Figure 1. 5d). (de Rosa *et al*., 1987).



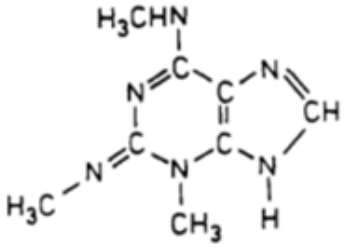
(a) Palytoxin



(b) Bunodosine 391



(c) Caissarone



(d) 1,2-iminomethyl-3-methyl-6-aminomethyl-9H-purine

Figure 1.5a : Molecular structure of Palytoxin (Ref: Pubchem); Figure 1.5b: Molecular structure of Bunodosine 391 (Zaharenko et al., 2011); Figure 1.5c: Molecular structure of

Caissarone (Cooper et al., 1995); Figure 1. 5d: Molecular structure of 1,2-iminomethyl-3-methyl-6-aminomethyl-9H-purine (de Rosa et al., 1987)

1.4.4 Bioprospecting of sea anemones

The ability of cnidarians to produce powerful venoms is well documented and has attracted many researchers. There are numerous studies reporting the toxic peptides and molecules present in the Actinarian venom (Jouiaei *et al*., (2015b); Prentis *et al.,* (2018); Madio *et al.,* (2019); D'Ambra & Lauritano (2020); Mariottini & Grice, (2019), Ashwood *et al.,* (2020)). The neurotoxic and cytotoxic nature of sea anemone venom can be exploited to develop novel drugs to treat several ailments . Neurotoxins function by blocking cellular processes in the nervous system and other tissues by binding to voltage-gated ion channels of the targeted organism. Since potassium channel toxins block potassium channels, they facilitate the release of the acetylcholine (Diochot & Lazdunski (2009)), which in humans can have analgesic and anaesthetic effects. They can also be therapeutic leads for the treatment of autoimmune diseases (Prentis *et al.,* (2018)). On the other hand, cytotoxins work by killing cells thereby having antimicrobial potential and can be employed in cancer treatment (Chatterjee, (2018)). Sintsova *et al*., (2015) reported the anti-inflammatory activity of the HCGS 1.20 recombinant polypeptide, a Kunitz-typeserine protease inhibitor from *Heteractis crispa.* Peptide Ms 9a-1 isolated from *M. senile* venom exhibited anti-inflammatory activity by potentiating TRPA1 (Logashina *et al*., 2017a). In a study conducted by Ramezanpour *et al.,* (2012), the venoms of *Heteractis crispa, Heteractis magnifica, Heteractis malu, Cryptodendrum adhaesivum* and *Entacmaea quadricolor* exhibited anti-cancer activity against human lung, breast and skin cancer cells. Loret and fellow workers (2018) reported the anti-angio activity of a low molecular weight protein from *Anemonia viridis.* Cytolysins Sticholysin I and II from *Stichodactyla helianthus* and equinatoxin II from *Actinia equina* displayed anti-parasitic activity against *Giardia duodenalis (*Tejuca *et al*., 1999*).* Ramezanpour and fellow workers (2014) reported that the venom of *Heteractis magnifica* killed human breast cancer cell lines via apoptosis. Actinoporin from *Heteractis crispa* exhibited anti-cancer activity *in vitro* as well as prevented the migration of HT-29 Colorectal Cancer Cell (Kvetkina *et al.,* (2020)). Actinoporin RTX-A from *Heteractis crispa* also has potential anti-cancer effects (Fedorov *et al.,* 2010).

Sea anemone venom that is effective in killing crustaceans has also been reported to be effective against insect pests given the close evolutionary relationship between insects and

crustaceans, therefore these peptides can be lead molecules in development of novel insecticides (Bosmans & Tytgat, 2007). Fu and fellow workers (2021) reported the anti-insecticidal activity of Peptide AP-GI from *Aiptasia pallida*. Neurotoxin BgII from *Bunodosoma granulifera* is potent on the insect sodium channel and can be used as a template to develop insecticides (Bosmans *et al*., 2002). On that account, the study of cnidarian toxin is rising as a promising field in the discovery of novel drug and therapeutic molecules as well as agriculturally and industrially important compounds.

Traditionally, bioactive compounds are extracted from a sample by bioassay-guided fractions and the structure, chemical composition and function are determined (Koehn & Carter (2009)). More recently, *in silico* methods are employed to unveil and identify genes, gene homologs, motifs and transcripts that express certain profiles (Johansen *et al.,* (2010); Yamaguchi *et al.,* (2010); Sperstad *et al*., (2011); (Urbarova *et al*., 2012)).

1.4.5 Sea anemone venom in healthcare

There are many sea anemone venom peptides explored for medical use. All these toxins are involved in neural channel manipulation within the body and cell death (Diochot & Lazdunski (2009); Chatterjee(2018)). Despite enormous research on sea anemone venom and the discovery of several novel toxins, the number of venom-derived peptides that have successfully advanced to clinical trials is currently limited.

In 1995, Olga Castañeda and her team discovered and characterized a potassium channel toxin ShK from the Caribbean sea anemone Stichodactyla helianthus (Castañeda et al., 1995). Extensive structure-activity studies led to the development of an ShK analog, ShK-186, as a selective Kv1.3. blocker (Beeton *et al*., 2005; Beeton *et al*., 2006; Pennington *et al*., 2009). ShK-186 (now known as Dalazatide) is the first drug derived from sea anemone venom that has completed Phase 1 clinical trials (Tarcha *et al*.,2017) and is ready to undergo Phase II clinical trials for treating several autoimmune diseases such as lupus erythematosus, psoriasis, rheumatoid arthritis, inflammatory bowel disease, type 1 diabetes, and multiple sclerosis (MS).

1.4.6 Other sea anemone bio-products in biomedical applications

Besides medicinal drugs, there are a few other sea anemone-derived molecules that have application in the biomedical industry. Aneroin, a recombinant protein originating from sea

anemone *Nematostella vectensis*, is being exploited as a hydrogel. It shows relatively stronger and stiffer properties than collagen, gelatin, and elastin, and four times stronger mechanical properties than silkworm silk. Aneroin finds its application for cell-containing biomaterials, cell-carrier patches, bio-artificial grafts, and burn dressing materials (Yang *et al*., 2013; Yang *et al*., 2015). A patent has been granted to the inventor for the method for manufacturing Aneroin (Patent no.US20150057435A1), Postech Foundation and Postech Academy Industry Foundation, Pohang-si, South Korea is the current assignee of the patent. Based on aneroin's durable mechanical properties and biodegradability a new 3DBP ink named aneroin ink has been developed. To improve the ink's printability and cell adhesiveness hyaluronic acid and mussel adhesive protein were respectively incorporated and a life-sized human ear, vascular graft, and rectangular multi-layered lattice were successfully bio-printed. Aneroin ink can further find an application in developing various 3D-shaped bioscaffolds in tissue and biomedical engineering fields (Park *et al.,* (2019). Similarly, Anegen hydrogel is a Load-Bearing Hydrogel Based on Engineered *Nematostella vectensis* Sea Anemone Skin-Derived Collagen-Like Protein that can be developed as a prospective load-bearing and cell-loading scaffolding biomaterial in the tissue and biomedical engineering area (Yang *et al*., 2018).

HcRed is a far-red fluorescent protein from the sea anemone *Heteractis crispa*, having absorption maxima at 590 nm and emission maxima at 650 nm, showing potential as a fluorescent marker in quantitative microscopy investigations (Lessard *et al.,* 2008).

1.4.7 Sea anemone as prospective Bio-control agents

Research on animal venom to a great extent is being used to develop biopesticides. Equistatin is a protease inhibitor from *Actinia equina*. It is found to be active against gut cysteine and aspartic proteases in common insect pests of crops, such as Coleopteran, Dipteran, and Thysanopteran insects. The gene coding for equistatin is isolated and cloned into vectors and transformed into agricultural plants, thus reducing the insect pest infestation (Lenarcic & Turk, 1999; Lenarcic *et al*., 1997; Outchkourov *et al.,* 2002; Outchkourov *et al*., 2003; Valizadeh *et al.,* 2013). There is a patent granted for a method of plant protection from pests using the above-mentioned technology (CA2294421A1) with Stichting Dienst Landbouwkundig Onderzoek as the current assignee. Sea anemone venom toxins, derived particularly from the acontia and acrorhagi, are being studied to develop novel anti-biofoulants. At the same time, laboratory trials on various species of sea anemones

as augmentative biocontrol are being conducted to minimize biofouling on marine artificial structures, whilst this work needs to be refined before its use.

1.5 Rationale and Gap in Knowledge

While venomous organisms have gained increasing interest in bioprospecting for therapeutic drugs, sea anemone venoms remain relatively underexplored compared to other venomous taxa like cone snails and snakes. This gap in knowledge is particularly evident in lesser-known species such as *Bunodosoma goanense*, which has not been studied beyond its taxonomy. The ecological interactions of *B. goanense*, suggest that venom peptides may play multifunctional roles beyond predation. However, the molecular mechanisms underlying these interactions remain unknown. Furthermore, the venom composition of *B. goanense* has not been characterized, leaving its pharmacological potential unexplored. This research aims to fill this gap by focusing on the unexplored sea anemone *Bunodosoma goanense*, examining its ecological interactions. Understanding the various ecological interactions of this anemone and the ecological roles of its venom can lead to the discovery of their functional roles and pave the way for translating these findings into biotechnological applications. This study targets *B. goanense* populations at Anjuna and Kunkeshwar, sites chosen for their ecological diversity and abundance of this species.

1.6 Study area

The present study was conducted at two intertidal rocky shore locations along the western coast of India — Anjuna, Goa and Kunkeshwar, Maharashtra — where natural populations of *Bunodosoma goanense* were observed and collected. These sites were selected due to their abundance of sea anemones and the presence of diverse ecological interactions, making them ideal locations to study the species' ecological behaviour, venom composition, and bioprospecting potential. Anjuna (15°34'02.7"N, 73°44'29.2"E) is a semi-exposed intertidal rocky shore along the central coast of Goa, characterized by basaltic rock platforms, tide pools, and patches of macroalgae. The site experiences moderate wave action and a tropical climate, with distinct monsoon and dry seasons. *Bunodosoma goanense* is commonly found in crevices and tide pools. Kunkeshwar (16°20'06.2"N, 73°23'17.2"E) is a wave-exposed

rocky intertidal shore along the Konkan coast. The shore consists of basaltic rock platforms interspersed with tide pools.

1.7 Research Objectives

1. **To Understand the Ecological Interactions of *Bunodosoma goanense* and the Molecular Mechanisms Involved:**

- Interaction studies of *B. goanense* with co-occurring marine organisms.
- Investigation of elected ecological interactions through field observations and molecular techniques

2. **To Characterize the Tissue-Specific Venom of the Sea Anemone:**

- Characterization of the venom from different tissues of *B. goanense* to understand it's functional role via proteomics and bioassay approaches.

3. **To obtain venom peptides via *in-vitro* and *in-silico* approaches:**

- Establishment of *in vitro* cultures of tentacle cnidocytes and ectodermal cells from *B. goanense* to check the production of venom *in-vitro.*
- *In-silico* determination of putative venom peptide using transcriptomic approach.
- Validation of structural and functional potential of the putative peptides using protein modeling technique.
- Synthesis of selected venom peptide and experimental validation of the function.

By integrating ecological observations with venom profiling and bioprospecting approaches, this study not only contributes to understanding the adaptive significance of venom in marine ecosystems but also highlights its potential in drug discovery. This knowledge lays the foundation for sustainable venom research by promoting non-destructive methods of peptide discovery.

Chapter 2

Identification of Sea Anemone: Morphology and Molecular Tools

2.1 Background of *Bunodosoma goanense* Vennam & den Hartog, 1993

The genus *Bunodosoma* Verrill, 1899 is distinguished by the presence of wart-like or bump-like structures on the column called vesicles. In this genus, the vesicles are non-adhesive and are arranged in neat rows along the body column of the anemones. The vesicles give the *Bunodosoma* Genus a distinctive appearance, aiding in their identification. The genus *Bunodosoma* comprises 14 species, among which is *Bunodosoma goanense* (Figure 2.1) ,commonly known as the Goa Sea anemone or Burgundy Sea anemone, which was first discovered and described by Vennam and den Hartog in 1993. The taxonomical classification is as follows.

Domain: Eukaryota

Kingdom: Animalia

Phylum: Cnidaria

Subphylum: Anthozoa

Class: Hexacorallia

Order: Actinaria

Suborder: Enthemonae

Superfamily: Actinioidea

Family: Actiniidae

Genus: *Bunodosoma*

Species: *goanense*



Figure 2.1 *Bunodosoma goanense*

The species was discovered on the rocky shores of Goa, India, and its specific name "*goanense*" is derived from this location. *B. goanense* is not only found along the west coast of India but also on the shores of Singapore. The adult polyp of *B. goanense* typically exhibits colours such as burgundy, maroon, or reddish, making it visually striking. When fully relaxed, an adult *B. goanense* can reach a size of approximately 6 to 10 centimetres. It is azooxanthellate, i.e., it doesn't harbour any symbiotic zooxanthellae. It is a shallow-water intertidal species, predominantly found in the mid-littoral to lower-littoral zones of the rocky shores. It usually attaches itself to rocks in tide pools but sometimes can also be found on unoccupied shells.

2.2 Part I: Identification of the Sea anemone

To ensure the accuracy of our research, we carried out an identification process for the sea anemone. This process included morphological analysis, cnidome characterization, and DNA barcoding. Morphological identification, based on external physical characteristics and internal anatomical features, provides a foundational understanding of an organism's appearance and structure. This method allows researchers to classify organisms into taxonomic groups and distinguish between closely related species. On the other hand, molecular identification, such as DNA barcoding, offers a precise means to confirm species identity by analyzing genetic markers. Combining both approaches enhances the robustness of species identification. This identification process forms the foundation of our subsequent research, ensuring that our findings are scientifically valid and reliable.

2.2.1 Methods

2.2.1.1 Study area and sample collection

The sea anemone specimens were collected from the rocky shores of Anjuna, Goa, India (15°34'02.7"N, 73°44'29.2"E), during low tide when the rocks are exposed. A metal spatula was used to gently detach the sea anemones from the substrate to avoid damaging the specimens. A total of 10 samples were collected, divided into two groups based on their preservation method and intended use:

a. Live Samples: Five live samples were placed in plastic sampling containers filled with seawater. These samples were used for studying morphological traits and cnidome characterization in a fresh, natural state. After which they were preserved in formalin.
b. Ethanol-Preserved Samples: Five samples were preserved in 100% ethanol to ensure high-quality DNA extraction. These samples were used for molecular studies.

2.2.1.2 Morphological identification

Preliminary diagnostics of anatomical characters, as described by den Hartog and Vennam (1993) and Fautin et al. (2015), were performed. Observations were made both in the natural habitat and in the laboratory. In the habitat, live specimens were observed for their behavior and physical characteristics. In the laboratory, live specimens were kept in seawater, and detailed morphological characteristics were documented. Additionally, characteristics of specimens preserved in formalin were noted for further comparison.

2.2.1.3 Cnidome analysis

The complement of cnidocysts in the specimen was studied and compared to those described by den Hartog and Vennam (1993) and Beneti (2016). For this analysis, smears of tentacles, acontia, acroragi, and column tissues were prepared and observed under a 100x microscope. The various types of cnidocysts present in these tissues were identified and documented. This detailed examination allowed for a thorough comparison with previously described cnidocyst complements.

2.2.1.2 Molecular identification

2.2.1.2.1 Genomic DNA extraction

Total genomic DNA was extracted from the sea anemone specimen preserved in 100% ethanol. The tissue was homogenized in liquid nitrogen using a mortar pestle. Approximately 80mg of the homogenized tissue was placed in 400 µl of lysis buffer. RNAse was added to reach a final concentration of 0.6 mg/ml, and the sample was incubated at 37ºC for 30. Proteinase K was then added to a final concentration of 1 mg/ml, and the mixture was incubated at 37ºC for 72 hours. After incubation, a mixture of phenol-chloroform-isoamyl alcohol was added. The sample was shaken for 15 minutes and centrifuged at 6500 rpm for 4 minutes. The supernatant was transferred to a clean tube and mixed with an equal volume of phenol-chloroform-isoamyl alcohol mixture, and the shaking and centrifugation were repeated. The supernatant was then combined with 30 µl of saturated NaCl solution and incubated at 4ºC overnight. DNA was precipitated by adding 2.5 volumes of cold absolute ethanol and centrifuged at 13,000 rpm for 4 minutes. The supernatant was discarded, and the pellet was washed with 70% ethanol and air-dried for 24 hours with the tube inverted. The DNA was then resuspended in TE buffer until fully dissolved. The extracted DNA was quantified using a NanoDrop spectrophotometer. To assess the integrity of the DNA, it was run on a 1.5% agarose gel stained with SYBR Safe

Green and visualized under UV light in a Gel documenting system. This is a modification of the method described by Pinto et al., 2000.

2.2.1.2.2 Polymerase chain reaction

For the PCR amplification of Cytochrome oxidase 1 (COX1 gene), a reaction mixture was prepared initially with a total volume of 25 µl during trial runs, which was later scaled up to a final volume of 50 µl. The reaction mixture comprised 100 ng of total DNA template, Takara Mastermix, Folmer's forward and reverse primers, and nuclease-free water. The PCR protocol commenced with an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of amplification. Each cycle consisted of denaturation at 94°C for 40 seconds, annealing at 40°C for 40 seconds, and extension at 72°C for 60 seconds. The final extension was carried out at 72°C for 5 minutes. For the PCR amplification of the 18S rRNA gene, the reaction mixture was prepared same as mentioned earlier. The reaction mixture comprised 100 ng of total DNA template, Takara Mastermix, forward and reverse primers, and nuclease-free water. The PCR protocol commenced with an initial denaturation step at 94°C for 4 minutes, followed by 35 amplification cycles. Each cycle consisted of denaturation at 92°C for 1 minute, annealing at 42°C for 1 minute, and extension at 72°C for 1 minute. The final extension was carried out at 72°C for 5 minutes. The amplified product was recovered using Promega Wizard® SV Gel and PCR Clean-Up System.

2.2.1.2.3 DNA Sequencing and Phylogenetic analysis

PCR products were sent to Eurofins Genomics India Pvt. Ltd, Bangalore for DNA sequencing using Sanger's sequencing method. Multiple sequence alignment was performed using BioEdit software, facilitating the alignment of the extracted DNA sequence and sequences of other species of *Bunodosoma* obtained from NCBI Genbank Nucleotide database. The sequences were imported into BioEdit, and ClustalW was used for the alignment. The phylogenetic tree was constructed using the Maximum Likelihood method in MEGA X software. The aligned sequences from BioEdit were imported into MEGA X, and the Maximum Likelihood analysis was initiated. The Tamura-Nei nucleotide substitution model was selected as the best-fitting model for the dataset for this analysis. To assess the reliability of the tree branches, bootstrap analysis with 1000 replications was performed. The resulting phylogenetic tree was visualized and edited within MEGA X.

2.2.2 Results and Discussion

2.2.2.1 Morphological identification

The preliminary diagnostics of anatomical characters are summarized in Table 2.1. The observations of the live appearance and external anatomy of the live sea anemone (Figure 2.2) is a solid deep red, maroon, or burgundy colour encompassing the column, tentacles, and oral disc, while the pedal disc exhibits a light red-pink hue. The column is covered with non-adhesive, small, spherical vesicular verrucae arranged in longitudinal rows. The anemone possesses 196 tentacles, with the oral disc, pedal disc, and column typically sharing a similar diameter, though the column may occasionally be narrower. The oral and pedal discs measure approximately 3 cm in diameter, and the tentacles range from 1 to 2 cm in length. When fully extended, the column can reach up to 10 cm in length. The preserved appearance of the sea anemone (Figure 2.3) exhibits notable colour changes. The oral disc appears brownish-grey, while the tentacles become shorter and display a dark grey-green colour. The column appears brown to tan, and the pedal disc turns whitish grey.



Figure 2.2: Live Sea anemone specimen



Figure 2.3: Preserved Sea anemone specimen

Table 2.1: Morphological characteristics Sea anemone specimen

Sr. no.	Morphological characters	Live specimen	Preserved specimen
1.	Colour	Burgundy, brick red	Pale Greyish black
2.	Length of column	6-9.5cm	3-5cm
3.	Width of basal disc	3.5-5cm diameter	2-3.5 cm diameter
4.	No. of tentacles	192	192
5.	Column protuberances	Non-adhesive vesicular verrucae	Non-adhesive vesicular verrucae
6.	Type of particles attached to protuberances	Mostly Sand and few specimens with tiny fragments of calcareous tubeworm exoskeleton	Particles detach after preservation
7.	Acrorhagi	Bright red, 2 rows	Greyish white, 2 rows

2.2.2.2 Cnidome analysis

The cnidome analysis involved the identification and documentation of the cnidocysts from various tissues of the sea anemone, including tentacles, acontia, acrorhagi, and column. Observations were made using light microscopy, and the identified cnidocysts were compared with those described by Vennam and Hartog (1993) and Fautin (2015). The types of the cnidocysts observed were consistent with the descriptions in these previous studies. The tentacles displayed a variety of cnidocysts, including Basitrich and Spirocyst. The acontia contained numerous Basitrich and Microbasic p-mastigophore, similar in size and morphology to those previously reported. The acrorhagi were observed to have an abundance Holotrich, Spirocyst and Basitrich. The column exhibited primarily Holotrich and Basitrich. The documented cnidocysts were photographed and are presented in the images below (Figure 2.4). Each type of cnidocyst was compared with the historical data provided by Vennam and Hartog (1993) and Fautin (2015), confirming the accuracy of the identification.

Acrorhagi



Holotrich Spirocyst Basitrich

Tentacles

Acontia



Basitrich Spirocyst Basitrich Microbasic p-mastigophore

Column



Holotrich Basitrich

Figure 2.4: Cnidocysts in the studied Sea anemone

2.2.2.3 Molecular Identification (DNA Sequencing and Phylogenetic analysis)

Genomic DNA of the sea anemone was successfully isolated and the COX1 gene and 18S rRNA gene were amplified via PCR (Figure 2.5).

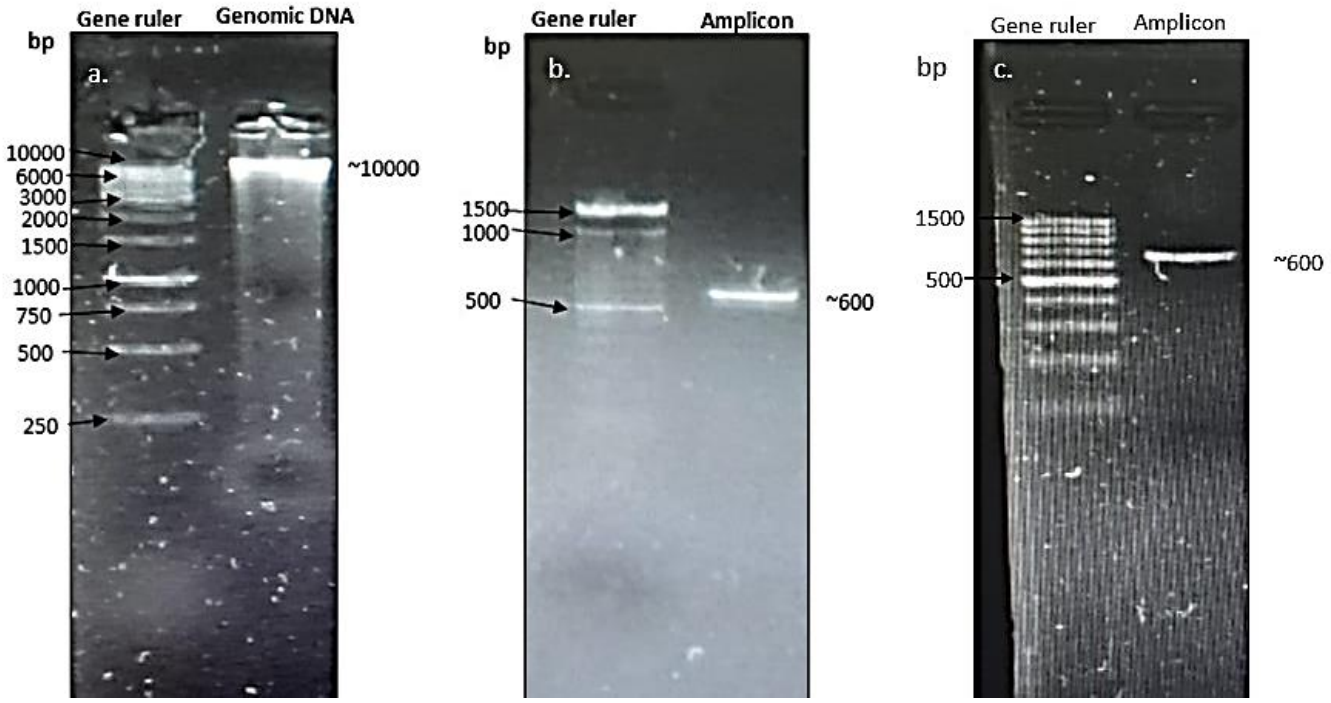


Figure 2.5 :(a) Isolated DNA of *Bunodosoma goenense* agarose gel, (b) Amplified COI gene product agarose gel (c) Amplified 18srRNA gene product in agarose gel

The amplified regions of the COX1 and 5.8S rRNA gene & ITS-2 sequence (part of 18rRNA) were subsequently submitted to the NCBI GenBank, receiving Accession Nos. OK396003.1 and ON892043.1, respectively. This submission marks the first COI gene sequence for the *Bunodosoma* genus and the first 5.8S rRNA gene & ITS-2 sequence of *B. goanense*. The sequences are as follows.

Sequence 1: Cytochrome oxidase I gene

1 GGAGAATTTG GTAATTGGTT AGTACCACTA TATATTGGTG CTCCCGATAT GGCCTTCCCA

61 CGACTAAATA ATATTAGTTT TTGGTTACTC CCTCCTGCGC TTATACTATT ATTAGGCTCT

121 GCCTTTGTTG AGCAAGGAGT AGGGACAGGG TGGACAGTTT ATCCTCCTCT TTCTGGCATT

181 CAAACGCATT CGGGAGGGGC GGTCGACATG GCCATCTTTA GCCTTCATTT AGCGGGTGCG

241 TCTTCTATAT TAGGGGCAAT GAATTTTATA ACAACCATAT TTAATATGAG AGCTCCGGGA

301 TTAACGATGG ATAGACTCCC ACTATTTGTG TGGTCCATTT TAATCACTGC CTTTTTATTA

361 TTACTTTCCC TACCAGTTTT AGCGGGTGGA ATAACCATGC TTTTAACAGA TAGGAATTTT

421 AATACGACTT TCTTTGACCC AGCAGGGGGT GGAGATCCCA TTTTATTCCA ACATTTATTT

481 TGATTCTTTG GTCACCC

Sequence 2: 5.8S rRNA gene & ITS-2 (Part of 18s rRNA gene)

1 AAACCCGCGC AGGACCCAAA AGAACCGTAA GAGTTGAAAA ATTCGAATCA CCGAATATTT

61 GTACACAAAC GGCGCTCTTG GGTTCTCCCA GGAGCACGTC TGTTTGAGTG TCGGCTTCCA

121 AACCATCGCA CGCCCCGCGT GCGGACTTGA GGCGTCGCGG CCCCCGGCAA CGTCGGGCCT

181 CGTCCCTTGA AATGCAGCAC GACCGATGGA AGCGGCCAGC GTCGCCACTC CGGGAAGGCC

241 AAAAAAGCAT CTTTGCCCTT TCCCGAGGCG TCGTCTGACC GGCCGTCCGG TCGGAGTTCG

301 GCAACAAGCC GTGCGCATCC ATTCTTGA

The COX1 gene could not be used for constructing a phylogenetic tree because no other *Bunodosoma* sequences were available in public databases. Our study represents the first COX1 submission for the genus *Bunodosoma*. Instead, we constructed the phylogenetic tree

using the 18S rRNA sequence. BLAST analysis of this sequence was performed using the NCBI GenBank database. Similar sequences were identified, and their FASTA files were downloaded. Multiple sequence alignment was conducted using BioEdit software, and a phylogenetic tree was constructed using the MegaX software application (Figure 2.6). The phylogenetic analysis positioned *B. goanense* closely with other *Bunodosoma* species, supporting its taxonomic classification, with a closest match of 97.18% identity with *Bunodosoma caissarum*. In sea anemones and other anthozoans, high sequence similarity in conserved molecular markers such as COI and 18S is commonly observed even among distinct, valid species. Therefore, molecular similarity alone is not sufficient for species delimitation. In the present study, species identification was based on an integrative taxonomic approach, combining molecular placement within the genus *Bunodosoma* with diagnostic morphological characters and cnidome composition, in accordance with the original species description of *Bunodosoma goanense* by Vennam and Hartog (1993). While *B. caissarum* represents a close molecular match, *B. goanense* is clearly distinguished by its morphological traits, and ecological attributes (Vennam and Hartog,1993).

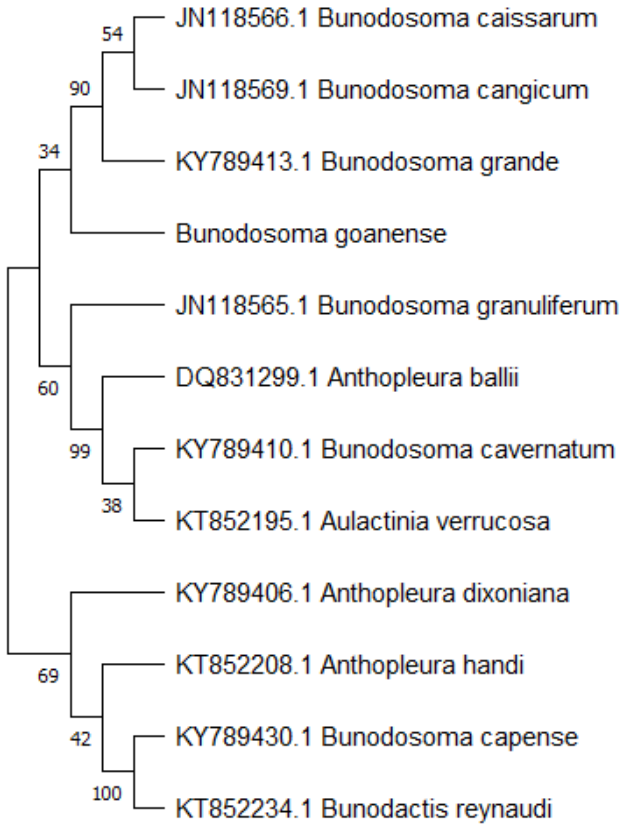


Figure 2.6 : Phylogenetic tree of *B. goanense* with the node values shown representing bootstrap support percentages

2.3 Part II: Discovery of asexual Reproduction: Observations of the likelihood of asexual reproduction in *Bunodosoma goanense*

Sea anemones are marine benthic invertebrates belonging to the class Anthozoa within the phylum Cnidaria that are known to reproduce sexually, asexually, or both (Chia 1976). Sexual reproduction involves fertilization, during which the male and female gametes fuse and form genetically unique offspring. Some sea anemones exhibit hermaphroditism which is mutually exclusive from asexual reproduction (Shick 1991). During asexual reproduction, according to Chia (1976), sea anemones can regenerate by cloning themselves through binary fission where an individual separates longitudinally or transversely to form two equal halves, pedal laceration during which small pieces of tissues of sea anemone separate from the base and form new individuals. Budding involves the formation of new individuals, without division of the parent, from primordial tissue by growth and cellular differentiation (Hughes 1989) and has been reported in very few species. According to Shick (1991), sea anemones rely upon asexual reproduction rather than sexual reproduction in harsh environmental conditions. Shick (1991) also noted that pedal laceration is predominantly found in littoral sea anemones, whereas binary fission is more common in intertidal sea anemones.

The genus *Bunodosoma* comprises 14 species of sea anemones, none of which had been described to reproduce asexually, until recently when Da Silva Junior et al. (2022) reported evidence for longitudinal fission in *B. cangicum* on the shores of Brazil. Den Hartog (1987) mentioned that there were so far no reports of longitudinal or transverse fission within the genus *Bunodosoma* Verrill, 1899. *Bunodosoma caissarum* reproduces exclusively sexually (Belem1987). The ability of *Bunodosoma californicum* to undergo fission is unknown (Daly 2004).

Bunodosoma goanense Den Hartog & Vennam, 1993, a shallow water inter-tidal sea anemone was first discovered on the rocky shores of Anjuna- Goa, on the West coast of India (Den Hartog & Vennam 1993). In their survey, Den Hartog & Vennam (1993) reported that *B.goanense* showed separate sexes and no indications of asexual reproduction. In the present study we provide evidence of possible asexual reproduction by longitudinal fission and pedal laceration in the sea anemone *Bunodosoma goanense.*

2.3.1 Methods

The findings reported herein were made in the mid intertidal zone of the rocky shores of Anjuna in Goa, India (15°34'02.7"N, 73°44'29.2"E) during low tide. The animals were observed and photographed *in situ* using a Samsung Galaxy M51 phone camera.

2.3.2 Result and Discussion

Longitudinal fission of *Bunodosoma goanense* (Fig.2.7 a) was observed on 21st November 2021. It is interesting to note two separate oral discs with a common body column and pedal disc (Mid-fission) indicating that the separation began at the oral disc, but the completion of the separation is not confirmed. Da Silva Junior et al. (2022) reported a similar observation in the case of *B.cangicum*. Although in *B. cangicum* and *B. goanense* the conclusion of fission was not visualized, in both, the process started at the oral end, keeping two completely individualized oral discs while the lower part of the column continued together. This mechanism of longitudinal fission is opposite of the documented method for several species where the process begins in the aboral part. On 19th December 2021, the possibility of cloning through pedal laceration was observed (Fig.2.7 b and 2.7 c). From this observation, we suspect that stretching and extension of the pedal tissue of the parental individual were seen, from which a new tiny clonal individual developed and separated from the parent. The photographs are observations and we, therefore, hypothesize the possibility of longitudinal fission in *B.goanense* and the likelihood of pedal laceration in the genus *Bunodosoma*.



Figure 2.7 a *Bunodosoma goanense* possibly undergoing longitudinal fission. The two separate oral discs (od1 and od2) with a common body column (bc) indicate that the separation began at the oral disc.

b The likelihood of pedal laceration observed in *B. goanense*. With the stretching and extension of the pedal tissue of the parental individual, a new tiny clonal individual (cl) developed and separated from the parent. Another region of pedal disc tissue (pd) stretched and extended to possibly form a new clone.

c A magnified image of the clone (cl) formed by pedal laceration showing its separation from the parental tissue. The tiny tentacles (tn) of the clone are clearly visible.

2.3.3 Conclusion

In this chapter, we successfully identified the sea anemone *Bunodosoma goanense* through a comprehensive approach that included morphological characterization, cnidome analysis, and DNA barcoding. Furthermore, we discovered two novel modes of asexual reproduction in *B. goanense,* expanding our understanding of its reproductive strategies. The identification and characterization of *B. goanense* provide a solid foundation for further ecological and biochemical studies, setting the stage for understanding its venom composition and ecological interactions in subsequent chapters.

Chapter 3

Ecological interactions and molecular mechanisms in *Bunodosoma goanense*

3.1 Ecological interactions of *Bunodosoma goanense*

The ecological interaction was observed along the West coast of India (Goa and Maharashtra). The sea anemone *Bunodosoma goanense* exhibits a remarkable diversity of ecological interactions. Through our observations in on the rocky shores of Goa and Maharashtra, we have noted several key interactions.



Figure 3.1: *B. goanense* feeding on Crab (Arthropod) and mussel (Mollusc) at Anjuna, Goa

The sea anemone is an opportunistic predator. We have observed it using its tentacles to capture unsuspecting shore crabs that wander too close by paralysing and immobilizing them and feeding upon them. Tiny mussels dislodged by wave action also drift towards the sea anemone, becoming a convenient prey for the anemone (Figure 3.1).



Figure 3.2: Facultative mutualism of the sea anemone *B. goanense* with sea snails *Purpura panama* and their eggs at Anjuna, Goa.

A facultative mutualism of the sea anemone with sea snails *Purpura panama* and their eggs. The snail is protected from its predators by the sea anemone. Any encroaching predator is fed upon by the sea anemone. The snail also lays its eggs around the sea anemone and uses it as a babysitter. The snail and its eggs are protected from predators, and in turn, the sea anemone receives food by preying on the predators (Figure 3.2).

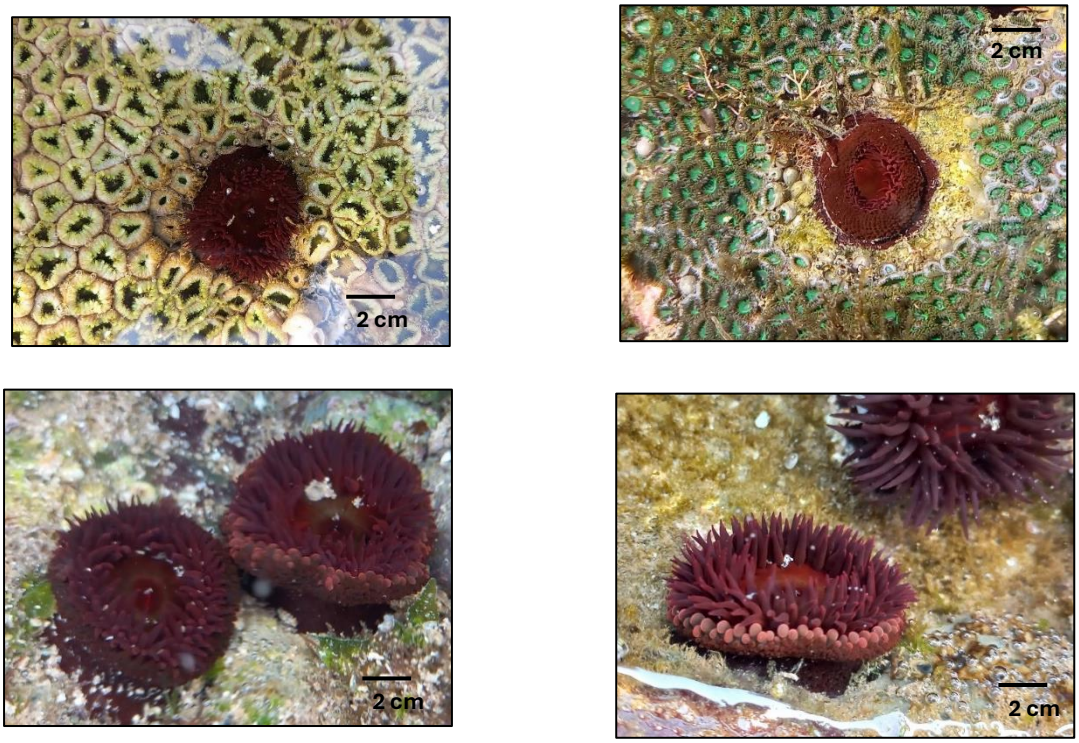


Figure 3.3: (a) Inter-specific spatial competition between *B. goanense* and *Palythoa* sp. (b) Inter-specific spatial competition between *B. goanense* and *Zoanthus* sp. (c) Intra-specific

non-clonal spatial competition between two *B. goanense* (d) *B. goanense* with inflated visible acrorhagi.

The sea anemone was also observed competing for space with soft corals *Palythoa* and *Zoanthus* (Figiure 3.3). A zone of clearance around the sea anemone in the mat of Zoanthus can be seen here, probably, caused by allelopathic interactions. Intraspecific spatial competition with non-clonal conspecifics was also observed. In such cases, specialized inflatable structures called acrorhagi are used to deter the encroachers.

This chapter explores two key ecological interactions of *Bunodosoma goanense*: its facultative mutualism with the marine snail *Purpura panama* and its spatial competition with soft corals *Zoanthus sp.* and *Palythoa sp.* Through field observations and transcriptomic analysis, we aim to elucidate the ecological strategies and molecular mechanisms underlying these interactions.

3.2 Part I: Facultative association of a Muricid snail *Purpura panama* with Sea anemone *Bunodosoma goanense*

3.2.1 Background

The tropical rocky intertidal zone is known for its high diversity and density of species compressed into the narrow inter-tidal area. Consequently, there is fierce competition for limited space and food resources. The inhabitants employ various ecological strategies to maintain their niche in this highly stressful ecosystem by coping with disturbances and interacting with other species and their environment (Southwood 1977). In ecosystems with relatively significant physical disturbance, stress, or predation, positive symbiotic interactions like mutualism and commensalism seem to be most prevalent, where species can boost the growth and survival of other species unable to exist in isolation. (Hacker & Gaines 1997).

3.2.2 Material and Methods

Monthly observations were made at the mid-intertidal zone of the rocky shores of Anjuna (15°34′02.7″N, 73°44′ 29.2″E), Goa, India, from June 2021 to March 2023 during low tide. The site is characterized by the presence of an extensive line of Basalt and granite rocks parallel

to the coast, which gets exposed during the low tide. The animals were observed and photographed in situ using a Samsung Galaxy M51 phone camera.

A total of seven snail specimens found attached to *B. goanense* were carefully detached with utmost care without disturbing the anemone and collected for subsequent analysis. These specimens were transported to the laboratory in live condition in plastic sample containers with seawater. The shells of the specimens were washed using artificial seawater and cracked open using a clamp. The snail's soft body was dissected in a petri dish. Stomach content was isolated and squashed preparations of this material were observed under a microscope (Olympus BX43) at 100×. The cnidocysts of the anemone were used as a reference to investigate their presence in the snail gut content. The other two snail specimens were preserved in 70% ethanol for identification.

3.2.3 Results and Discussion

In our study, *P. panama* were firmly attached to *B. goanense* during the non-monsoon period (Figure 3.4 a). We observed *B. goanense* feed on mussels and crabs (Figure 3.4 d). It ejects out and discards the empty mussel shells with tiny bits of mussel tissue still stuck onto them. *Purpura panama* opportunistically feeds on these leftovers ejected by *B. goanense*. *P. Panama* belongs to the family Muricidae; Rafinesque, 1815. Muricids are carnivorous sea snails that prey upon oysters, barnacles, limpets, etc. (Poutiers 1998). When carrion is present, some predator species act opportunistically as facultative scavengers (Morton 2006; Moleón and Sánchez-Zapata 2015). Species of the gastropod family Muricidae exhibit the opportunistic behavior of scavenging the leftovers of other predators. (Morton 2006; de Carvalho-Souza & González-Ortegón 2022). Due to such proximity to *B. goanense*, we suggst that *P. panama* could opportunistically feed on the leftovers or partially digested / undigested food boluses (esp. mussels) ejected by the sea anemone.

The snail gut-content analysis highlighted the presence of both discharged and undischarged nematocysts (Figure 3.4 c & d). The undischarged nematocysts resembled those present in *B. goanense*. The absence of *B. goanense* tissue, besides the cnidocysts, in the *P. panama* gut content rules out the possibility of predation or parasitism by the snail. So, we assume that they were selectively sequestering or stealing them from the body column of the sea anemone. We, therefore, hypothesize the occurrence of Kleptocnidism in this interaction. Kleptocnidism is a phenomenon commonly reported in Sea slugs (Gastropoda: Nudibranchia). The sea slugs

sequester the undischarged cnidocysts from their prey and store these kleptocnidae in their cnidosacs which are used for their defense (Greenwood 2009). So far there have been no reports of kleptocnidism observed in sea snails except for the report by Ohkawa (1975) who observed that the eggs of the parasitic sea snail *Alexania inazawai* were coated with the cnidocytes of the host sea anemone *Haliplanella luciae.* Herein, we report for the first time the presence of intact cnidocysts in the gut of a sea snail.

The snails did not adhere to the sea anemone during monsoons (June-October 2021). From August-September 2021, the snails mated and deposited egg capsules as mats on rocky surfaces (Figure 3.4 e). Interestingly, we observed three *P. panama* individuals start to deposit egg-capsules on rocky surfaces around the pedal disc of a *B. goanense*, eventually proceeding outwards to form a mat of egg-capsules around it. Along a stretch of approximately 150 meters of this rocky shore, we observed patches of egg-capsule mats deposited around ~20 individual sea anemones. This egg-capsule deposition event was not observed in succeeding monsoons.

Predatory crabs on the rocky shores of Anjuna are predators of gastropod eggs. These crabs are one of the prey species of *B. goanense (*(Figure 3.4 f). Therefore, we hypothesize that *P. panama* uses *B. goanense* as a "babysitter" to protect its egg-capsules from predatory crabs. Any potential predators of snail eggs would either be preyed upon by the sea anemone or deterred by its venomous sting. Nonetheless, egg capsules were also deposited on surfaces devoid of *B. goanense*. Shimek (1981) reported a similar observation in Alaska about the snail *Neptunea pribiloffensis* (Dall, 1919) that deposited its corncob-shaped egg-capsules near a "babysitter" sea anemone *Urticina grebelnyi* Sanamyan & Sanamyan, 2006, to protect eggs from predatory sea urchins.

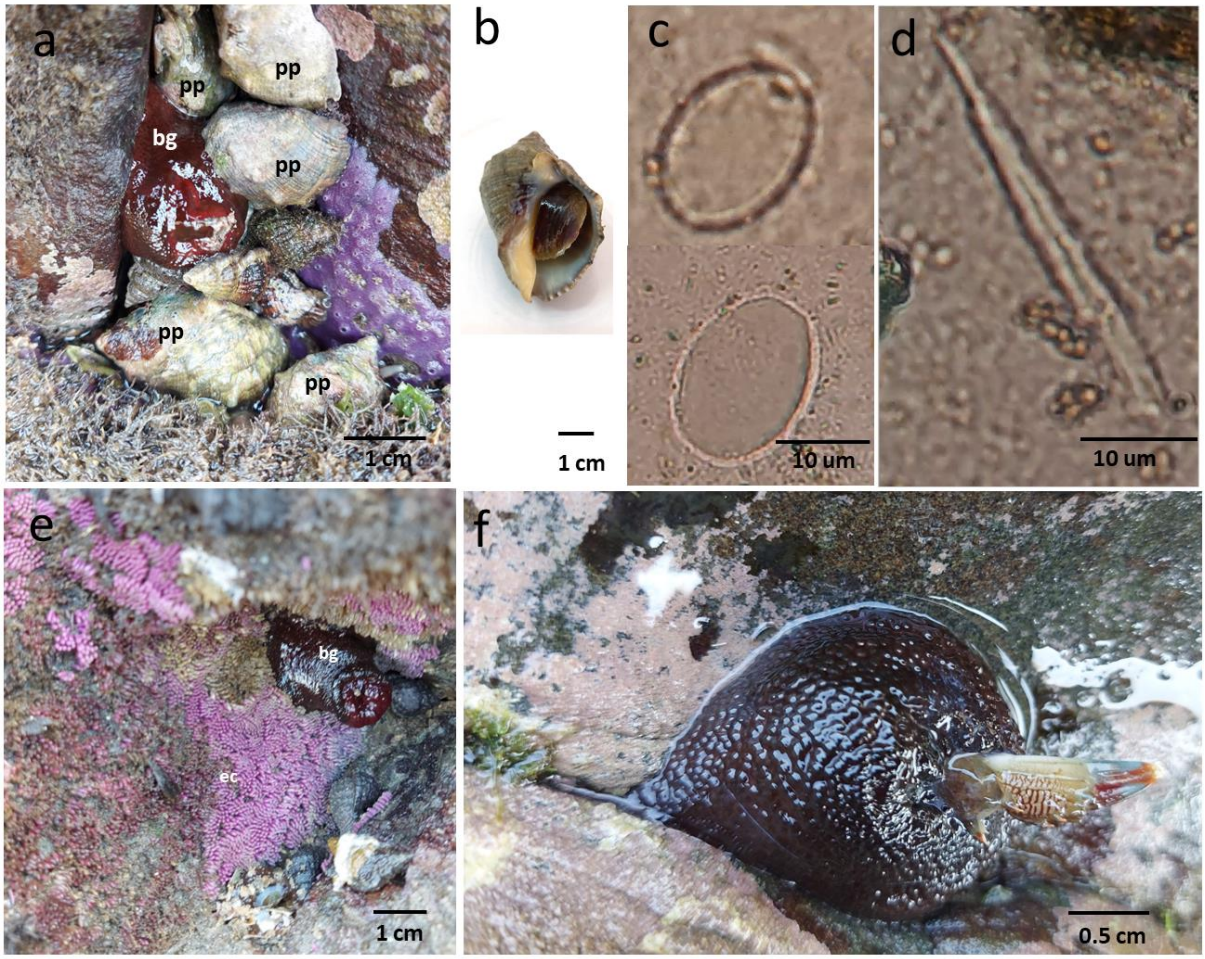


Figure 3.4 a. Three marine snails *Purpura panama* (pp) attached to the body column of *Bunodosoma goanense* (bg) and others in close proximity. b. Shell of *P. panama* with live snail inside. c. Discharged nematocysts in gut content of *P. panama*. d. A nematocyst in the gut content of *P. panama*. e. A mat of *P. panama* egg-capsules (ec) deposited around *B. goanense* to protect their egg capsules from predators. f. A *B. goanense* sea anemone feeding on a crab

3.2.4 Conclusion

In this study, we report an in-situ observation of an association between the marine snail *Purpura panama* Röding, 1798, and the sea anemone *Bunodosoma goanense* den Hartog & Vennam, 1993, both common inhabitants on the rocky shores of Anjuna, Goa. Our findings reveal that *P. panama* opportunistically feeds on the leftovers ejected by *B. goanense*, displaying a unique example of facultative mutualism. Gut-content analysis of the snail showed the presence of undischarged nematocysts, indicating kleptocnidism, a phenomenon typically observed in sea slugs. Furthermore, *P. panama* deposits egg capsules around *B. goanense*

during the monsoon season, suggesting that the sea anemone provides protection from predatory crabs, acting as a "babysitter." This study provides valuable insights into the ecological interactions on tropical intertidal rocky shores, highlighting the adaptive strategies of marine organisms to enhance their survival.

Having explored the mutualistic relationship between *B. goanense* and *P. panama*, we now turn our attention to the competitive interactions of *B. goanense* with soft corals, which reveal a different facet of its ecological adaptability.

3.3 Part II: A Transcriptomic Exploration into the Interspecies Conflict between Sea Anemone and Soft Corals

3.3.1 Background

Tropical intertidal rocky shore habitats exemplify ecosystems subjected to extreme environmental fluctuations (Helmuth & Hofmann, 2001; Tomanek & Helmuth, 2002). These habitats shaped by tidal oscillations, present many challenges that demand specialized adaptations for survival. Organisms within these habitats navigate a dynamic landscape characterized by desiccation stress, temperature extremes, salinity fluctuations, and fierce ecological interactions, necessitating physiological and behavioural strategies for survival (Petraitis, Fisher & Dudgeon, 2008; Newell, 1976; Connell, 1972). Ecological interactions within such ecosystems are intricate and important. Mutualistic associations and nutrient cycling mechanisms highlight species' interconnectedness, contributing to these ecosystems' resilience and sustainability (Toby Kiers, 2010). Predation, a cornerstone of these ecosystems, shapes community structure and influences population dynamics (Dunning, Danielson& Pulliam, 1992). Within the rocky tidepools, competition among species for space, food, and shelter is fierce. Organisms have evolved diverse strategies to minimize direct competition (Southwood, 1977). Intense spatial competition can lead to competitive exclusion, where one species outcompetes others for a particular niche or space. However, coexistence also occurs through niche differentiation, where species partition resources or adapt to slightly different microenvironments, allowing them to thrive alongside competitors without directly competing (Tokeshi, (1997)). With limited space and resources, sessile organisms like sea anemones and soft corals are particularly vulnerable to spatial competition (Chadwick & Morrow, 2011), which presents an intriguing ecological dynamic within marine ecosystems, particularly in rocky intertidal environments where these organisms often exist. Both sea anemones and soft corals belong to the class Anthozoa and share similarities in their ecological niches, leading to competition for space and resources. Sea anemones and soft corals often compete for suitable substrate and attachment sites within the rocky shore. This competition for attachment points can be intense, especially in areas with favourable conditions for both species. To gain an edge in the competition, sea anemones and soft corals may employ chemical warfare, which influences the settlement, growth, and survival rates of both organisms. Soft corals produce and release chemical compounds into the surrounding water, a strategy known as allelopathy

(Sammarco et al., 1983). These compounds can inhibit the growth or settlement of nearby competitors, including other soft corals or encrusting organisms. They also possess a remarkable ability for rapid growth and encrustation, spreading across surfaces to outcompete neighbouring organisms. They use specialized structures like stolons or creeping mats to encrust available substrate efficiently. This growth strategy allows them to occupy space and shade out competitors, reducing their access to light and resources. Some soft corals have physical defenses that deter competitors. By dominating available substrate, soft corals effectively reduce the chances of other organisms establishing themselves in the same area (Wood,1995; Suchanek & Green,1981), Pouryousef et al., 2020)). They might have spiny structures, rough textures, or produce mucus coatings that discourage other organisms from settling or growing nearby. Some species possess specialized nematocysts or stinging cells that defend against predators and competitors (Schmidt, Daly & Wilson, 2019). Similarly, sea anemones possess defensive mechanisms against competitors. The tentacles of sea anemones are armed with cnidocytes, which, when triggered by physical contact, explosively discharge, releasing a barbed thread that injects venom into the target organism (Jenner & Undheim, 2017). This venom contains a mix of peptide toxins, which can paralyze or deter competitors (Madio, King & Undheim, 2019; Menezes & Thakur, 2022). Besides the nematocysts, sea anemones also produce venomous ectodermal mucus secretions. The mucus contains toxins that serve as an additional defense (Moran et al., 2012). Acrorhagi are specialized structures found in some sea anemones, particularly employed during non-clonal intraspecific spatial competitions. When in contact with neighbouring competitor sea anemones, acrorhagi can be inflated and used in aggressive warring interactions (Daly, 2003).

The rocky shores along the West Coast of India harbour diverse benthic communities, including sea anemones and soft corals. Among these, sea anemone *Bunodosoma goanense* (Cnidaria: Actinaria) and soft corals *Zoanthus sansibaricus* (Cnidaria: Zoantharia) and *Palythoa sp*. (Cnidaria: Zoantharia) often stand out, engaging in spatial competition for territory in this habitat (Figure 3.5). This study aims to illuminate the molecular battlefield of the spatial competition between *Bunodosoma goanense* and the soft corals through an investigation combining field observations and transcriptomic techniques. Our research aligns with recent investigations into sponge competition along the West coast of India, the work of Deshpande et al. (2021), highlighting the emergence of transcriptomic approaches in unveiling the complex molecular interplay in sessile organism interactions. Our study presents a novel investigation into the competitive strategies of the sea anemone *Bunodosoma goanense*. We

combine field observations with a comprehensive transcriptomic analysis, making it the first study to report on both the in-situ spatial competition between sea anemones and soft corals and the underlying gene expression patterns associated with this interaction. By analyzing the differential gene expression patterns of *B. goanense* during these competitive interactions, we aim to decipher the strategies employed by this sea anemone. This approach transcends traditional ecological observations, offering a deeper understanding of the molecular language of adaptation and resilience in an organism facing competition.

3.3.2 Transcriptomics and Differential gene expression

The transcriptome refers to the complete set of RNA transcripts produced by an organism under specific conditions, providing insights into which genes are actively expressed. Transcriptomic analysis is a powerful molecular biology technique used to study the entire set of RNA transcripts (transcriptome) produced by the cells or tissues of an organism at a specific time under particular conditions. Unlike genomic sequencing, which focuses on an organism's complete DNA sequence, transcriptomics provides insights into which genes are actively expressed and to what extent. This method is crucial for understanding gene regulation, identifying novel genes, exploring alternative splicing events, and deciphering cellular responses to environmental changes or stimuli. The process typically begins with RNA extraction from cells or tissues of interest. The extracted RNA is then converted into complementary DNA (cDNA) using reverse transcriptase, which serves as a template for subsequent sequencing. Next-generation sequencing (NGS) technologies, such as Illumina sequencing platforms, are commonly employed to generate millions of short RNA sequences, known as reads. These reads are then aligned to a reference genome or assembled de novo to reconstruct the transcriptome. Bioinformatics tools are used to analyze the data, including quantifying gene expression levels, identifying differentially expressed genes between conditions, and predicting functional annotations. Transcriptomic analysis has diverse applications across biological research, including in fields such as developmental biology, disease mechanisms, evolutionary studies, and ecological interactions. By revealing the dynamic gene expression patterns within an organism, transcriptomics provides fundamental insights into the molecular basis of biological processes and can guide further experimental investigations. (Lowe et al., 2017; Wang et al., 2009).

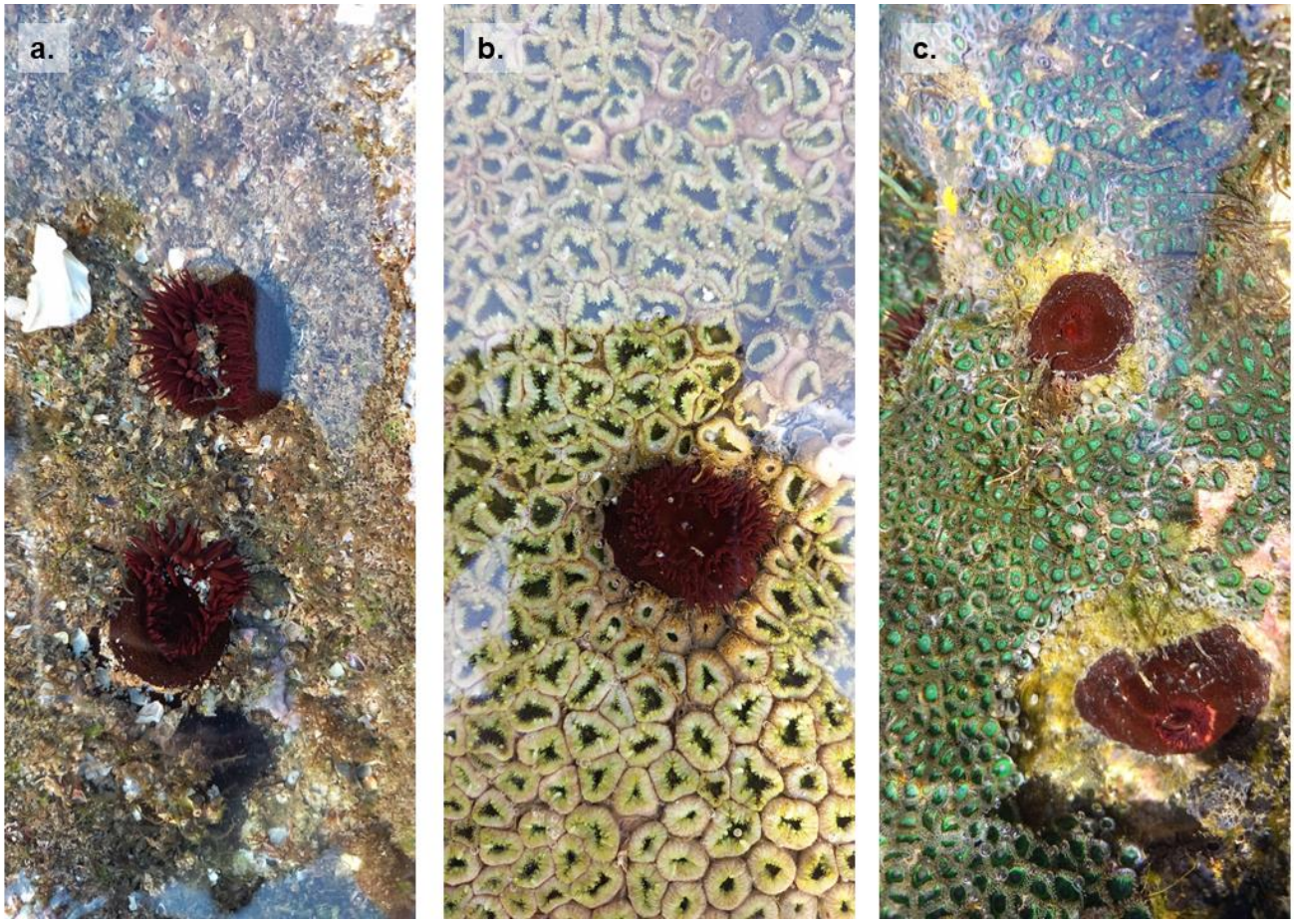


Figure 3.5 a. *B. goanense* without cnidarian competitors/neighbours (control) b. *B. goanense* individual co-occurring with the *Palythoa sp*. c. *B. goanense* individuals co-occurring with the zoanthid *Zoanthus* sp. with a presence of a clearance zone around the sea anemone on the rocky shores of Kunkeshwar.

3.3.3 Methods

3.3.3.1 Study site and sample collection

The field observations and sampling were conducted during low tide in the mid-littoral zone along the rocky beach of Kunkeshwar, Maharashtra, India, in April 2023. This zone, marked by a heterogeneous rocky substrate, is distinguished by, extensive mats of *Zoanthus* and *Palythoa* colonies interspersed with scattered solitary *Bunodosoma goanense* sea anemones naturally coexisting throughout the year. This specific littoral zone offers a unique ecological context for investigating the spatial competition dynamics between sea anemones and soft corals. The sea anemone samples were divided as (i) Sea anemone without competition (control set referred to hereafter as C), (ii) Sea anemone in competition with *Zoanthus sansibaricus* (referred to hereafter as Z), and (iii) Sea anemone in competition with *Palythoa sp.* (referred

hereafter as P) (refer Figure 3.5). Triplicates of each sample were collected and instantly flash-frozen in liquid nitrogen. Samples were stored at −80°C until RNA extraction.

3.3.3.2 RNA extraction, library preparation and sequencing

Total RNA extraction was conducted through the traditional TRIzol method. Illumina TruSeq stranded mRNA sample prep kit was employed for the preparation of paired-end RNA-Seq sequencing libraries. The analysis of libraries was carried out on 4200 TapeStation systems. Illumina paired-end libraries were sequenced on NovaSeq 6000.

3.3.3.3 De novo transcriptome assembly

The analysis was performed using the tools available on the Galaxy platform. Raw sequencing reads ([dataset] Menezes & Thakur, 2024) were subjected to quality control using FastQC (Galaxy Version 0.74+galaxy0) (Andrews, 2023) and preprocessing using Trimmomatic (Galaxy Version 0.38.1) **(**Bolger et al., 2014**)** to remove low-quality bases and adapters. Quality assessment of cleaned reads was conducted to ensure the removal of low-quality sequences, utilizing FastQC for evaluation. De novo transcriptome assembly was performed using Trinity (Galaxy Version 2.15.1+galaxy1) (Grabherr,2011). Reads were remapped to the raw transcriptome to validate the assembly and assess its compatibility with the original data using the Align reads and estimate abundance tool (Galaxy Version 2.15.1+galaxy0). Using the tool Build expression matrix (Galaxy Version 2.15.1+galaxy0), mapping tables were merged, and read counts were normalized. Additional assembly statistics, such as contig Ex90N50 and Ex90 transcript count, were computed to provide insights into the assembly's quality using Compute contig Ex90N50 statistic and Ex90 transcript count (Galaxy Version 2.15.1+galaxy0). The completeness of transcriptome assembly was evaluated using BUSCO (Galaxy Version 5.5.0+galaxy0) (Simão et al., 2015). Transcripts with low expression levels were filtered out to Filter low expression transcripts (Galaxy Version 2.15.1+galaxy0).

This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GKWR00000000. The version described in this paper is the first version, GKWR01000000.

3.3.3.4 Annotation and Functional Analysis

A gene-to-transcript map was generated to establish relationships between genes and their corresponding transcripts using the tool Generate gene to transcript map (Galaxy Version

2.15.1+galaxy0). Protein-coding sequences were predicted from transcripts using TransDecoder (Galaxy Version 5.5.0+galaxy2) (Haas et al., 2013). Predicted peptides were subjected to a similarity search against Uniprot database using NCBI BLAST+ BLASTp (Galaxy Version 2.14.1+galaxy1) (Altschul et al., 1997; Camacho et al., 2009; Cock et al., 2015). Annotation of transcripts was carried out using Trinotate (Galaxy Version 3.2.2+galaxy0) (Grabherr et al., 2011).

3.3.3.5 Differential Expression Analysis

Reads were remapped to the filtered set of transcripts to focus on the most relevant transcripts using Align reads and estimate abundance (Galaxy Version 2.15.1+galaxy0) (Grabherr et al., 2011). Mapping tables were merged, and read counts were normalized using the TMM method using Build expression matrix (Galaxy Version 2.15.1+galaxy0) (Grabherr et al., 2011). Differential expression analysis was performed using Differential expression analysis (Galaxy Version 2.15.1+galaxy0) (Grabherr et al., 2011) tool using the DESeq2 method. Differentially expressed transcripts were extracted and clustered based on expression patterns using Extract and cluster differentially expressed transcripts (Galaxy Version 2.15.1+galaxy0) (Grabherr et al., 2011). Genes were grouped into expression clusters using tool Partition genes into expression clusters (Galaxy Version 2.8.4+galaxy1). The accession numbers corresponding to transcripts were extracted from the Trinotate output and a comprehensive search was conducted utilizing the UniProt ID mapping tool to retrieve the corresponding Gene Ontology (GO) terms associated with these accession numbers. The acquired GO terms were then systematically amalgamated with their respective transcripts.

3.3.3.6 Gene ontology enrichment analysis

Gene Ontology enrichment analysis was executed employing Panther 18.0 software (accessed online on 08 January 2024). A stratified approach was adopted, segregating the upregulated and downregulated genes into distinct analyses. The selection criteria for upregulated and downregulated genes were determined based on fold change values. The statistical assessment of enrichment was accomplished through the application of Fisher's exact test. The filtering of results was conducted based on a stringent False Discovery Rate (FDR) threshold, specifically FDR $P < 0.05$.

3.3.4 Results and Discussion

The sea anemone, *Bunodosoma goanense*, displayed a zone of clearance around its base when inhabiting mats of the zoanthid, *Zoanthus sp*. This clearance zone, devoid of *Zoanthus* polyps, formed a distinct halo around the sea anemone. Interestingly, this phenomenon was not observed when *B. goanense* co-occurred with mats of the *Palythoa* sp. This initial field observation sparked a deeper investigation into the potential mechanisms underlying these contrasting competitive interactions.

Triplicates of *B. goanense* individuals co-occurring with the zoanthid *Zoanthus* sp. (Z), *B. goanense* individuals co-occurring with the *Palythoa sp*. (P), control samples of *B. goanense* individuals (C) were collected from areas devoid of any immediate competitors. The collected RNA samples from each group were then subjected to RNA sequencing. This process generated millions of short sequence reads representing the transcripts present in the samples. The sequencing data was processed on the Galaxy platform. The initial read count was approximately 25,000,000. Following trimming, 90% of reads remained, indicating minimal data loss. The average base quality score after trimming was Phred 33, which corresponds to a base call accuracy of 99.9%. This high score suggests that the trimmed reads are of excellent quality and suitable for downstream analyses. The trimmed reads from each RNA-seq sample were assembled into a de novo transcriptome. The resulting transcriptome comprised a total of 309954 assembled transcripts. To assess the completeness of the transcriptome assembly, we employed the BUSCO (Benchmarking Universal Single-Copy Orthologs) software. BUSCO analyzes the presence and integrity of a set of highly conserved genes expected to be found in a complete transcriptome. The BUSCO analysis for our transcriptome assembly yielded a score of 93.3%, indicating a high degree of completeness. This score signifies that a large proportion of the expected universal single-copy genes were successfully identified within the assembled transcripts, suggesting a high-quality transcriptome suitable for further analyses. Transdecoder predicted 82,146 peptide sequences. Among these annotated sequences, 29,916 exhibited significant matches to proteins within the Swiss-Prot database. These matches provide valuable insights into the potential functions of the corresponding genes expressed by *B. goanense* in the analyzed samples.

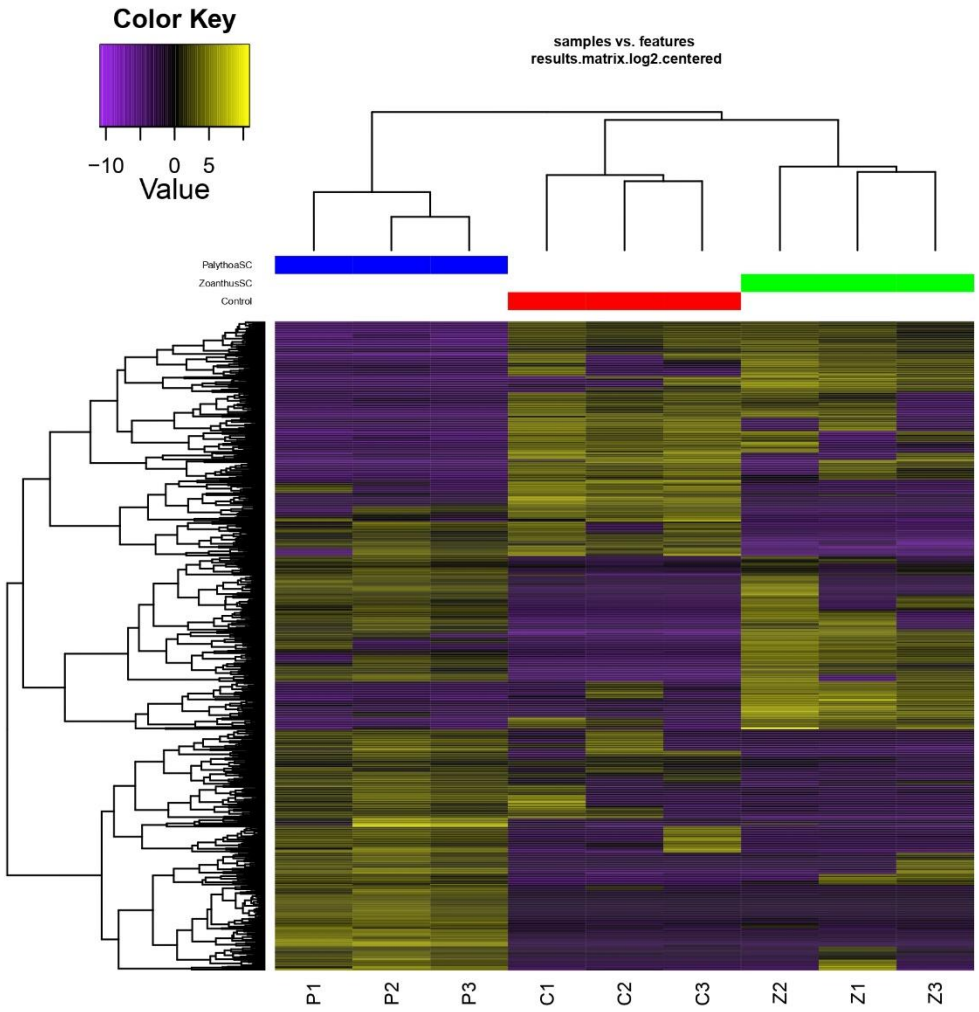


Figure 3.6. The heatmap indicating differential gene expression profiles of the sea anemone *Bunodosoma goanense* competing with *Palythoa* and *Zoanthus* compared to a control sample.

The heatmap (Figure 3.6) shows the results of a differential gene expression analysis comparing the gene expression profiles of the sea anemone *Bunodosoma goanense* under three conditions. Each row in the heatmap represents a gene, and each column represents a sample. The colour intensity in each square represents the level of gene expression, with yellow indicating upregulation and purple indicating downregulation. The higher the intensity of the colour, the greater the change in gene expression. The heatmap shows clear differences in gene expression patterns between the three groups, indicating that *B. goanense* modulates its gene expression depending on the competitor it encounters.

During interaction with *Zoanthus sp*, 21 (annotated) genes were upregulated, and 27 (annotated) genes were downregulated in *B. goanense*. From the upregulated genes, a total of 10 GO terms were enriched in the CC (cellular component) classification, 5 in MF (molecular

function) and 26 in the BP (biological process). From the downregulated genes, a total of 12 in MF (molecular function), 10 in the BP (biological process) and a total of 19 GO terms were enriched in the CC (cellular component) classification. Whereas during interaction with *Palythoa sp.* 87 (annotated) genes were downregulated and 89 (annotated) genes were upregulated. From the upregulated genes, no GO terms were enriched in the CC (cellular component) classification, 31 in MF (molecular function) and 268 in the BP (biological process). From the downregulated genes, a total of 25 GO terms were enriched in the CC (cellular component) classification, 20 in MF (molecular function) and 182 in the BP (biological process).

In the case of Sea anemone interacting with *Zoanthus sp*., the significant enriched upregulated MF were associated with galactokinase activity, galactose binding, carbohydrate kinase activity, monosaccharide binding, carbohydrate binding. The enriched BP were associated with galactose catabolic process, protein localization to nucleoplasm, carbohydrate catabolic process, DNA replication. Whereas the significant downregulated BP were associated with glutamate metabolic process, establishment of protein localization to plasma membrane, heterochromatin formation, ribosomal large subunit biogenesis, dicarboxylic acid metabolic process, amide metabolic process.

While, in the case of Sea anemone interacting with *Palythoa sp*., the significant enriched upregulated BP were associated with Positive regulation of inflammatory response to wounding, Regulation of defense response to bacterium, Inflammatory response. The MF were associated with Neuropeptide Y receptor activity, neuropeptide binding and Protein kinase activity. Whereas, the enriched downregulated BP were associated with regulation of growth, gamete generation, cellular processes, developmental processes. The figure 3.7, figure 3.8 and figure 3.9 show the GO terms upregulated and downregulated by *B. goanense* during interaction with *Zoanthus sp.* and *Palythoa sp.*

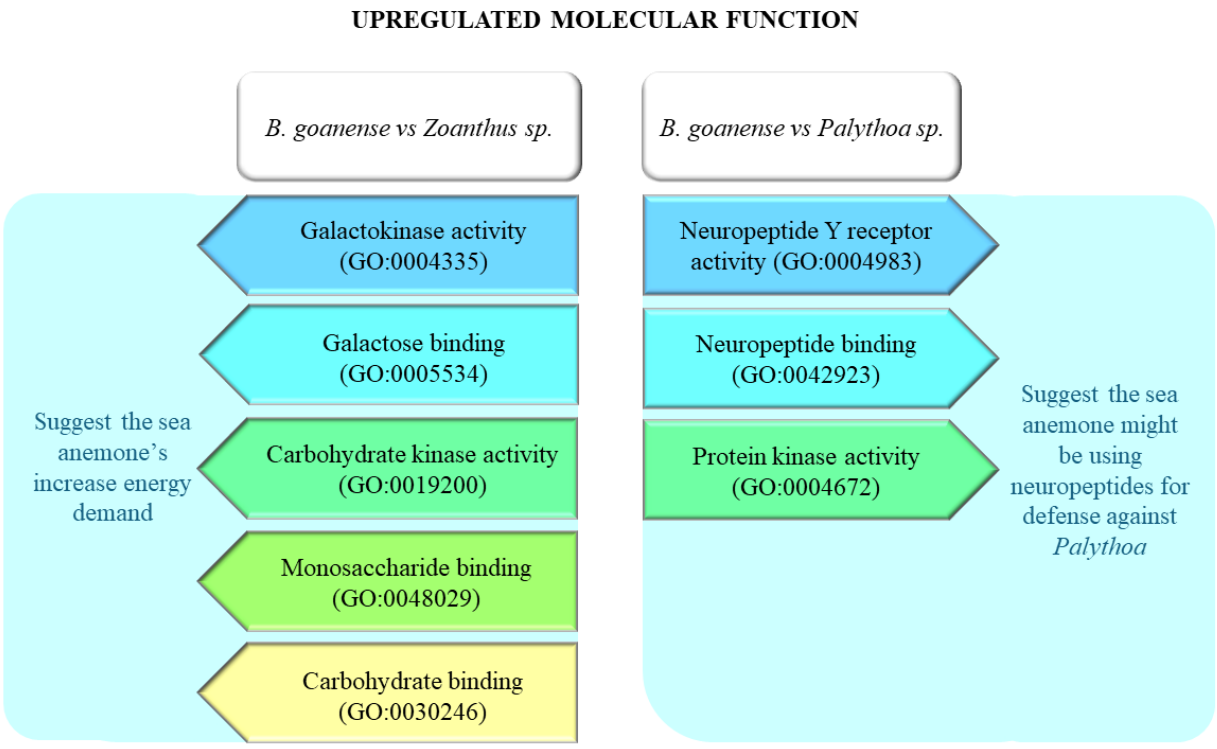


Figure 3.7: A comparison between upregulated Molecular Function GO terms between *B. goanense* vs *Zoanthus sp.* and *B. goanense* vs *Palythoa sp.*

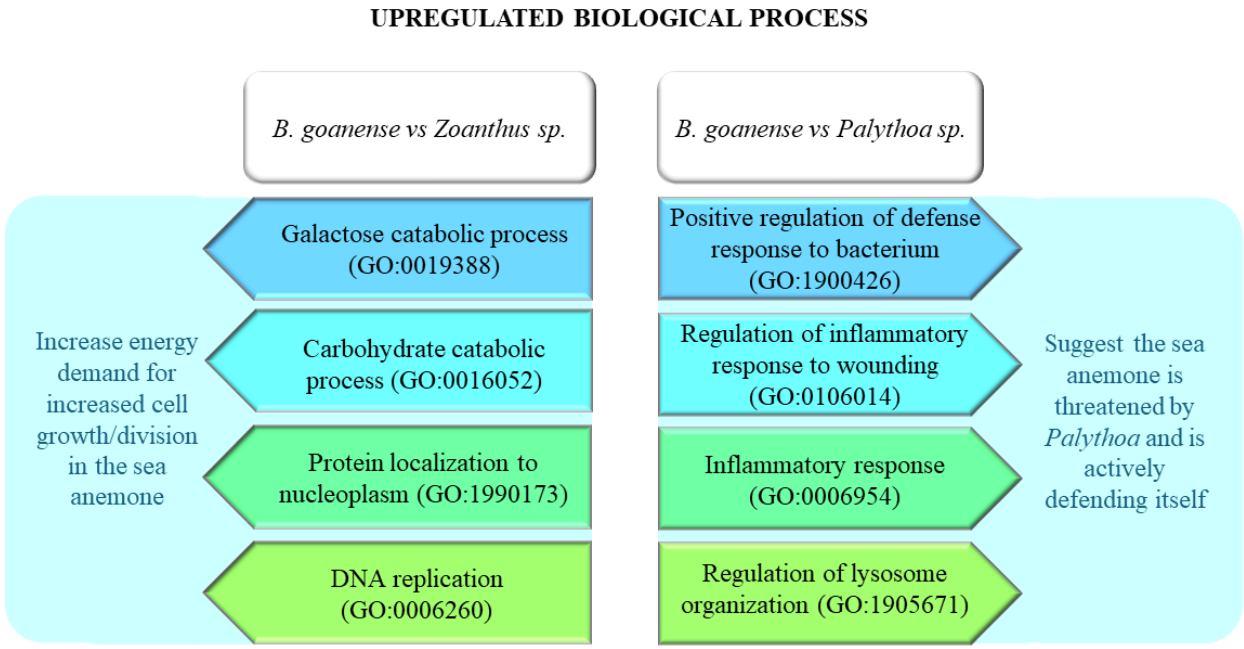


Figure 3.8: A comparison between upregulated Biological Process GO terms between *B. goanense* vs *Zoanthus sp.* and *B. goanense* vs *Palythoa sp.*

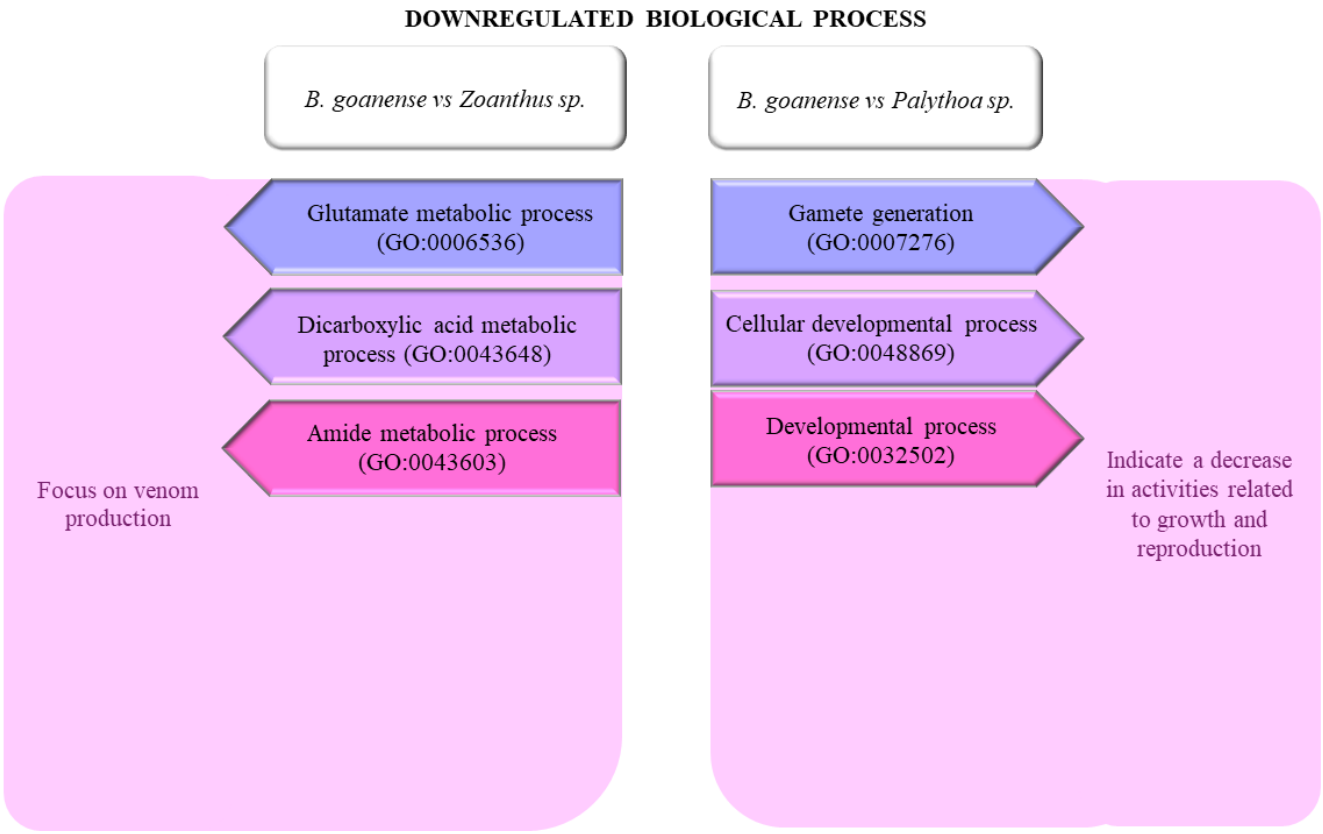


Figure 3.9: A comparison between downregulated Biological Process GO terms between *B. goanense* vs *Zoanthus sp.* and *B. goanense* vs *Palythoa sp.*

Discussion: Our study at the Kunkeshwar rocky shores revealed intriguing insights into the competitive strategies employed by the sea anemone, *Bunodosoma goanense*. We observed a distinct difference in its interactions with two co-occurring cnidarians: *Zoanthus sp.* and *Palythoa sp.* A striking observation was the consistent presence of a clearance zone around *B. goanense* when inhabiting *Zoanthus* mats. This halo, devoid of *Zoanthus* polyps, suggested a potential competitive edge held by the sea anemone. On the other hand, when coexisting with *Palythoa*, *B. goanense* lacked a clearance zone, and the two cnidarians appeared to coexist in closer proximity. This observation aligns with the known aggressive spatial competition exhibited by *Palythoa sp*. To gain deeper insights into the underlying mechanisms driving these contrasting behaviors, we analyzed the transcriptome of *B. goanense* during competition with each rival.

The heatmap reveals a fascinating diversity in gene expression patterns across the three groups. This suggests that *B. goanense* exhibits remarkable adaptability, tailoring its gene expression profile to the specific competitor it encounters, highlighting its sophisticated strategies for

navigating the competitive environment. Both the control and *Zoanthus* competing groups might share some similar expression patterns compared to the *Palythoa* competition group. This could be because they represent scenarios where the anemone is not actively competing for space, unlike the *Palythoa* interaction. However, the heatmap also suggests distinct differences between the control and *Zoanthus* competition groups. The *Zoanthus* competition group appears to have more pronounced changes in gene expression (higher color intensity) compared to the control. This suggests that even though they might share some similar patterns, the anemone still exhibits a unique gene expression response when facing *Zoanthus* compared to the absence of any competitor. The *Palythoa* interaction appears to trigger a more distinct response at the gene expression level.

The GO term analysis reveals distinct strategies the sea anemone employs when interacting with *Zoanthus sp*. and *Palythoa sp*. During the interaction with *Zoanthus sp*., the enriched upregulated Molecular Functions (MF) like carbohydrate and galactose metabolism terms suggest the sea anemone's increase energy demand. Biological Processes (BP) like galactose catabolism, carbohydrate metabolism, protein localization to the nucleoplasm, and DNA replication support the increase energy demand for increased cell growth/division. Downregulated BP, like Glutamate metabolism, could free up these amino acids for promoting venom peptide production.

In the case where the Sea anemone interacts with *Palythoa sp*., the enriched upregulated BP such as inflammatory response, defense response to bacteria, and positive regulation of inflammatory response suggest the sea anemone is threatened by *Palythoa* and is actively defending itself. MF such as Neuropeptide Y receptor activity and neuropeptide binding suggest the sea anemone might be using neuropeptides for defense against *Palythoa*. The downregulated BP like regulation of growth, gamete generation, cellular processes, and developmental processes indicate a decrease in activities related to growth and reproduction.

3.3.5 Conclusion

The gene expression analysis revealed distinct patterns, supporting the field observations. When competing with *Zoanthus sp.*, *B. goanense* exhibited upregulated pathways associated with energy production, cell proliferation, and protein synthesis. This suggests an active strategy focused on growth, tissue repair, and potentially the production of defense molecules to outcompete *Zoanthus sp*. This aligns with the observed clearance zone, as increased activity

and potential toxin production could suppress *Zoanthus* growth in the anemone's vicinity. In contrast, the transcriptome analysis revealed a resource-conserving strategy by *B. goanense* when facing *Palythoa* sp. The upregulation of inflammatory response indicates stress induced during this interaction. Downregulated growth, cell proliferation and gamete generation suggest a focus on maintenance and survival (Refer Figure 3.10 for brief graphical summary). Overall, the study highlights *B. goanense*'s remarkable adaptability. Our study uniquely sheds light on the intricate relationship between sea anemones and soft corals by being the first to report on both the field observation of their spatial competition and the transcriptomic analysis of the sea anemone's response at the gene expression level. This paves the way for a deeper understanding of the molecular mechanisms underlying these interspecies interactions in their natural environment.

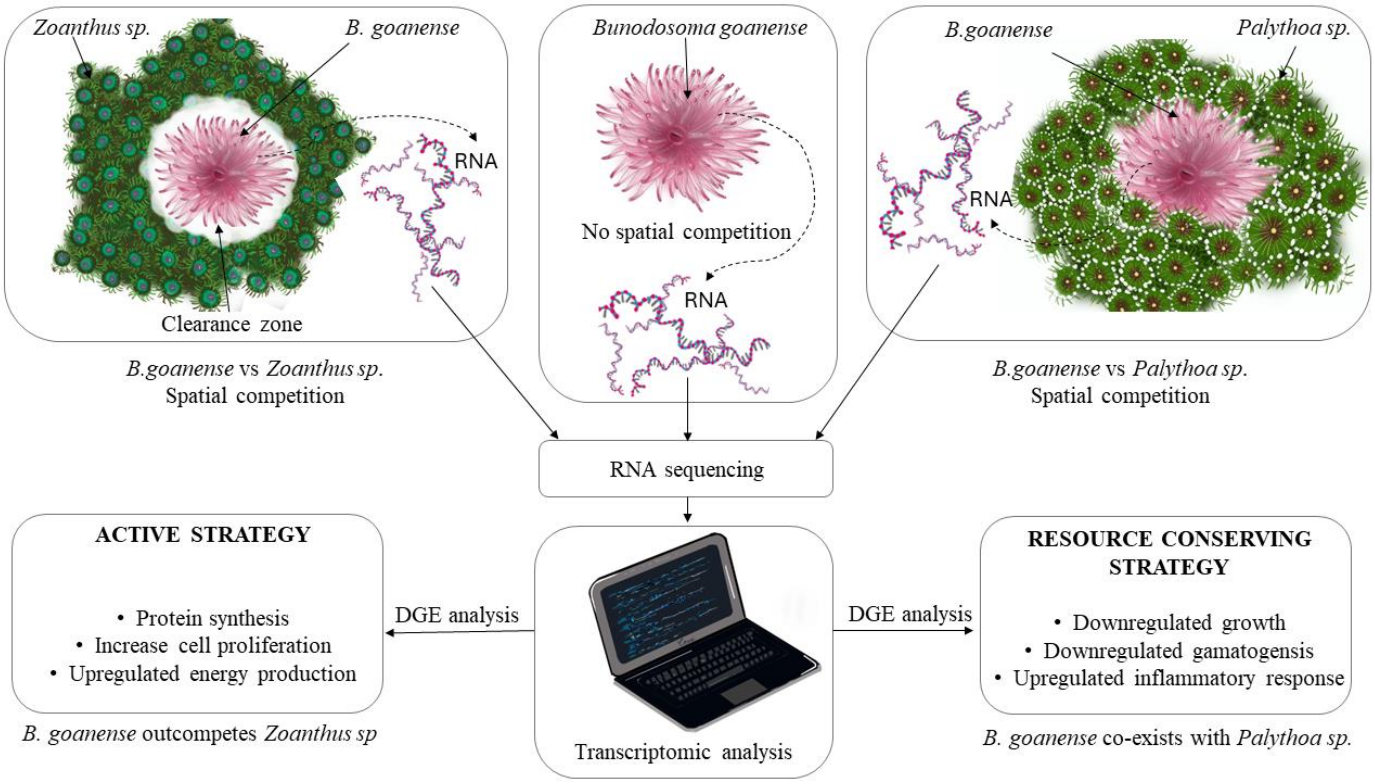


Figure 3.10: Graphical summary of Transcriptomic Exploration into the Interspecies Conflict between Sea Anemone and Soft Corals

3.4 Conclusion of the Chapter

In this chapter we have discussed about the two interesting ecological interactions of the sea anemone *Bunodosoma goanense*, that is, facultative mutualism with the marine gastropod *Purpura Panama* and Spatial competition with soft corals *Zoanthus sp*. and *Palythoa sp*.

Part 1: Facultative Mutualism with *Purpura panama*

In this part, we reported an in-situ observation of an association between the marine snail *Purpura panama* and *B. goanense* on the rocky shores of Anjuna, Goa. Our findings reveal that *P. panama* opportunistically feeds on the leftovers ejected by *B. goanense*, showcasing a unique example of facultative mutualism. Gut-content analysis of the snail revealed undischarged nematocysts, indicating kleptocnidism, a phenomenon typically observed in sea slugs. Additionally, *P. panama* deposits egg capsules around *B. goanense* during the monsoon season, suggesting that the sea anemone provides protection from predatory crabs, acting as a "babysitter." This study underscores the adaptive strategies of marine organisms to enhance their survival, offering valuable insights into the ecological interactions on tropical intertidal rocky shores.

Part 2: Spatial Competition with *Zoanthus sp*. and *Palythoa sp*.

In the second part, we explored the spatial competition between *B. goanense* and soft corals *Zoanthus sp*. and *Palythoa sp*. on the same rocky shores. Gene expression analysis revealed distinct patterns supporting our field observations. When competing with *Zoanthus sp*., *B. goanense* exhibited upregulated pathways associated with energy production, cell proliferation, and protein synthesis, indicating an active strategy focused on growth, tissue repair, and potential production of defense molecules. This suggests that *B. goanense* can actively suppress *Zoanthus* growth in its vicinity, aligning with the observed clearance zone. The transcriptome analysis showed a resource-conserving strategy when *B. goanense* faced *Palythoa sp*., with upregulated inflammatory responses indicating stress, and downregulated growth, cell proliferation, and gamete generation suggesting a focus on maintenance and survival.

Overall, this chapter highlights *B. goanense's* adaptability in its ecological interactions. The study uniquely describes the relationship between sea anemones and their ecological partners. By being the first to report both field observations and transcriptomic analyses of these interspecies interactions, our study paves the way for a deeper understanding of the molecular mechanisms underlying these complex relationships in their natural environment.

Chapter 4

Characterization of the Tissue-Specific crude Venom of the Sea Anemone

4.1 Background

Sea anemones are soft and sedentary in nature, making them very vulnerable to predation. This is the same reason why prey capture is also more challenging. Due to their benthic nature, they face competition for space in their habitat. To overcome these challenges, these animals have evolved to produce powerful venom arsenals. Venom is a rich source of toxic peptides and proteins which are predominantly cytotoxic and neurotoxic in nature. This venom is produced in specialized cells called nematocytes. Sea anemones are armed with nematocytes in various body parts, including tentacle, acrorhagi, acontia, and mesenterial filaments, along with toxin-secreting glands on the epidermal tissue on the body column (Moran et al., 2012). We have observed several ecological interactions of *Bunodosoma goanense* employing venom. It captures and immobilizes mussels and crabs by stinging them with its tentacles and feeding on them. It can break open tightly closed shells of mollusks and tough crustaceans by secreting proteolytic peptides and other molecules in its gastric cavity. To maintain its territory, it fights with the sea anemone *Anthopleura anjunae and* deters it by stinging it with its tentacles. It also competes with non-clonal *B.goanense* for space by stinging it with its acrorhagi. We have also observed clearance zones around *B.goanense* surrounded by a mat of soft-coral *Zoanthus sp.,* probably the venomous mucus from the body column deterring the zoanthids. As yet, there are no bioactive molecules reported from *B.goanense*; however, the venom of other *Bunodosoma spp*. studied around the world report the discoveries of potent neurotoxins. Granulitoxin, Cangitoxin II and Cangitoxin III isolated from *Bunodosoma cangicum* (Santana et al.,1998), BcsTx1, BcsTx2 and BcsTx3 from *B.caissarum* (Orts et al., 2013a; Orts et al., 2013b), Bgk, U-AITX-Bg1a-e from *B.granuliferum* (Rodríguez et al., 2012; Aneiros et al., 1993) are KTxs. Cangitoxin isolated from *B.cangicum* (Cunha et al.,1998), BcIII and BcIV a NaTxs from *B.caissarum* (Oliveira et al., 2006) are NaTxs. This warrants the need to study the venom

profiles of various venom-producing tissues of *B.goanense* and determine the pharmacological potential. Analysis of the tissue-specific venom constituents will provide data on the peptides involved in various ecological interactions. Pharmacological screening of these peptides will identify those involved in paralysis, immobilization, or death of predator, prey, or competitor by causing neurotoxicity and cytotoxicity. This study can potentially pave the path for discovering novel neuro-therapeutic compounds.

4.2 Variation in Venom composition across different tissues

Venom toxins of sea anemones are perhaps the most functionally distinct (Schendel et al., (2019)). Sea anemones are covered with nematocytes and toxin-secreting epidermal gland cells and do not have a centralized venom delivery system (Moran et al., (2012)). The venom varies dramatically across the body Macrander & Daly (2016) and even tentacle types (Ashwood et al., (2021a)). Therefore, the ecological role of toxins can be understood from the distribution of venom across the body of the sea anemone. Ashwood and co-workers (2021b) reported that the regionalization of toxin production remains consistent with the segregation of the ecological functions of venom across the envenomating structures. For example, venom toxins in the tentacles aid in prey capture and defense, whereas those in the acrorhagi are responsible for intra-specific aggression, in the mesenterial filaments for food digestion, in acontia for self-defense, and in the gametes for protection of the eggs and zygote (Basulto et al.,2006; Beckmann & Özbek (2012); Surm et al., (2019), Madio et al., (2018); Macrander et al., (2015)). Additionally, sea anemones have different venom toxins during different stages of their life cycle (Columbus-Shenkar et al. (2018)).

In this study, we determine whether the venom composition differs across various venom producing tissues with the hypothesis that venom composition is tailored to the ecological functions of each tissue. To understand the ecological role of venom toxins, it is important to know for what purpose the venom is employed, and this knowledge can then be translated for bioprospecting applications. This chapter aims to characterize the tissue-specific venom of *Bunodosoma goanense*, exploring its composition, bioactivity, and ecological significance. Through biochemical and functional analyses, we investigate how venom variation across tentacles, acontia and mesenterial filaments, and mucus supports ecological interactions and assess its potential for drug discovery.

4.3 Methods

4.3.1 Crude Venom Extraction from *Bunodosoma goanense*

Sea anemones *Bunodosoma goanense* were collected from the rocky shores of Anjuna, Goa, India. The collected animals were maintained in an aquarium for four days, with fresh seawater changed daily to ensure a clean environment and to remove any mucus discharge and excretory waste. During this period, the anemones were not fed to prevent contamination from food residues and to ensure venom production in high concentration. The sea anemones were then sacrificed, and venom was extracted from three specific tissues: tentacles, mesenterial filaments and acontia, and ectodermal tissue mucus. Each tissue type was subjected to sonication in distilled water to disrupt the cells and release the venom components (Carrette & Seymour, 2004). The resulting sonicated tissue suspensions were centrifuged at 15000 rpm for 5 minutes to separate the cell debris from the proteinaceous supernatant. The supernatants were then lyophilized to obtain freeze-dried crude venom extracts, which were subsequently stored for further analysis.

4.3.2 Separation of peptides using SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was employed as a preliminary tool to qualitatively assess the presence of proteins and their molecular sizes in the crude venom samples (Simpson, 2006; Kohno et al., 2009; de Oliveira et al., 2006). The SDS-PAGE gel preparation began with the separation gel. To prepare this gel, 4.1 mL of distilled water, 3.3 mL of acrylamide/bis solution (30% 37.5:1, Bio-Rad), 2.5 mL of Tris–HCl (1.5 M, pH 8.8), 100 µL of 10% SDS, 10 µL of N,N,N′,N′-tetramethylethylenediamine (TEMED) (Bio-Rad), and 32 µL of 10% ammonium persulfate (APS) were mixed. TEMED and APS were added last to initiate polymerization. The mixture was poured into the gel apparatus, leaving approximately 2 cm from the bottom of the comb to accommodate the stacking gel. The top of the gel was overlaid with isopropanol to prevent bubbles and drying during polymerization. After about 30 minutes, the separation gel was fully polymerized, and the isopropanol was removed, and the gel washed with distilled water.

The stacking gel was then prepared by mixing 6.1 mL of distilled water, 1.3 mL of acrylamide/bis solution (30%, 37.5:1), 2.5 mL of Tris–HCl (0.5 M, pH 6.8), 100 µL of 10% SDS, 10 µL of TEMED, and 100 µL of 10% APS. This mixture was poured on top of the polymerized separation gel, and combs were inserted to form wells. After another 30 minutes,

the stacking gel was completely polymerized. The gel was then clamped into the electrophoresis apparatus, and the buffer chambers were filled with running buffer as per the apparatus instructions. Protein samples and molecular mass markers were loaded into the wells, and the gel was subjected to electrophoresis.

4.3.3 Silver staining

The gel was fixed in a solution of 50% methanol and 10% acetic acid for 30 minutes at room temperature with gentle agitation. After fixation, the gel was rinsed with distilled water for 5 minutes to remove residual fixative. The gel was immersed in a sensitizing solution containing 0.02% sodium thiosulfate for 1 minute. Following sensitization, the gel was thoroughly rinsed with distilled water for 1 minute to remove any excess sodium thiosulfate. For staining, the gel was immersed in a solution of 0.1% silver nitrate, supplemented with 0.5% formaldehyde for 30 minutes at room temperature with gentle agitation. The gel was then rinsed briefly with distilled water to remove excess silver nitrate. The developing process was initiated by placing the gel in a developing solution of 2.5% sodium carbonate mixed with 0.05% formaldehyde. Development continued until the protein bands became clearly visible, which took between 5 minutes. To stop the development, the gel was placed in a stopping solution of 1% acetic acid for 5 minutes. Finally, the gel was briefly washed with distilled water to remove any remaining acetic acid and excess silver stain. The stained gel was then photographed using a gel documentation system (Chevallet et al., 2006).

4.3.4 FTIR analysis

The venom extracts were dissolved in chloroform. Approximately 100ul of this solution was carefully loaded onto NaCl plates (sample holder). A background spectrum was recorded using only chloroform on the NaCl plate to account for any interference or baseline noise. After establishing the blank background, the sample-loaded NaCl plates were scanned over the wavenumber range of 4000 to 400 cm^{-1}. The resulting FTIR spectrum was analyzed to identify characteristic peaks corresponding to different functional groups present in the venom.

4.3.5 Peptide fractionation

Crude venom fractionation was performed using size-exclusion HPLC (Superdex 30 column,10/30 GL). During the analysis, peptide fractions were eluted with a 30% acetonitrile solution) over 60 min. at a flow rate of 0.5 mL/min. The fractions were freeze-dried and tested for bioactivities. This was followed by further separation of the active fractions by gradient

HPLC (C-18 column) using a gradient of water and acetonitrile over 120 min. at a flow rate of 1 mL/min (Orts et al., 2013).

4.3.6 Cytotoxicity assay

4.3.6.1 Anti-cancer Activity

The anti-cancer potential of the venom fractions (M1, T1, and A1) from mucus, tentacle, and acontia and mesenterial tissues was evaluated against human cancer cell lines A549 (lung cancer) using the MTT assay. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin under standard conditions at 37°C in a humidified 5% CO_2 incubator. Cells were seeded in 96-well plates and treated with venom fractions at concentrations of 10, 50, and 100 µg/mL for 24 hours, along with untreated cells as the negative control and Doxorubicin and Levofloxacin as the Standard positive control. Cell viability was determined using the MTT assay.

% Proliferation= (OD control/OD sample)×100

% Inhibition=100−% Proliferation

4.3.6.2 Antibacterial Activity

The antibacterial activity of the venom fractions was assessed against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* using the agar well diffusion method. Wells were loaded with 20 µL of venom fractions at 10 µg/mL, while Ampicillin (10 µg/mL) served as the positive control and Methanol as the negative control. After incubation at 37°C for 24 hours, inhibition zones were measured.

4.3.7 Neurotoxicity assay: Voltage gated ion channel activity

4.3.7.1 Isolation of *Xenopus laevis* Oocytes by Partial Ovariectomy

All experiments involving *Xenopus laevis* frogs were approved by the Ethical Committee for Animal Research of KU Leuven and were conducted in accordance with the European Union guidelines on the welfare of laboratory animals (Directive 2010/63/EU). Adult female *Xenopus laevis* frogs were immersed in an aqueous solution containing 0.1% buffered tricaine (ethyl 3-aminobenzoate methanesulfonate, 1 g/L, Sigma-Aldrich, Rockville, MD, USA) and NaHCO3

(1 g/L; Sigma-Aldrich, Rockville, MD, USA) in aquarium water (pH 7.5) for 15 minutes. Post-recovery, the frogs were monitored daily and returned to their tanks at the Aquatic Facility of KU Leuven. Ovarian lobes were surgically removed and enzymatically defolliculated in a Ca2+-free ND96 solution (96 mM NaCl, 2 mM KCl, 2 mM MgCl2, and 5 mM HEPES) supplemented with collagenase from *Clostridium histolyticum* type IA (1.5 mg/mL; Sigma-Aldrich, Rockville, MD, USA) on a rocker platform at 16°C for 2 hours and 30 minutes. The defolliculated oocytes were transferred to a calcium-containing ND96 buffer (96 mM NaCl, 2 mM MgCl2, 2 mM KCl, 5 mM HEPES, and 1.8 mM CaCl2, pH 7.5) supplemented with geomycine (100 mg/L; Schering-Plough, Heist-op-den-Berg, Belgium) and theophylline (90 mg/L; ABC Chemicals, Nazareth, Belgium) at 16°C (Peigneur et al., 2019).

4.3.7.2 Heterologous Expression of Ion Channels in *Xenopus laevis* Oocytes

For the expression of ion channels in *Xenopus laevis* oocytes, specific Kv and Nav channels were utilized. Kv channels including Kv1.1, Kv1.3, and Kv1.5 from the roundworm *Caenorhabditis elegans*, as well as Shaker channels from *Drosophila melanogaster*, were chosen for this study. Additionally, Nav channels Nav1.4 and Nav1.5 from the cockroach *Blattella germanica* were also employed. The linearized plasmids containing the respective channel genes were transcribed using the SP6 mMESSAGE mMACHINE transcription kit (Ambion, Austin, TX, USA). Stage V–VI oocytes of *Xenopus laevis* were selected for microinjection. Oocyte microinjection was performed using a microinjector (Drummond Scientific®, Broomall, PA, USA), with a programmed cRNA injection volume of 10-20 nL, depending on the channel subtype. Following injection, the oocytes were incubated for 1–3 days at 16 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 2 mM MgCl2, and 5 mM HEPES, pH 7.4), supplemented with 50 mg/L of gentamicin sulphate to maintain sterility and promote optimal expression conditions for the ion channels (Peigneur et al., 2019).

4.3.7.3 Electrophysiological Recordings

Electrophysiological measurements were conducted at room temperature (18–22°C) using the two-electrode voltage-clamp (TEVC) technique. A GeneClamp 500 amplifier (Axon Instruments®, Burlingame, CA, USA) was utilized for data acquisition, which was subsequently stored using Clampex9 software (Axon Instruments®, USA). Glass micropipettes were crafted from borosilicate glass capillaries (WPI 1B120-6) using a manual stretcher from World Precision Instruments® (Sarasota, FL, USA). The bath and perfusion solutions comprised either the standard ND96 solution (for Nav and Kv channels). Whole-cell

currents of the oocytes were recorded 1 to 3 days post-RNA injection. Both the current and voltage electrodes were filled with 3 M KCl, with resistance adjusted between 1.0 to 2.5 MΩ. Currents were sampled at 20 kHz for Nav channels and 10 kHz for Kv channels, filtered using a four-pole Bessel low-pass filter at 1 kHz for sodium channels and 500 MHz for potassium channels. Kv1.x currents were elicited by 500 ms depolarizations to 0 mV, followed by a 500 ms pulse to -50 mV from a holding potential of -90 mV. Sodium currents were evoked by a 100 ms depolarization to 0 mV. Current-voltage relationships were determined by 50 ms step depolarizations between -90 and +65 mV, using 5 or 10 mV increments. Each experiment was performed on oocyte triplicates (Peigneur et al., 2019).

4.3.8 Peptide mass determination

For peptide mass determination, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) was employed using a timsTOF Flex instrument operating in positive ion mode. For the mass determination of venom peptides, samples were resuspended in a solution of 50% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA). A 1 µL aliquot of each sample was mixed with 1 µL of matrix solution, consisting of α-Cyano-4-hydroxycinnamic acid (CHCA), specifically prepared for MALDI-TOF MS analysis. The mixture was then dispensed onto a 384 Anchorchip target plate. Mass spectrometric analysis was performed using a timsTOF Flex instrument operating in MALDI positive mode. (Peigneur et al., 2019).

4.4 Results and Discussion

4.4.1 SDS-PAGE Analysis of crude Venom Extracts

SDS-PAGE analysis was conducted to qualitatively determine the proteinaceous components present in the venom samples extracted from tentacles, ectodermal mucus and acontia of *B. goanense*. The results revealed distinct protein bands, indicating the presence of multiple peptides and proteins ranging in molecular weight from 3.5 to 260 kDa (Figure 4.1). Notably, we observed bands corresponding to cytotoxins and neurotoxins which as similar to those reported in literature. Pore-forming toxins (~20 kDa) bands are observed. Macrander & Daly (2016) reported also reported the presence of actinoporins in *Bunodosoma cavernatum* of molecular weight 21 kDa and 20.36 kDa. Actinoporins with similar molecular weight such as DELTA-actitoxin-Oor1a (17.88 kDa) from *Oulactis orientalis* (Il'ina et al., 2005), DELTA-stichotoxin-Hmg2b (19.20 kDa) from *Heteractis magnifica* (Samejima et al., 2000), DELTA-stichotoxin-She4b (19.28 kDa) from

Stichodactyla helianthus (Blumenthal & Kem, 1983), DELTA-actitoxin-Afr1e (19.77 kDa) from *Actinia fragacea* (Morante et al., 2019), DELTA-actitoxin-Aas1a (23.31 kDa) from *Anthopleura asiatica* (Kohno et al., 2009), DELTA-actitoxin-Aeq1b (23.61 kDa) from *Actinia equina* (Pungerčar et al., 1997), Nigrelysin (23.73 kDa) from *Anthopleura nigrescens* (Alvarado-Mesén et al., 2019) have been previously reported. Bands corresponding to Phospholipases (13-18kDa) are observed. Phospholipase A2 A2-actitoxin-Cgg2a (13.63 kDa) from *Condylactis gigantea* (Romero et al., 2010), Phospholipase A2 A2-actitoxin-Ucs2a (17.31 kDa) from *Urticina crassicornis* (Razpotnik et al., 2010), Phospholipase A2 A2-hormotoxin-Apt1a (17.81 kDa) from *Adamsia palliata* (Talvinen & Nevalainen, 2002), DELTA-actitoxin-Aas1a (23kDa) from *Anthopleura asiatica* (Kohno et al., 2009) have been reported. Multiple bands between 5060 kDa probably corresponding to sea anemone Membrane attack complex as similar molecular weight peptides have been reported earlier, such as DELTA-alicitoxin-Pse2b (54.4 kDa) from *Phyllodiscus semoni* (Satoh et al., 2007), DELTA-thalatoxin-Avl2a (55.4 kDa) from *Actineria villosa* (Oshiro et al., 2004), DELTA-alicitoxin-Pse2a (56.0 kDa) from *Phyllodiscus semoni* (Nagai et al., 2002). Peptide bands corresponding to Neurotoxic peptides (3-6.5 kDa) are also observed in the SDS-PAGE gel, that are similar to reported neurotoxic peptides like Delta-actitoxin-Bcg1d (4.7kDa) from *Bunodosoma cangicum* (Zaharenko et al., 2012), Delta-actitoxin-Bcs1a (4.9kDa) from *Bunodosoma caissarum* (Malpezzi et al., 1993), Delta-actitoxin-Bgr2a (5.0 kDa) from *Bunodosoma granuliferum* (Loret et al., 1994), Kappa-actitoxin-Bcs4a (5.7 kDa) from *Bunodosoma caissarum* (Orts et al., 2013), U-actitoxin-Bgr3a (4.5 kDa) from *Bunodosoma granuliferum* (Rodríguez et al., 2012), Toxin Bcg III 28.78 (3.8 kDa) from *Bunodosoma cangicum* (Zaharenko et al., 2008).

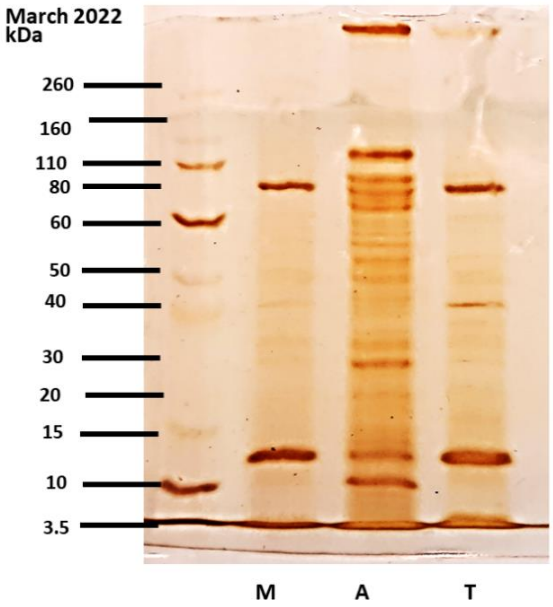


Figure 4.1: SDS-PAGE analysis of crude venom extracts (M=Mucus, A= acontia and mesenterial filaments, T= tentacles) showing distinct protein bands ranging in molecular weight from 3.5 to 260 kDa

4.4.2 FTIR Analysis of Venom Extracts

The FTIR analysis of the crude venom extract from *Bunodosoma goanense* revealed characteristic peaks corresponding to multiple functional groups, suggesting the presence of both proteinaceous and non-proteinaceous compounds (Figure 4.2, 4.3 & 4.4). The strong, broad absorption observed between 3400-3300 cm^{-1} corresponds to N-H (amines) and O-H (carboxylic acids, alcohols) stretching vibrations, indicative of peptides, proteins, and possible secondary metabolites. The presence of medium absorption at 1620-1626 cm^{-1} suggests C=O stretching from carboxyl or amide groups, reinforcing the presence of peptide bonds in venom proteins. Additionally, the peak at 1250-1020 cm^{-1} corresponds to C-N or C-O stretching, further supporting the presence of amides. Notably, a strong absorption at 730-665 cm^{-1} indicates C-H out-of-plane bending vibrations, which are characteristic of alkenes or aromatic rings. This suggests that in addition to peptides, the venom may contain non-peptidic bioactive molecules compounds. Given that compounds like palytoxin, caissarone and bunodosine-known marine toxins-contain alkene moieties (Moore & Bartolini, 1981; Cooper et al., 1995; Zaharenko et al., 2002), it is plausible that similar non-peptide toxins may be present in this venom. (Summarized in Table 4.1)

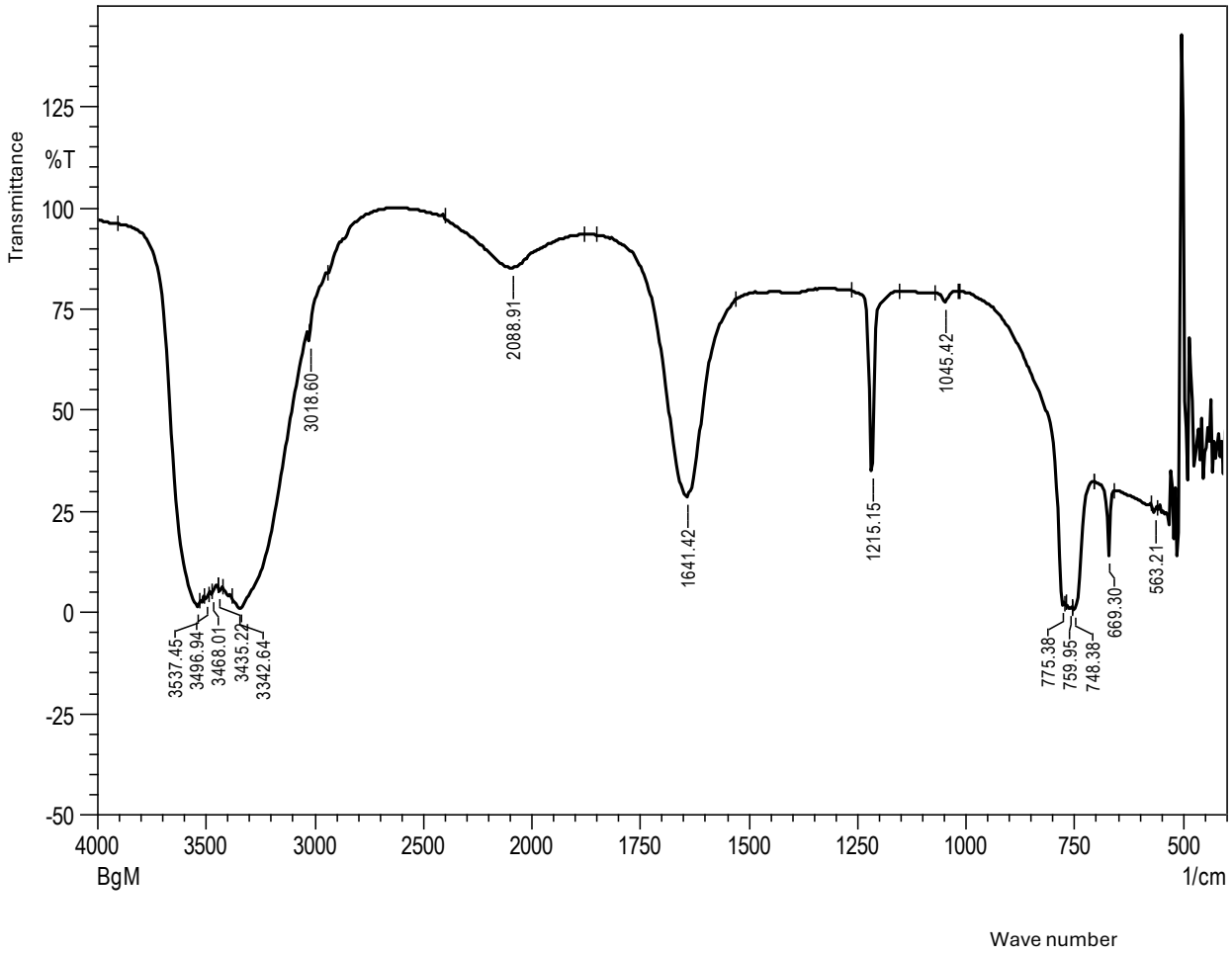


Figure 4.2: FTIR analysis of Crude Venomous mucus showing the presence of functional groups: carboxylic acids, amines and alkenes. (x-axis: wave number (1/cm), y-axis: transmittance (%))

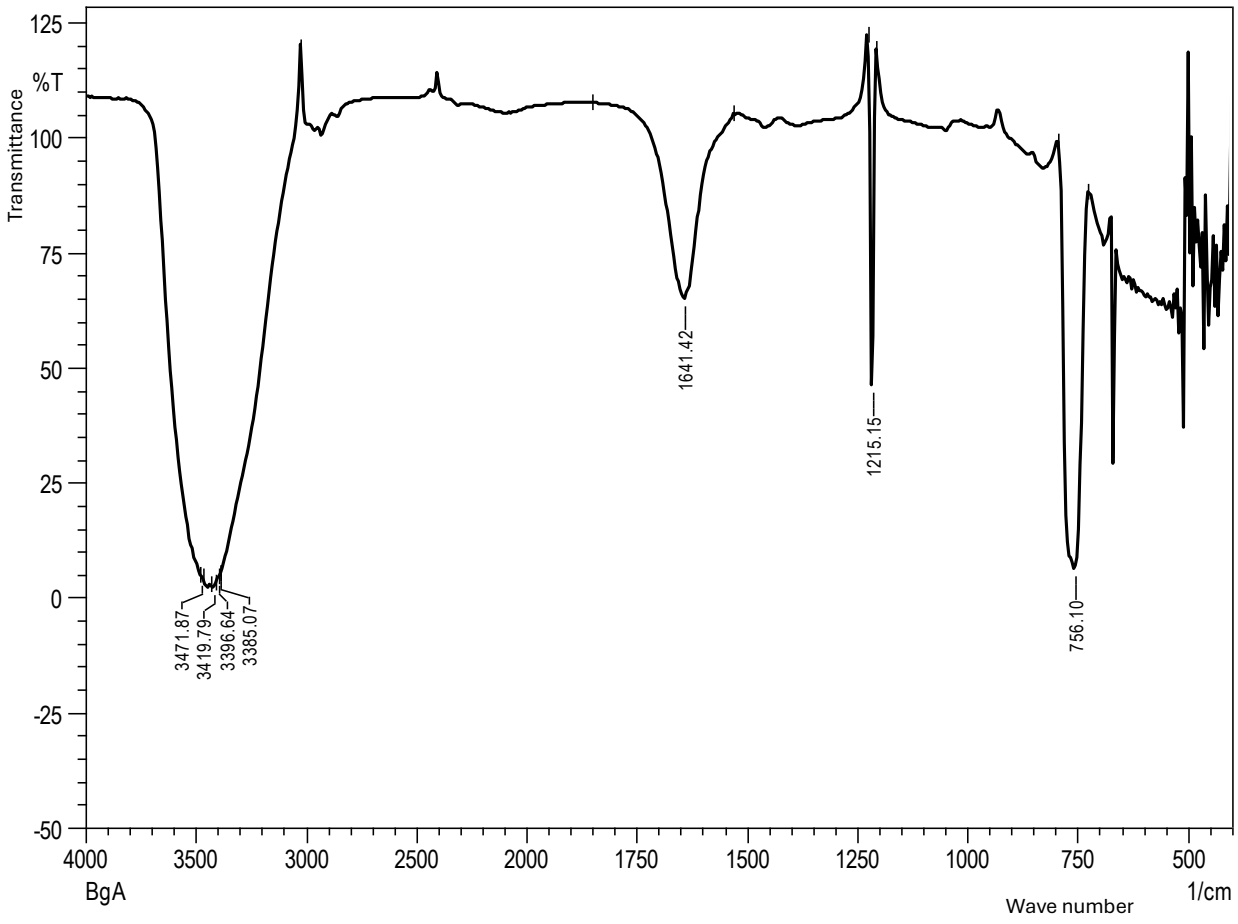


Figure 4.3**:** FTIR analysis of Crude Acontial and mesenterial venom showing the presence of functional groups: carboxylic acids, amines and alkenes.

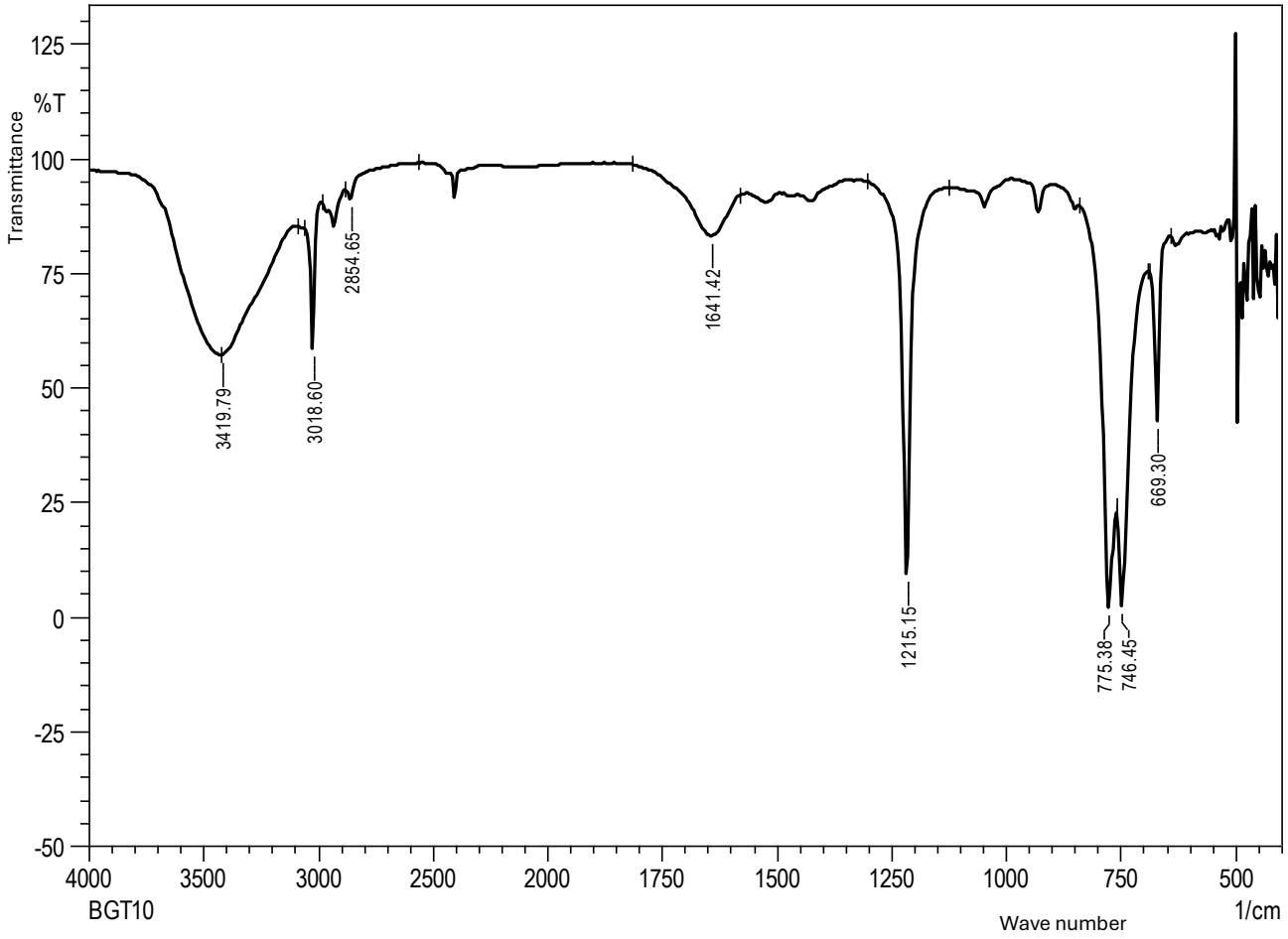


Figure 4.4: FTIR analysis of crude tentacle venom showing the presence of functional groups: carboxylic acids, amines and alkenes.

Table 4.1: FTIR spectrum analysis of crude venom of mucus, acontia and mesentrial venom, and tentacle venom show the presence of the same functional groups carboxylic acids, amines and alkenes

Frequency range cm-1	Appearance	Compound class
3400-3300	Strong broad	Carboxylic acid
1662-1626	Medium	Alkene
1250-1020	Medium	Amine
730-665	Strong	Alkene

4.4.3 Gel Filtration and Reverse phase Chromatography HPLC of Venom Extracts:

To further elucidate the complexity and tissue-specific variation of the venom composition in *Bunodosoma goanense*, we performed gel filtration high-performance liquid chromatography (HPLC) on crude venom extracts from different tissues. The ectoderm mucus venom was separated into 7 fractions (Figure 4.5), the acontia venom into 8 fractions (Figure 4.6), and the tentacle venom into 7 fractions (Figure 4.7) based on their molecular size. Each of these 22 fractions was then subjected to further fractionation using Reverse phase gradient chromatography based on their polarities (Figure 4.8 to 4.29).

The chromatography profiles of these fractions displayed significant differences, validating our hypothesis that venom composition varies among different tissues of *B. goanense*. This tissue-specific variation was evident in the distinct chromatographic patterns observed for each type of venom extract.

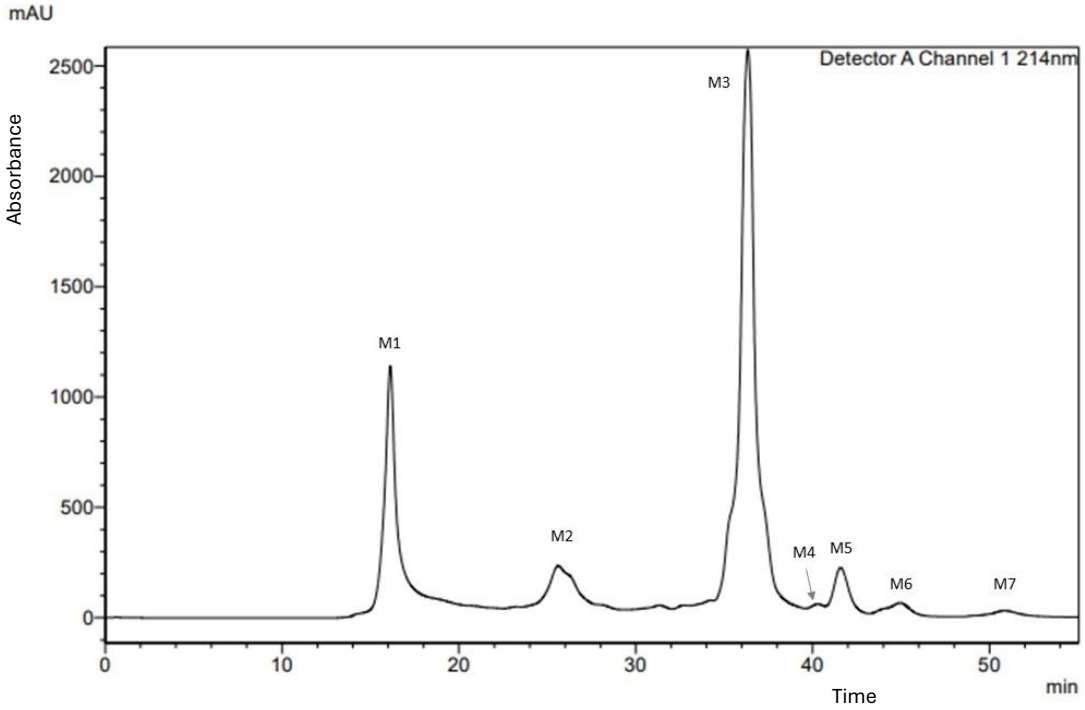


Figure 4.5: Gel filtration high-performance liquid chromatography (HPLC) profile of Crude mucus venom separated into 7 distinct fraction peaks ((x-axis: time (minutes), y-axis: milli-absorbance units (mAU))

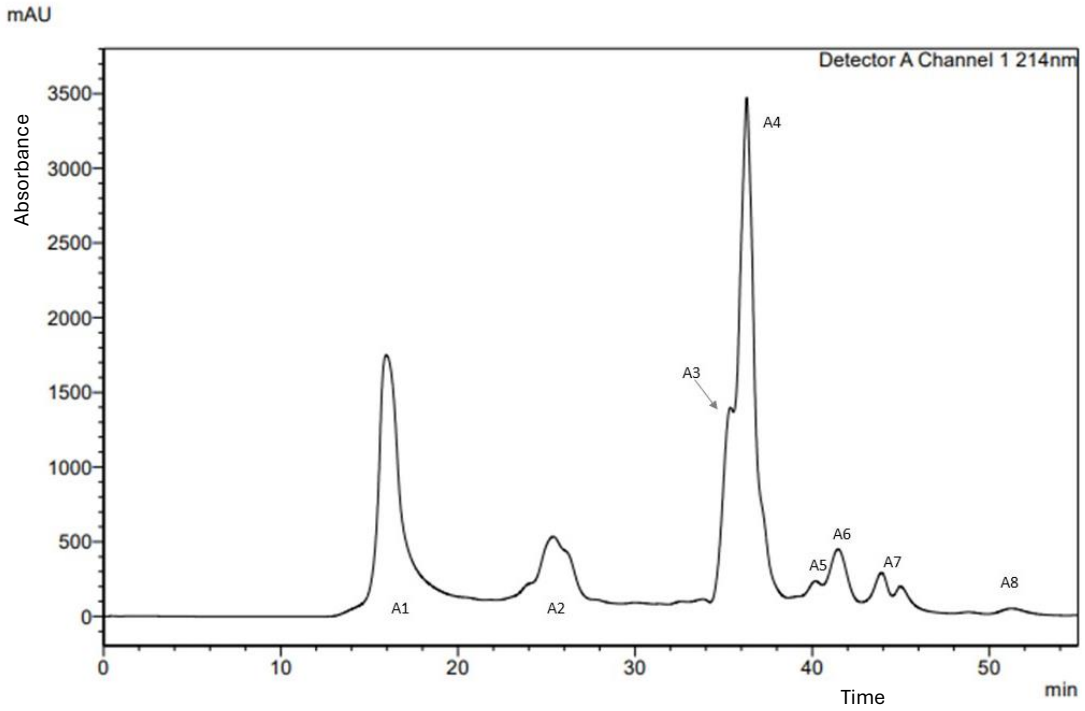


Figure 4.6: Gel filtration high-performance liquid chromatography (HPLC) profile of Crude Acontia venom separated into 8 distinct fraction peaks

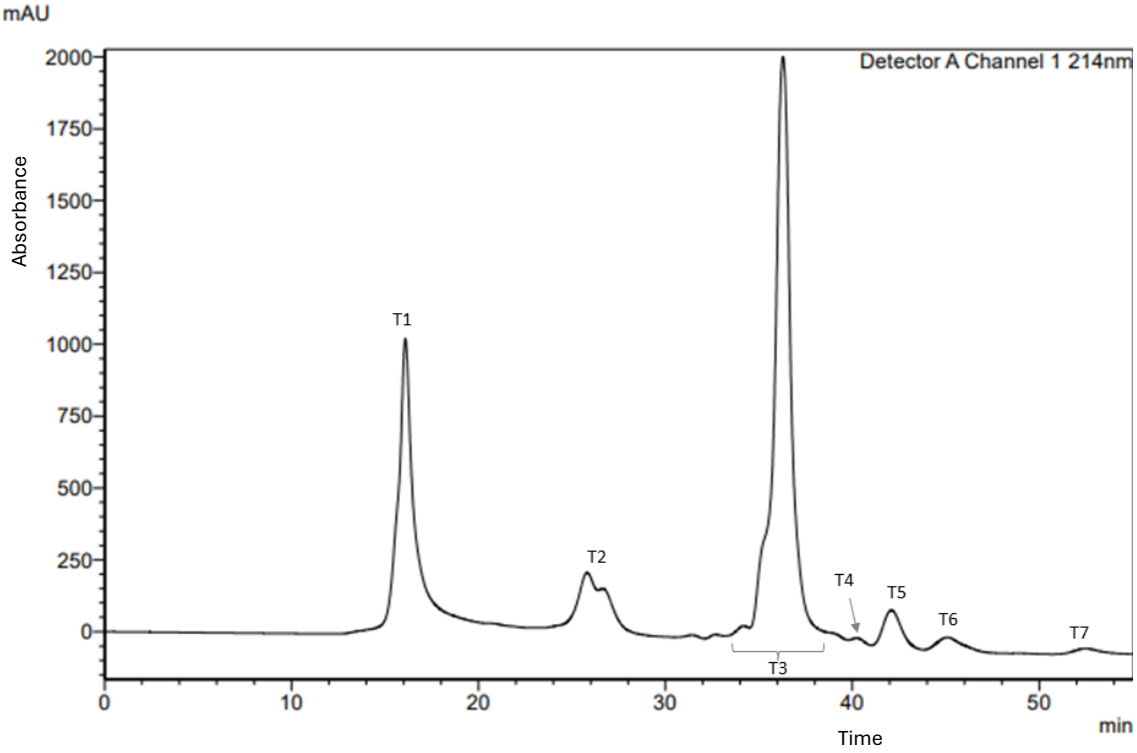


Figure 4.7: Gel filtration high-performance liquid chromatography (HPLC) profile of Crude tentacle venom separated into 7 distinct fraction peaks

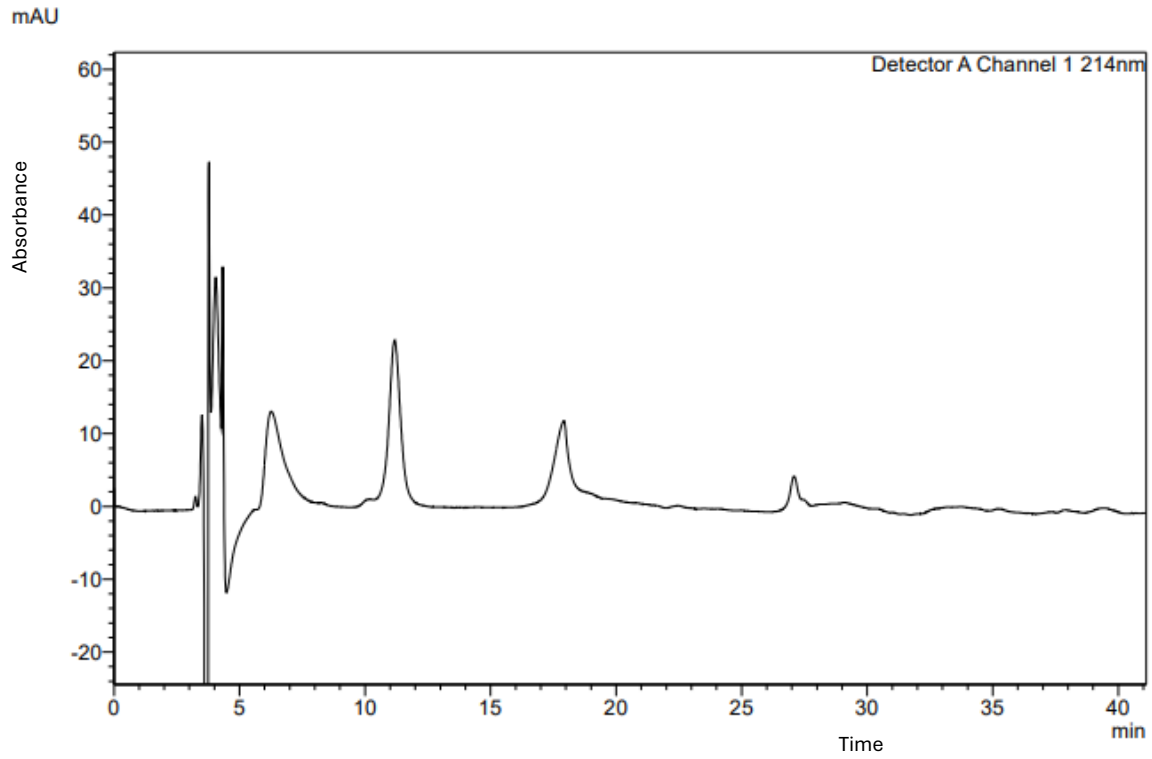


Figure 4.8: Reverse phase high-performance liquid chromatography (HPLC) profile of M1 mucus venom fraction

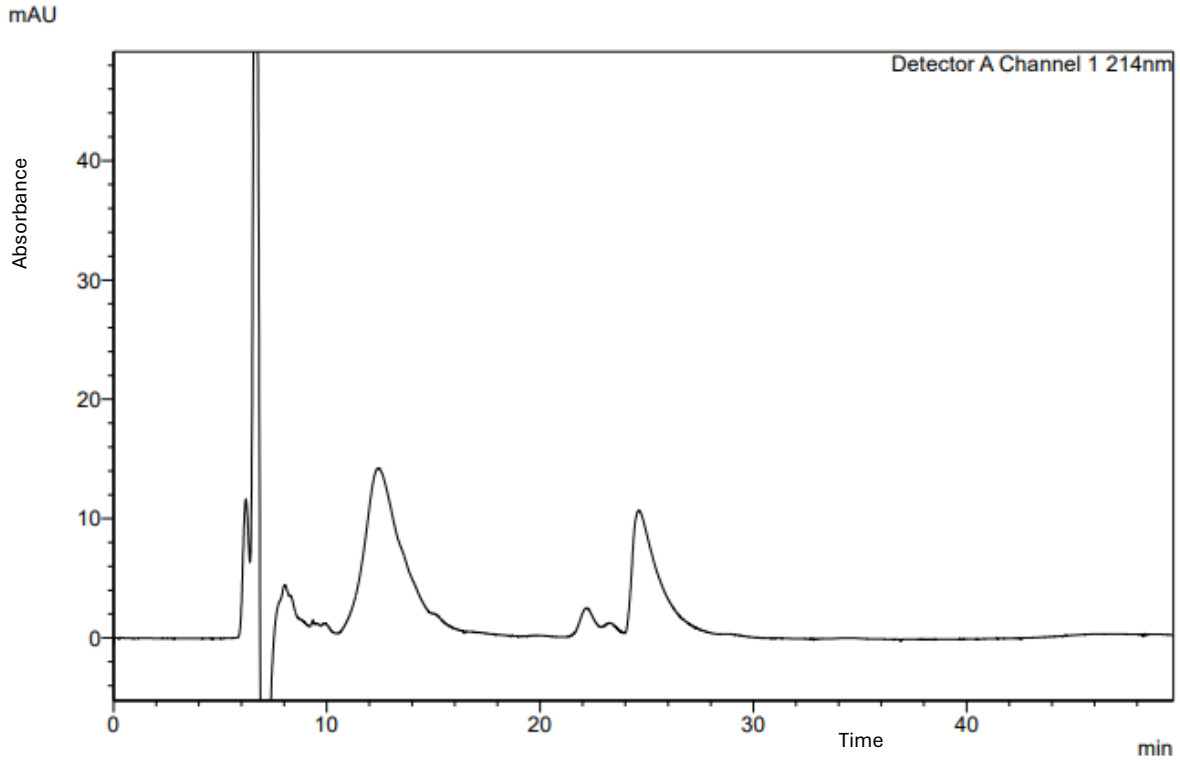


Figure 4.9: Reverse phase high-performance liquid chromatography (HPLC) profile of M2 mucus venom fraction

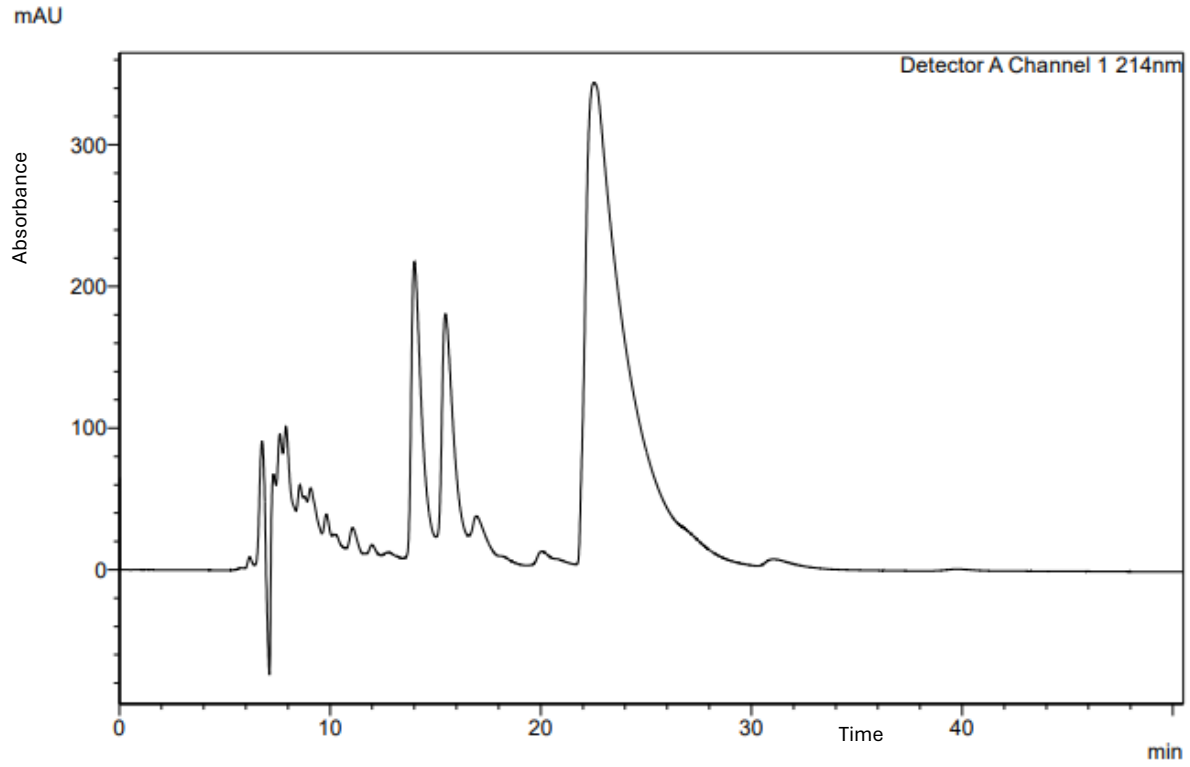


Figure 4.10**:** Reverse phase high-performance liquid chromatography (HPLC) profile of M3 mucus venom fraction

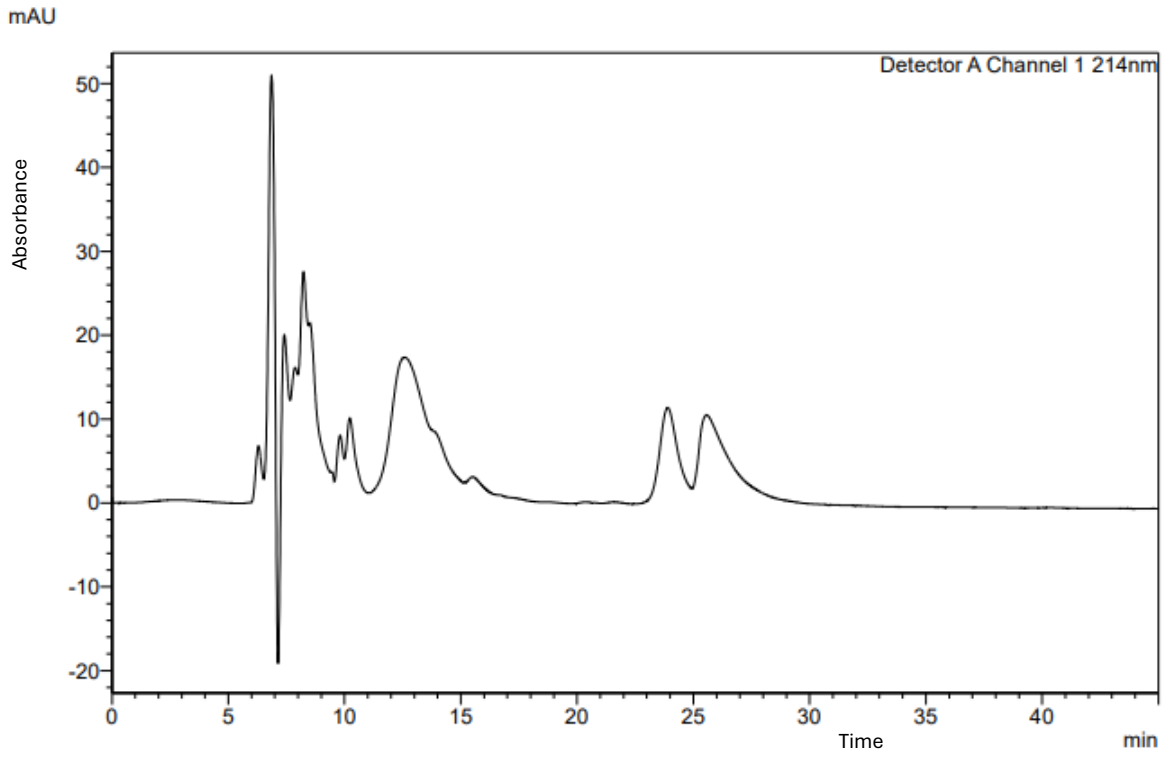


Figure 4.11: Reverse phase high-performance liquid chromatography (HPLC) profile of M4 mucus venom fraction

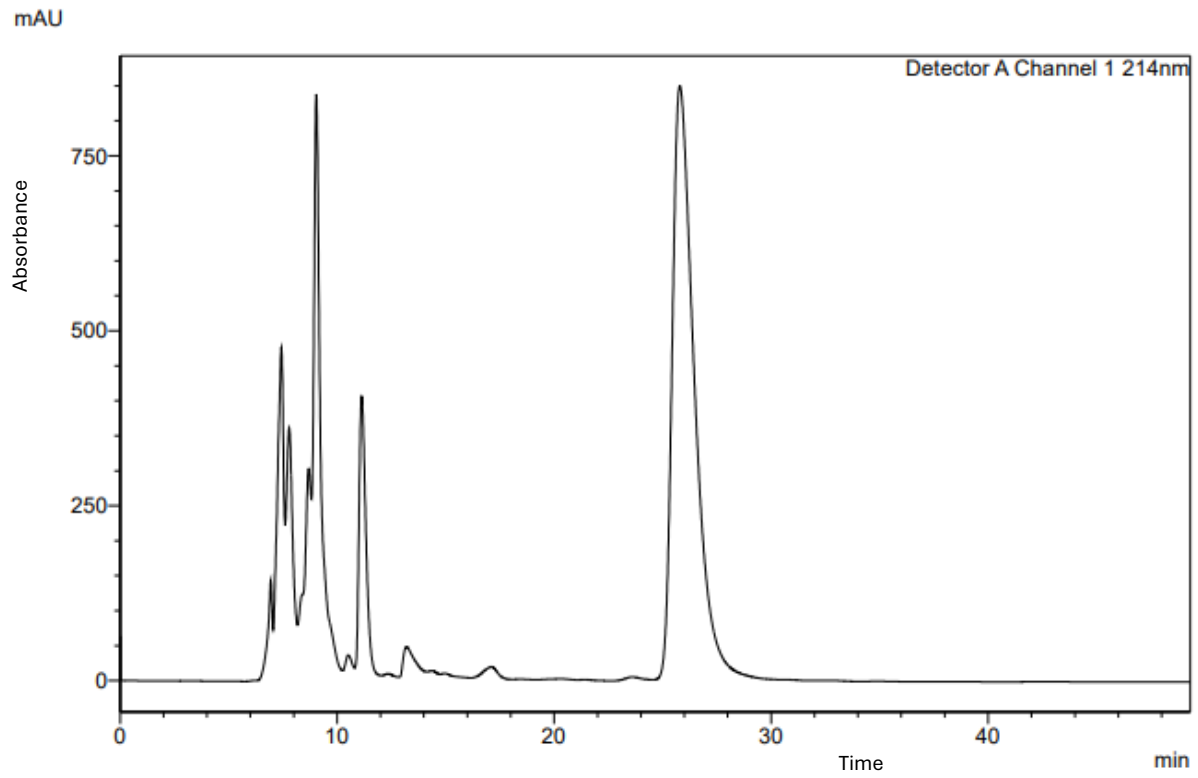


Figure 4.12: Reverse phase high-performance liquid chromatography (HPLC) profile of M5 mucus venom fraction

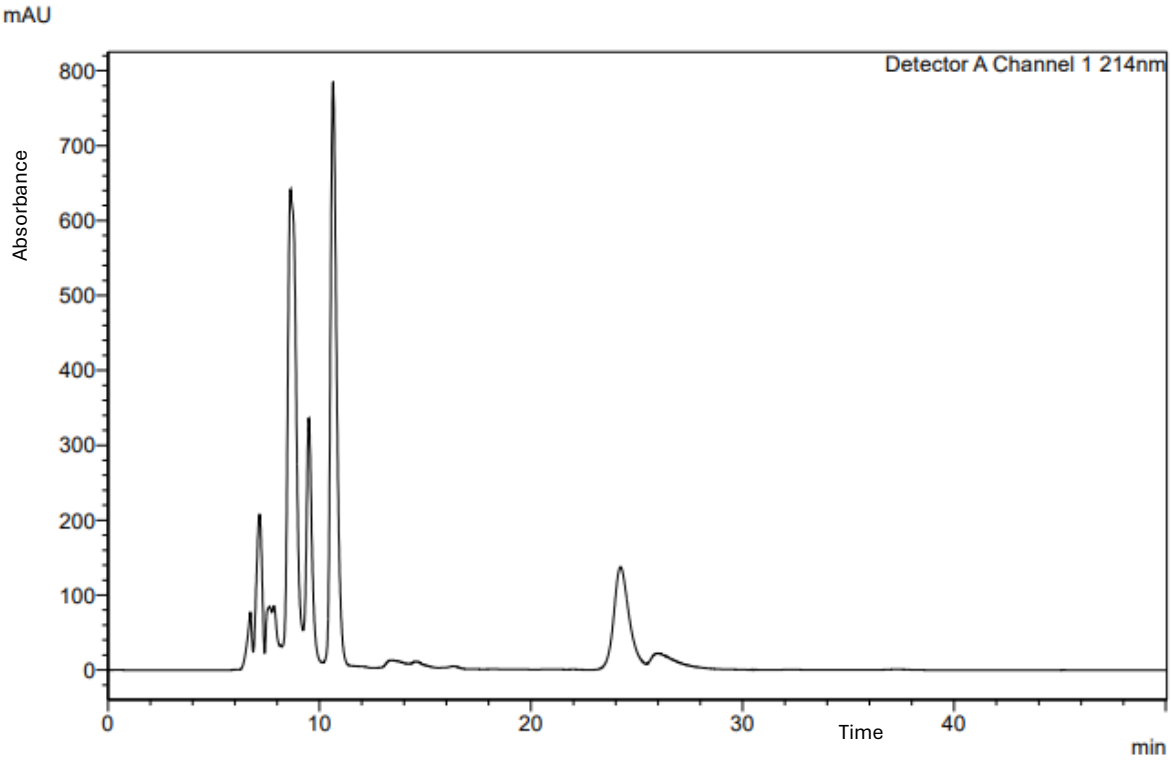


Figure 4.13: Reverse phase high-performance liquid chromatography (HPLC) profile of M6 mucus venom fraction

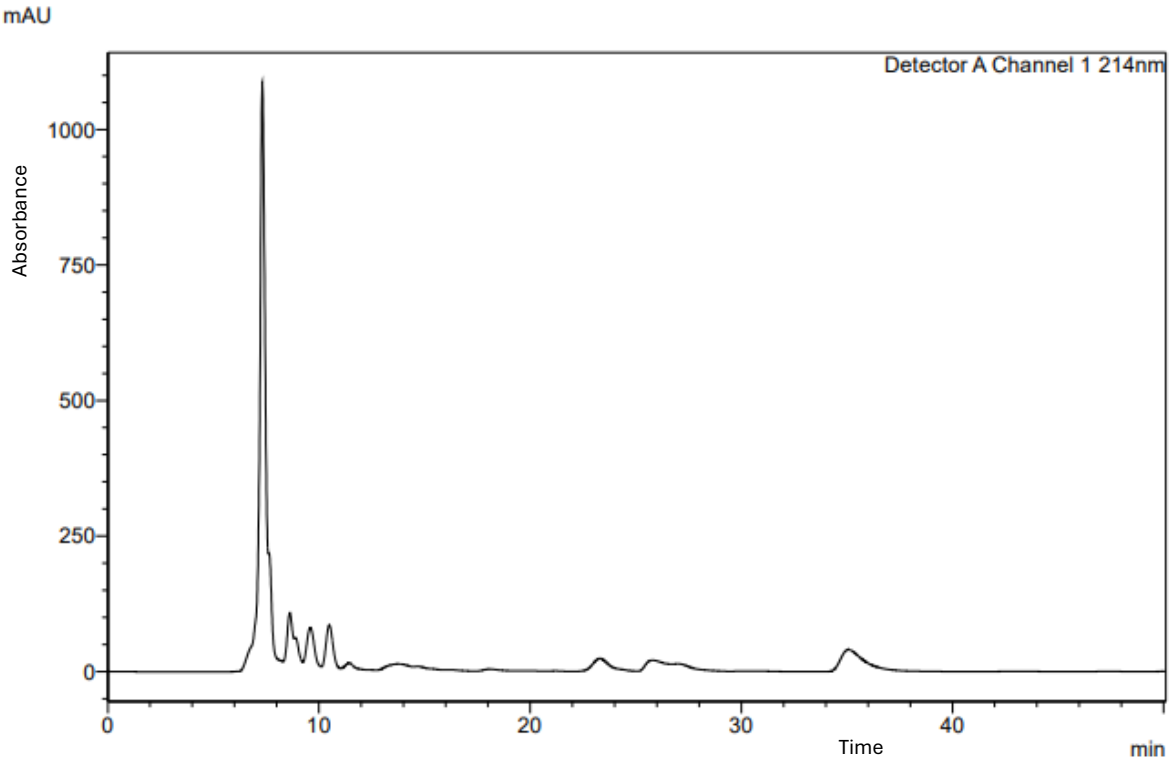


Figure 4.14: Reverse phase high-performance liquid chromatography (HPLC) profile of M7 mucus venom fraction

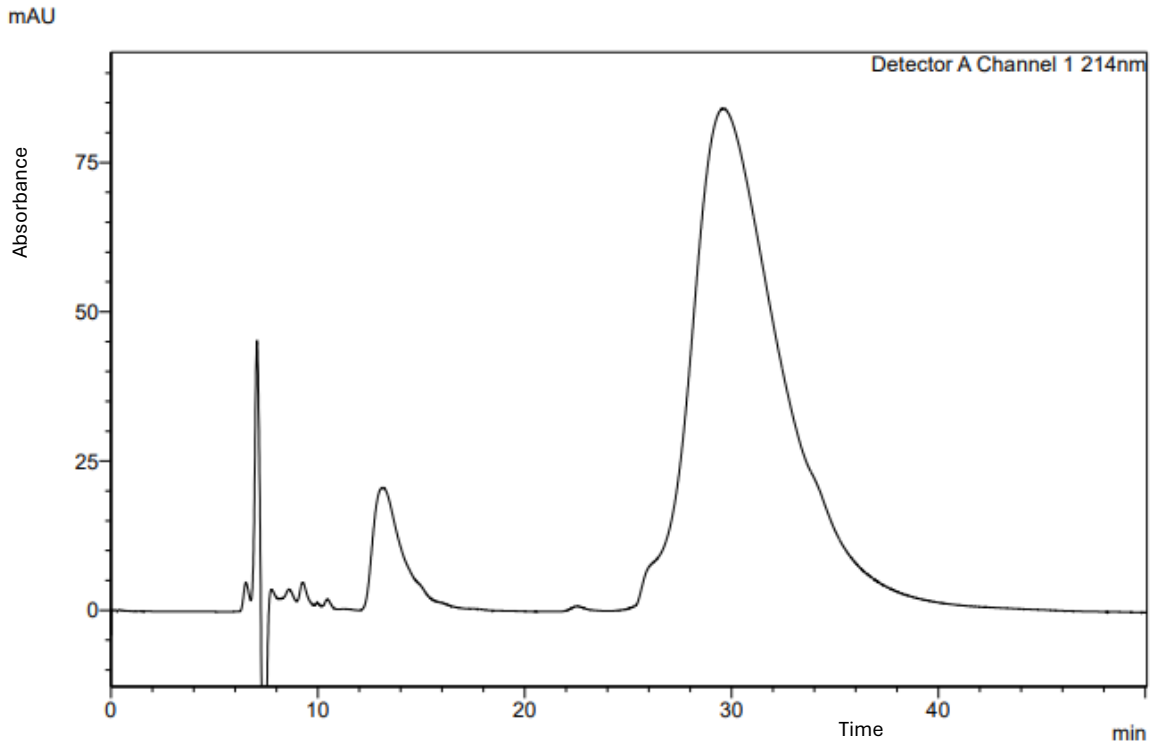


Figure 4.15: Reverse phase high-performance liquid chromatography (HPLC) profile of A1 Acontia and mesenterial filaments venom fraction

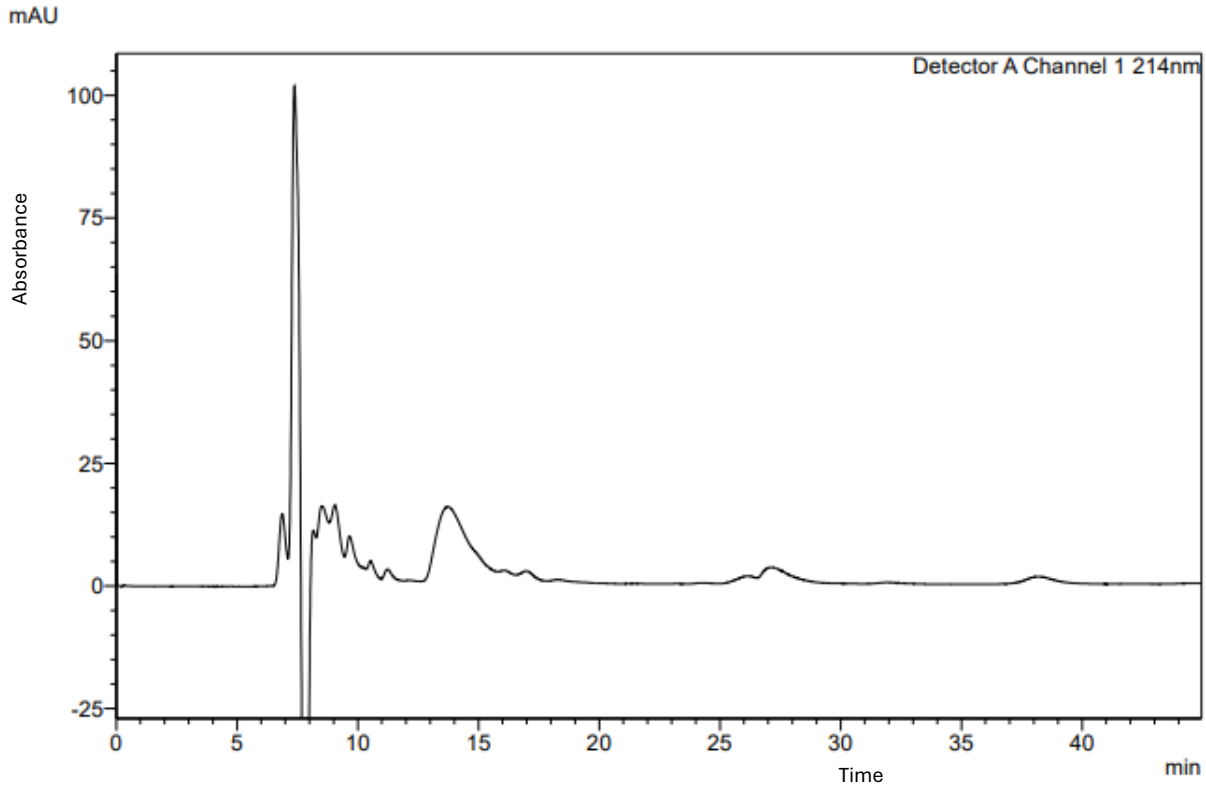


Figure 4.16: Reverse phase high-performance liquid chromatography (HPLC) profile of A2 Acontia and mesenterial filaments venom fraction

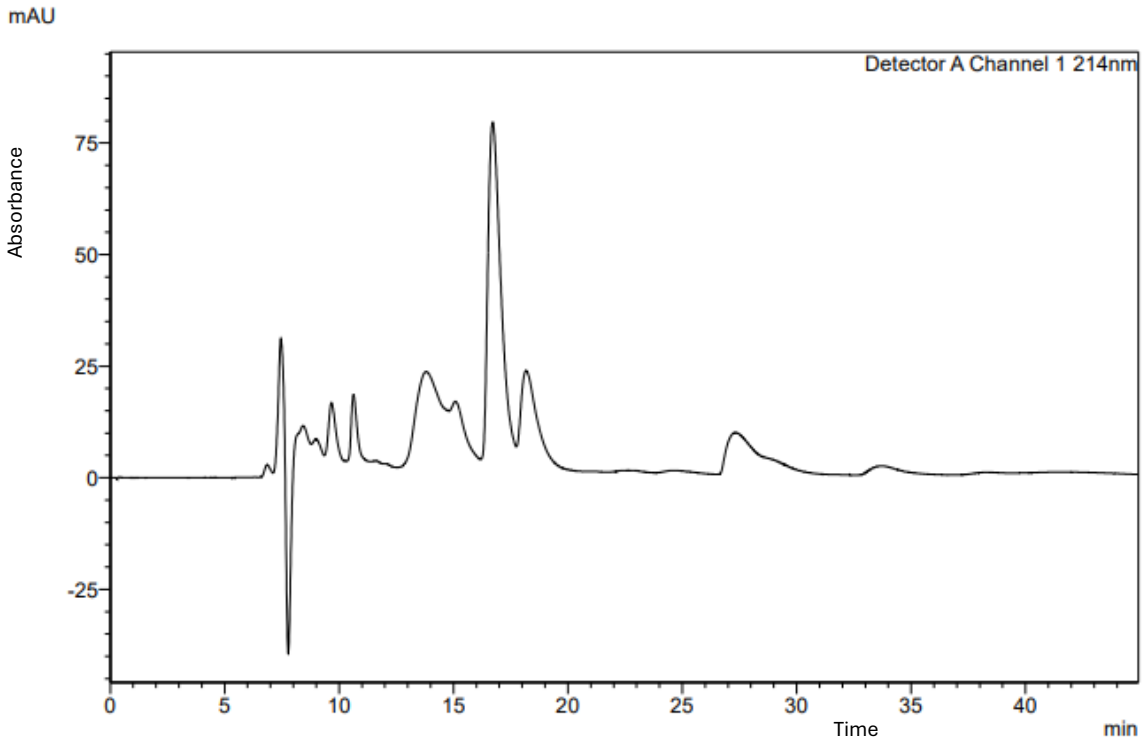


Figure 4.17: Reverse phase high-performance liquid chromatography (HPLC) profile of A3 Acontia and mesenterial filaments venom fraction

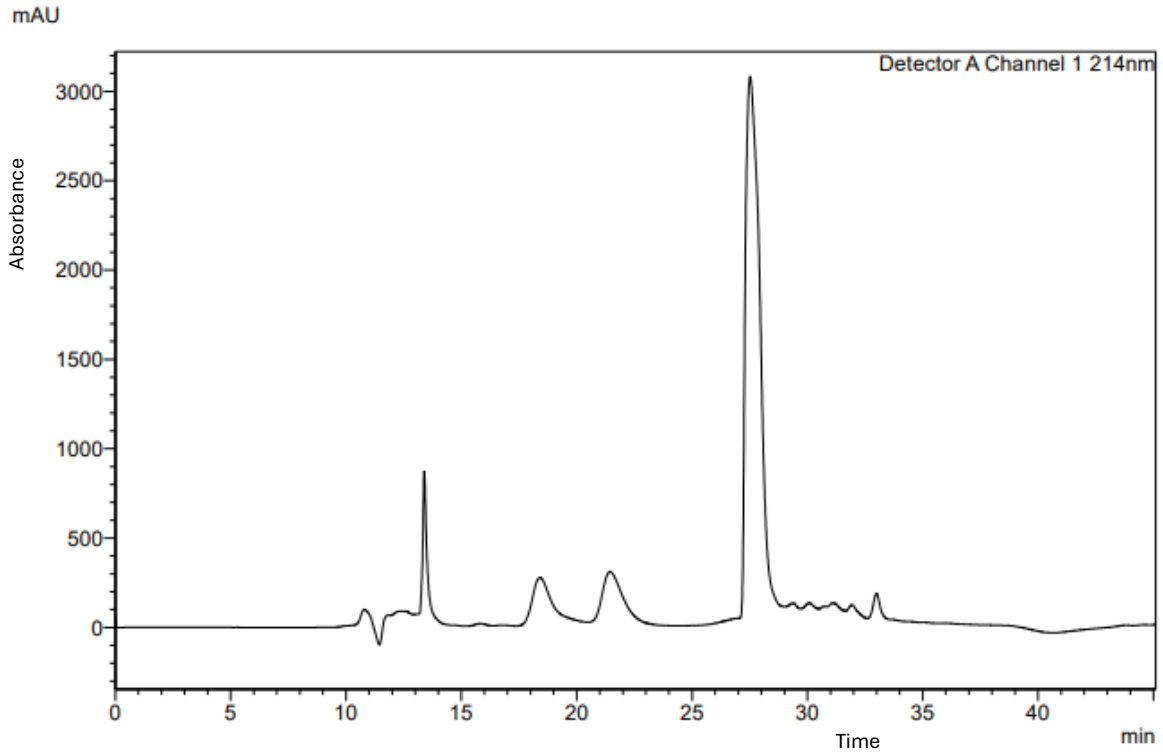


Figure 4.18: Reverse phase high-performance liquid chromatography (HPLC) profile of A4 Acontia and mesenterial filaments venom fraction

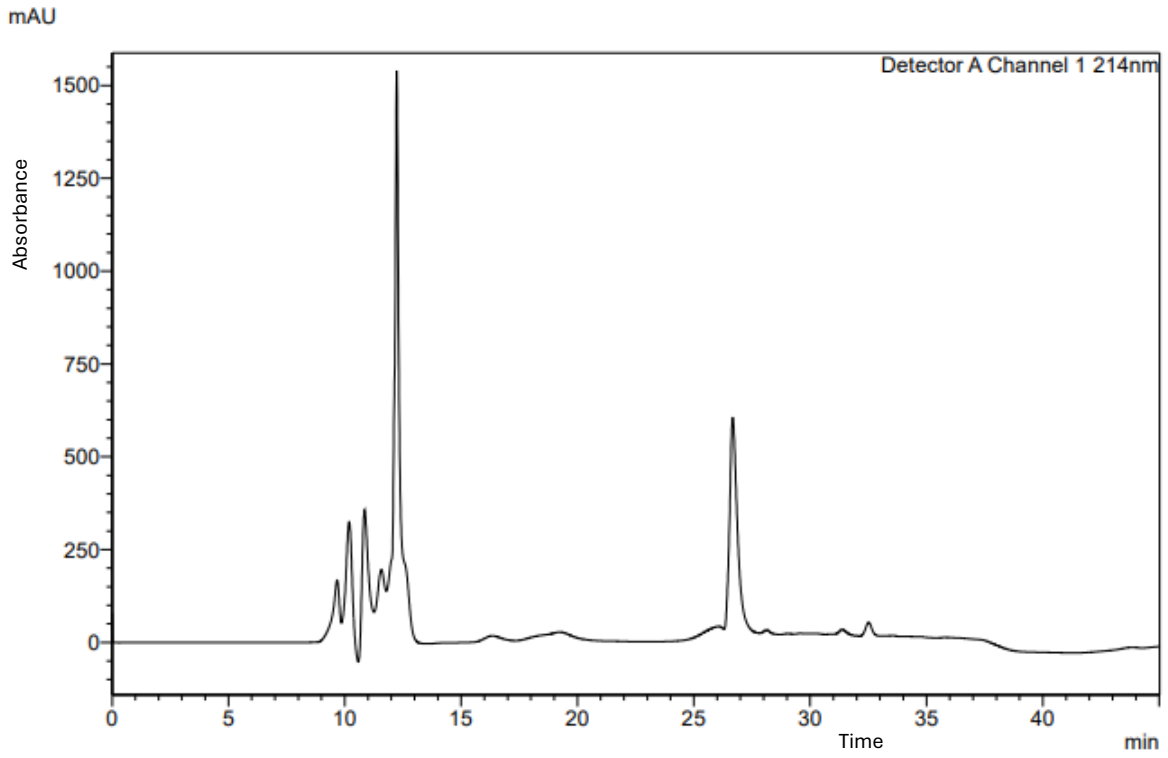


Figure 4.19: Reverse phase high-performance liquid chromatography (HPLC) profile of A5 Acontia and mesenterial filaments venom fraction

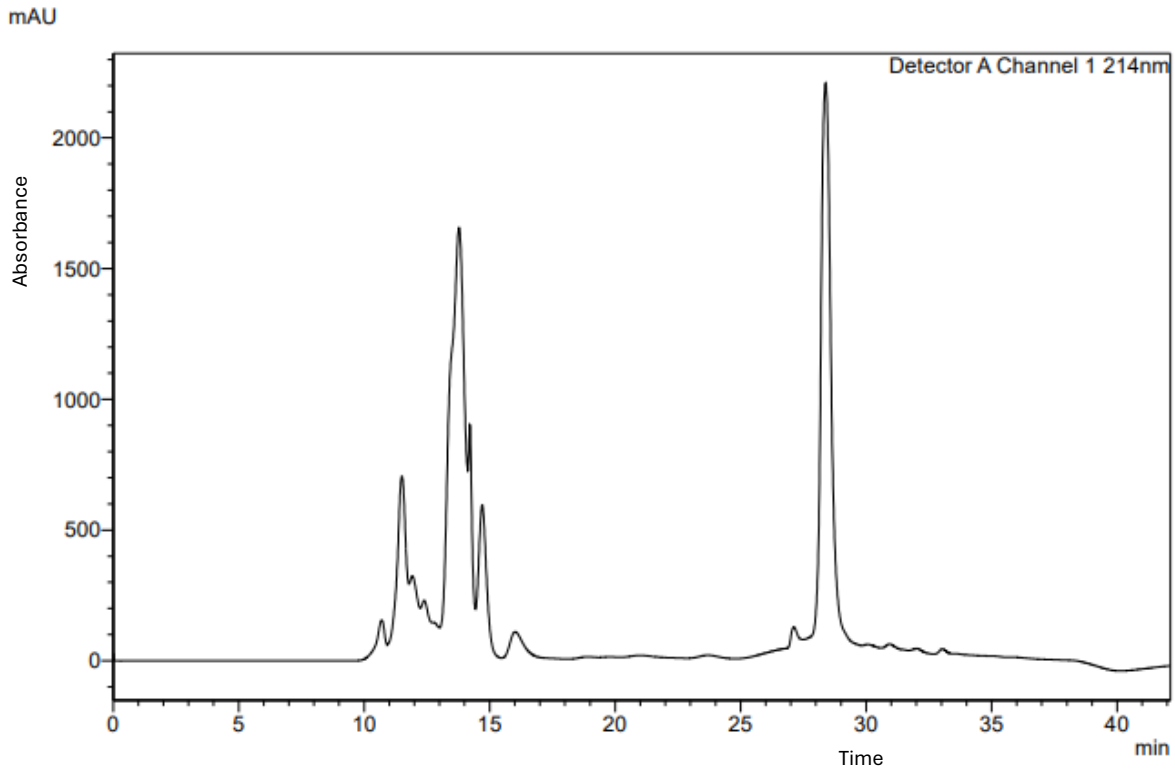


Figure 4.20: Reverse phase high-performance liquid chromatography (HPLC) profile of A6 Acontia and mesenterial filaments venom fraction

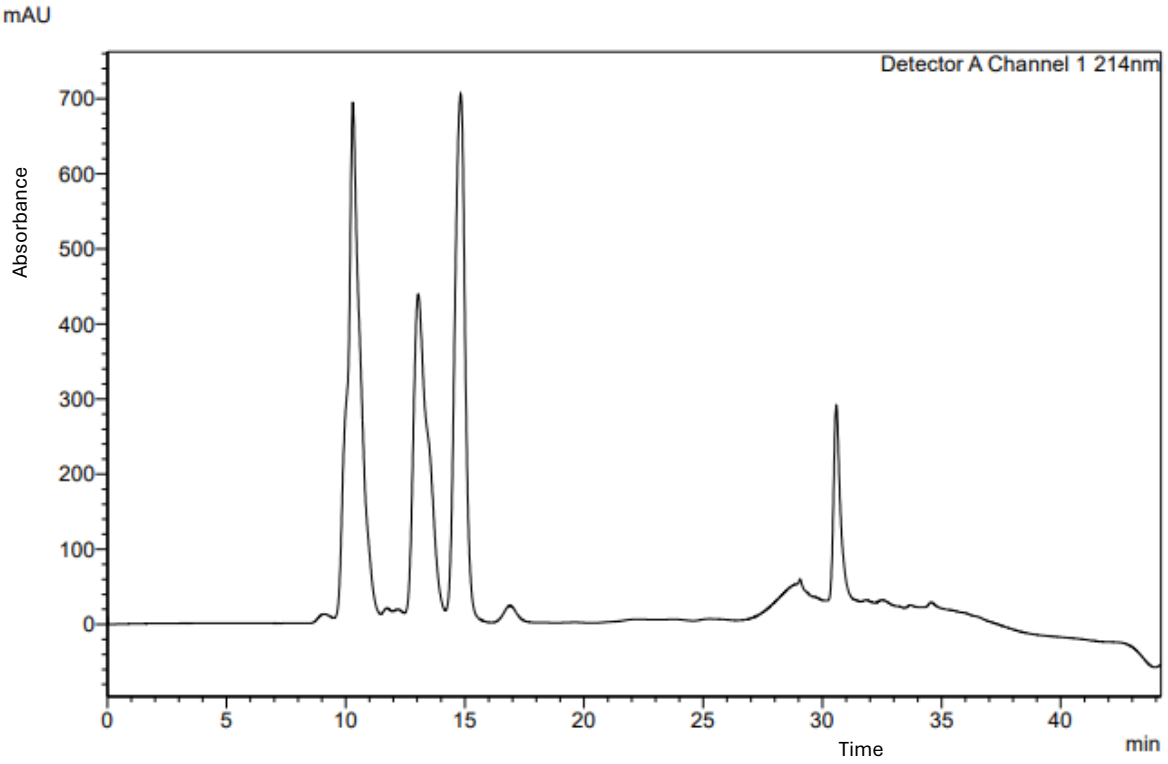


Figure 4.21: Reverse phase high-performance liquid chromatography (HPLC) profile of A7 Acontia and mesenterial filaments venom fraction

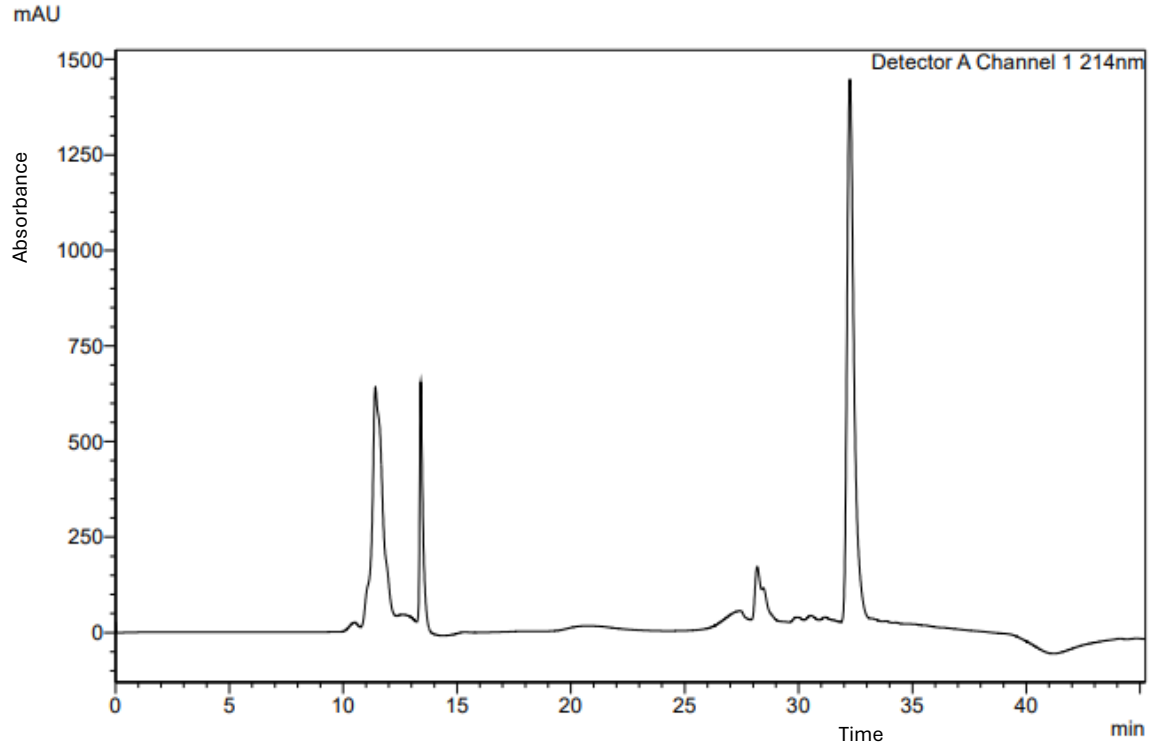


Figure 4.22: Reverse phase high-performance liquid chromatography (HPLC) profile of A8 Acontia and mesenterial filaments venom fraction

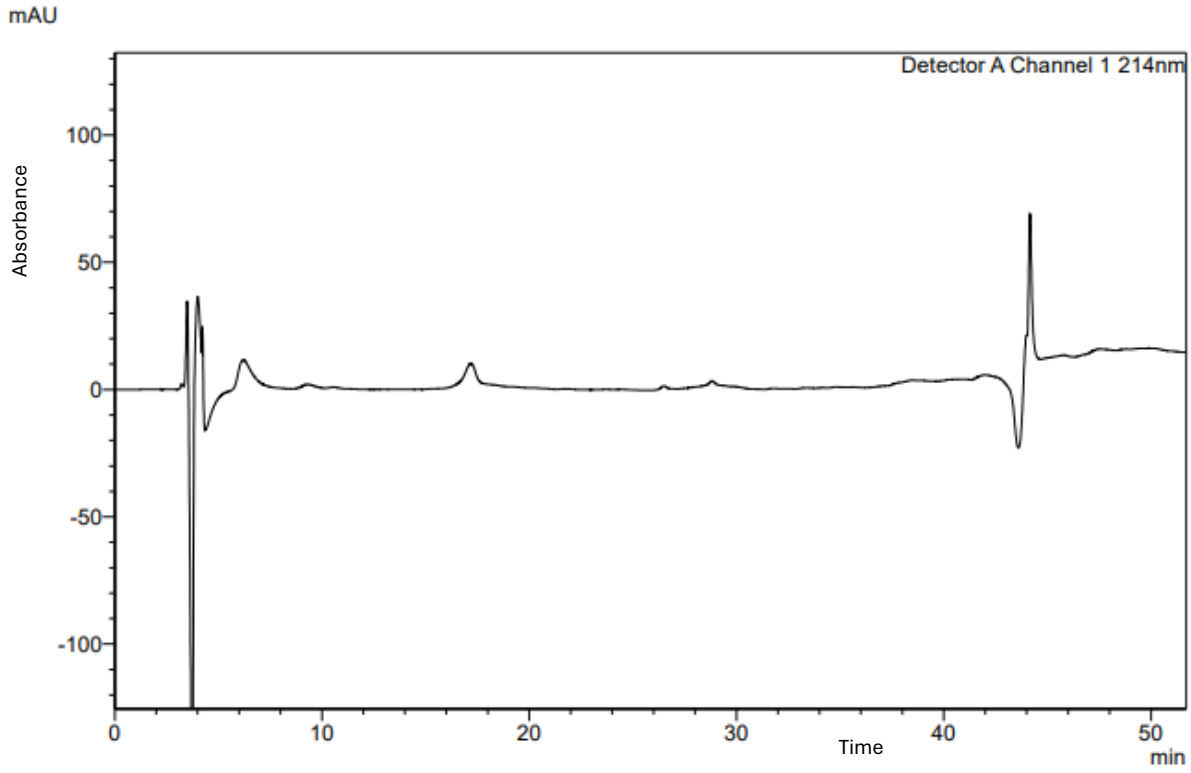


Figure 4.23: Reverse phase high-performance liquid chromatography (HPLC) profile of T1 Tentacles venom fraction

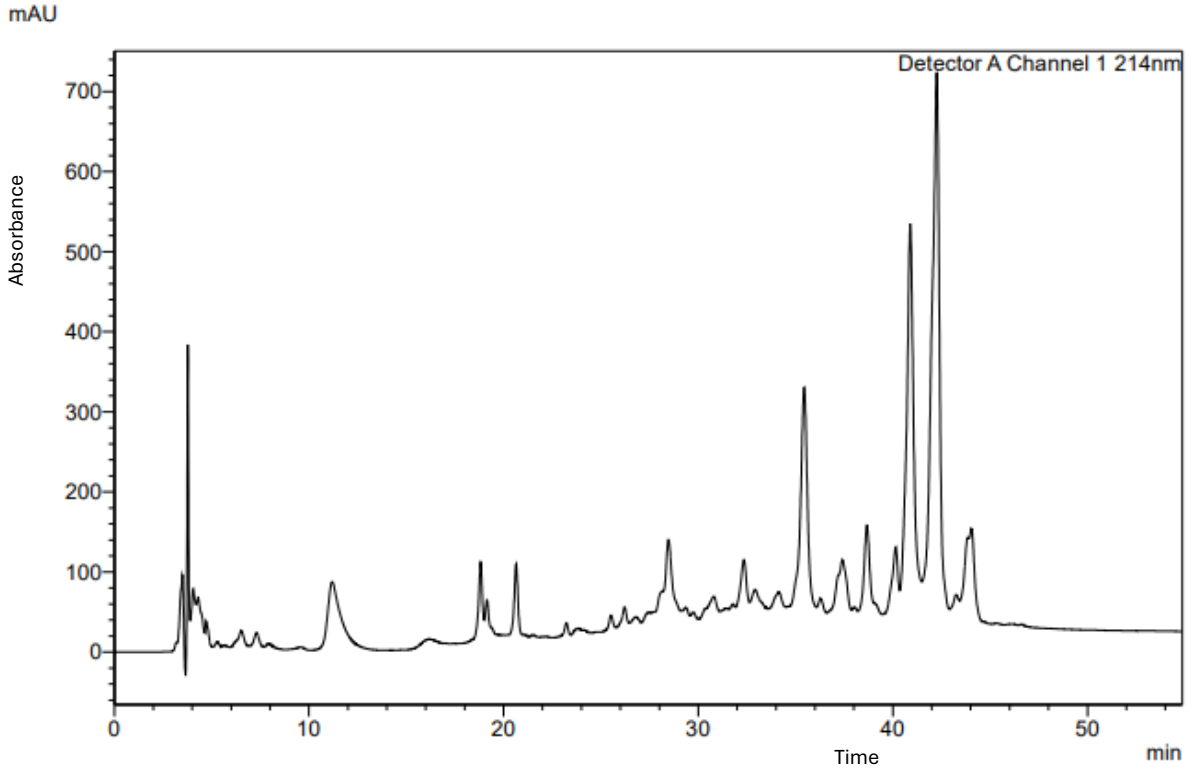


Figure 4.24: Reverse phase high-performance liquid chromatography (HPLC) profile of T2 Tentacles venom fraction

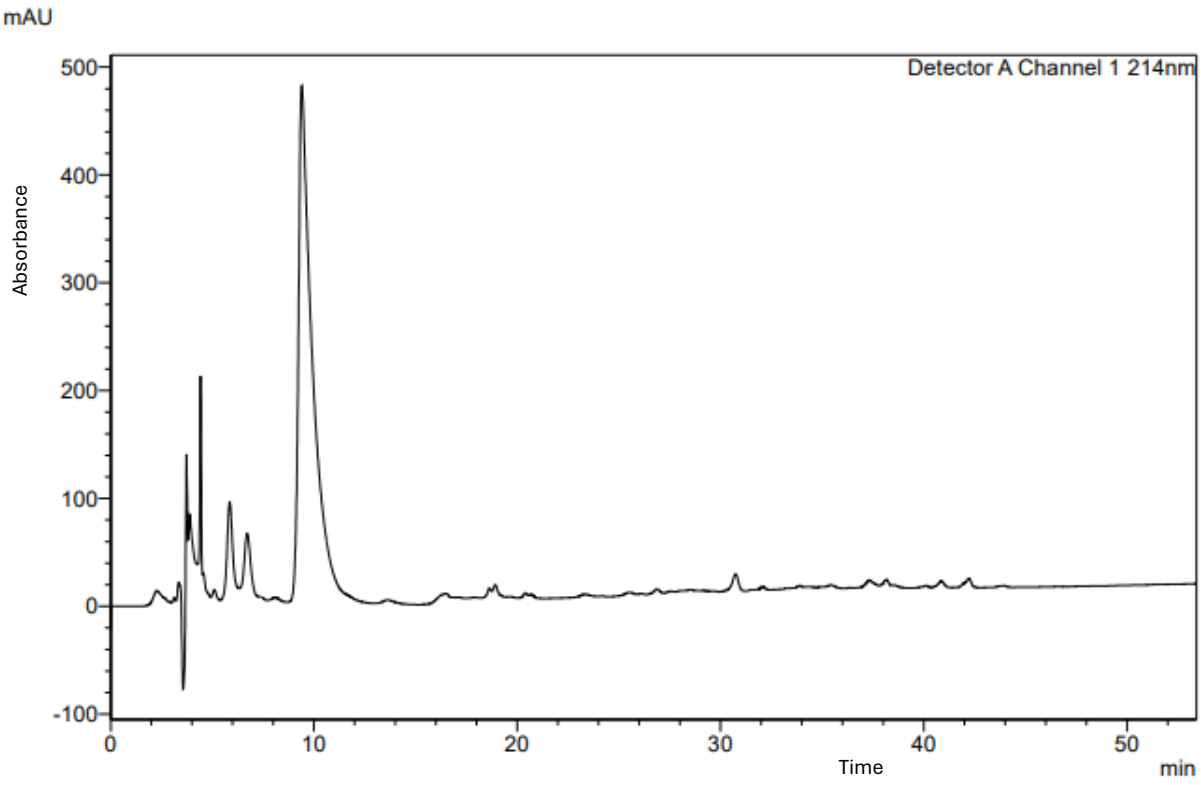


Figure 4.25: Reverse phase high-performance liquid chromatography (HPLC) profile of T3 Tentacles venom fraction

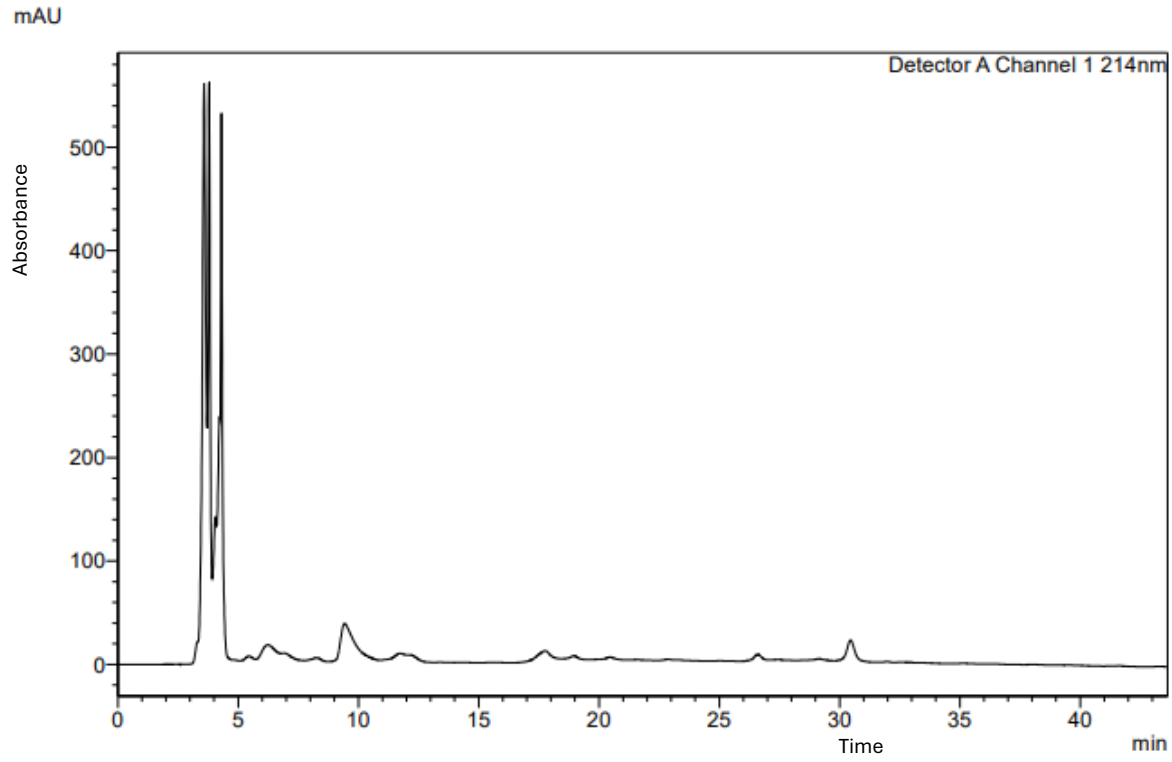


Figure 4.26: Reverse phase high-performance liquid chromatography (HPLC) profile of T4 Tentacles venom fraction

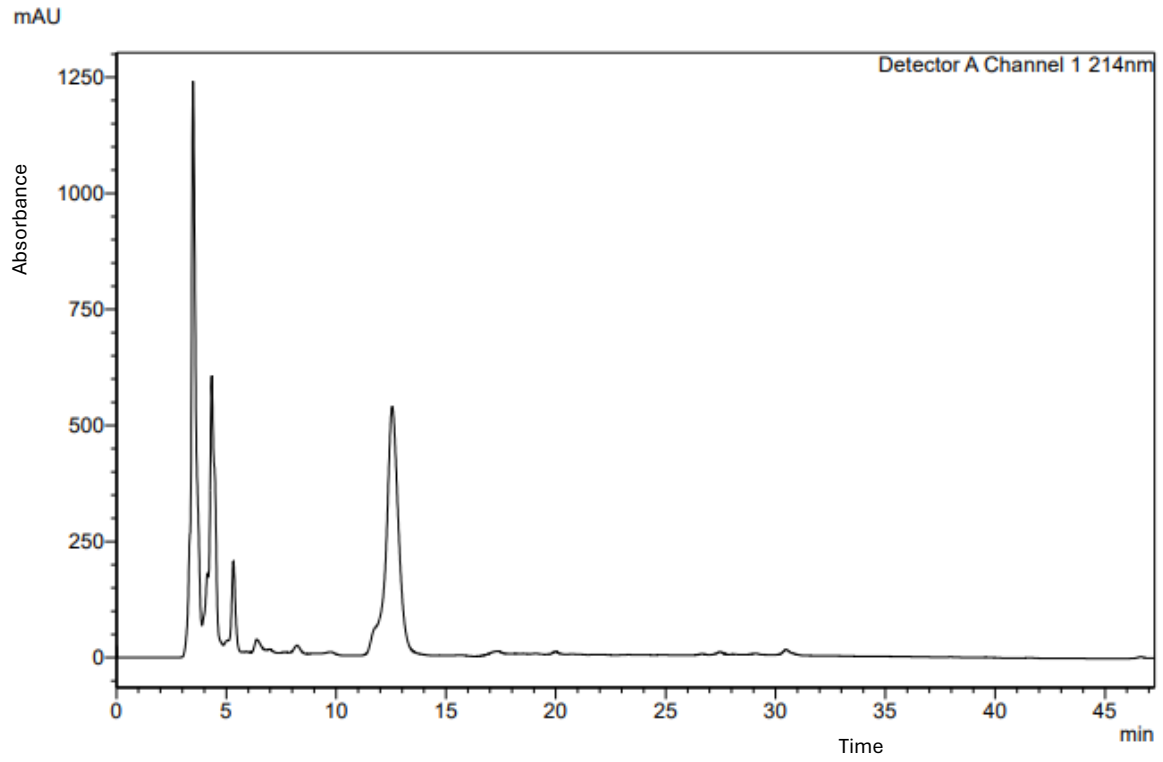


Figure 4.27: Reverse phase high-performance liquid chromatography (HPLC) profile of T5 Tentacles venom fraction

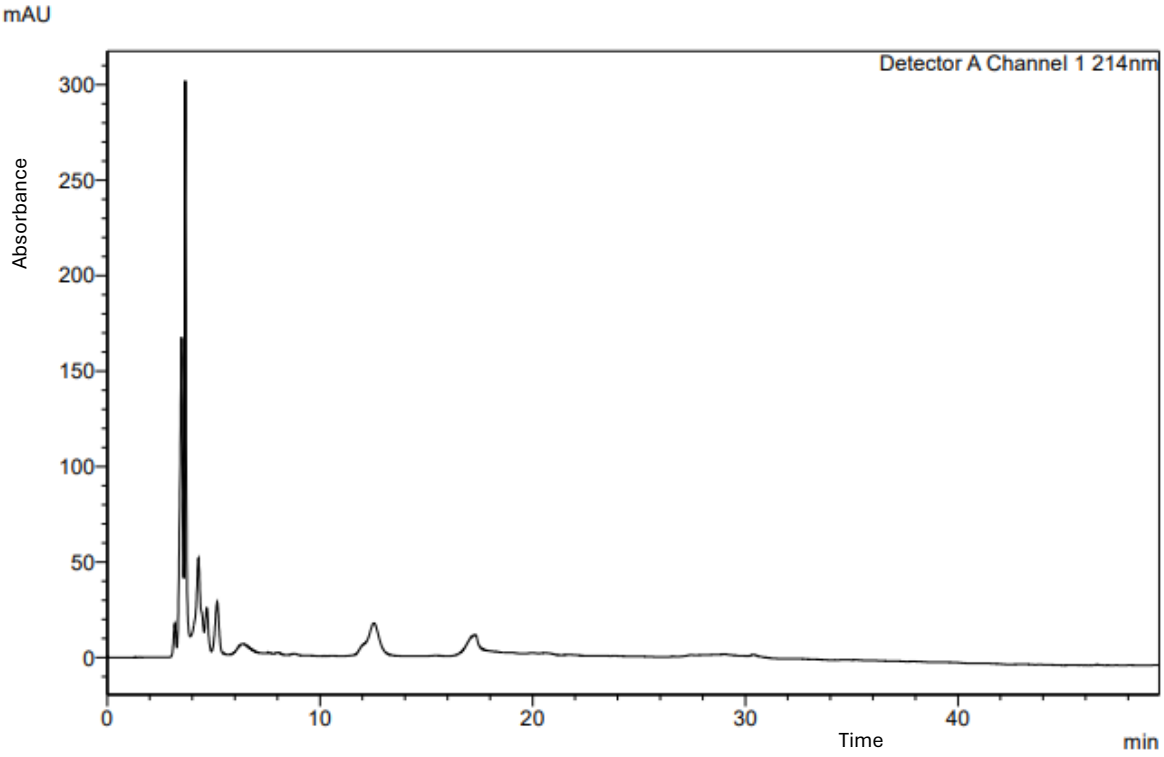


Figure 4.28: Reverse phase high-performance liquid chromatography (HPLC) profile of T6 Tentacles venom fraction

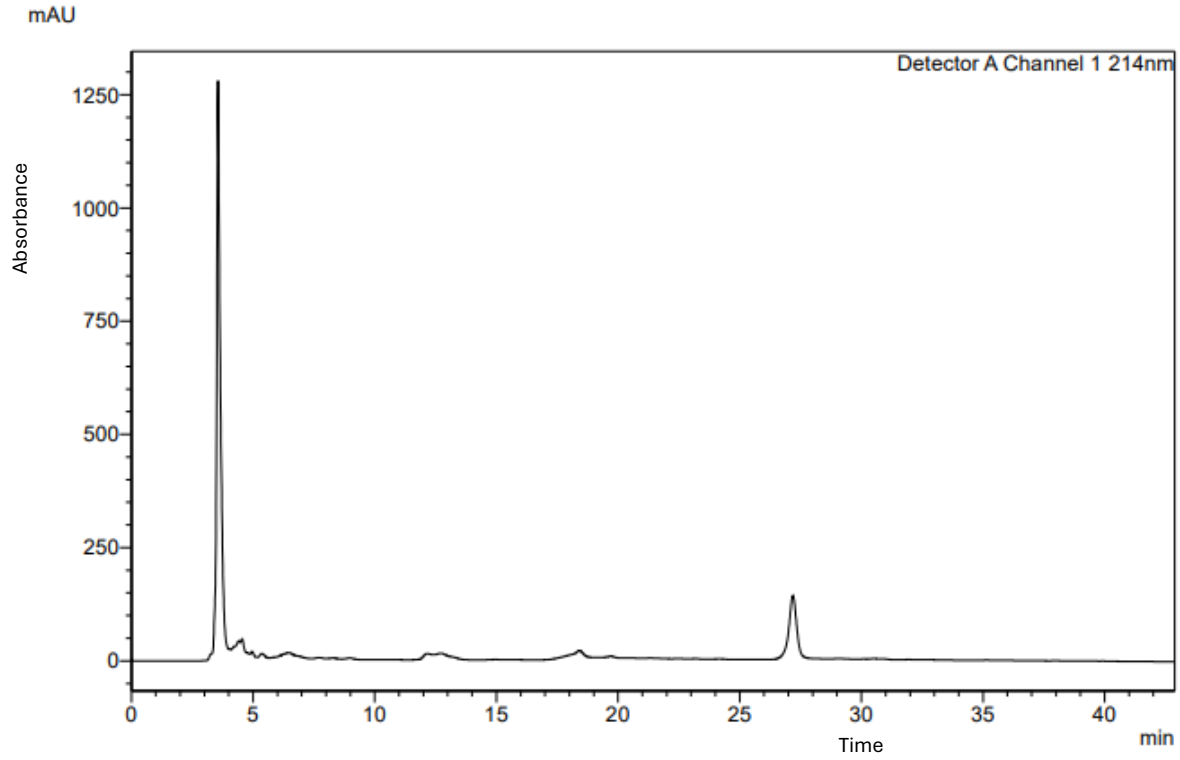


Figure 4.29: Reverse phase high-performance liquid chromatography (HPLC) profile of T7 Tentacles venom fraction

The distinct chromatographic profiles obtained from different tissues of *Bunodosoma goanense* confirm our hypothesis of tissue-specific venom composition. In cnidarians, venom is primarily stored and delivered through nematocysts, which are distributed throughout the body but vary in abundance and type across different tissues. The venom composition of most sea anemone species remains largely unexplored. Our results align with previous studies showing that sea anemone venom is not a uniform mixture but rather varies across different anatomical regions, likely reflecting functional specialization for predation, defence, and digestion (Macrander et al., 2016). Tissue-specific venom composition has been documented in actiniarians, where toxin expression is closely linked to the ecological role of different body regions. The transcriptomic and proteomic analyses of *Telmatactis stephensoni* revealed distinct venom profiles in tentacles, epidermis, and gastrodermis, each adapted to a specific ecological function (Ashwood et al., 2022). Our chromatographic data similarly indicate that venom components in *B. goanense* are regionally compartmentalized, supporting the idea that toxin production is functionally partitioned across envenomating structures. Furthermore, comparative studies on *Actinia fragacea* have demonstrated that venom composition extends beyond nematocyst-derived toxins and includes bioactive compounds secreted in mucus and various tissues (Barroso et al., 2025). In our study, tissue-specific chromatography profiles

suggest that *B. goanense* venom includes a diverse range of biomolecules, some of which may not be exclusively associated with nematocysts but rather secreted in the mucus or stored in other cellular compartments. This could explain the presence of multiple peaks corresponding to different classes of peptide toxins, including actinoporins, phospholipases, and ion channel inhibitors, as reported in previous proteomic studies (Barroso et al., 2025). Overall, our chromatographic findings provide evidence that sea anemone venom is specialized and regionally adapted to distinct ecological functions.

4.4.4 Cytotoxicity assay

4.4.4.1 Anti-cancer activity assay

The cytotoxic activity of venom fraction (T1, M1, A1) from *Bunodosoma goanense* was evaluated against human cancer cell lines A549 (lung cancer) using the MTT assay. The results revealed that the venom fractions exhibited dose-dependent cytotoxicity, with the highest inhibitory activity observed in the tentacle fraction (T1), followed by similar activity int the Acontial and mesenterial venom fraction (A1) and mucus venom fraction M1) and were compared to standard cancer chemotherapeutics Levofloxacin and Doxorubicin (Table 4.2).

Table 4.2: The cytotoxic activity of venom fraction (T1, M1, A1) from *Bunodosoma goanense* evaluated against human cancer cell lines

		Conc. tested (µg/mL)	OD			Average	% Proliferation	% Inhibition
1	Cells alone					2.0186	100	0
2	T1	10	1.4173	1.5353	1.5748	1.509133	74.76138578	25.23861422
		5	1.635	1.6114	1.6576	1.634667	80.98021731	19.01978269
		2.5	1.717	1.7186	1.7573	1.730967	85.75085042	14.24914958
3	M1	10	1.7272	1.7156	1.7581	1.733633	85.88295518	14.11704482
		5	1.7537	1.7468	1.7682	1.756233	87.00254302	12.99745698
		2.5	1.7764	1.7621	1.7968	1.778433	88.10231514	11.89768486
4	A1	10	1.6681	1.7822	1.7258	1.725367	85.47343043	14.52656957

		5	1.7197	1.8021	1.7613	1.761033	87.24033158	12.75966842
		2.5	1.766	1.8061	1.7671	1.779733	88.16671621	11.83328379
5	Levofloxacin	100	1.4201	1.3192	1.3939	1.377733	68.25192378	31.74807622
		50	1.451	1.375	1.4588	1.428267	70.75530896	29.24469104
6	Doxorubicin	10	0.9041	0.9419	0.9295	0.925167	45.83209485	54.16790515
		5	1.0477	1.0737	1.0814	1.0676	52.8881403	47.1118597

Sea anemones, as sessile marine organisms, rely on venom for survival, using it for prey capture, defense against predators, and competition for space. One of the critical ecological roles of sea anemone venom is its cytotoxic potential, which may function in subduing prey, deterring predators, and inhibiting the overgrowth of competing organisms (Menezes and Thakur, 2022). Studies on various sea anemones, including *Heteractis magnifica*, *Stichodactyla haddoni*, *Heteractis crispa*, and others, have demonstrated their venom's ability to disrupt cellular integrity and induce apoptosis in multiple mammalian cell lines. Several investigations have highlighted the cytotoxic effects of sea anemone venom on human cancer cell lines, particularly breast (T47D, MCF7), lung (A549), colorectal (HT-29), melanoma (SK-MEL-28), and brain cancer cells. The venom from *H. magnifica* and *S. haddoni* exhibited strong dose-dependent cytotoxicity, significantly reducing viable cancer cell numbers. Furthermore, venom fractions from *S. haddoni* showed specificity in cytotoxic action displaying high activity against breast, brain, and colon cancer cells while exhibiting minimal toxicity toward normal cells. Similarly, *H. crispa* venom-derived actinoporins, known for their strong membranolytic properties, demonstrated significant anti-proliferative and anti-migratory effects against MDA-MB-231 breast cancer, HT-29 colorectal cancer, and SK-MEL-28 melanoma cells. These actinoporins disrupted cancer cell adhesion and migration by downregulating matrix metalloproteinases (MMP-2 and MMP-9. Venom from *H. magnifica* effectively induced apoptosis in lung and breast cancer cells, its effect on normal cells was less pronounced, with cell death occurring primarily via necrosis rather than apoptosis. Collectively, these findings indicate the cytotoxic nature of sea anemone venom. The observed apoptotic and cytolytic activities likely contribute to their ability to immobilize prey, defend against predation, and establish dominance in benthic ecosystems.

4.4.4.2 Antibacterial Activity of Venom Fractions

The antibacterial activity of venom fractions (M1, A1, T1) from *Bunodosoma goanense* was evaluated using the well diffusion assay against *Staphylococcus aureus* and *Escherichia coli*. The venom fractions showed stronger antibacterial activity against Gram-positive *S. aureus* compared to Gram-negative *E. coli*. The solvent control (Methanol) did not produce any inhibition zones, confirming that the observed antibacterial activity was attributed to the venom fractions and not the solvents (Table 4.3).

Table 4.3 The antibacterial activity of venom fractions (M1, A1, T1) from *Bunodosoma goanense* evaluated using the well diffusion assay

Sr. No.	Sample	Conc. Tested (µg/mL)	Zone of Inhibition (mm)	
			S. aureus	*E. coli*
1.	M1	10	+(10)	+(8)
2.	A1	10	+(10)	+(10)
3.	T1	10	++(12)	+(8)
4.	Positive Control (Ampicillin)	10	+++(20)	+++(15)
5.	Negative Control (Methanol)	-	0	0

Our findings align with previous reports, confirming the idea that sea anemone venom has the potential to kill bacterial cells. These observations are consistent with prior studies, where sea anemone extracts exhibited inhibitory effects on *Staphylococcus aureus*, *Bacillus subtilis, Salmonella typhi, Klebsiella pneumoniae, Vibrio cholerae*, and *Pseudomonas aeruginosa* (Kvetkina et al., 2021; Kumar et al., 2020). Notably, extracts from *Corallimorphus cf. pilatus* and *Stomphia coccinea* demonstrated strong antimicrobial effects against *Candida albicans*, a fungal pathogen (Kvetkina et al., 2021). Further supporting our results, transcriptomic and proteomic studies on *Cnidopus japonicus* identified multiple antimicrobial peptides (AMPs) within the venom, three of which exhibited confirmed antimicrobial activity against bacterial strains (Grafskaia et al., 2018).

The ability of sea anemone venom to kill cells, as demonstrated in our study, provides an understanding of both, its ecological role and its therapeutic potential. By bridging ecological function with biomedical application, we can use venom-derived peptides as natural templates for drug development. The same bioactive peptide that paralyzes prey in the wild can be developed into therapeutics to treat various human diseases. Given the rise in antibiotic resistance and cancer-related mortality, marine venoms represent an essential resource for discovering novel therapeutics.

4.4.5 Activity of venom against voltage gated ion channels

Based on the molecular size of the peptides, the venom from the tentacles was separated into seven fractions (T1-T7) (Figure 4.7), the venom from mesentery filaments and acontia was separated into eight fractions (A1-A8) (Figure 4.6) and the ectodermal tissue mucus venom was separated into seven fractions (M1-M7) (Figure 4.5). The activities of the venom fractions were tested against voltage-gated ion channels: Kv1.1, Kv1.3, Shaker, Nav1.4 and Nav1.5. The activity against Kv1.1 was seen in fractions T2, T3, A2, A3, A4, A5, M2, M3, M4 (Figure 4.30). The activity against Kv1.3 was seen in fractions T2, T3, A2, A3, A4, M2, M5 (Figure 4.31). The fractions T2, T3, A2, A3, A4, A8, M3, M4 showed activity against Shaker (Figure 4.32). Whereas only fraction T2 was active against Nav1.4 and Nav1.5 (Figure 4.33). This result is summarized in Table 4.4. The active fractions T2 and T3 were further fractionated by C-18 gradient Reverse phase HPLC. This separation resulted in sixteen T2 fractions (T2.1-T2.16) and eleven T3 fractions (T3.1-T3.11). These fractions were freeze-dried, and their activities were tested against the same voltage-gated ion channels mentioned above. The active fractions were T2.8, T2.12, T2.15, T3.1, T3.5, T3.7. The fraction T2.8 was active against Kv1.1, Kv1.3 and Shaker. The fraction T2.12 was active against Nav 1.4 and Nav 1.5. The fractions T2.15, T3.1, and T3.5 were only active against Kv1.3, whereas fraction T3.7 was active against Shaker. This result is summarized in Table 4.5.

Table 4.4: The activity of venom fractions obtained by gel filtration HPLC against Voltage gated

Voltage gated Ion channels		Fraction										
		T2	T3	A2	A3	A4	A5	A8	M2	M3	M4	M5
	Kv1.1	+	+	+	+	+	+		+	+	+	
	Kv1.3	+	+	+	+	+				+		+
	Shaker	+	+	+	+	+		+		+	+	
	Nav1.4	+										
	Nav1.5	+										

Table 4.5: The activity of fractions of T2 and T3 against Voltage gated ion channels

Voltage-gated Ion channels		Fraction						
		T2.8	T2.12	T2.15	T3.1	T3.5	T3.6	T3.7
	Kv1.1	+				+	+	
	Kv1.3	+		+	+	+		
	Shaker	+						+
	Nav1.4		+					
	Nav1.5		+					

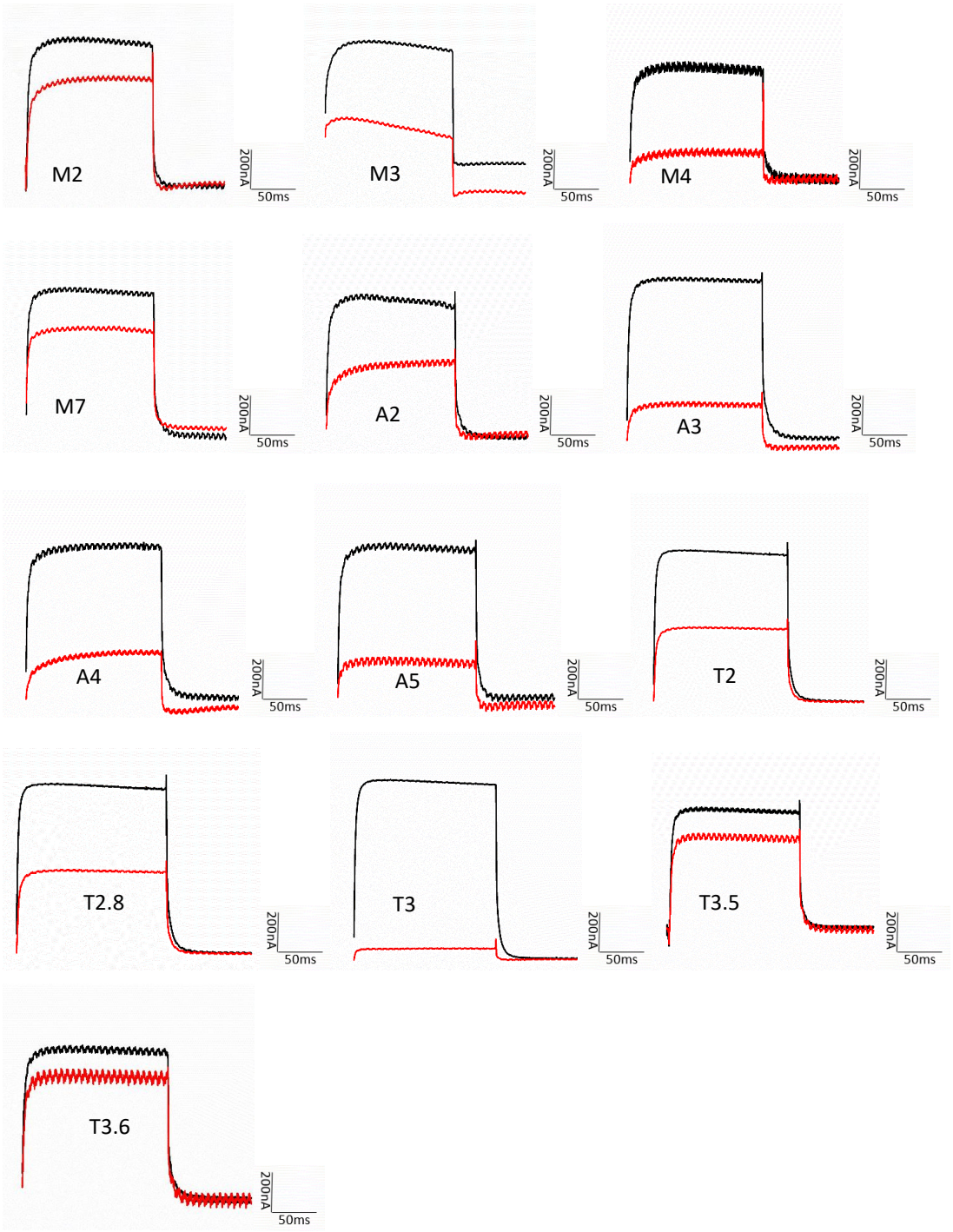


Figure 4.30: The effect on Kv1.1channels induced by venom fractions on currents of the cloned insect Kv1.1channels expressed in *Xenopus* oocytes. ((x-axis: time (ms), y-axis: current (nA))

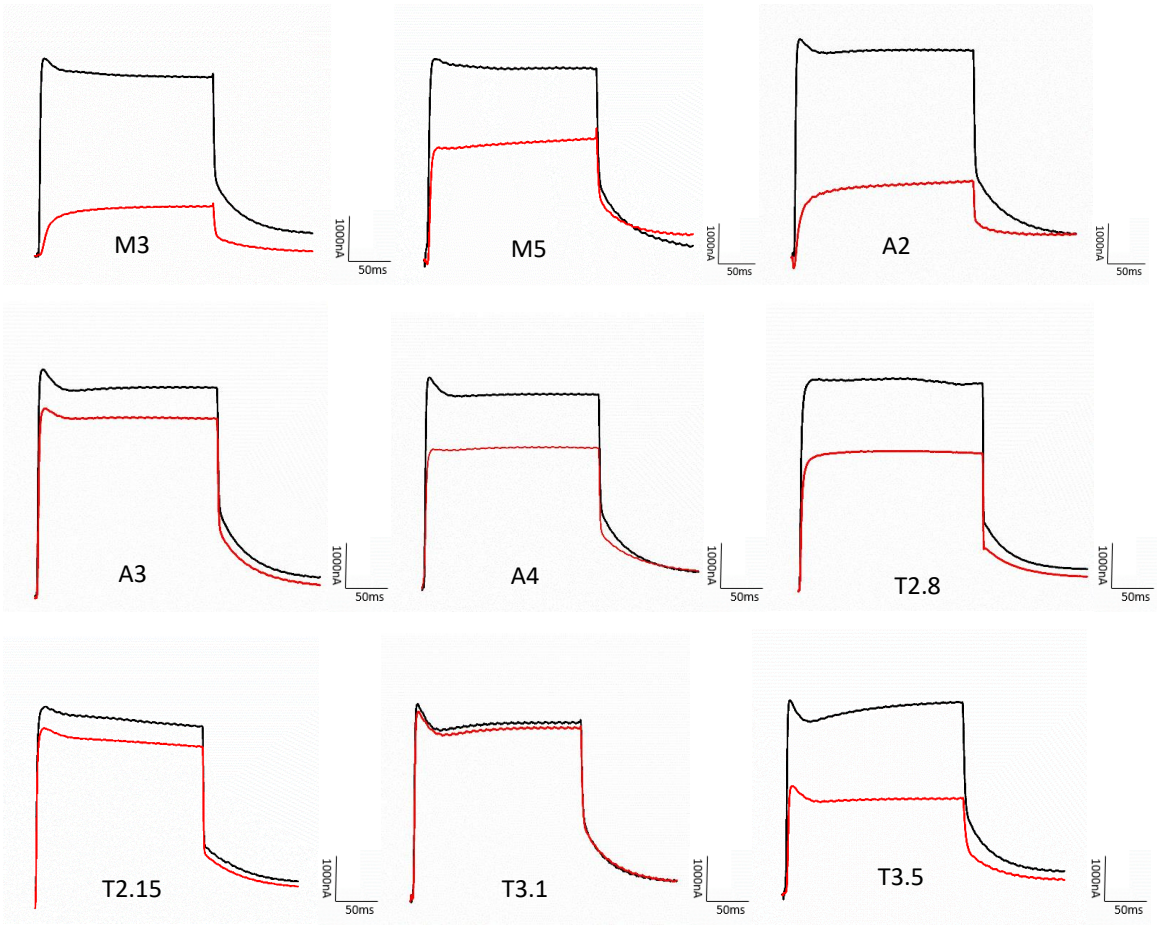


Figure 4.31: The effect on Kv1.3 channels induced by venom fractions on currents of the cloned insect Kv1.3 channels expressed in *Xenopus* oocytes. ((x-axis: time (ms), y-axis: current (nA))

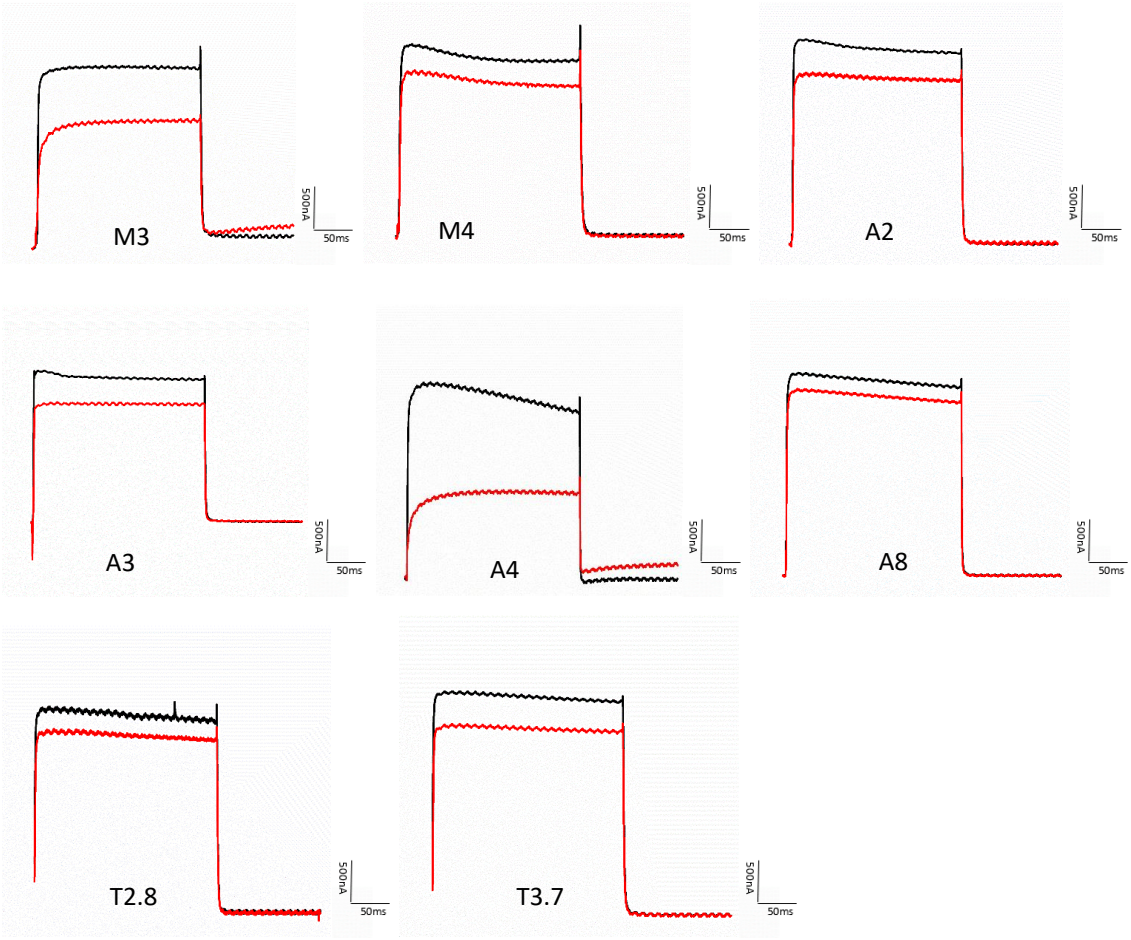


Figure 4.32: The effect on insect Shaker channel induced by venom fractions on currents of the cloned insect Shaker channels expressed in *Xenopus* oocytes. ((x-axis: time (ms), y-axis: current (nA))

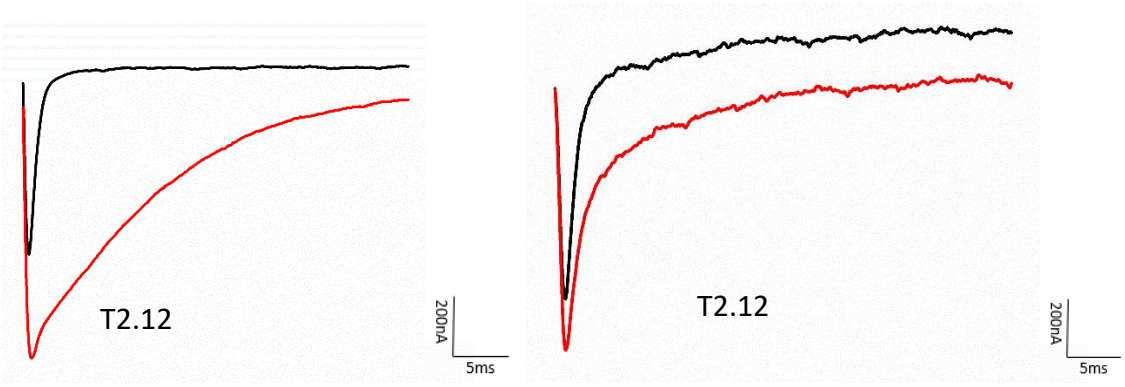


Figure 4.33: The effect on Nav1.5 and Nav1.4 channels induced by venom fractions on currents of the cloned Nav1.4 and Nav1.5 channels expressed in *Xenopus* oocytes. ((x-axis: time (ms), y-axis: current (nA))

Among these fractions, only T2.12 showed activity against voltage-gated sodium channels, while T2.15 and T2.8 exhibited the best activity against voltage-gated potassium channels. Venom from the sea anemones from the *Bunodosoma* genus have also shown similar activities against voltage-gated ion channels. Kappa-actitoxin-Bgr1a from the venom from *Bunodosoma granuliferum* showed activity against Kv 1.1, Kv1.2, Kv 1.3, Kv 1.6 (Aneiros et al., 1993). Kappa-actitoxin-Bcs4a from *Bunodosoma caissarum* was active against Kv 1.1, Kv1.2, Kv 1.3, Kv 1.6 and Shaker channel, and causes paralysis in crabs (Orts et al., 2013). Delta-actitoxin-Bcg1d from *Bunodosoma cangicum* was active against Nav1.1, Nav 1.5 and Nav 1.6 (Zaharenko et al., 2012). Delta-actitoxin-Bcs1a from *Bunodosoma caissarum* showed activity against Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.5 and Nav1.6 (Malpezzi et al., 1993). Peptide from *Bunodosoma granuliferum*, Delta-actitoxin-Bgr2a, showed activity against Nav1.2, Nav1.4 and Nav1.5 (Loret et al., 1994).

In our study, the tentacle venom of *B. goanense* showed the broadest range of ion channel modulation, with activity against both Kv1.1, Kv1.3, Kv1.5 (potassium channels) and Nav1.4 and Nav1.5 (sodium channels). This diverse activity suggests that tentacle venom is optimized for prey capture, where rapid immobilization of motile prey like small crustaceans and fish is essential. They can also be involved in defense. (Moran et al., 2009). Toxins that target sodium channels are typically fast acting, since Na+ ion channels are faster than K+ ion channels in terms of activation and inactivation (Barnett & Larkman, 2007) and thus can rapidly disrupt signalling and muscle function. Tentacles are directly involved in prey capture, and the presence of these fast-acting toxins in tentacle venom makes ecological sense, as sea anemones rely on the rapid immobilization of prey to ensure successful capture. This rapid action is essential for predatory success in marine environments where prey may be agile and quick to escape. In contrast, the venom from mucus and acontia tissues exhibited a more selective activity against potassium channels (Kv1.1, Kv1.3, Shaker), but not sodium channels (Nav1.4 and Nav 1.5). This suggests that these tissues may play a more defensive role. Potassium channel blockers are typically associated with pain induction, irritation, and inflammation, which can deter potential predators or competitors (Madio et al., 2017; Menezes & Thakur, 2022). In sea anemones, the secretion of venomous mucus serves as a chemical shield, discouraging predation and competitive overgrowth by neighboring organisms (Frazão et al., 2012).

Additionally, these findings have significant biomedical/pharmacological implications. Na+ and K+ ion channels are crucial therapeutic targets in treating neurological disorders,

cardiovascular diseases, and autoimmune conditions. Since Kv1.1 is predominantly found in the central nervous system (CNS), including the brain, spinal cord, and peripheral nerves, where it regulates neuronal excitability by controlling action potential repolarization and firing rates (Wang et al., 1994). Kv1.1 dysfunction is linked to episodic ataxia type 1 (EA1), epilepsy, and neuropathic pain, making its modulators potential candidates for treating these conditions by stabilizing neuronal excitability (Servettini et al., 2023). Kv1.3 is mainly expressed in T-lymphocytes (T-cells), where it is essential for T-cell activation during immune responses (Jang et al., 2015). Kv1.3 has emerged as a promising therapeutic target for immune modulation as the first and only (to date) sea anemone venom derived peptide ShK-186 (now known as Dalazatide) is undergoing Phase II clinical trials for treating several autoimmune diseases such as lupus erythematosus, psoriasis, rheumatoid arthritis, inflammatory bowel disease, type 1 diabetes, and multiple sclerosis (MS) (Tarcha *et al*.,2017). Shaker-related channels are expressed in the nervous system, heart, and muscles, and are involved in neuronal excitability, neurotransmitter release, and heart rhythm stabilization (Li et al., 1994) and can offer targets for drugs that could treat neuropathic pain, epilepsy, and cardiac arrhythmias (Mathie, 2010; Wulff & Zhorov, 2008). On the other hand, Nav1.4 is highly expressed in skeletal muscle fibers, where it is critical for skeletal muscle contraction by generating action potentials that trigger movement (Nathan et al., 2020). Nav1.4 is associated with hyperkalemic periodic paralysis and congenital myotonia, suggesting that specific modulators could help manage these disorders by stabilizing muscle excitability (De Bellis et al., 2023). Nav1.5 primarily found in cardiac muscle cells, is essential for heart rhythm regulation by controlling the action potential propagation in the heart (Nathan et al., 2020) and can be used as a target for anti-arrhythmic drugs used in treating conditions like long QT syndrome and Brugada syndrome (Saint, 2008; Perez-Neut et al., 2015). The venom's ability to modulate ion channels not only enables rapid prey immobilization but also positions these peptides as potential lead compounds for neurological and cardiological therapeutics.

4.4.6 Molecular masses of the venom peptides

Due to difficulty in resolving and obtaining a pure single peak for T2.8, only T2.12 and T2.15 were selected for further characterization. For T2.12, the observed mass was 4973.1925 Da, and this was confirmed by the presence of a double-charged species at 2463.78 Da (Figure 3.34). Despite the detection of a major species, other minor species were also present. Fraction T2.15 was found to be a pure peptide with an observed mass of 4372.7635 Da (Figure 3.35).

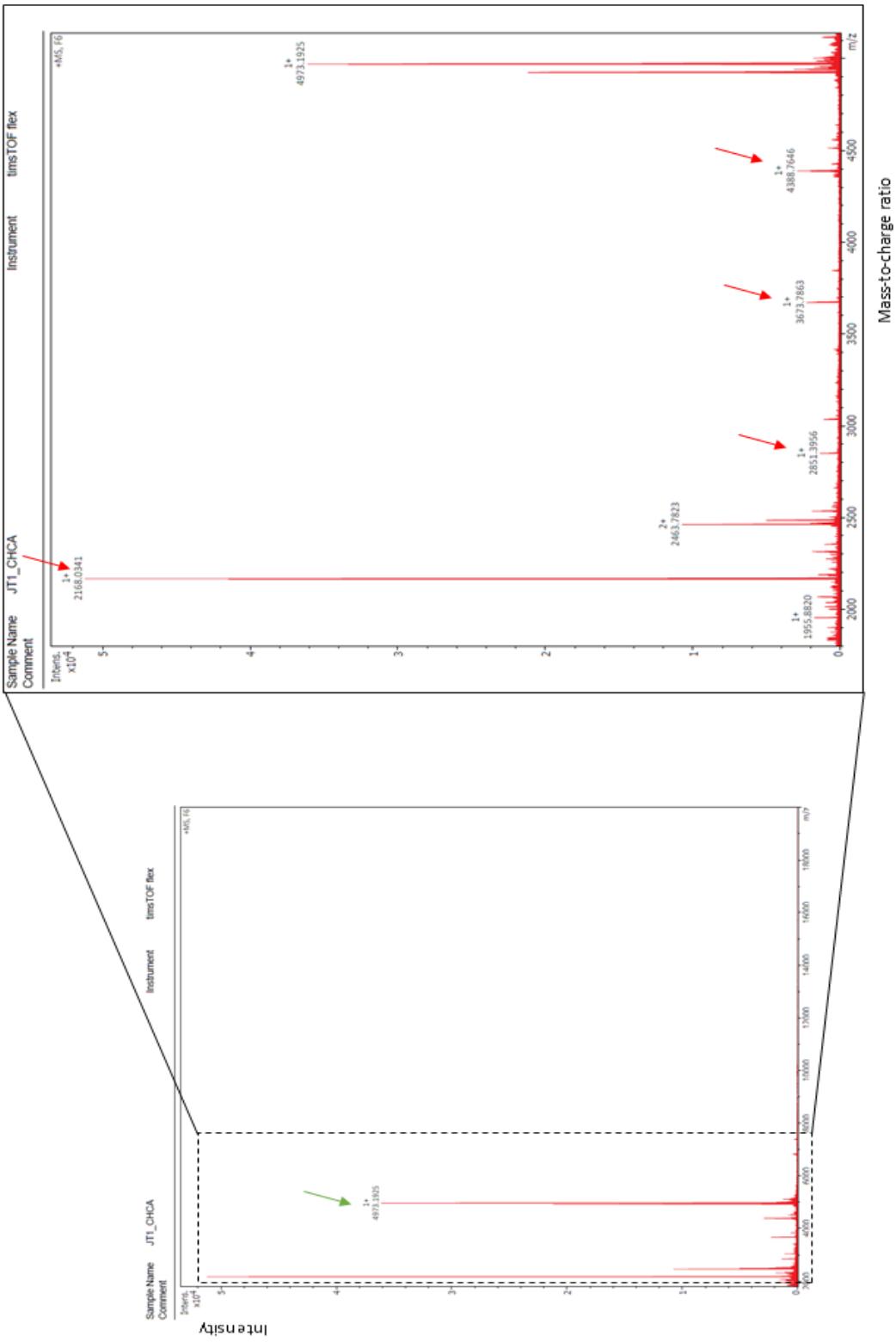


Figure 4.34: Mass spectrometry profile of fraction T2.12 with an observed mass was 4973.1925 Da, which was confirmed by the presence of a double-charged species at 2463.78 Da, corresponding to the bioactive peptide isolated from the tentacle venom of *Bunodosoma goanense.* ((x-axis: Mass-to-charge ratio (m/z), y-axis: intensity x10^4)

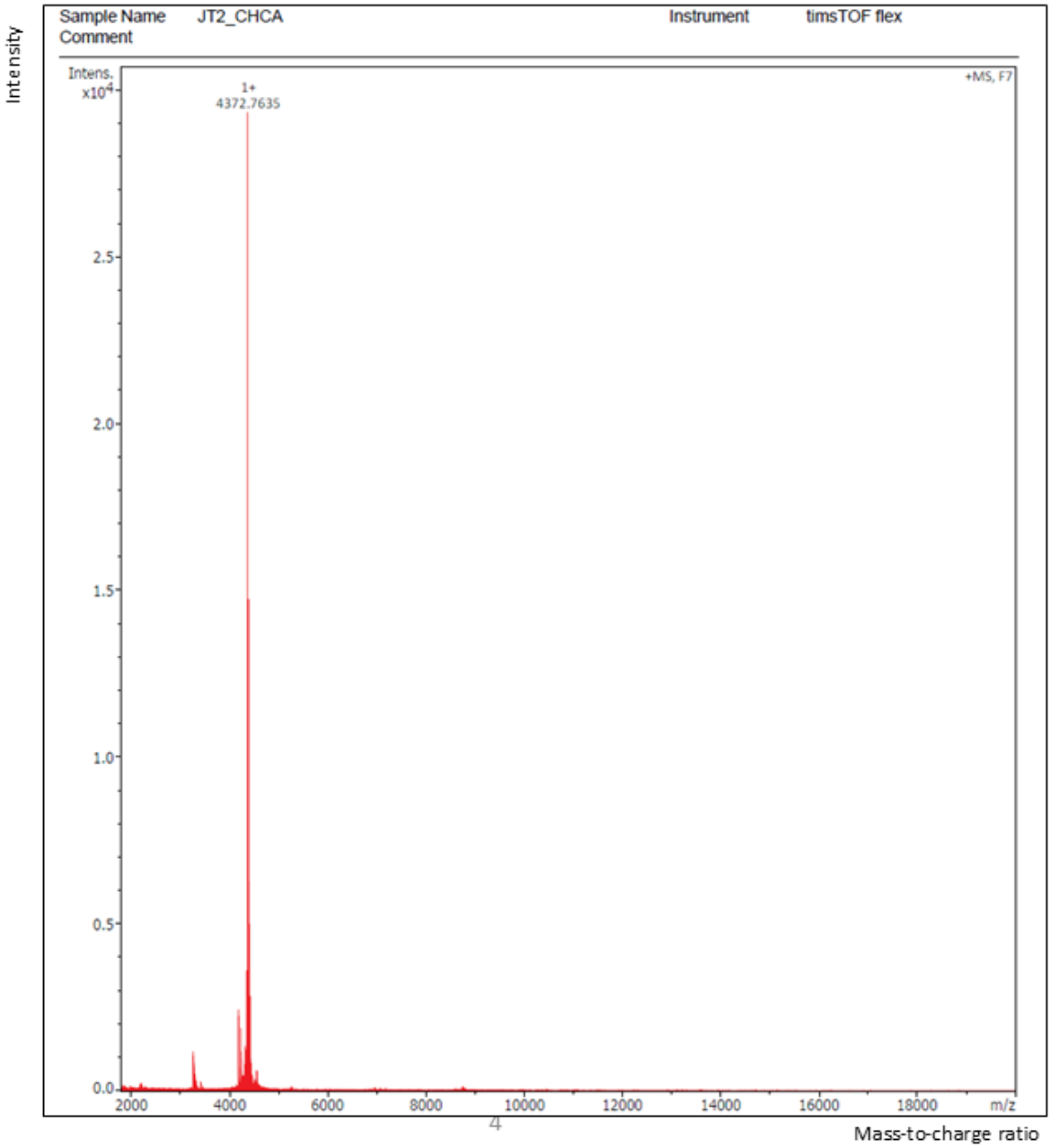


Figure 4.35: Mass spectrometry profile of fraction T2.15 showing a molecular ion peaks at 4372.7635 Da, corresponding to the bioactive peptide isolated from the tentacle venom of *Bunodosoma goanense* with Kv1.3 potassium channel inhibitory activity. ((x-axis: Mass-to-charge ratio (m/z), y-axis: intensity x10^4)

4.5 Conclusion

The study of the venom composition of *Bunodosoma goanense* revealed that its venom is tissue-specific, indicating the diverse ecological roles of the sea anemone's anatomical structures. We demonstrated that venom from tentacles, acontia and mesenterial filaments, and ectodermal mucus exhibits distinct toxin profiles, supporting the hypothesis that venom composition is functionally adapted to different ecological interactions. The venom fractions exhibited antibacterial activity against Gram-positive bacteria Gram-negative bacteria, and dose-dependent cytotoxicity against cancer cell lines. This indicates the cytotoxic role of peptides in the sea anemone venom responsible for cell lysis and cell death of the prey or predators. Additionally, the tentacle venom, optimized for prey capture, exhibited the most diverse activity by targeting both sodium and potassium ion channels, facilitating the rapid immobilization of motile prey. In contrast, mucus and acontia and mesenterial filament venom, which showed selective activity against potassium channels, are likely involved in defence mechanisms. The cytotoxic and neurotoxic peptides of *Bunodosoma goanense* venom act synergistically to induce immobilization, paralysis, and cell death, playing a crucial role in prey capture, predator deterrence, and other ecological interactions. The observed activity reflects general cytotoxicity rather than selective anti-cancer activity, and that additional studies using normal cell lines would be required to assess specificity or therapeutic relevance. This knowledge has enabled the discovery of peptide fractions with antibacterial, anticancer activities, and two novel venom peptides having ion channel-modulating properties, indicating their potential as lead molecules for drug discovery. Our study provides the first comprehensive understanding of the venom composition of *Bunodosoma goanense*, supporting its value as a new model species for venom research and a promising source of bioactive compounds for drug discovery.

Chapter 5

Isolation and characterization of venom peptides through experimental and computational methods

5.1 Need for sustainable alternatives for animal derived venom production

While natural venoms have proven to be invaluable for biotechnological and pharmacological advancements, relying solely on venom extracted from animals poses significant ethical and environmental concerns. Large-scale extraction often requires sacrificing organisms, which can disrupt ecosystems, particularly for species with restricted habitats or low population densities. This dependency also limits the scalability of venom-based applications, as natural venom yields are often minimal and variable. To address these challenges, there is a growing need to explore alternative methods for obtaining venom or generating its components in laboratory settings. By developing in vitro systems for venom production, we can reduce the reliance on live animals. This approach not only ensures sustainability but also enables more controlled and scalable production for research and therapeutic applications.

This chapter investigates two alternative strategies for obtaining venom peptides from *Bunodosoma goanense* without traditional extraction from live animals. Part 1 explores *in vitro* cell culture as a potential venom production method, while Part 2 leverages transcriptomic analysis and peptide synthesis to identify and validate venom peptides. These approaches aim to advance sustainable venom research and therapeutic discovery.

5.2 Establishment of *in-vitro* culture of tentacle nematocytes and ectodermal cells of *Bunodosoma goanense* for venom production (Part 1)

Actinarian cell culture is a growing area of research that helps to understand the developmental biology, symbiosis, regeneration, and stress responses of these animals. Unlike vertebrate cell lines, cnidarian cell cultures face challenges related to cell adhesion, proliferation, and long-term viability, but recent advances have demonstrated successful methods for culturing various cnidarian cell types. One such interesting report is the *in vitro* culturing of ectodermal monolayers from the model sea anemone *Nematostella vectensis*. Rabinowitz et al. (2016) developed a method using N-acetyl-L-cysteine (NAC) to separate the ectoderm from the mesoglea, allowing for the formation of 2D monolayers that transformed into 3D tissue structures within 24 hours. These structures were sustained for months in culture, releasing cells into the medium and retaining proliferative activity. This system provided a valuable platform for studying tissue organization, actin filament dynamics, and cell proliferation. Similarly, efforts to culture cells from the symbiotic sea anemone *Anemonia viridis* have been driven by the need to understand host-symbiont interactions and responses to environmental stress. Barnay-Verdier et al. (2013) established primary cell cultures from *A. viridis* tentacle cells, obtaining a heterogeneous culture containing both host epithelial-like cells and free-living zooxanthellae. These cultures lasted up to four weeks and provided a model to study symbiosis breakdown (bleaching) under environmental stressors. In a study, Ventura et al. (2018) developed a gastrodermal cell culture from regenerating tentacle tissues, demonstrating that host cells alone can survive thermal stress, but the presence of symbionts increases vulnerability to bleaching. These studies show how actinarian primary cell cultures can be powerful tools for investigating symbiosis, stress responses, and cellular physiology. Building on these previous studies, we attempted to culture tentacle and ectodermal cells from *Bunodosoma goanense* to assess their ability to produce venom *in vitro*.

5.2.1 Methods:

5.2.1.1 Cell culture: *Bunodosoma goanense* were maintained in an aquarium at room temperature (29-32℃) with the water changed every three days. The dissociation protocol for

Bunodosoma goanense was adapted from Barnay-Verdier et al. (2013) to establish a primary cell culture. Regenerating tentacles (15–20) were collected in sterile calcium-free artificial seawater (SCaASW) and incubated for 20 minutes in 1 mM glycine in sterile artificial seawater (SASW) to induce cnidocyte discharge. After briefly drying on a sterile paper towel, the tentacles were rinsed twice for 2 minutes in SASW enriched with 1% antibiotic-antimycotic solution and MycoKill AB® (1× in final medium). For tissue Ectodermal cell culture, the ectoderm was gently scraped from the body column following the method described by Richier et al. (2003). Cell dissociation was carried out in 0.15% collagenase type I in SASW for 30 minutes at room temperature under agitation. The cell suspension was centrifuged at 200 g for 5 minutes, and the resulting pellet was resuspended in 1 mL of culture medium before seeding into tissue culture plates. The cells were incubated at 20°C. The culture medium used was Grace's Insect Medium (GIM; Gibco), supplemented with 23.9 g L^{-1} NaCl (Grace's Modified Insect Medium, GMIM) to meet the salinity requirements of marine invertebrate cells, following the recommendations of Khalesi (2008). The regenerated cells were observed under light microscope 100X .

5.2.2 Results and Discussion: The current study aimed to investigate the feasibility of establishing *in vitro* cultures of *Bunodosoma goanense* tentacle and ectodermal cells and to assess their potential to produce venom under laboratory conditions. A primary confluent cell layer was successfully established from regenerated tentacle cells, whereas ectodermal cells did not survive in culture. Microscopic observations at 100X magnification revealed the presence of round cells with large dark vacuoles in the regenerated tentacle cell cultures Figure 5.1. However, the cultures failed to be sub-cultured and also could not generate nematocytes *in vitro*, consequently, venom production could not be detected. The inability to differentiate nematocytes from tentacle cells indicates a major limitation in replicating venom synthesis pathways outside the organism. Establishing primary cell cultures from marine invertebrates presents significant challenges due to the delicate nature of their tissues and their sensitivity to environmental conditions (Domart-Coulon & Ostrander, 2015). In this study, tentacle cells of *B. goanense* remained viable in culture for three consecutive days, forming a confluent cell layer, but further optimization is required to achieve nematocyte differentiation and venom production.

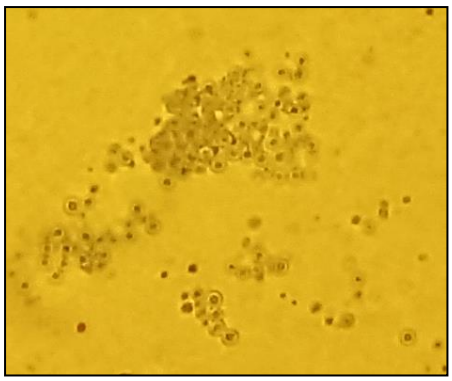


Figure 5.1: Round cells with large dark vacuoles formed from regenerated tentacle cells culture

5.2.3 Conclusion: Despite establishing tentacle cell culture, the ectodermal cells did not survive in culture. This indicates the complexity of replicating venom production under artificial conditions. Since *in vitro* venom production proved unfeasible, we shifted to a transcriptomic approach to identify and synthesize venom peptides, as described in Part 2.

5.3 *In-silico* determination of putative venom peptide using transcriptomic approach and synthesis of selected venom peptides and experimental validation of their function (Part 2)

Sea anemone venom contains a diverse array of functional peptides and proteins, many of which modulate ion channels and exhibit antimicrobial, cytolytic, or enzymatic activities. Despite more than a century of research on sea anemone toxins, recent high-throughput omics approaches have revealed that much of the venom proteome remains unexplored. The application of transcriptomics has significantly advanced our understanding of sea anemone venom diversity. For instance, Fu et al. (2022) identified 533 putative venom peptides and proteins from different developmental stages of *Exaiptasia diaphana*, assigning them to 75 superfamilies. Similarly, Mitchell et al. (2020) analyzed tentacle transcriptomes of *Oulactis sp.*, identifying nearly 400 venom-related components, including pore-forming toxins, peptidases, and ion channel modulators. The combination of transcriptomics and proteomics has further refined toxin characterization, as demonstrated by Madio et al. (2017), who used both approaches to identify 23 validated toxin families in *Stichodactyla haddoni*. Venom composition is highly variable between species, tissues, and even individuals. Guo et al. (2024) explored the tissue-specific transcriptomes of *Heteractis crispa*, revealing 1049 toxin-

related transcripts, with ShK domain peptides, β-defensins, and Kunitz-type inhibitors being prominent. Likewise, Li et al. (2024) applied integrative venomics to *Heteractis magnifica*, identifying 728 putative toxin sequences, 101 of which were validated by proteomics. Notably, venoms also contain antimicrobial peptides (AMPs), as evidenced by Grafskaia et al. (2018), who discovered novel AMPs in *Cnidopus japonicus* through in silico screening and experimental validation. Transcriptomics has also been used to explore the functional significance of venom peptides in ecological interactions. Macrander et al. (2015) examined *Anthopleura elegantissima*, identifying venom components specific to intraspecific competition in acrorhagi-bearing polyps. This study states the importance of venom beyond predation and defense, revealing its role in competitive interactions. The present study employs a transcriptomic approach to identify putative venom peptides in *Bunodosoma goanense*. This study uses *in-silico* methods to identify putative venom peptides from sea anemone transcriptome. By analyzing RNA sequencing data, toxin-like sequences are predicted and classified into known venom superfamilies. This method reduces reliance on wild specimens and uncovers known and novel venom peptides, that could accelerate the discovery of peptides for pharmacological and biotechnological applications.

5.3.1 Methods:

5.3.1.1 Identification of Venom peptides from *Bunodosoma goanense* using Transcriptome analysis: The de novo transcriptome assembly of *Bunodosoma goanense*, previously performed (Chapter III), was utilized for venom peptide identification. This transcriptome assembly served as the foundational dataset for the subsequent annotation and characterization of venom-related transcripts. A database specifically for cnidarian was obtained from the UniProt-ToxProt database https://www.uniprot.org . This database included peptide/protein sequences from various cnidarian. The assembled *B. goanense* transcriptome was queried against the custom cnidarian database using BLASTx and BLASTp tools using the galaxy platform. An E-value cut-off of 1e-5 was employed to ensure the selection of high-confidence matches. Only the Transcripts associated with the GO term for toxin activity GO: 0090729 were extracted. Annotation of these putative venom peptide toxin transcripts was performed using the ID mapping tool on the UniProt website https://www.uniprot.org/id-mapping. Accession numbers obtained from the BLAST results were input into the tool to retrieve detailed annotations, including peptide toxin names, toxin families, and sequences

which provided information about the nature and classification of the venom peptides present in *Bunodosoma goanense.*

5.3.1.2 Protein modeling: To model the three-dimensional structures of venom peptides, amino acid sequences obtained through the transcriptomic analysis of *Bunodosoma goanense* were used. These sequences were first curated to ensure completeness and accuracy. The curated sequences were then input into the SWISS-MODEL online platform (https://swissmodel.expasy.org), which performed an automated search for homologous templates in its database using BLAST. Suitable templates with high sequence similarity and adequate resolution were selected, and the platform aligned these templates with the input sequences to ensure optimal coverage. Using the selected templates, SWISS-MODEL generated 3D structures of the venom peptides through homology modeling. The finalized 3D structures were visualized to examine their conformations, active sites, and overall structural integrity. Based on the *in-silico* predictions from the transcriptome of *B. goanense*, a putative sodium channel toxin was selected for synthesis. The peptide was synthesized commercially by Priveel Peptides (Tamil Nadu, India) using solid-phase peptide synthesis.

5.3.1.3 Contraction paralysis assay: To assess the bioactivity of the synthesized peptide and crude mucus, tentacle, acontia and mesenterial venom extracts, we conducted in vivo toxicity assays using juvenile Gambusia fish (n = 3 per condition, in triplicates). Each fish was exposed to 0.5 mg/mL of the respective venom fraction or synthesized peptide in water. Fish were continuously observed for up to 120 minutes post-exposure for signs of neuromuscular effects, including paralysis, erratic movement, or loss of equilibrium or death. Control fish were maintained in identical conditions without venom exposure (Sachkova et al., 2020).

5.3.2 Results and Discussion:

5.3.2.1 Peptide Family from transcriptome: To overcome the challenges of venom production *in-vitro*, a transcriptomic approach was employed to identify putative venom peptides from *B. goanense*. The annotation of venom-related transcripts revealed 16 different venom peptide families (Tables 5.1 and 5.2), including neurotoxins, and cytotoxins, which are characteristic of sea anemone venoms (Madio et al., 2019; Sachkova et al., 2020. This approach is advantageous over traditional venom extraction methods, which often require sacrificing the organism and collecting venom directly. Transcriptome analysis is relatively non-invasive, reducing ethical concerns and minimizing the impact on marine populations

that may be vulnerable to over-collection (Brown et al., 2018). Furthermore, it provides a broad view of the venom peptide repertoire, capturing both abundant and rare transcripts that might be missed in physical venom samples. This approach aligns with contemporary studies emphasizing the efficacy of transcriptomics in elucidating the venom profiles of cnidarians. For instance, a multi-omics investigation into *Bunodosoma caissarum* identified numerous venom-related transcripts and proteins, highlighting the complexity and richness of sea anemone venoms (Mazzi Esquinca et al., 2023; Li et al., 2024; Moutinho Cabral et al., 2024).

Table 5.1 List of transcripts identified with GO Term for Toxin activity (GO: 0090729)

Transcript ID	**Toxin Peptide family**	**Amino Acid Sequence of Putative peptide**
TRINITY_DN118343_c0_g1	Actinoporin family, Sea anemone subfamily	SLALAGTIIEGASLTISVLEKVLQALGD ISRKIAIGIDNE
TRINITY_DN118343_c0_g1	Actinoporin family, Sea anemone subfamily	EEEKD-KKSLALAGTIIEGASLTISVLEKVLQAL GDISRKIAIGIDNESGREWTAINTYFR AGTSDVVLPYTVPHGKALLYDGQKS RGPVATGVVGVVAYVSSNDAYTVAV MFSVPFDYNLYSNWWNVKIYKGKR RADQNVYEDLYYYASPFRGDNGWH SRNLGYGLKSRGFMNSSGQAVLQIK VEQ
TRINITY_DN119504_c0_g1	Actinoporin family, Sea anemone subfamily	FSREVSIGVDNETHKRWTASNVYFYS GTSDAVLPHTVPSSKALLYTARKT-GWSTNGSEGVFTYHMSDGNTLAVL FSVPYNQIWNKNRWNVKIYRGHRR AGKRMFKAMFY-SKAIKGNNHWRFRYLGYGRRMRGY MTSSSKAALKIKVF
TRINITY_DN1378_c0_g1	Actinoporin family, Sea anemone subfamily	GPVATGVVGVLAYAMSDGNTLAVM FSVPYDYNLYKNWWNAKVYSGRRQ ADQKMYEELYYNNSPFRGDNGWH QKKIGYGTSACRGFMNSSGEAILQIK V
TRINITY_DN1780_c2_g1	Actinoporin family, Sea anemone subfamily	SLALAGTIIEGAALTISVLNNVLQALG DISRKIAIGIDNE

TRINITY_DN1780_c2_g1	Actinoporin family, Sea anemone subfamily	MTRLFIVFIVITMMCAAIALSPAKKT NEKEKD-KKSLALAGTIIEGAALTISVLNNVLQA LGDISRKIAIGIDNESGREWTAINTYF RAGTSDVVLPYTVPQGKALLYNGQK SRGPVATGVVGVVAYVSSNDAYTVA VMFSVPFDYNLYSNWWNV
TRINITY_DN215889_c0_g1	Actinoporin family, Sea anemone subfamily	AATGSSGIVTYKLQHATNLHIMWDC PFNFDYSDNFIGLMLTSNSSRLNPDE SLFKNMY--QSP-PGDQDWNDMNL
TRINITY_DN3494_c0_g1	Actinoporin family, Sea anemone subfamily	SAAVAGAVIQGATLTFQVL
TRINITY_DN3494_c0_g1	Actinoporin family, Sea anemone subfamily	AVAVAGAVISGGTLTFKVLDRILTAIG DVNRKIAIGIENQ
TRINITY_DN3494_c0_g1	Actinoporin family, Sea anemone subfamily	SAAVAGAVIQGATLTFQVLDRILKAL SDVSRKIAIGIDNE
TRINITY_DN6703_c0_g1	Actinoporin family, Sea anemone subfamily	SRKIDIGIDNESVREWTAINTYFRAGT SDVVLPHTVPHGKALLYDGQKNRGP EATGVVGVVAYVSSNDAYTVAVMFS VPFDYNLYSNWWNVKIYSGIKRADQ KM
TRINITY_DN6703_c0_g1	Actinoporin family, Sea anemone subfamily	GGTLTFKVLDRILTAIGDVTRKIAIGIE NQSGKEWWALNAYYRTGTSDIVLP QTVPYGKALLYKGQKDRGPVATGVV GVLAYAMTDGNTLAVMFSVPYDYN LYKNWWNVRIYSGRKRADQKMYEE LYYN
TRINITY_DN6703_c0_g1	Actinoporin family, Sea anemone subfamily	SGGTLTFKVLDRILTAIGDVTRKIAIGI ENQ
TRINITY_DN87706_c2_g1	Actinoporin family, Sea anemone subfamily	GASLTISLLEKVLQALGDVSRKIAIGFD NE
TRINITY_DN87706_c2_g1	Actinoporin family, Sea anemone subfamily	GASLTISLLEKVLQALGDVSRKIAIGFD NESGREWTALNTYFRAGTSDVVLPY TVPHGKALLYNGQKSRGPVATGVVG VVAYVSSNDAYTEAVMFSVPFDYNL YSNWWNVKIYKGKRRADQNVYEDL YYYASPFRGDNGWLSRNLGYGLKSR GFMN

TRINITY_DN11037_c0_g1	Cnidaria small cysteine-rich protein (SCRiP) family	MDFKIQMFSLVLALVVMSVQS----GPEEMNNEKRAVNCYRINGYCFPRNNTCPRGKQDCYQYDCNLVNQKCCCP
TRINITY_DN39933_c0_g1	Cnidaria small cysteine-rich protein (SCRiP) family	MDFKIQMFLLVLVLVAMSVQSA--PGERDNDEKRTLS-CYTMKGYCMSRNDTCPTGYQDFHHYFCNQVDTKCCSPT
TRINITY_DN5154_c1_g1	Cnidaria small cysteine-rich protein (SCRiP) family	FAGLSLALNAASNRICNAKYGKCPKQPAKPAECPADEPDQCSQDKDCKAPGDLCC
TRINITY_DN736_c0_g1	Cnidaria small cysteine-rich protein (SCRiP) family	LVVMSVQSGPDET-NNEKRLLKCSSIGGVCMSNALRCPRGQNVCPSY-GCRGFNNKCCCGPNVPNVG
TRINITY_DN10023_c2_g2	EGF domain peptide family	CIKECASTPCQNGGSC--IDKIGGFDCECPDGYSGNTCQ
TRINITY_DN10158_c0_g1	EGF domain peptide family	ECAKNPCQNGATC--VDGINDYSCQCAPGYTGRDCD
TRINITY_DN10158_c0_g1	EGF domain peptide family	NPCLGGGTCAVGSQNRHWYCICPPGKTGLRCEK
TRINITY_DN11061_c0_g1	EGF domain peptide family	ASNPCQNGGTC--VDTWFDYYCQCTDGYSGRNCQK
TRINITY_DN11061_c0_g1	EGF domain peptide family	CLNNGTCKP-QKDGTFICICHANYTGELCE
TRINITY_DN124662_c0_g1	EGF domain peptide family	CLNGGLC--LERSDDFYCDCKIGFTGPRC
TRINITY_DN14238_c0_g1	EGF domain peptide family	ECSSNPCRNGGTCK--DRVNGYYCACPAGLTGKHCEN
TRINITY_DN153040_c0_g1	EGF domain peptide family	CLNGATC--ISSRKKYLCICASGYTGESCQ

TRINITY_DN1776_c0_g1	EGF domain peptide family	APGFCLNGGTCVFADP---QYCICPPGWGGVRCE
TRINITY_DN1776_c0_g1	EGF domain peptide family	HNPCLNGGTCHDSVPYGYVICECPREYHGPRCEQ
TRINITY_DN1854_c0_g1	EGF domain peptide family	ECLRNPCKNGGSCRNTN--GDYTCTCRSGYQGKNCEQ
TRINITY_DN24586_c0_g1	EGF domain peptide family	ASRPCLNGGTC--VESVDENGKNIFYCRCPPEFQGTLCD
TRINITY_DN27433_c0_g1	EGF domain peptide family	CGGRCDDNYCLNGGLC-----IGENKCKCTDKYSGKRCDR
TRINITY_DN3065_c0_g1	EGF domain peptide family	ECAKNPCQNGATC--VDGINDYSCVCAAGYNGRDCE
TRINITY_DN32157_c0_g3	EGF domain peptide family	ECASNPCQNGARCTNL--LNNYYCACPLGWTGKNCE
TRINITY_DN41678_c0_g1	EGF domain peptide family	ECAKNPCQNGATC--VDGINGYSCICAPGYNGQNCE
TRINITY_DN4573_c1_g1	EGF domain peptide family	CQNGGNCTALAGGHQYNCSCNKDFTGDNCEK
TRINITY_DN4801_c0_g1	EGF domain peptide family	QAHNPCKNGGSCIDGAHYGSFECRCPQGFSGRLCE
TRINITY_DN4801_c0_g1	EGF domain peptide family	QAHNPCKNGGSCIDGAHYGSFECRCPQGFSGRLCE
TRINITY_DN5172_c0_g1	EGF domain peptide family	CLNGGQCVPHPYHPRGSGLYQCKCINGFSGDKCER

TRINITY_DN5211_c0_g1	EGF domain peptide family	ECASSPCANGGLC--VDKLGEYKCLCKAGYSGDRCQ
TRINITY_DN52536_c0_g1	EGF domain peptide family	CLNGGQCVPHPYHPRGSGLYQCKCVNGFTGDRCEK
TRINITY_DN5395_c0_g1	EGF domain peptide family	CTAK--TDFCSNSPCKRGNCRN--TLGTYFCKCPEDYGGKNCD
TRINITY_DN6246_c0_g1	EGF domain peptide family	NPCLNGGSCHSDQ--NHYYCNCYHGFTGRRCE
TRINITY_DN9821_c0_g1	EGF domain peptide family	ECSTNPCQNGGTC--TDQVNGYSCTCSAGYNGTRCE
TRINITY_DN9909_c0_g1	EGF domain peptide family	ASNPCLNGGMCSS-QANGSYSCTCAPGYTGQRCE
TRINITY_DN13772_c0_g1	Jellyfish toxin family, Type II subfamily	IKGVEDVVKTSMTSLDDLTSGDPARVIKGCLNIASSVLTIVGGPYGVAASSICNIIVAIISHSSPRKPDMATEIIQEVRQAIENKLHEHNLMIQDQKFQGLIDRVKNMV-AYMRCLNKVSKDEPIPD-------QALFETDLPQLIGELASSFNMYA-SCKNEEAYKRCVEFVVFYCKAKTAHVLLLANITAAFKSTG
TRINITY_DN20433_c0_g1	Jellyfish toxin family, Type II subfamily	EPGRAGQFVIEEM-PGYLLISSKQWPDWFLFVDE--LGRLRAEPGDPGNKGHF
TRINITY_DN212727_c0_g1	Jellyfish toxin family, Type II subfamily	NAGSDPIGAIRAALDIGAAF-SSLAGPKGQVVSIVLSFISGFLSLFGKGEQQPRPVGEIVREEIEKWYSRDLSNQARGAIGDFQISKDF

TRINITY_DN42959_c0_g1	Jellyfish toxin family, Type II subfamily	WLEKVLAKAKAKYQRTRPSKNELRA WDTAQNDSPPGDVITSLESSLNKVE TANNKQWITRTKNGLNELKNKWLET PEERRGEIIEALKTAAGSLETFANAGE DPVGAIRAAIDVTASFAS-LAGPKGQVIGEILGLLSGLLSLFGEGP SPKPVDQVVRQEIERWYSRALTNQA EGAIKAFKNSKVFLNGVAKSGRSLSD SEQAALASHVPVYRGVEFMGTLASEI RNIIRNNQENYEEAKKCLKYIELYATQ ATLKDMILQQMAALLTDSHA
TRINITY_DN1066_c0_g1	NEP3 family	KHAECKSWKD-RGECGNNNAWMSSNCPKSCRVCG EPPSDCKDEFPVACEEYAQRYECSYN PGWMLP-NCPKSCGV
TRINITY_DN10740_c0_g1	NEP3 family	DIDWKELNIAPEDCNDQDPEECSEL ADDGQCLKQFERMKTECPWSCRFC SKKGLSTKQCEDL-NEGNCPRWAQSKDCVRLPDYMLQ NCKKSCQLCGSEN--KYEDKDVRCPDWAKAGFCRSTPDID LKCPYSCRKYKYIPEAPKPYPYPVSAL HPYQYQ
TRINITY_DN124097_c0_g1	NEP3 family	RYCGSWARNGECRRNPHYMRQHC PKSCRICGGGSLCR--DMNRYCSSWARRGECRRNRNYMY SYCKRSCR
TRINITY_DN142057_c0_g1	NEP3 family	CRDLLDYCAQWPDK-YCQDYKDYMKTNCPRKCGYCSGPTA -PPVCADKS--QHCSGWKASGFCDPSNQWHSYMK SNCAKTCGFCG
TRINITY_DN142784_c0_g1	NEP3 family	EGCKDKRDDCAAYAFPEYCATYWGF MLKHCRGSCRFCDAVHVNGEETTP QPDTTPTTVARLPTFSSECEDVLDDC RRQISDPKYCLSNLAFMTKYCRKTCQ LC
TRINITY_DN15860_c0_g1	NEP3 family	KNCAYWAGKGECQKNPTYMLKECK KSCNQCGD-----CVDGDRNCAHWASIGECDKNPEW MKSNCKKSCRQ
TRINITY_DN1785_c0_g1	NEP3 family	CPRWAKAGYCKKGPYIRYMRKKCRK SCGICKGGGGGVNPKCNDKRSSCPR WASKGYCKKYSGYMKKNCPKSCNK
TRINITY_DN190677_c0_g1	NEP3 family	QRHCRYWASRGQCRRNPNYMKVN CKRSCGLCG---GGGCVDKHRNCSGWARYGYCRTNS YVRSNCRRSCR

TRINITY_DN1917_c0_g1	NEP3 family	CKRWANIGECLKNADWMAKHCKRS CRRCKTAVIPTTEMFTLPLTKETRPLC FDNDPIQCPLWAQRGECTKNPVYM NKNCAKSC
TRINITY_DN19983_c0_g1	NEP3 family	CKDKS-- RSCGYWAKKGFCQMREDFMKKYCI KSCNKCYPEESDECIDRDTRCKSWAR ARYCTSS
TRINITY_DN21952_c0_g1	NEP3 family	CRKTCNLCGKKRVVFKAECRDKYRFP EHCKAWARQGTCKRNPVFMYRNCI KSCGRCS
TRINITY_DN224825_c0_g1	NEP3 family	WKLIDIDPEECEDMSEDCENFADEG YCLEKFKYALKTCPWSCRFCKRTEEH ETEKCRDLAGTESCRQWASNSDCFH LPDYMMQNCKRSCELCGPETGFRY
TRINITY_DN292_c1_g1	NEP3 family	CKDKNAVQCPLWAKRGECSKNRIY MGKNCAKSCEFCK----- APVCKDNPKFKHKCPLWALKDECTL KNKKWMQVNCPKSCNVCVC------ ADNNPRCQKWAKAGECDKNTDYM HVNCKKSCKK
TRINITY_DN3394_c0_g1	NEP3 family	YCSQWPDK- YCQDYKDYMKTNCPMKCGYCTGPT QAPVCQDKSSNCPGWKQNGFCSTS SQWHSYMKSNCAK
TRINITY_DN34224_c0_g1	NEP3 family	CVKNPGWMRPNCAKSCKVCSVAPT ACKDKDPVQCPAWARMGECTKNP GYMRANCAKSCNV
TRINITY_DN36985_c0_g1	NEP3 family	CRD-- SDRRCSRWASRGECRNNPFWMQL NCKRSCRSCHVKTYDDVPLPPVIPTP FPGCSNYDKSCKYWAYNGYCSRGNP GFVSYVRYECPLSC
TRINITY_DN4170_c0_g1	NEP3 family	CPLWAAKGYCTKNKAYMHKNCSKS CNICKGVCEDSNKSQ------- CPSWAKNGECTKNKQYMDKYCKKS CGKC------ KVSCEDDSAYQSKCPTWAATGECTK NKTWMHKHCAKSCK
TRINITY_DN4648_c2_g1	NEP3 family	PKQCKDSNVIQCPLWAKKGECEKNK GYMHKNCAKSCKIC-----IPKACKD- SNTVQCPLWAKKGECEKNKDSMHK NCAKSCKICQVSV--- CVDINPKCSKWAKSGECKANPIYMH VYCKKSCNK

TRINITY_DN48464_c0_g2	NEP3 family	GEKKCKLWEEDGDCIRLPDYMQQN CKKSCELCGPESSNQYVDEDVRCPF WGKNGYCNINAEIKNKCPHSCTKYK NVPAAIQPYPYPIIALYPYQPKP-------- --TPTPPPTTKPGQTT
TRINITY_DN59323_c0_g1	NEP3 family	CPQWADSGECHKNLPWMSANCPK SCRTCGEPASD---CKDEY- PRACEHWKNTGECQNNPGWMNIY CEKSCGKC
TRINITY_DN607_c0_g1	NEP3 family	CADNNKNCAYWAGKGECQKNPTY MLKECKKSCNQC----GD---CVD-- GDKNCAHWASIGECEKNPEWMKS NCKKSCRQC
TRINITY_DN607_c0_g2	NEP3 family	DWMKANCKKTCSLCGGGGGGSC QDTDNRCRYW-- TGYCRSNAYVMKNCKKTCR
TRINITY_DN630_c0_g1	NEP3 family	ISA-HCKNYEYECIQWAKEGK----- CQTEKEKMKLYCPLSCKFCNKCTKPK PTPSLAPRTGVGLRCLD-KYAKC- PDYAHLNQCGTTAWIDFNCRKSC--- ----RTKCDTYP
TRINITY_DN6572_c0_g1	NEP3 family	HRSCRYWASIGECNKNPGYMKANC KKSCRICGGGGGGGQCYDRNARC SSWANRGECRRNPGYMRLYCKKSC
TRINITY_DN11513_c0_g1	Phospholipase A2 family	MIYKALLLFVLAILVYGQVQGRSPVR YRIMPSEWDANEDEQSPVRKRSIVE FAKMVACATRRDPFDYHNYGNWCR IGGGNGTLPVDETDGCCRTHNMCY YNDRMGDCTKKVPPYQYQANQTD QECRIACKDSLGSCERDVCECDRASA LCFASHPYNPENK
TRINITY_DN121394_c0_g1	Phospholipase A2 family	IYQFGLMIHCETNRWASEYVGYGC WCGLGGKGTPVDETDRCCKNHDKC YYAVHRSGK-- CSYMGAAYTTIYHRKGCSDCDKQQN NPCEQAVCECDSVAVKCFAKAKFND ANKRYSQSKC
TRINITY_DN2647_c0_g1	Phospholipase A2 family	SLYEFYQMITCETGRSWQDYNLYGC FCGKGGTGTPVDALDQCCFIHDKCY ERAAATVCPWP---- LQIYVDSYWHENCSDCVASKNTACE QALCECDSKAAKCFKDNKWDPQYD DYPQEKC
TRINITY_DN34612_c0_g2	Phospholipase A2 family	GIYEFGQMISCLTNRSSLDYADYGC WCGLGGSGRPVDQLDRCCHTHDRC YDDIIASGICGYSWQV- YFRTYEYSGCTKCDPLSSYPSDDQYAL CRTKLCECDSTAASCFSNSKFNPQHV NYNLTMC

TRINITY_DN372_c0_g1	Phospholipase A2 family	MKGSVFMWVLLFCGAASLQFVFSM EDEDESIQDLHEVGSETDLREDEQSP VQKRNLVQFANMVRCATRRNPFDY NDYGNWCGVGGGGRPVDGVDRCC RAHDMCYRRSRRYRRRCYPKWRTY RYRARRRRRRCRITCKNRWGSCEK AVCECDRVAALCFARNRYHRRYK
TRINITY_DN4685_c0_g1	Phospholipase A2 family	MIFKVLTLAVLAAVLVQGHRRNDYKI -- ASADRPAAQKRNLVQFGLMVNCMT GRFALDYNNYGNYCGVGGSGTPVD ALDRCCKVHDECYGRYEHCSPYYISYI SYRTGWHPNCKLTCGDT- DPCKKAVCICDKEAAECFAKQNYNP DN
TRINITY_DN47_c0_g1	Phospholipase A2 family	VVQFGAMIRSHTGRSPADYNGYGC WCGYGGKGTPRDGTDRCCRRHDKC YNRLVR-- RRTCGRWQIYHKNYYYWGLKCWKG RGSSRCGRSLCACDVAAVKCFK
TRINITY_DN47021_c0_g1	Phospholipase A2 family	MIECATGRSTFDFVNYGNYCGWGG SGIVKDDLDSCCKVHDECYGQWNEC RPKTNIYAYHLSGYHPNCEITCSAVPP NTECERAVCLCDKVAADCFARSHYN PEHK
TRINITY_DN6207_c0_g1	Phospholipase A2 family	IMLAILLGMAFLVDGLSVNKLEGDQ MMDVKRDYVRA- EKRDLLQFRDMIKCATGRRAWDYN RYGNWCGIGGSGTPVDGVDRCCRA HDWCYDSHNSCSPKWTSYDYSISGS HPWCSITCNGSNDHCKMSVCNCDK VAAECFARNTYNSNNKR
TRINITY_DN18600_c0_g1	Sea anemone 8 toxin family	MGCARLVVLLIIGTFFLSQAIGFMQE ELLEER------ KKPCINEYGTKICGGVIREIDCFKPPD SRMGGFARRKCCKFC
TRINITY_DN28170_c0_g1	Sea anemone 8 toxin family	KGVCDNVFSDYICGEVIRRDLDCVGN EKRRMNKFARRFCQKLCGLC
TRINITY_DN3531_c0_g1	Sea anemone 8 toxin family	MASVRMLVLLLIGTVLLCQVSADSEL LNEILAAHAEEDMPGKRCINKYKGNI CGGVIRPIDCMRKKSRMGKFARNYC QHFCGIC
TRINITY_DN9340_c0_g1	Sea anemone short toxin (type III) family	LFASVMCAALAVRPSIEDHHKNAQV KADVHEHIHSEPDEKSCCATWTAKT VDSPKHCWVKGSCFKWGQS

TRINITY_DN2210_c0_g1	Sea anemone sodium channel inhibitory toxin family, Type I subfamily	GVPCRCDSDGPSVRGNTLSGTVWV FGCPSGWNKCARNGPTIGSCCK
TRINITY_DN2210_c0_g1	Sea anemone sodium channel inhibitory toxin family, Type I subfamily	GVPCRCDSDGPSVRGNTLSGTVWV FG-CPSGWNKCARNGPTIGSCCKQ
TRINITY_DN2210_c0_g1	Sea anemone sodium channel inhibitory toxin family, Type I subfamily	CRCDSDGPSVRGNTLSGTVWVFGCP SGWNKCARNGPTIGSCCK
TRINITY_DN2210_c0_g1	Sea anemone sodium channel inhibitory toxin family, Type I subfamily	GVPCRCDSDGPSVRGNTLSGTVWV FGCPSGWNKCARNGPTIGSCCKQ
TRINITY_DN2210_c0_g1	Sea anemone sodium channel inhibitory toxin family, Type II subfamily	VPCRCDSDGPSVRGNTLSGTVWVF GCPSGWNKCARNGPTIGSCCKQ
TRINITY_DN2210_c0_g1	Sea anemone sodium channel inhibitory toxin family, Type II subfamily	CRCDSDGPSVRGNTLSGTVWVFGCP SGWNKCARNGPTIGSCCKQ
TRINITY_DN16286_c0_g1	Sea anemone structural class 9a family	NIIDSPCRPCYYRDSSGNCVYDKWSC GG
TRINITY_DN8546_c0_g1	Sea anemone structural class 9a family	NIIDVPCRPCYYRDSSGNCVYDQLGC GA
TRINITY_DN36141_c0_g3	Sea anemone type 1 potassium channel toxin family, Type 1a subfamily	SARVSLEDVNDNDEALYKRSTCKDKL HESSCQWYRDRGYCDKNNQYYNYF ATNCASTCGVC
TRINITY_DN8398_c0_g1	Sea anemone type 1 potassium channel toxin family, Type 1a subfamily	MVSKAIILLVVLALSTCVKGDPRNTN RKKNTGPARSARSPQRWVFKTQGST CRDRV-- ANCNQLRGYNWCRYYVNYMSTNC RKTCGFC
TRINITY_DN142057_c0_g1	Sea anemone type 1 potassium channel toxin family, Type 1b subfamily	MAAKWWVAFFILALAILSY- ATNPDENTEIDEEEEEEPRERIRRQSG CRDLLDYCAQWPDKYCQDYK------- DYMKTNCPRKCGYC
TRINITY_DN1681_c0_g1	Sea anemone type 1 potassium channel toxin family, Type 1b subfamily	CQDKLPYCKCKSYKKHGYCTTYPDD MKRRCKKTCSFC

TRINITY_DN17665_c0_g1	Sea anemone type 1 potassium channel toxin family, Type 1b subfamily	DIDSRCAGYAKRNFCKLGSLVPWMK KNCKKSCNIC
TRINITY_DN189453_c1_g1	Sea anemone type 1 potassium channel toxin family, Type 1b subfamily	SCADKYPGAKCKQFVSKDTCHTKEA RENCGVTCEAC
TRINITY_DN454_c0_g1	Sea anemone type 1 potassium channel toxin family, Type 1b subfamily	CKDKWYQVFCKVVKAAGRCDSPKG KEKCAKTCGHC
TRINITY_DN6600_c0_g1	Sea anemone type 1 potassium channel toxin family, Type 1b subfamily	CGDRIWNARCARLKKQGYCDDPTTI DEMKMNCAWTCGYC
TRINITY_DN7843_c0_g1	Sea anemone type 1 potassium channel toxin family, Type 1b subfamily	ILQANLLYGCDGSKRLTDEQREELEE DYSDPMTGPCGDVYDSNSCLAVKSL GYC--ERYPRTLRRACGQTCQLC
TRINITY_DN26110_c0_g1	Sea anemone type 3 (BDS) potassium channel toxin family	AVSCNCGGQQGHYHFGRDSCPFVR DQNGRKVYSGTECGQALGVCC
TRINITY_DN33088_c0_g2	Sea anemone type 3 (BDS) potassium channel toxin family	CSCDGHSGTYWWNRISCPSKYGYYG TCAKFNGVCC
TRINITY_DN33088_c0_g2	Sea anemone type 3 (BDS) potassium channel toxin family	LGCSCDGHSGTYWWNRIS-CPSKYGYYGTCAKFNGVCCW
TRINITY_DN33088_c0_g2	Sea anemone type 3 (BDS) potassium channel toxin family	CSCDGHSGTYWWNRISCPSKYGYYG TCAKFNGVCCW
TRINITY_DN4563_c0_g1	Sea anemone type 3 (BDS) potassium channel toxin family	ASCYCGKKKGTYWFLHLKRCPGGHG YTGTCRYFAGICCYK
TRINITY_DN4563_c0_g1	Sea anemone type 3 (BDS) potassium channel toxin family	SCYCGKKKGTYWFLHLKRCPGGHGY TGTCRYFAGICCYK
TRINITY_DN4563_c0_g1	Sea anemone type 3 (BDS) potassium channel toxin family	SCYCGKKKGTYWFLHLKRCPGGHGY TGTCRYFAGICC

TRINITY_DN4563_c0_g1	Sea anemone type 3 (BDS) potassium channel toxin family	CYCGKKKGTYWFLHLKRCPGGHGYTGTCRYFAG
TRINITY_DN4563_c0_g1	Sea anemone type 3 (BDS) potassium channel toxin family	SCYCGKKKGTYWFLHLKRCPGGHGYTGTCRYFAG
TRINITY_DN1348_c0_g2	Sea anemone type 5 potassium channel toxin family	MKMKGMVVLCLVLLMLSLVVAESKEETFDDYGMQLEKRCKGRGARCSSSSECC
TRINITY_DN195054_c0_g1	Sea anemone type 5 potassium channel toxin family	GRKLRCLTQCDEGGCLKYKQCLFYAGLQ
TRINITY_DN195054_c0_g1	Sea anemone type 5 potassium channel toxin family	SCKNKLAKCEKDNECCDKKDWGGRKLRCLTQCDEGGCLKYKQCLFYAGLQ
TRINITY_DN290_c0_g1	Sea anemone type 6 potassium channel toxin family, PHAB family	QCKKCSKGRCRPVPNCG
TRINITY_DN462_c0_g1	Sea anemone type 6 potassium channel toxin family, PHAB family	CKKCSKGRCRPVPNCG
TRINITY_DN104_c8_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GPCRAAFRRWYFDTTKNKCRTFIYGGCQANGNNFLKKRSCESLC
TRINITY_DN104_c8_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	CFQRKKVGPCRAAFRRWYFDTTKNKCRTFIYGGCQANGNNFLKKRSCESLC
TRINITY_DN12705_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GPCRASLPRFYFNRKSRKCSRFVFGGCKGNGNNFRTRSECKLKCR
TRINITY_DN12705_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	NVCDLPKFTGPCRASLPRFYFNRKSRKCSRFVFG
TRINITY_DN12705_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	CDLPKFTGPCRASLPRFYFNRKSRKCSRFVFGGCKGNGNNFRTRSECKLKC

TRINITY_DN158375_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	SSGPCFGYFEMWYYNSTTKKCERFV YGGCEGNRNSFESARECKAMCAV
TRINITY_DN158375_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	ICLLAPSSGPCFGYFEMWYYNSTTKK CERFVYGGCEGNRNSFESARECKAM C
TRINITY_DN17057_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	TGFCRGMVPRWYYNSGTGKCEKFY WGGCPMGSNDNRFEYHYQCNNM CQV
TRINITY_DN17057_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	CLLPRKTGFCRGMVPRWYYNSGTG KCEKFYWG
TRINITY_DN17057_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	NSEDVCLLPRKTGFCRGMVPRWYY NSGTGKCEKFYWG
TRINITY_DN17057_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	DVCLLPRKTGFCRGMVPRWYYNSG TGKCEKFYWGGCPMGSNDNRFEYH YQCNNMC
TRINITY_DN1862_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GNCKAKIERYYYDKPNDSCKRFWYT GCNGNANNFETREACQDAC
TRINITY_DN1862_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	PGTCKGNFQRYYYDNKTKQCKTFTY GGCGGNKNNFKVKDDCLRECRVH
TRINITY_DN1862_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	CSKPLVVGNCKAKIERYYYDKPNDSC KRFWYTGCNGNANNFETREACQDA C
TRINITY_DN1862_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	FCLFPPAPGTCKGNFQRYYYDNKTK QCKTFTYG
TRINITY_DN26825_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	DVGVCQVPFVRYYFDTTVGYCKSFFY LGCKGNANRFSTLKECLEKC
TRINITY_DN26825_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	TDVSYGIDSSCILPKDVGVCQVPFVR YYFDTTVGYCKSFFYLGCKGNANRFS TLKECLEKCMPDLFSYDSQEEN

TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GKCRGYFPRYYYSTEKGSCQMFIYGG CGGNRNNFETLDECNATCN
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GRCRGRFPRYHYSTEKGRCEIFIYGGC GGNRNNFETLDDCNATCN
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GRCRGRFPRYYYSTEKGRCEIFIYGGC GGNRNNFKTLDECRSTCR
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GRCRGRFPRYYYSTEKGSCQMFIYG GCGGN
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GRCRGRFPRYYYSTEKGSCQMFIYG GCGGNRNNFETLDECNATCN
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GRCRGRFPRYYYSTEKGSCQMFIYG GCGGNRNNFKTLDECRSTCR
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	ESFCTLAPVTGRCRGRFPRYYYSTE
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	ESFCTLAPVTGRCRGRFPRYYYSTEK GRCEIFIYG
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	ESFCTLAPVTGRCRGRFPRYYYSTEK GSCQMFIYG
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	SFCTLAPVTGKCRGYFPRYYYSTEKGS CQMFIYG
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	SFCTLAPVTGRCRGRFPRYHYSTEKG RCEIFIYG
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	SFCTLAPVTGRCRGRFPRYYYSTEKG RCEIFIYG

TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GRCRGRFPRYYYSTEKGSCQMFIYG
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	SFCTLAPVTGRCRGRFPRYYYSTE
TRINITY_DN303_c1_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	SFCTLAPVTGRCRGRFPRYYYSTEKG SCQMFIYG
TRINITY_DN3599_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	CLKPVKQGRCVPYRLNVRWHFNQQ KKDCFPFIYLGCAGTDVSDDNNFETK ARCLSACQ
TRINITY_DN50309_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	ETGPCRSKIIRWYYSPHRGKCLKFIYG GCRGNLNRFLTKSSCRTICQ
TRINITY_DN50309_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	PGPCRAYFERWYYDSAKGRCLKFVY GGCDGNHNNFKTTKECETAC
TRINITY_DN50309_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	CLQPKIPGPCRAYFERWYYDSAKGR CLKFVYGGCDGNHNNFKTTKECETA C
TRINITY_DN5078_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GSCNGHTARYYFDTASKSCKKFMYS GCDGNANRFYSLKRCERKCK
TRINITY_DN5078_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	PGPCFARFQKWFYDTRAAKCKMFV YGGCLGNANNFDNKKACLQACSAK
TRINITY_DN5078_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	YIVRFFFNSATRLCEPFVYSGDGGNE NNFRDKESCRRACS
TRINITY_DN5078_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	SDICNLPKTPGPCFARFQKWFYDTRA AKCKMFVYG
TRINITY_DN5078_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	ASDICNLPKTPGPCFARFQKWFYDTR AAKCKMFVYG

TRINITY_DN5078_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	RFFFNSATRLCEPFVYSGDGGNENN FRDKESCRRAC
TRINITY_DN5111_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GFCRAAFPRFYYDTTTRRCTYFTYGG CGGNANNFESWWECYQTCT
TRINITY_DN5111_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	NPICLLPRVVGFCRAAFPRFYYDTTTR RCTYFTYG
TRINITY_DN5111_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	MVFLLCFFLVADVSYGINPICLLPRVV GFCRAAFPRFYYDTTTRRCTYFTYGG CGGNANNFESWWECYQTC-TPGAIVFPEDSPKEN
TRINITY_DN57_c2_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	VRKWYHDAATSSCKEFDFLGCYGNE NSFDSQSQCQSACIA
TRINITY_DN5999_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	AHMPRYYYNPWTRRCQTFIYGGCH GNANRFPTRKRCEANC
TRINITY_DN5999_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	DPGPCYAHMPRYYYNPWTRRCQTFI YGGCHGNANRFPTRKRCEANC
TRINITY_DN5999_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GPCRAAIPRYYYNSKAGKCERFIYGG CRGNANNFYTKKRCNMAC
TRINITY_DN5999_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	KDICRLPKVVGPCRAAIPRYYYNSKA GKCERFIYG
TRINITY_DN5999_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	DICQLPKVVGPCRAAIPRYYYNSKAG KCERFIYG
TRINITY_DN5999_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	NICRLPKVVGPCRAAIPRYYYNSKAG KCERFIYG
TRINITY_DN62108_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GSCSRTLDRWHYDASKKSCVFFVYS GCGGNNNNFVSKAECQRTC

TRINITY_DN62108_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	VPAECLLPKQVGSCSRTLDRWHYDASKKSCVFFVYSGCGGNNNNFVSKAECQRTC
TRINITY_DN66000_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	GPCRGAFIRYYYNSKENECKKFHYGGCRGNANNF
TRINITY_DN66000_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	SSICDLPISEGPCRGAFIRYYYNSKENECKKFHYG
TRINITY_DN66000_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	CDLPISEGPCRGAFIRYYYNSKENECKKFHYGGCRGNANNFETKEECEETC
TRINITY_DN68726_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	CWKPPKYGSCTDEYIRFGYNPLIGACTAYLWSGCGGGDNRFVTQYECHARC
TRINITY_DN728_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	CRGYFPRYFYNSTSQQCQYFIAGGCHPSGWNGFYTEQECNQTC
TRINITY_DN728_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	NPGPCTQRVSRWAFNATTGRCKKFNYGGCFGNENNFLSKELCQRRCR
TRINITY_DN728_c0_g1	Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	CRGYFPRYFYNSTSQQCQYFIAG

Table 5.2 Summary of venom peptide families identified from the *Bunodosoma goanense* transcriptome

Toxin Peptide family	**No. of transcripts**
Actinoporin family, Sea anemone subfamily	8
Cnidaria small cysteine-rich protein (SCRiP) family	4
EGF domain peptide family	22
Jellyfish toxin family, Type II subfamily	4
NEP3 family	24
Phospholipase A2 family	9
Sea anemone 8 toxin family	3
Sea anemone short toxin (type III) family	1
Sea anemone sodium channel inhibitory toxin family	1
Sea anemone structural class 9a family	2
Sea anemone type 1 potassium channel toxin family, Type 1a subfamily	2
Sea anemone type 1 potassium channel toxin family, Type 1b subfamily	7
Sea anemone type 3 (BDS) potassium channel toxin family	3
Sea anemone type 5 potassium channel toxin family	2
Sea anemone type 6 potassium channel toxin family	2
Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	19

5.3.2.2 Protein modelling: From the 16 families, only complete sequences belonging to neurotoxin and cytotoxic peptide families were selected for further in silico analysis, while partial sequences and other toxin families were excluded. The three-dimensional models of the selected peptides were predicted using Swiss-Model to analyse their conserved and essential structural features. The presence of conserved motifs and structural completeness strongly suggests that these peptides are likely functional toxins.

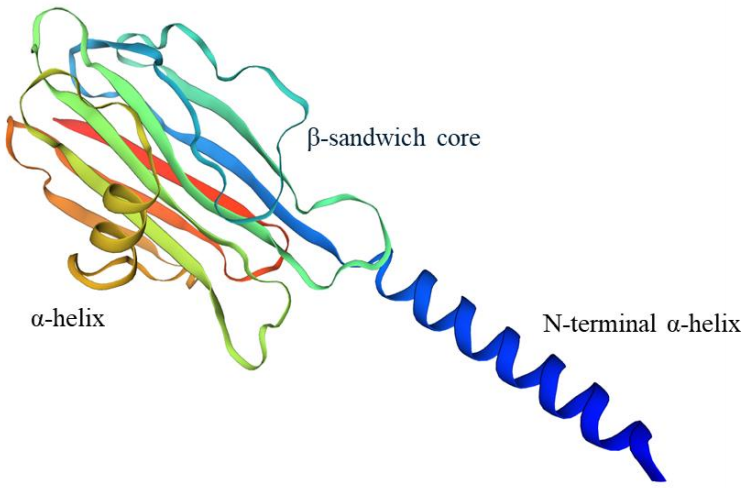


Figure 5.2: Actinoporin Sequence 1 TRINITY_DN118343_c0_g1

EEEKD-
KKSLALAGTIIEGASLTISVLEKVLQALGDISRKIAIGIDNESGREWTAINTYFRAGTSD
VVLPYTVPHGKALLYDGQKSRGPVATGVVGVVAYVSSNDAYTVAVMFSVPFDYNLY
SNWWNVKIYKGKRRADQNVYEDLYYYASPFRGDNGWHSRNLGYGLKSRGFMNSS
GQAVLQIKVEQ

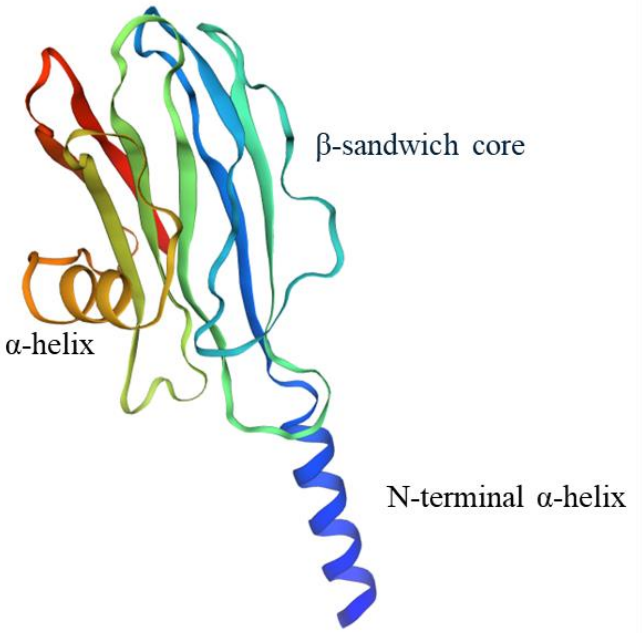


Figure 5.3: Actinoporin Sequence 2 TRINITY_DN87706_c2_g1

GASLTISLLEKVLQALGDVSRKIAIGFDNESGREWTALNTYFRAGTSDVVLPYTVPHG
KALLYNGQKSRGPVATGVVGVVAYVSSNDAYTEAVMFSVPFDYNLYSNWWNVKIYK
GKRRADQNVYEDLYYYASPFRGDNGWLSRNLGYGLKSRGFMN

The N-terminal region of both sequences exhibited hydrophobic residues, including stretches such as LALAGTIIEGA and GASLTISLL, suggesting the presence of a potential pore-forming α-helix, which is crucial for membrane insertion. The RGD motif, short for Arg-Gly-Asp, is a highly conserved sequence found in many sea anemone actinoporins. This motif plays a critical role in the oligomerization of actinoporin monomers, which is essential for the formation of functional pores in the target cell membrane. The structural model of actinoporin peptides have a typical β-sandwich core composed of two antiparallel β-sheets, flanked by two α-helices on either side. This structural architecture is characteristic of actinoporins, which form membrane pores by inserting their N-terminal α-helix into the lipid/water interface of the target membrane. The hydrophobic residues on the N-terminal α-helix facilitate membrane penetration, leading to pore formation and cytolytic activity (Rojko et al., 2016). Our predicted venom peptides exhibit conserved motifs and structural features characteristic of actinoporins (Figure 5.2 & 5.3).

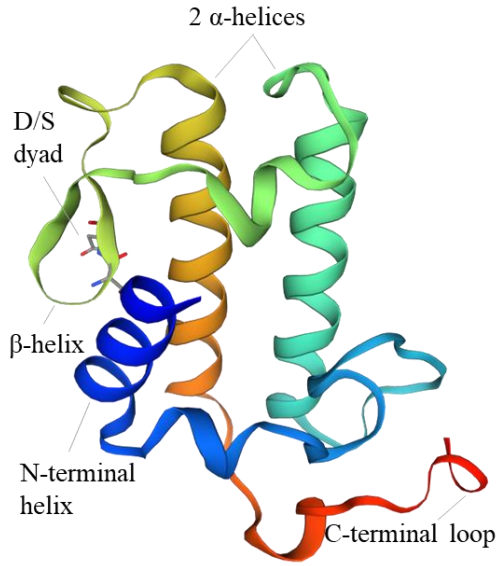


Figure 5.4: Phospholipase Sequence 1 TRINITY_DN121394_c0_g1

IYQFGLMIHCETNRWASEYVGYGCWCGLGGKGTPVDETDRCCKNHDKCYYAVHRSGK—
CSYMGAAYTTIYHRKGCSDCDKQQNNPCEQAVCECDSVAVKCFAKAKFNDANKRYSQSKC

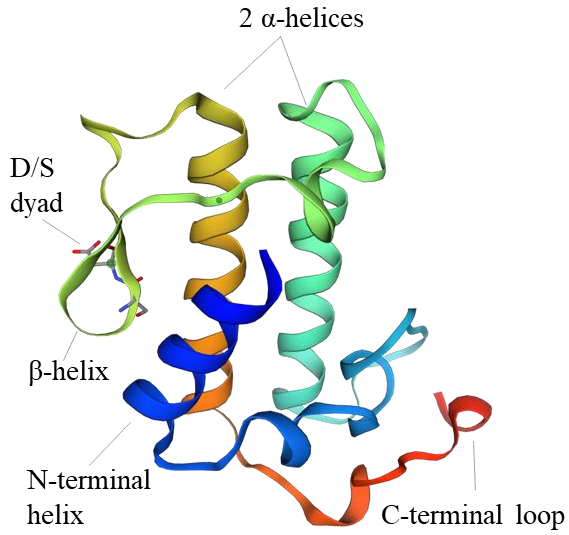


Figure 5.5: Phospholipase Sequence 2 TRINITY_DN2647_c0_g1

SLYEFYQMITCETGRSWQDYNLYGCFCGKGGTGTPVDALDQCCFIHDKCYERAAATVCPWP-
---LQIYVDSYWHENCSDCVASKNTACEQALCECDSKAAKCFKDNKWDPQYDDYPQEKC

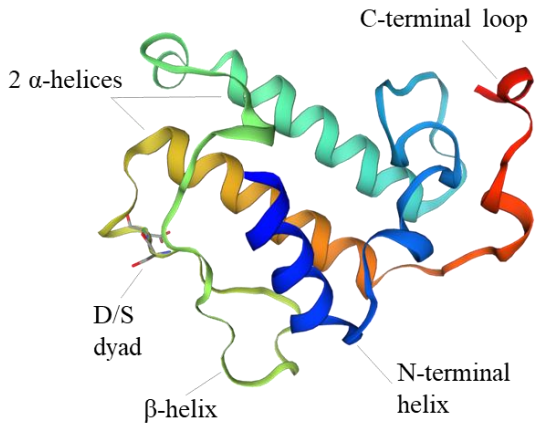


Figure 5.6: Phospholipase Sequence 3 TRINITY_DN34612_c0_g2

GIYEFGQMISCLTNRSSLDYADYGCWCGLGGSGRPVDQLDRCCHTHDRCYDDIIASGICGYS
WQV-
YFRTYEYSGCTKCDPLSSYPSDDQYALCRTKLCECDSTAASCFSNSKFNPQHVNYNLTMC

The predicted phospholipase A_2 (PLA_2) sequences exhibit several conserved structural features and motifs characteristic of sea anemone PLA_2-like toxins (Figure 5.4 to 5.6). The presence of an N-terminal α-helix suggests its role in membrane binding and substrate interaction (Oliveira et al., 2018). A catalytic DS dyad, comprising conserved Asp (D) and Ser (S) residues, is observed, which is essential for enzymatic activity and lipid hydrolysis (Khan & Ilies, 2024). Additionally, multiple conserved cysteine (C) residues indicate the formation of disulfide bridges, contributing to structural stability. The β-sheet core supports substrate recognition and enzymatic function. The C-terminal loop, which plays a role in maintaining structural integrity and facilitating substrate binding, is also well-conserved (Burke & Dennis, 2009). These conserved features suggest that the identified PLA_2 sequences may function as active and structurally stable venom components in *Bunodosoma goanense.*

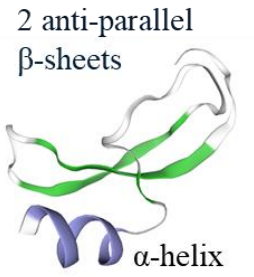


Figure 5.7: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN303_c1_g1_i1.p2

GRCRGRFPRYYYSTEKGSCQMFIYGGCGGNRNNFKTLDECRSTCR

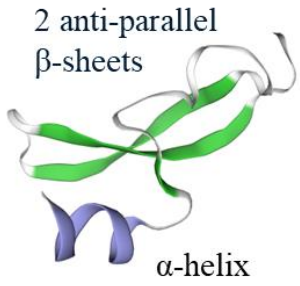


Figure 5.8: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN728_c0_g1_i4.p1

NPGPCTQRVSRWAFNATTGRCKKFNYGGCFGNENNFLSKELCQRRCR

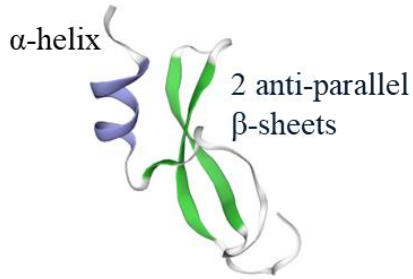


Figure 5.9: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN1862_c0_g1_i1.p2

FCLFPPAPGTCKGNFQRYYYDNKTKQCKTFTYG

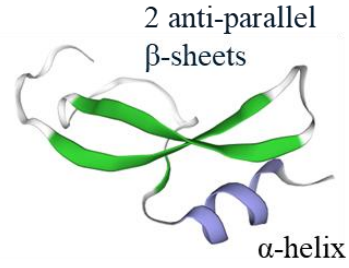


Figure 5.10: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN5078_c0_g1_i2.p1

GSCNGHTARYYFDTASKSCKKFMYSGCDGNANRFYSLKRCERKCK

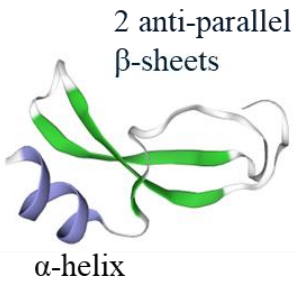


Figure 5.11: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN5111_c0_g1_i1.p1

GFCRAAFPRFYYDTTTRRCTYFTYGGCGGNANNFESWWECYQTCT

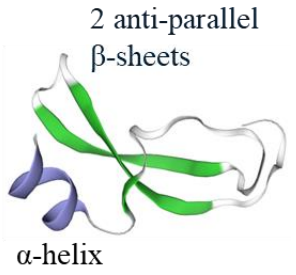


Figure 5.12: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN5999_c0_g1_i1.p1

DPGPCYAHMPRYYYNPWTRRCQTFIYGGCHGNANRFPTRKRCEANC

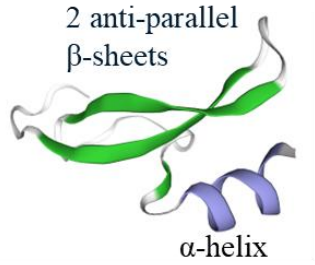


Figure 5.13: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN12705_c0_g1_i3.p1

NVCDLPKFTGPCRASLPRFYFNRKSRKCSRFVFG

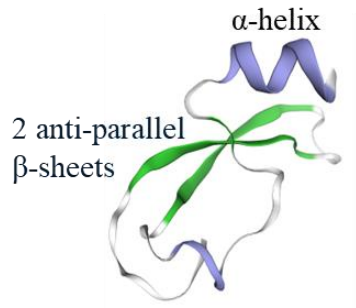


Figure 5. 14: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN17057_c0_g1_i14.p1

NSEDVCLLPRKTGFCRGMVPRWYYNSGTGKCEKFYWG

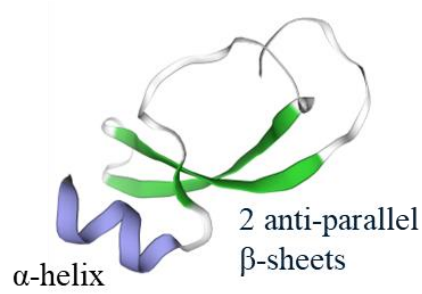


Figure 5.15: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN26825_c0_g1_i2.p1

DVGVCQVPFVRYYFDTTVGYCKSFFYLGCKGNANRFSTLKECLEKC

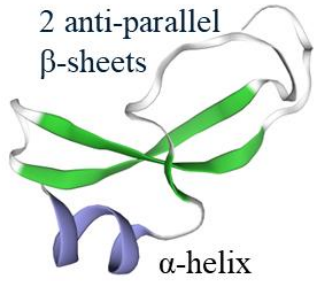


Figure 5.16: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN50309_c0_g1_i1.p1

ETGPCRSKIIRWYYSPHRGKCLKFIYGGCRGNLNRFLTKSSCRTICQ

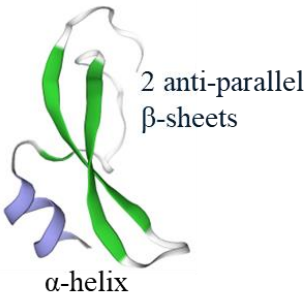


Figure 5.17: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN62108_c0_g1_i1.p1

GSCSRTLDRWHYDASKKSCVFFVYSGCGGNNNNFVSKAECQRTC

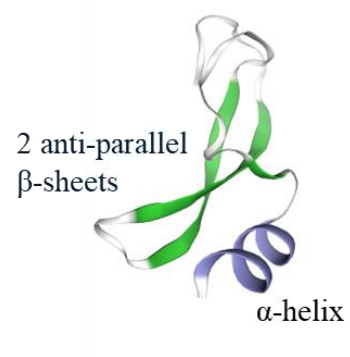


Figure 5.18: Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily TRINITY_DN158375_c0_g1_i1.p1

ICLLAPSSGPCFGYFEMWYYNSTTKKCERFVYGGCEGNRNSFESARECKAMC

The structural model of Kunitz-type toxins (Figure 5.7 to 5.18) revealed a conserved fold composed of one α-helix and two antiparallel β-sheets arranged in a three-stranded β-hairpin configuration (Madio et al., 2019). The protein was stabilized by three disulfide bridges linking six cysteine residues in the Cys-Cys-Cys motif. This scaffold is commonly associated with serine protease inhibition and potassium channel blockade (Norton & Pallaghy, 1998).

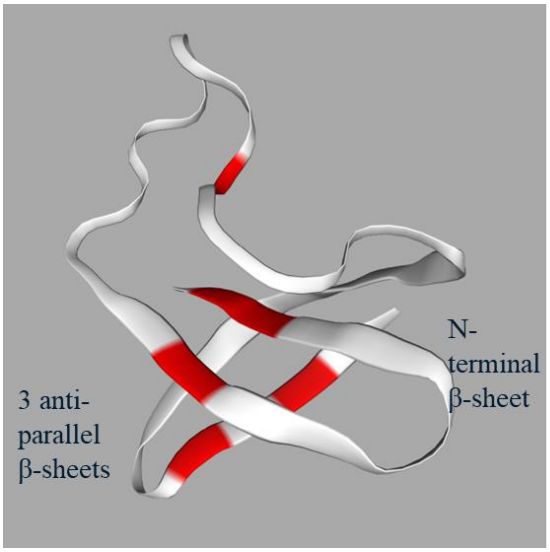


Figure 5.19: Sea anemone type 3 (BDS) potassium channel toxin family Sequence 1 TRINITY_DN4563_c0_g1

ASCYCGKKKGTYWFLHLKRCPGGHGYTGTCRYFAGICCYK

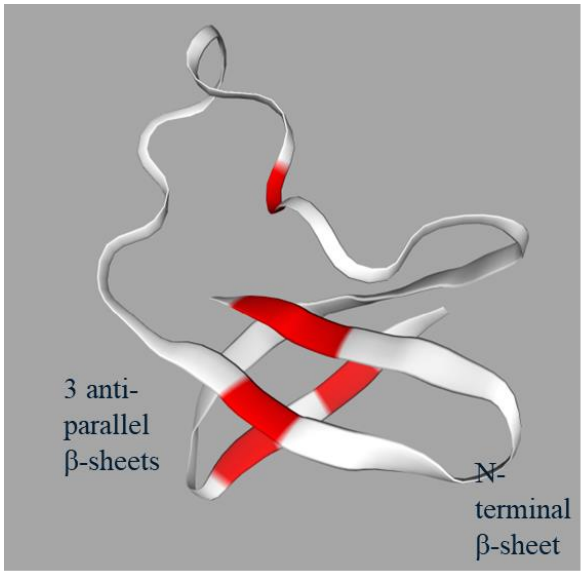


Figure 5.20: Sea anemone type 3 (BDS) potassium channel toxin family Sequence 3 TRINITY_DN33088_c0_g2

LGCSCDGHSGTYWWNRIS-CPSKYGYYGTCAKFNGVCCW

The BDS-like potassium channel toxin (Figure 5.19 & 5.20) exhibited a structural fold comprising three antiparallel β-sheets and one short N-terminal β-sheet, stabilized by six cysteine residues forming three disulfide bridges (Driscoll et al., 1989; Frazão et al., 2012). This compact structure is commonly associated with potassium channel blockers, where the β-sheet hairpin loop interacts with the channel's extracellular vestibule.



Figure 5.21: K-channel toxin (PHAB) TRINITY_DN290_c0_g1

QCKKCSKGRCRPVPNCG

The PHAB-like potassium channel toxins showed a minimalistic structure composed of 15-17 amino acids forming a β-hairpin loop (Figure 5.21). The flexible loop is stabilized by disulfide bridges, which likely enable interaction with potassium channels (Madio et al., 2018).

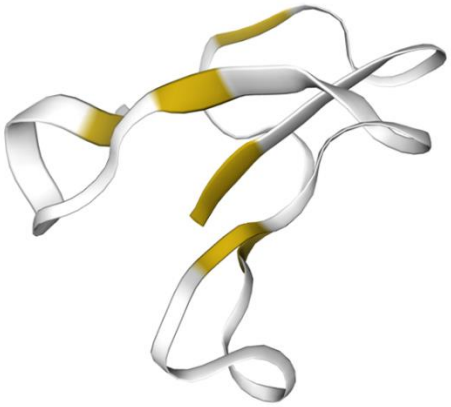


Figure 5.22: Cnidaria small cysteine-rich protein (SCRiP) family TRINITY_DN11037_c0_g1

MDFKIQMFSLVLALVVMSVQSGPEEMNNEKRAVNCYRINGYCFPRNNTCPRGKQDCYQYDC
NLVNQKCCCP



Figure 5.23: Cnidaria small cysteine-rich protein (SCRiP) family TRINITY_DN39933_c0_g1

MDFKIQMFLLVLVLVAMSVQSA--PGERDNDEKRTLS-
CYTMKGYCMSRNDTCPTGYQDFHHYFCNQVDTKCCSPT

The predicted model of SCRiPs revealed a small, globular structure with six conserved cysteine residues (Highlighted in yellow) forming a disulfide-stabilized fold (Figure 5.22 & 5.23). This motif is a hallmark of cnidarian-specific proteins involved in innate immunity and antimicrobial activity (Madio et al., 2019).

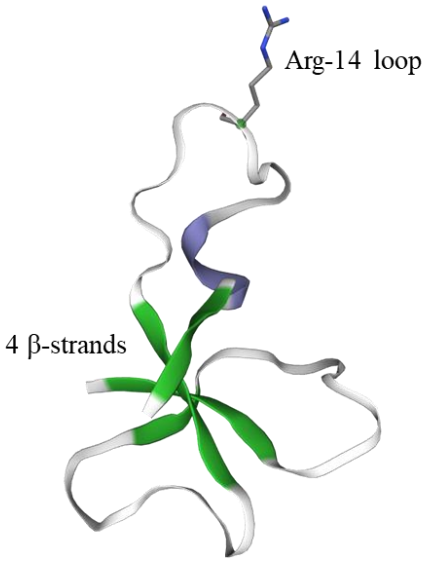


Figure 5.24 Sea anemone sodium channel inhibitory toxin family TRINITY_DN2210_c0_g1

GVPCRCDSDGPSVRGNTLSGTVWVFGCPSGWNKCARNGPTIGSCCKQ

The predicted structures of sodium channel toxins exhibited the canonical fold of Type I and Type II Sea anemone toxins, comprising an antiparallel β-sheet made up of four β-strands and a flexible Arg-14 loop (Figure 5.24) . This Arg-14 loop plays a critical role in binding to the

voltage sensor domain of sodium channels, causing channel inhibition or modulation (Moran et al., 2009). The nucleotide sequence encoding the selected venom peptide candidate (TRINITY_DN2210_c0_g1_i2) was submitted to the NCBI GenBank database and assigned the accession number PZ111315.

These structural predictions are consistent with prior studies on sea anemone venom peptides, reinforcing their reliability.

5.3.2.3 Contraction paralysis assay: Mucus venom induced contractive paralysis in 7 minutes. Tentacle venom was the most potent, inducing paralysis within 3 minutes. Acontia venom had a delayed effect, causing paralysis after 15 minutes. The synthesized peptide did not induce any observable effects. But after enabling the peptide into blood steam of the fish, instant contraction paralysis was observed. The contraction paralysis assay demonstrated that venom extracted from different tissues of *B. goanense* exhibits potent neurotoxic activity. The rapid onset of paralysis by tentacle venom (within 3 minutes) suggests the presence of sodium or potassium channel toxins that interfere with neuromuscular transmission, as observed in other sea anemones (Madio et al., 2019; Sachkova et al., 2020).The delayed effect of acontia venom (15 minutes) may be due to the predominance of cytolytic toxins such as actinoporins, which require longer exposure to disrupt cell membranes (Belmonte et al., 2014). The weaker paralytic effect of mucus venom suggests that it contains lower concentrations of neurotoxins, possibly serving as a chemical deterrent against predators or fouling organisms. URL of fish contractive paralysis assay video https://youtube.com/shorts/kfQN4GTxfws?feature=share

Interestingly, the synthesized sodium channel toxin peptide did not induce any observable effect when administered through water exposure, but it caused instant contraction paralysis when injected into the bloodstream of Gambusia fish. The observed discrepancy in the efficacy of the synthesized sodium channel toxin peptide between water exposure and direct bloodstream injection in Gambusia fish can be attributed to the route of administration. Peptide toxins often require direct access to the circulatory system to effectively reach their molecular targets. This phenomenon aligns with findings in peptide drug delivery research, where the administration route significantly influences the bioavailability and therapeutic efficacy of peptides (Reis et al., 2006). Conversely, direct injection into the bloodstream circumvents these barriers, allowing the peptide to maintain its structural integrity and

promptly interact with its intended targets, thereby eliciting the desired physiological response (Ibraheem et al., 2014).

5.3.3 Conclusion:

This study explored an ethically sustainable approach to venom peptide discovery by integrating in vitro, in silico, and synthetic peptide validation methods. Initially, we attempted to obtain venom peptides through cell culture, but venom production was not observed. To overcome this limitation, we utilized transcriptomic analysis to predict putative venom peptides, followed by chemical synthesis of one selected peptide for functional validation. The transcriptomic analysis revealed novel peptides from 16 known sea anemone peptide families which are primarily neurotoxins and cytotoxins. If the putative peptide sequence is structurally accurate and complete, having the necessary tertiary structures and conserved residues/ motifs, then it should ideally be functional. Therefore, the putative peptides obtained from the transcriptome can be a reliable method to discover novel venom peptides, which can be further studied for their therapeutic applications. This approach offers a sustainable, ethical alternative to traditional venom studies. Bioassays on juvenile *Gambusia* fish revealed that crude venom from different tissues induced contractive paralysis at varying times, with tentacle venom acting the fastest, followed by mucus and acontia venom. However, the synthesized peptide did not induce any observable effects. But after enabling the peptide into blood steam of the fish, instant contraction paralysis was observed.

While transcriptome analysis effectively identifies expressed genes, it does not confirm the presence or abundance of these peptides in the venom itself, a gap that proteomic analysis could address. Similarly, the accuracy of 3D models hinges on template quality, and novel peptides may require experimental validation, such as X-ray crystallography or NMR spectroscopy, to confirm their structures.

Summary of the Thesis

This thesis explores the chemically mediated ecological interactions of the sea anemone *Bunodosoma goanense*, a unique and understudied anthozoan species found along the western coast of India. The study integrates ecological fieldwork, molecular biology, transcriptomics, biochemical assays, and functional validation to provide a holistic understanding of the species' ecological behavior and bioactive venom components, with implications for drug discovery. In Chapter 1, we begin with a comprehensive review of ecological interactions in sea anemones, emphasizing the role of chemical cues in their survival, reproduction, and defense. The chapter also outlines the composition of sea anemone venoms—largely composed of cytolytic and neurotoxic peptides—and discusses their relevance in pharmaceutical and biotechnological applications. This literature-based chapter lays the conceptual foundation for the experimental work in subsequent chapters. Chapter 2 details the identification and characterization of *Bunodosoma goanense* using morphological features, cnidome analysis, and DNA barcoding. Importantly, this chapter also reports the discovery of two previously unrecorded modes of asexual reproduction in this species, thereby contributing to our understanding of sea anemone reproductive ecology. Chapter 3 presents two distinct ecological interactions involving *B. goanense*. The first part describes a facultative mutualism with the gastropod *Purpura panama*, where the snail benefits by feeding on ejected food remnants and laying egg capsules near the anemone, potentially gaining protection and the sea anemone get food by predating on the gastropod' predators.. Gut content analysis revealed undischarged nematocysts, suggesting kleptocnidism. In the second part, we document spatial competition between *B. goanense* and soft corals (*Zoanthus* sp. and *Palythoa* sp.) using field observations supported by transcriptomic data. *B. goanense* adopts different molecular strategies based on the competitor: an active growth and repair response against *Zoanthus*, and a resource-conserving, stress-tolerant response against *Palythoa*. These findings illustrate the ecological plasticity and context-dependent responses of *B. goanense*. Chapter 4 investigates the venom composition of *B. goanense*, revealing tissue-specific venom profiles from the tentacles, acontia, mesenterial filaments, and ectodermal mucus. The tentacle venom displayed broad-spectrum activity against both sodium and potassium ion channels, highlighting its role in prey capture. In contrast, venom from the mucus and acontia-filament complex showed more selective potassium channel activity, suggestive of defensive roles. Bioactivity assays revealed antibacterial and cytotoxic effects. Two novel peptides with ion channel-modulating properties were identified, highlighting the therapeutic potential of these molecules. Chapter 5 introduces an ethical and sustainable approach to venom research by combining transcriptomic prediction and chemical synthesis of venom peptides. Although *in vitro* venom production via cell culture was unsuccessful, the transcriptome revealed diverse peptides from 16 known toxin families. A selected peptide was synthesized and functionally tested in juvenile *Gambusia* fish, where direct bloodstream administration caused contractile paralysis, confirming

bioactivity. This approach demonstrates the potential of transcriptome-guided discovery of venom peptides, while also highlighting the need for further proteomic validation and structural confirmation.

Future Prospects

The findings presented in this thesis open several promising avenues for future research. A key next step is the proteomic validation of transcriptome-predicted venom peptides to confirm their presence and relative abundance in different tissue-specific venoms of *Bunodosoma goanense*. Expanding the electrophysiological characterization of venom components against a wider panel of ion channel isoforms, including those of clinical relevance, may help identify peptides with therapeutic potential. High-throughput screening of bioactive peptides for antibacterial, anticancer, and anti-inflammatory properties may contribute to the development of novel lead compounds for drug discovery.

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Annexure I

List of Publications

1. Menezes, C., & Thakur, N. L. (2022). Sea anemone venom: Ecological interactions and bioactive potential. *Toxicon*, 208, 31-46. https://doi.org/10.1016/j.toxicon.2022.01.004
2. Menezes, C., & Thakur, N. L. (2022). Observations of the likelihood of asexual reproduction in sea anemone *Bunodosoma goanense* den Hartog & Vennam, 1993. *Marine Biodiversity*, 52, 49. https://doi.org/10.1007/s12526-022-01299-9
3. Menezes, C., & Thakur, N. L. (2023). Facultative association of *Purpura panama* with *Bunodosoma goanense*. *Marine Biodiversity*, 53(6), 84. https://doi.org/10.1007/s12526-023-01394-5
4. Menezes, C., Thakur, N., Carleer, K., Peigneur, S., & Tytgat, J. (2026). Identification and Functional Characterization of a Nav-Targeting Peptide NavTx-Bg1 from Sea Anemone *Bunodosoma goanense* (Vennam & den Hartog, 1993) Transcriptome. *Toxicon*, 109016. https://doi.org/10.1016/j.toxicon.2026.109016
5. Menezes, C., & Thakur, N. L. (2026). Natural co-habitation with soft corals influences gene expression in *Bunodosoma goanense* den Hartog & Vennam, 1993. *Hydrobiologia*, 1-16. https://doi.org/10.1007/s10750-026-06145-8

Conferences

1. "Sea anemone venom: Unraveling ion channel interactions for therapeutic insights" Oral presentation at **4th World Ocean Science Congress (WOSC) 2024Venue**: IIT-Madras Research Park, Tamil Nadu, India from 27th to 29th February 2024, Organized by NIOT, IIT-Madras, VIBHA
2. "Venomous Insights: Understanding ecological dynamics of Sea anemone *Bunodosoma goanense* through tissue specific venom correlation" Oral presentation at **20th Marine Biological Association Post-graduate Conference 2024 (MBA-PG 2024)** Venue: Plymouth Marine Laboratory, Marine Biological Association, University of Plymouth, England, United Kingdom from 23rd to 25th April 2024, Organized by PML, MBA, University of Plymouth
3. "Sea anemone venomics in drug discovery." Poster presentation at **International Conference on Emerging Trends in Translational Bioinformatics 2024.** Venue: Department of Bioengineering and Biotechnology, BIT Mesra, Ranchi, Jharkhand, India from 5th to 7th December 2024 organised by Department of Bioengineering and Biotechnology, BIT Mesra, Ranchi, Jharkhand, India
4. "Overlooked but Essential: The Ecological Importance and Conservation of Sea Anemones" Poster presentation at **9th National Conference of Ocean Society of India (OSICON-25)**

2025 Venue: CSIR-NIO, Goa, India from 5th to 7th February 2025,Organized by CSIR, CSIR-NIO, OSI.

5. "Therapeutic potential of Marine venom" Poster presentation at **9th Current Trends in Drug Discovery Research (CTDDR) 2025** Venue: CSIR-CDRI, Lucknow, Uttar Pradesh, India from 19th to 22nd February 2025, Organized by CSIR-CDRI

Workshops, Seminars and Training courses

1. National Webinar on "**Marine bioresources and bioprospecting**" organized by PG and Research Department of Biochemistry, Bharathidasan College of Arts and Science, Tamil Nadu, India on 14th June 2021.
2. Workshop cum Training Programme on "**Natural Products from Marine Bioresources**" (Virtual Mode), organized by CSIR-NIO, Dona Paula, Goa, India during 30th -31st March 2021
3. Online course in "**Untargeted Metabolomics using High resolution Mass spectroscopy**" organized by Centre for cellular and molecular platform (C-CAMP) during 28th -29th October 2021.
4. Hands-on Training course in "**MS-based Proteomics**" organized by Centre for cellular and molecular platform (C-CAMP), Bangalore, during 26th -28th September 2022.
5. Participated in "**INYAS-Saransh Thesis competition for Ph.D**." Students organized by INYAS.
6. Online course in "**Technical Hands-on Online Training in Next Generation Sequencing RNASeq Transcriptome Analysis**" organised by BDG Life Science during 29 - 31 March 2023.
7. Online course in "**Research Integrity (Core1-8)**" organised by Epigeum Online Course System via European molecular biology organisation (EMBO) on 15 May 2023.

Grants and Awards

1. **DST-Inspire Fellowship** (IF190436) - Department of Science and Technology, Govt. of India
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Sea anemone venom: Ecological interactions and bioactive potential

Cecelia Menezes, Narsinh L. Thakur *

CSIR-National Institute of Oceanography, Dona Paula, 403004, Goa, India

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ABSTRACT

This article provides a brief overview of the literature related to the chemical ecology of sea anemones, focusing on their venom, and summarizing their bioprospecting potential. Sea anemones have been known as a copious source of peptides and other molecules having bio-medical potential, however very little is known about the ecological role of these molecules. From an ecological prospect, these molecules are involved in prey capture, protection against predators, or deterring competitors of the sea anemone. Whereas, in bioprospecting, these toxic molecules have potential biotechnological applications. Herein, we present the diversity of sea anemone venom toxins reported to date and describe the role of venom in various chemically mediated ecological interactions of the sea anemone. This paves a path for continuing and broadening efforts to evaluate their functional and ecological importance.

1. Introduction

Sea anemones are predatory marine animals belonging to the invertebrate Phylum Cnidaria (order Actiniaria, Class Anthozoa) which are usually attached to rocks or suitable substrata. They are mostly sessile animals, but if required they can move short distances by gliding along the seafloor. They are present from the tropical to the polar regions and occupy the intertidal zone of all oceans and are found up to the depths of more than 10,000 m. The adult polyp consists of a cylindrical column attached to the substratum via the pedal disc at one end. At the opposite end lies the oral disc which consists of the centrally located actinopharynx and a crown of tentacles (Fig. 1).

Sea anemones have an important role in the marine ecosystem. They are predatory animals that prey on free-living organisms like crustaceans, molluscs, and fish, thus acting as a link between the pelagic and benthic ecosystems (Brusca and Brusca, 2003). They play key biogeochemical roles in benthic ecosystems. Mostly, they are solitary animals, but some are also known to show symbiosis, especially in the coral reef ecosystem where they offer refuge and protection to other reef animals and in exchange receive leftover food and fish metabolic waste as a source of nitrogen (Cleveland et al. (2011)).

The sea anemone is one of the most venomous animals in the world and is known to be a rich source of toxic proteins and peptides (Madio et al., 2017, 2019). These venomous molecules are more than just toxins as they are involved in various ecological interactions of the sea anemone such as in predatory feeding, spatial competition and aggressive encounters, self-defence, and even in larval recruitment (Schendel et al. (2019)). There are numerous reviews of sea anemone toxins concerning their bioactivity and pharmacological applications, but the knowledge of the ecological role of these molecules is limited. O'Hara et al. (2021) reviewed literature studying the effect of ecological factors such as environmental temperature, salinity, ontogeny, geographic location, and diet on cnidarian venom and stated that venom ecology research is necessary for pharmacologically important cnidarians to facilitate the advancement of medical treatments. Similarly, Schendel et al. (2019) in their review on the importance of behaviour and venom system morphology in understanding its ecology and evolution mentioned that more studies of behaviour, morphology and ecologically relevant toxin pharmacology will boost our understanding of the evolution of venoms toxins, and enable the discovery of their potential as sources of molecular tools and therapeutic and agrochemical lead compounds. Ashwood et al. (2020) reviewed the diversity of venom composition between Meduzoa and Anthozoa. They suggest that the temporal and spatial expression of protein and peptide toxins is a

* Corresponding author.
E-mail address: thakurn@nio.org (N.L. Thakur).

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consequence of the functional and ecological factors. In their review, Surm and Moran (2021) provided insights into how the development and life-history dynamics of animals shape the evolution of their venom and its ecological implications. There is significant scope for studies to understand the chemical ecology of these animals and the ecological role of their toxins as it can pave the way for the discovery of novel and unexpected biotechnological applications. In this review, we focus on sea anemone venom to understand its role in the various chemically mediated ecological interactions and the current biotechnological application.

2. Sea anemone venom tissue

Marine venoms are probably as myriad as marine biodiversity. As a result of the co-evolution of processes between the host, parasite, predator, and prey, these venomous animals have developed various glands or tissues for the production storage, and dispersal of their venom. The most distinct characteristic of their phylum cnidaria is the presence of stinging cells called Cnidocytes or nematocytes which play a particularly important role in prey capture and anti-predatory defense of the animal. Cnidocytes contain a stinging capsule known as a Cnida. Among anthozoans there are three categories of cnidae: (1) Nematocysts, which have harpoon-like structures that inject venom into the prey and paralyzes it (atrichs, isorhizousholotrichs, anisorhizousholotrichs, b-mastigophores/basitrichs/spirullae, microbasic p-mastigophores, microbasic p-mastigophores) (2) Spirocysts, which consists of a string that is fired and entangle the prey and (3) Ptychicysts, these release sticky substances to hold onto prey, also build protectoral tube in tube-dwelling sea anemones (England and Robson, 1991; Zenkert and Takahashi, 2011). Based on their functions, these cnidae sub-types are differentially located of the animal. The number of cnidae and their distribution is influenced by the body size, prey type, and selectivity in prey capture. The quantity of cnidae subtypes present in various locations of the animal is conserved and unique to every species, therefore playing an important role in the taxonomical identification of the anthozoan species (Carlgren, 1949). Among the three cnidae subtypes, only the nematocysts are known to contain venom. The production of venom from other classes of cnidae is uncertain. Nematocysts are present on tentacles, acrorhagi, acontia and mesentrial filaments and their distribution differs (Macrander et al., 2016; Kass-Simon and Scappaticci 2002). The nematocyst has a small vesicle loaded with venom, inner filament, and external sensory hair. A touch to the hair mechanically sets off a cell explosion, that releases a harpoon-like structure that pins to and injects a dose of venom in the predator (Beckmann and Özbek, 2012)). Besides nematocysts, venom is also secreted from the ectodermal cells (Moran et al., 2012b).

3. Role of venom and other chemical cues in the ecological interactions of sea anemone

All organisms produce, emit, detect, and respond to the chemical signals in their environment. These molecular levels of interactions play an important role in fine-tuning processes that increase the survival of a species in its habitat, such as feeding or prey capture, reproduction, optimizing the survival conditions for the offspring, or avoiding potential predators (Klaschka, 2009).

To understand the role of venom toxins, it is important to know when and for what purpose the venom is employed. Venom toxins of sea anemones are perhaps the most functionally distinct (Schendel et al. (2019)). Sea anemones are covered with nematocytes and toxin-secreting epidermal gland cells and do not have a centralized venom delivery system (Moran et al. (2012b)). The venom varies dramatically across the body Macrander et al. (2016) and even tentacle types (Ashwood et al. (2021a)). Therefore, the ecological role of toxins can be understood from the distribution of venom across the body of the sea anemone. Ashwood et al. (2021b) reported that the regionalization of toxin production remains consistent with the segregation of the ecological functions of venom across the envenomating structures. For example, venom toxins in the tentacles aid in prey capture and defense, whereas those in the acrorhagi are responsible for intra-specific aggression, in the mesenterial filaments for food digestion, in acontia for self-defense, and in the gametes for protection of the eggs and zygote

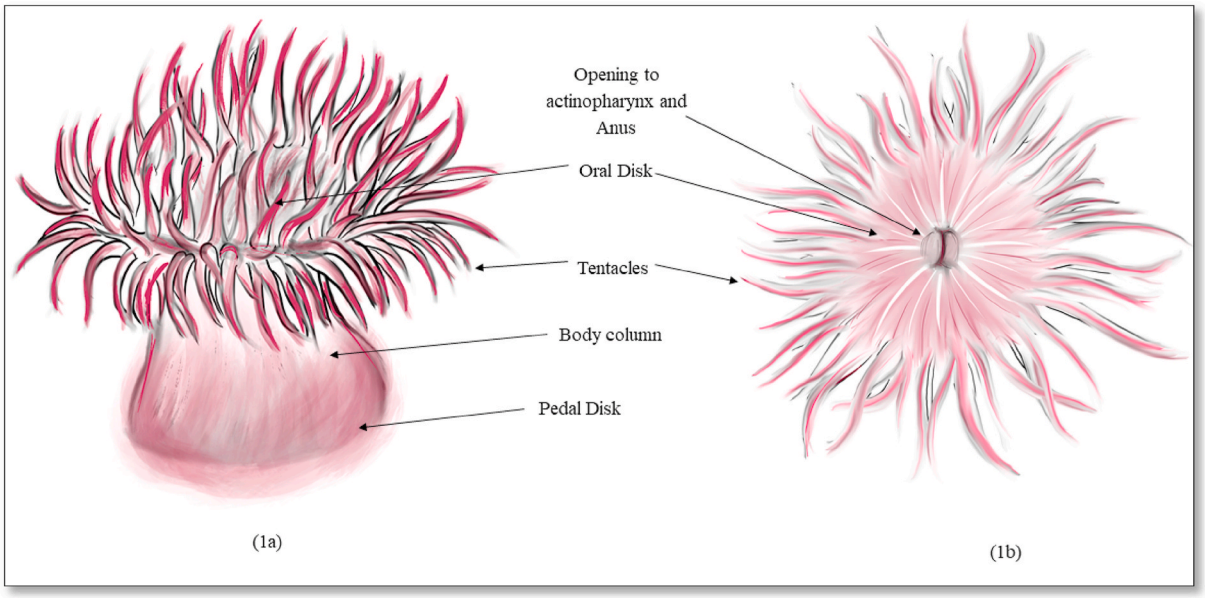


Fig. 1. a: External side-view of the anatomy of a Generalized Sea anemone. The body is a cylindrical column with the oral disk comprising of a common opening to the actinopharynx and anus, and a ring of tentacles, and pedal disk that attaches to the substratum; Fig. 1b: Top-view of a sea anemone showing the oral disc with tentacle and a common opening to the actinopharynx and anus.

(Basulto et al.,2006; Beckmann and Özbek (2012); Surm et al. (2019), Madio et al. (2018); Macrander et al. (2015)). Additionally, sea anemones have different venom toxins during different stages of their life cycle (Columbus-Shenkar et al. (2018)).

3.1. Feeding

Sea anemones are typically predators that capture and feed on smaller prey, but some species also show symbiosis with other organisms in their habitat species (Baker (1931)), while few others are known to be parasitic during their larval stages (Panikkar (1938)).

3.1.1. Predation

Sea anemones rely upon chemical and mechanical cues to detect prey (Watson et al. (2009); Watson & Mire (2004); Watson & Hessinger (1989)). Chemoreceptors and mechano-receptors located on the supporting cells of the tentacles (Watson and Hessinger, 1988) control the nematocyst (Thorington and . Hessinger, 1990). Thorington and Hessinger (1988) successfully triggered the discharge of nematocyst by *Diadumene lineata* and *Exaiptasia diaphana* when chemosensitized by N-acetylneuraminic acid (NANA) and proline. Weir et al., 2020 proposed that the Cav 2.1 voltage-gated calcium channel orthologue (nCaV) plays a role as molecular filters for sensory discrimination and demonstrated that nematocytes from *Nematostella vectensis* used this specialized (nCaV) to integrate dynamic voltage signals produced by distinct sensory stimuli. Some deep-sea sea-anemone species use lures to attract their prey. A novel fluorescent protein cjFP510 had been isolated from the tentacles of deep-sea anemone *Cribrinopsis japonica.* The biological role of this protein has been proposed to be the attraction of prey (Tsutsui et al., 2016). Frank and Bleakney (1978) reported that exposure to *Nematostella vectensis* Nv1 toxin causes paralysis in arthropods. Macrander, Moran & Reitzel (2017) and others evaluated the effects of *Nematostella vectensis* Nv1, NvePTx1, NEP3 toxins on *Fundulus* larvae, nematode and *Artemia* copepod preys. The extracellular NANA and glycine, originating from wounded prey, cause the release of captured prey to allow ingestion (Thorington and Hessinger, 1998). Sea anemones are rich in neurotoxins and cytolytic toxins that are responsible for the paralysis, immobilization and death of the prey. Granulitoxin and Cangitoxin from *Bunodosoma cangicum* is potent neurotoxin responsible for the paralysis and immobilization of the prey (Santana et al. (1998); Zaharenko et al. (2008b)). Crassicorin reported from the pharynx of *Urticina crassicornis* is a neurotoxin responsible for the paralysis of prey (Kim et al. (2017)). Likewise, Kaliseptine from Anemonia sulcata (Schweitz et al. (1995); Ishida et al. (1997) are voltage-gated ion channel toxins that are responsible for the paralysis and immobilization of prey. A lethal toxin (AvTX-60A) was discovered in sea anemone *Actineria villosa* which kills its prey by a pore-forming mechanism (Oshiro et al., 2004). Alvarado-Mesén et al. (2019) reported the role of a cytotoxin Nigrelysin in the prey capture of *Anthopleura nigrescens.* Similarly, Caissarolysin I (Bcs I), Bandaporin, Tenebrosins, Equinatoxin and Magnificalysin are actinoporins reported in the venom of *Bunodosoma caissarum, Anthopleura asiatica*, *Actinia tenebrosa*, *Actinia equina* and *Heteractis magnifica* respectively that are involved in cytotoxic cell death of the prey (de Oliveira et al. (2006); Kohno et al. (2009); Norton et al. (1990); Ferlan and Lebez (1974); Šuput (2014), Khoo et al. (1993)).

Some sea anemone preys have developed a mechanism to escape the predator when captured. Kicklighter and Derby (2006) reported that the ink and other metabolites in *Aplysia californica* secretions were aversive to the predator anemone *Anthopleura sola*. The sea hare escapes from *A. sola* by eliciting tentacle shrivelling and/or retraction, which lead to anemones dropping ensnared sea hares.

3.1.2. Parasitism

The larval stages of certain species of sea anemones are known to be parasitic to other marine organisms. Spaulding (1972) reported that the tentacles of jellyfish *Clytia gregaria, Clytia hemisphaerica, Aequorea forskalea, itrocoma cellularia,* and *Mitrocomella polydiodemata* are parasitized by the larvae of burrowing sea anemone *Peachia quinquecapitata,* where it feeds on the gonads and other tissues of its host before liberating as juveniles. Similarly, the planula of *Edwardsiella lineata* is an endoparasite that infects the ctenophore *Mnemiopsis leidyi* (Reitzel et al., 2006). To date, there are no reports on the kairomones that induce parasitic behaviour in the cnidarian.

3.1.3. Symbiosis

Even though sea anemones produce toxic compounds, many species of these cnidarians have mutualistic relationships with other marine organisms. The most common symbionts are the single-celled algae, either Zooxanthalae and/or Zoochlollerae that live in the gastro-dermal cells of the oral disc and tentacles (Fig. 2a) where they receive protection and ensured availability of sunlight and nutrients from the metabolic waste from its host and in turn, the cnidarians receive oxygen and food such as glycerine, glucose, and alanine (Ruppert, 2004). Terminal sugar residues, Mannose-Mannose, Galactose-β-(1–4)-N-acetylglucose amine along with other glycoproteins present on the cell wall of symbiotic zooxanthellae are responsible for the establishment of symbiosis between the single-celled algae and sea anemone *Exaiptasia diaphana* (Lin et al., 2000).

Sea anemones also exhibit symbiosis with some species of fish and invertebrates which are immune to their sting (Fig. 2c). This immunity can be innate as well as acquired via the process of acclimatization which leads to chemical camouflaging in the fish (Elliott and Mariscal, 1997). The fish receives protection from the predator. The fish aids in better circulation of oxygen helps in removal of parasites and necrotic tissue from the cnidarian and exchange of nutrients also occurs (Mariscal, 1970). Murata et al. (1986) studied the species-specific partnership between *Heteractis crispa* (Sea anemone) and *Amphiprum perideraion* (Anemonefish), and *Stichodactyla gigantea* and *A. ocellaris* (Anemonefish). This study revealed the presence of a new pyridinium compound, Amphikueumin from *Heteractis crispa* which induces characteristic attracted swimming in *A. perideraion*. Similarly, the sea anemone *Stichodactyla gigantea* released tyramine and tryptamine that incites the symbiotic fish *A. ocellaris* to swim towards it with a tail-wagging and active searching habit. Another study was conducted by Miyagawa and Hidaka (1980) to understand the chemical attraction of *Amphiprion clarkii* juvenile by symbiotic sea anemones *Exocoelactis actinostoloides, Exocoelactis actinostoloides,* and *Stichodactyla haddoni* . The results of this caging experiment demonstrated that the juvenile *Amphiprion clarkii* is attracted to symbiotic sea anemones by some chemical substances released from them and not by any visual cues and that they can discriminate the symbiotic sea anemone species by some chemical substances released from their symbiotic sea anemone.

Many species of crabs and gastropods harbour sea anemones on their appendages or carapace/shells (Fig. 2b) to camouflage in their environment and the sea anemone in turn receives tiny chunks of food while

the host is feeding and protecting from predators (Guinot et al. (1995). *Hepatus ephiliticus* initiated the transfer of the sea anemone *Calliactis tricolor* onto its body by rubbing the tentacles of the cnidarian on its carapace (Guinot et al., 1995). Brooks and Rittschof (1995) reported that the porcellanid crab *Porcellana sayana* is also strongly attracted chemically to the symbiont sea anemone *Calliactis tricolor*, however, the active molecules that emanate from *C. tricolor* and attract the crabs is still unknown. The capability of the hermit crabs *Dardanus venosus* and *Pagurus pollicaris* to identify their symbiotic sea anemone *Calliactis tricolor* chemically was reported by Brooks (1991). Izumi et al., 2018, reported for the first time a symbiotic relationship between a homoscleromorph sponge (*Oscarella* sp.) and Sea anemone (*Tempuractis rinkai*) (Fig. 2d). The sea anemone hides inside the sponge during a potential attack by a predator. In turn, the sea anemone helps to deter the sea slug *Berthella* sp. which is known to feed upon homoscleromorph sponges.

3.2. Larval recruitment

Recruitment is the process by which young individuals undergo larval settlement and become part of the adult population. This is achieved through the detection of physical and/or chemical cues. Space for larval recruitment of *Condylactis gigantea* is ensured in a space-restricted environment due to the toxins it produces (Bak and Borsboom, 1984). The toxin NvePtx1of sea anemone *Nematostella vectensis* protects eggs and planulae from fish predators. (Columbus-Shenkar et al., 2018). Mussel beds of *Mytilus californianus* serve as an important refuge site for the settlement of the *Anthopleura xanthogrammica* juvenile population. This indicates that the juveniles' ability to detect the location of mussel beds via chemical cues. The juveniles later migrate from juvenile habitat to adult habitat, dislodge and prey upon the mussels (Sebens, 1981). Weis et al. (2005) attempted to induce settlement and metamorphosis of *Anthopleura elegantissima* and *A. artemisia* larvae using various inducers such as Metamorphosin A, 12-O-tetradecanoylphorbol- 13-acetate, Phorbol 1 2,13- dibutyrate, KCl, NH_4Cl, γ -aminobutyric acid (GABA) and 3,4–di hydroxyphenylalanine (DOPA). They were not successful in their attempts probably due to other biological and physical parameters such as light intensity, duration, age of the larvae, and combination of chemical cues which were not considered during the study.

3.3. Spatial competition

Marine organisms compete for space in crowded ecosystems such as tidepools (Fig. 3) and coral reefs. The organisms may crowd, undercut, crush, overshadow, overgrow, digest, or poison their neighbors (Quinn, 1982; Branch, 1984).

Aggression involves very exquisite intra-and interspecific recognition (Bigger, 1982). Most cnidarians use their venom to inhibit growth or kill their spatial competitors. Catch tentacles were used in intraspecific and interspecific aggression among *Cereus pedunculatus, Cylista elegans, and Cylista troglodytes* (Williams (1975)). The acrorhagi are specialized nematocysts filled sacs containing a cocktail of venom that is used to cause damage during spatial competition with non-clonal intraspecific individuals (Jenner and Undheim (2017); Surm et al. (2019); Ayre and Grosberg (1995)) (Fig. 4). An injured anemone closes, moves away, and sometimes may release its pedal disc from the substratum (Brace (1990)). In the anemones, *Cereus pedunculatus, Cylista elegans, and Cylista troglodytes*, the catch tentacles were used offensively against members of their species from different localities and other species (Purcell (1977)). Bak and Borsboom (1984) studied the allelopathic interaction between the giant sea anemone *Condylactis gigantea* and benthic algae. Algal biomass and spore germination was inhibited by the cnidarian exudates consisting of terpenes.

3.4. Defence against predators

Sea anemones are soft and stationary organisms making them very vulnerable to predation. The anti-predatory mechanism can be defensive as well as offensive. The sea anemones can either actively defend themselves by using their tentacles to sting the predator or it can retract their tentacles and column, exposing the predator to its ectodermal mucus on the body column.

The sea anemone venom contains various compounds of proteinic nature including pore-forming toxins or Cytolysin, acid-sensing ion channel toxins, phospholipases Na^+ channel toxins, K^+ channel inhibitors, other neurotoxins, haemolytic toxins, and proteinase inhibitors (Hessinger and Lenhoff, 1988). The defensive behaviour of sea anemones, when attacked by the nudibranch *Aeolidia papillos*, was studied by Edmunds et al. (1976). They reported that *Anemonia sulcata* uses its tentacles to defend itself against the predator, whereas *Actinia equina* responds by tentacle and column retraction, and inflating its column, exposing its acrorhagi to the eolid. *Anthopleura elegantissima* responds in similar ways. *Anemonia* crawls away but it does not detach. Exposure to the Nv1 toxin of the starlet sea anemone *Nematostella vectensis* leads to contraction paralysis of its predator the grass shrimps (Kneib, 1985). Similarly, Christomanos (1953) isolated purple pigment and protein threads from *Adamsia rondeletii* and suggested that its possible function was in self-defence of the sea anemone. Sea anemones are known to disperse alarm pheromones to alert their conspecifics against predators as a strategy to reduce predation. Howe and Sheikh (1975) reported that anthopleurin acts as a toxin in addition to an alarm pheromone. When attacked by a predator, *Anthopleura elegantissima* immediately withdraws its tentacles and oral disc. Now the predator is exposed only to the body of the sea anemone that contains the highest amount of anthopleurin. After feeding upon the sea-anemone, the predator travels through the water and aids in the spread of anthopleurin, alerting conspecifics to defend themselves (Howe, 1976). *Anthopleura* sp. moves away from injured or crushed conspecifics, indicating the release and detection of alarm pheromones (Howe and Sheikh, 1975). Similarly, the attack by *Clinocottus globiceps* leads to the alarm response in nearby *A. xanthogrammica* (Hand, 1996). Acontia, a unique defence tissue consisting of thread-like structures containing numerous stinging cells, located in the gastrovascular cavity of anemone. When threatened, the sea anemone ejects the acontia through its actinopharynx, and toxins are injected into the predator (Lam et al. (2017)). Edmunds et al. (1976) studied the behaviour of sea anemones when attacked by Aeolidia and reported that *Cylista elegans, Cereus pedunculatus, Aiptasia couchii, Actinothoe sphyrodeta* and *Metridium senile* eject acontia as a defence response.

4. Types of sea anemone venom

The venom composition is not known in detail, but it mainly consists of proteinaceous substances such as peptides, proteins, enzymes, and proteinase inhibitors and non-proteinaceous molecules like purines, quaternary ammonium compounds, biogenic amines, and betaines

(Martins et al., 2009; Jouiaei et al., 2015b; Frazão et al., 2012; Mariottini and Pane, 2014). This venom cocktail varies from species to species but predominantly consists of molecules that act as neurotoxins, cytotoxins and induce pain and inflammation (Surm et al., 2019; Mariottini and Pane, 2010; Honma and Shiomi, 2006; Madio et al., 2019).

4.1. Cytotoxins

Cnidarian venom is known to exhibit cytotoxic activity, which induces necrosis, apoptosis, prevents cell growth. Therefore, the ecological function of cytotoxins is to aid in effective prey capture and defence against predators and competitors (Anderluh and Macek (2002)). Table 1.

4.1.1. Pore-forming toxins (PFT)

These are proteins that disrupt biological membranes by lesion or pore formation. The plasma membrane permeability of the target cells is altered, possibly causing cell death (Los et al., 2013).

4.1.1.1. Actinoporins. The 'sea anemone pore-forming proteins' are collectively referred to as Actinoporins, a sub-class of Pore-forming toxins, a type of cytolysin.

4.1.1.2. Membrane attack complex/Perforins (MACPF). Perforins are cytolytic pore-forming proteins that bind to cell membranes and oligomerize to form pores up to the size of 20 nm in the cell, leading to cytolysis (Liu et al., 1995). MACPF is used by sea anemones to kill prey and deter predators (Oshiro et al., 2004)).

4.1.1.3. Hydralysins-related toxin. Hydralysins are non-nematocyte-derived beta-pore-forming toxins in cnidaria, discovered in the digestive endodermal cells of Chlorohydra viridissima (Zhang et al., 2003; Sher et al., 2005). They play a role in the lysis and digestion of prey by binding to specific receptors and forming pores in the tissue (Sher et al., 2005; Sher et al., 2008; Moran et al., 2012b).

4.1.2. Phospholipases

Enzymes such as phospholipases constitute a small component of sea anemone venom but are very poorly studied (Madio et al., 2019). These are enzymes that catalyse the hydrolysis of 2-acyl ester bonds of 3-sn-phospholipids leading to fatty acids and lysophospholipids production (Yu et al., 1993).

4.1.3. Metalloprotease

These are metal-dependent proteases that degrade the extra-cellular matrix causing necrosis and haemorrhage and preventing blood clot formation in the prey or predator (Fox and Serrano (2005); Fry et al. (2009)).

4.2. Neurotoxins

Neurotoxins are low molecular weight peptides that disrupt nerve tissue (Madio et al., 2019). Neurotoxicity is caused by blocking neuron cellular processes such as inter-neuron communication and membrane depolarization by the toxin. The major ecological role of neurotoxins is the paralysis and immobilization of the prey (Madio et al., 2019). Table 1.

4.2.1. Voltage-gated potassium (Kv) channel toxins (KTxs)

These toxins inhibit the potassium current through channels Kv1. and Kv3. subfamilies and intermediate conductance calcium-activated potassium channels (Frazão et al., 2012). PHAB is a type of KV toxins and is the shortest sea anemone toxin comprising of 17 residues, κ-actitoxin-Ate1a (Ate1a) peptide. It plays a role in prey immobilization that is achieved through inhibition of Shaker-type KV channels (Madio et al., 2018).

4.2.2. Voltage-gated sodium (Nav) channel toxins (NaTxs)

Voltage-gated ion channels are transmembrane proteins that facilitate the electrical signalling of cells. NaTxs bind to the Nav channels resulting in the interference in generation and conduction of action potential and release of neurotransmitters in synapses (Gilchrist et al. (2014)).

4.3. Kunitz peptides

The Kunitz-type protease inhibitors belong to the serine protease inhibitors family. Kunitz peptides reported in sea anemones display Kv blocking activity and protease inhibiting activity (Peigneur et al.,2011; Schweitz et al., 1995). Table 1.

4.4. Acid sensing ion channel blockers/inhibitors

ASICs are a family of voltage insensitive and proton-sensing channels which are responsible for Na^+ influx and play important roles in mediating pain sensation (Waldmann et al., 1997, Dibas et al. (2019)). Peptides with ASIC inhibition potential have been recently discovered in Cnidarians (Osmakov et al. (2013); Rodríguez et al. (2014)). Their ecological role is to repel potential predators by inducing pain (Jami et al. (2018)) Table 1.

4.5. TRPV1 inhibitor

TRPV is a highly calcium-selective family of transient receptor potential cation channels (TRP channels) in animals (Winston and Lutz, 1988). TRPVs are molecular triggers of pain stimulus and TRPV inhibitors attenuate the expression of TRPV1 and thus can be used to develop analgesics (Jouiaei et al., 2015b) Table 1.

4.6. Acrorhagin

Acrorhagins are di-sulphide-rich peptides isolated from the Acrorhagi of sea anemone *Actinia equinia* (Krishnarjuna et al., 2021). Acrorhagin I have no homologies with any known toxins, while acrorhagin II is similar to spider neurotoxins hainantoxin-I and Tx 3–2 and cone snail neurotoxin ω-conotoxin MVIIB (Honma et al., 2005b). The acrorhagi toxins of *Actinia equina* induce tissues necroses by the intracellular formation of reactive oxygen species while lacking the neurotoxic activity (Bartosz et al., 2008). The ecological function of Acrorhagin is to deter encroaching intra-specific spatial competitors by causing paralysis and necrosis of the tissue Table 1.

Table 1
List of Venom peptides with the source organism, toxin type, and ecological function.

Sr. No.	Peptide	Source organism	Toxin type	Ecological function	Reference
1	Fra-E	*Actinia fragacea*	Actinoporins	Prey capture or/and defense against predators and competitors	Morante et al. (2019)
2	Nigrelysin	*Anthopleura nigrescens*	Actinoporins	Prey capture or/and defense against predators and competitors	Alvarado-Mesén et al. (2019)
3	CJTOX I and CJTOX II	*Cribrinopsis japonica*	Actinoporins	Prey capture or/and defense against predators and competitors	Tsutsui and Sato (2018)
4	RTX-A, RTX-S, RTX-G, RTX-S II, Hct-A and Hct-S4	*Heteractis crispa*	Actinoporins	Prey capture or/and defense against predators and competitors	Leichenko et al. (2014); Tkacheva et al. (2011); Klyshko et al. (2004); Monastyrnaia et al. (1999); Monastyrnaya et al. (2002).
5	Fragaceatoxin C	*Actinia fragacea*	Actinoporins	Prey capture or/and defense against predators and competitors	Bellomio et al. (2009)
6	Gigantoxin-4	*Stichodactyla gigantea*	Actinoporins	Prey capture or/and defense against predators and competitors	Hu et al. (2011)
7	Caissarolysin I (Bcs I)	*Bunodosoma caissarum*	Actinoporins	Prey capture or/and defense against predators and competitors	de Oliveira et al. (2006)
8	Avt-I	Globular vesicles of the tentacles of *Actineria villosa*	Actinoporins	Prey capture or/and defense against predators and competitors	Uechi et al. (2005)
9	Or-A and Or-G	*Oulactis orientalis*	Actinoporins	Prey capture or/and defense against predators and competitors	Il'ina et al. (2005)
10	Src I	*Sagartia elegans*	Actinoporins	Prey capture or/and defense against predators and competitors	Jiang et al. (2003)
11	PsTX-20A	*Phyllodiscus semoni*	Actinoporins	Prey capture or/and defense against predators and competitors	Nagai et al. (2002)
12	Bandaporin	*Anthopleura asiatica*	Actinoporins	Prey capture or/and defense against predators and competitors	Kohno et al. (2009)
13	Unnamed pore-forming protein	*Anthopleura dowii*	Actinoporins	Prey capture or/and defense against predators and competitors	Ramírez-Carreto et al. (2019)
14	Tenebrosins TN-A, TN-B, TN-C	*Actinia tenebrosa*	Actinoporins	Prey capture or/and defense against predators and competitors	Norton et al. (1990)
15	Equinatoxin II	*Actinia equina*	Actinoporins	Prey capture or/and defense against predators and competitors	Ferlan and Lebez (1974)
16	U-AITx-Ate 1	*Actinia tenebrosa*	Cytotoxin	Prey capture or/and defense against predators and competitors	Elnahriry et al. (2019)
17	Magnificalysin I, Magnificalysin II, HMg III, Cytolysin HmT	*Heteractis magnifica*	Actinoporins	Prey capture or/and defense against predators and competitors	Khoo et al. (1993); Wang et al. (2000); Samejima et al. (2000).
18	Src-1	*Sagartia elegans*	Actinoporins	Prey capture or/and defense against predators and competitors	Jiang et al. (2002)
19	SmT-2,SmT-2	Stichodactyla mertensii	Actinoporins		Samejima et al. (2000)

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Table 1 (*continued*)

Sr. No.	Peptide	Source organism	Toxin type	Ecological function	Reference
				Prey capture or/and defense against predators and competitors	
20	UpI	Urticina piscivora	Actinoporins	Prey capture or/and defense against predators and competitors	Cline et al. (1995)
21	AvTX60	Nematocysts of *Actineria villosa*	Membrane attack complex/Perforins	Prey capture or/and deter predators	Oshiro et al. (2004)
22	PsTX-60B	*Phyllodiscus semoni*	Membrane attack complex/Perforins	Prey capture or/and deter predators	Satoh et al. (2007)
23	Sticholysin I and II	*Stichodactyla helianthus*	Phospholipase	Prey capture or/and deter predators	del Monte-Martinez et al. (2014); Anderluh and Macek (2002)
24	Unnamed	Tentacle Venom of *Condylactis gigantea*	Phospholipase	Prey capture or/and deter predators	Romero et al. (2010)
25	PLA2	*Bunodosoma caissarum*	Phospholipase	Prey capture or/and deter predators	Martins et al. (2009)
26	Aerolysin	*N. vectensis*	Hydralysin-related toxin	Lysis and digestion of prey	Moran et al. (2012a)
27	Zinc dependent metalloproteinase	*Epiactis japonicus*	Metalloproteinase	Prey capture or/and deter predators	Babenko et al. (2017).
28	Disintegrin/Zinc dependent metalloproteinase	*Aulactinia verrucose*	Metalloproteinase	Prey capture or/and deter predators	Domínguez-Pérez et al., 2018.
29	Granulitoxin	*Bunodosoma cangicum*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Santana et al. (1998)
30	AeK	*Actinia equina*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Minagawa et al. (1998)
31	APETx1, APETx2, APETx3	*Anthopleura elegantissima*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Peigneur et al. (2012)
32	Kappa-actitoxin-Ate1a	*Actinia tenebrosa*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Madio et al. (2018)
33	Cangitoxin II and Cangitoxin III	*Bunodosoma cangicum*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Zaharenko et al. (2008a)
34	Blood-depressing substance-1 (BDS-1), also known as kappa-actitoxin-Avd4a,	*Anemonia sulcata*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Diochot et al. (1998); Liu et al. (2012)
35	Crassicorin-I and Crassicorin-II	The pharynx of *Urticina crassicornis*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Kim et al. (2017)
36	AnerK, AETX I, AETX II, AETX III, AETX-K	Anemonia erythraea	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Hasegawa et al. (2006); Shiomi et al. (1997)
37	Kaliseptine	Anemonia sulcata	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Schweitz et al. (1995).
38	BcsTx1, BcsTx2 and BcsTx3	Bunodosoma caissarum	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Orts et al. (2013a); Orts et al. (2013b)
39	Bcg III 21.75	*Bunodosoma cangicum*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Osmakov et al. (2013)
40	Bgk, U-AITX-Bg1a-e	*Bunodosoma granuliferum*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Aneiros et al. (1993); Rodríguez et al. (2012)
41	Ca I and CaK	*Cryptodendrum adhaesivum*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Maeda et al. (2010); Yamaguchi et al. (2010)
42	Halcurin	*Halcurias carlgreni*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Ishida et al. (1997)
43	HmK	*Heteractis magnifica*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Gendeh et al. (1997)
44	OspTx2a	Oulactis sp	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Sunanda et al. (2018)
45	Metridin	Metridium senile	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Krebs & Habermehl (1987)

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Table 1 (*continued*)

Sr. No.	Peptide	Source organism	Toxin type	Ecological function	Reference
46	SgK, SHTX I/SHTX II, shtx-k	*Stichodactyla gigantea*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Yamaguchi et al. (2010); Honma et al. (2008)
47	SmK	*Stichodactyla mertensii*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Yamaguchi et al. (2010)
48	ShK	*Stichodactyla helianthus*	Voltage-gated Potassium Channel Toxins	Paralysis and immobilization of prey	Castañeda et al. (1995)
49	Gigantoxin-II and Gigantoxin-III	*Stichodactyla gigantea*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Shiomi et al. (2003)
50	Av3	*Anemonia viridis*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Moran et al. (2007)
51	Hk7a and Hk2a	*Anthopleura* sp.	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Xiang et al. (2008)
52	Cangitoxin	*Bunodosoma cangicum*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Cunha et al. (2005)
53	BcIII and BcIV	*Bunodosoma caissarum*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Oliveira et al. (2006)
54	Nv1	Ectodermal gland cells of *Nematostella vectensis*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Moran et al. (2012a)
55	NvePtx1	Eggs and Planula of *Nematostella vectensis*	Voltage-gated Sodium Channel Toxins	Protection of eggs and planula from predator	Columbus-Shenkar et al. (2018)
56	CgNa	*Condylactis gigantea*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Billen et al. (2010)
57	ApC	*Anthopleura elegantissima*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Salceda et al., 2006
58	Av3	*A. elegantissima*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Gur Barzilai et al., 2014
59	RpII	*Heteractis magnifica* mucus	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Sintsova et al. (2018)
60	Calitoxin I and Calitoxin II	*Calliactis parasitica*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Cariello et al. (1989); Spagnuolo et al. (1994)
61	ATX-I, ATX-II and ATX-III	*Anemonia sulcata*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Alsen, (1983)
62	Peptide toxin Am I, Peptide toxin Am II and Peptide toxin Am III	*Antheopsis maculata*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Honma et al., 2005a
63	Toxin AFT-I and Toxin AFT-II	*Anthopleura fuscoviridis*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Sunahara et al. (1987)
64	Anthopleurin-B, Toxin PCR1, Toxin PCR2, Toxin PCR3, Toxin PCR4, Toxin PCR5, Toxin PCR6, Toxin PCR7	*Anthopleura xanthogrammica*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey. Alarm pheromone	Norton et al., 1981; Kelso and Blumenthal, 1998; Howe and Sheikh. (1975)
65	Ade-1	*Exaiptasia diaphana*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Nesher et al. (2013)
66	Bc-III and Bc-IV	*Bunodosoma caissarum*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Oliveira et al. (2004); Oliveira et al. (2006)
67	Delta-actitoxin-Bca1a	*Bunodosoma capens*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	van Losenoord et al. (2019)
68	Bg II and Bg III	*Bunodosoma granuliferum*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Loret et al. (1994)
69	Toxin AnmTx Cj 1c-1, Toxin CjTL7, Toxin CjTL8	*Epiactis japonica*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Babenko et al. (2017)
70	Re–I, RpII, RpIII, Neurotoxin-I, Neurotoxin-II, Neurotoxin-III, Neurotoxin-IV, and Hcr 1b-3, Hcr 1b-2, Hcr 1b-4.	*Heteractis crispa*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Shiomi et al., 1996; Zykova, 1985; Zykova et al. (1988a); Zykova et al. (1988b); Zykova and Kozlovskaia (1989); Kalina et al. (2018); Kalina et al. (2020); Metrione et al. (1987)
71	SHTX IV	Stichodactyla haddoni	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Honma et al. (2008)
72	U-SHTX-Sdd1	*Homostichanthus duerdeni*	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Cassoli et al. (2013)
73	MS9.1	Metridium senile	Voltage-gated Sodium Channel Toxins	Paralysis and immobilization of prey	Logashina et al. (2017a).
74	APEKTx1	*Anthopleura elegantissima*	Kunitz peptides	Paralysis and Death of prey	Peigneur et al. (2011)
75	AXPI-I and –II	*Anthopleura xanthogrammica*	Kunitz peptides	Paralysis and Death of prey	Minagawa et al. (1997)
76	Unammed Kunitz-type peptides	The mucus of *Heteractis magnifica*	Kunitz peptides	Paralysis and Death of prey	Sintsova et al. (2018)
77	HC1, HC2, HC3, HCRG, HCRG1, HCRG2, HCRG21	*Heteractis crispa*	Kunitz peptides	Paralysis and Death of prey	Kozlov et al. (2009); Gladkikh et al. (2020); Monastyrnaya et al. (2016); Gladkikh et al. (2015); Gladkikh et al. (2012)
78	AEAPI,	Acrorhagi venom of *Actinia equina*	Kunitz peptides	Paralysis and Death of prey	Minagawa et al. (2008)
79	AXAPI		Kunitz peptides		Minagawa et al. (2008)

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Table 1 (*continued*)

Sr. No.	Peptide	Source organism	Toxin type	Ecological function	Reference
		Acrorhagi venom of *Anthopleura xanthogrammica*		Paralysis and Death of prey	
80	AFAPI-I and AFAPI-III	Acrorhagi venom of *Anthopleura fuscoviridis*	Kunitz peptides	Paralysis and Death of prey	Minagawa et al. (2008)
81	Iq-peptide	*Heteractis magnifica*	Kunitz peptides	Paralysis and Death of prey	Kvetkina et al. (2018)
82	ShPI-1, also known as the bovine pancreatic trypsin inhibitor (BPTI)-Kunitz-type protein	*Stichodactyla helianthus*	Kunitz peptides	Paralysis and Death of prey	García-Fernández et al. (2016)
83	Kalicludine-1, Kalicludine-2, and Kalicludine-3	Anemonia sulcata	Kunitz peptides	Paralysis and Death of prey	Schweitz et al. (1995)
84	SHTX II	*Stichodactyla haddoni*	Kunitz peptides	Paralysis and Death of prey	Honma et al. (2008)
85	Gigantoxin I, SHTX V	*Stichodactyla gigantea*	SCRiP	Bacterial defence	Honma et al. (2008); Shiomi et al. (2003).
86	Ueq 12-1	*Urticina eques*	SCRiP	Bacterial defence	Logashina et al. (2017b)
87	Msen-SCRiP	*Metridium senile*	SCRiP	Bacterial defence	Jouiaei et al. (2015a)
88	π-AnmTX Ugr 9a-1	*Urticina grebelnyi*	ASIC inhibitors	Repel potential predators	Osmakov et al. (2013)
89	PhcrTx1	*Phymanthus crucifer*	ASIC inhibitors	Repel potential predators	Rodríguez et al. (2014)
90	τ-SHTX-Hcr2b (APHC1), (τ-SHTX-Hcr2c (APHC2) and τ-SHTX-Hcr2d (APHC3)	*Heteractis crispa*	TRPV1 Inhibitors	Trigger pain in predator or competitor	Andreev et al. (2008); Kozlov et al. (2009).
91	Acrorhagin I, II	*Actinia equina*	Arorhagin	Deter encroaching intraspecific competitor	Honma et al. (2005b)
92	κ-Actitoxin-Ate1a	*Actinia tenebrosa*	PHAB	Paralysis and immobilization of prey	Madio et al. (2018).

4.7. Non-proteinaceous molecules

Heteractis crispa has been reported to produce Palytoxin (Fig. 5a), a polyether, that is a vasoconstrictor and one of the most poisonous non-proteinaceous toxins known to date (Mahnir et al., 1992). Bunodosine 391 is an acylated amino acid discovered from *Bunodosoma cangicum* (Fig. 5b). It is reported to act on serotonin receptors and has analgesic activity (Zaharenko et al., 2011). Caissarone (3,9-dimethyl-6-methylimino-8-oxo-3,6,8,9-tetrahydropurine) is an iminopurine from *Bunodosoma caissarum* which is the first marine compound reported as an adenosine receptor antagonist (Fig. 5c). Caissarone is reported to increase motility in isolated mammalian gut preparations (Cooper et al., 1995). 1,2-iminomethyl-3-methyl-6-aminomethyl-9H-purine was extracted from *Cylista troglodytes* (Fig. 5d). (de Rosa *et al.*, 1987).

5. Bioprospecting of sea anemones

The ability of cnidarians to produce powerful venoms is well documented and has attracted many researchers. There are numerous studies reporting the toxic peptides and molecules present in the Actinarian venom (Jouiaei et al. (2015b); Prentis et al. (2018); Madio et al. (2019); D'Ambra and Lauritano (2020); Mariottini & Grice (2019), Ashwood et al. (2020)). The neurotoxic and cytotoxic nature of sea anemone venom can be exploited to develop novel drugs to treat several ailments. Neurotoxins function by blocking cellular processes in the nervous system and other tissues by binding to voltage-gated ion channels of the targeted organism. Since potassium channel toxins block potassium channels, they facilitate the release of the acetylcholine (Diochot and Lazdunski (2009)), which in humans can have analgesic and anaesthetic effects. They can also be therapeutic leads for the treatment of autoimmune diseases (Prentis et al. (2018)). On the other hand, cytotoxins work by killing cells thereby having antimicrobial potential and can be employed in cancer treatment (Chatterjee (2018)). Sintsova et al. (2015) reported the anti-inflammatory activity of the HCGS 1.20 recombinant polypeptide, a Kunitz-typeserine protease inhibitor from *Heteractis crispa.* Peptide Ms 9a-1 isolated from *M. senile* venom exhibited anti-inflammatory activity by potentiating TRPA1 (Logashina et al., 2017a). In a study conducted by Ramezanpour et al. (2012), the venoms of *Heteractis crispa, Heteractis magnifica, Heteractis malu, Cryptodendrum adhaesivum* and *Entacmaea quadricolor* exhibited anti-cancer activity against human lung, breast and skin cancer cells. Loret et al., 2018 reported the anti-angio activity of a low molecular weight protein from *Anemonia viridis.* Cytolysins Sticholysin I and II from Stichodactyla helianthus and equinatoxin II from Actinia equina displayed anti-parasitic activity against Giardia duodenalis (Tejuca et al., 1999). Ramezanpour et al., 2014 reported that the venom of *Heteractis magnifica* killed human breast cancer cell lines via apoptosis. Actinoporin from *Heteractis crispa* exhibited anti-cancer activity *in vitro* as well as prevented the migration of HT-29 Colorectal Cancer Cell (Kvetkina et al. (2020)). Actinoporin RTX-A from *Heteractis crispa* also has potential anti-cancer effects (Fedorov et al., 2010).

Sea anemone venom that is effective in killing crustaceans has also been reported to be effective against insect pests given the close evolutionary relationship between insects and crustaceans, therefore these peptides can be lead molecules in development of novel insecticides (Bosmans and Tytgat, 2007). Fu and fellow workers (2021) reported the anti-insecticidal activity of Peptide AP-GI from *Aiptasia pallida* (Fu et al., 2021). Neurotoxin BgII from *Bunodosoma granulifera* is potent on the insect sodium channel and can be used as a template to develop insecticides (Bosmans et al., 2002). On that account, the study of cnidarian toxin is rising as a promising field in the discovery of novel drug and therapeutic molecules as well as agriculturally and industrially important compounds.

Traditionally, bioactive compounds are extracted from a sample by bioassay-guided fractions and the structure, chemical composition and function are determined (Koehn and Carter (2009)). More recently, in *silico* methods are employed to unveil and identify genes, gene homologs, motifs and transcripts that express certain profiles (Johansen et al. (2010); Yamaguchi et al. (2010); Sperstad et al. (2011) (Urbarova et al., 2012);).

5.1. Sea anemone venom in healthcare

There are many sea anemone venom peptides explored for medical use. All these toxins are involved in neural channel manipulation within the body and cell death (Diochot and Lazdunski (2009); Chatterjee

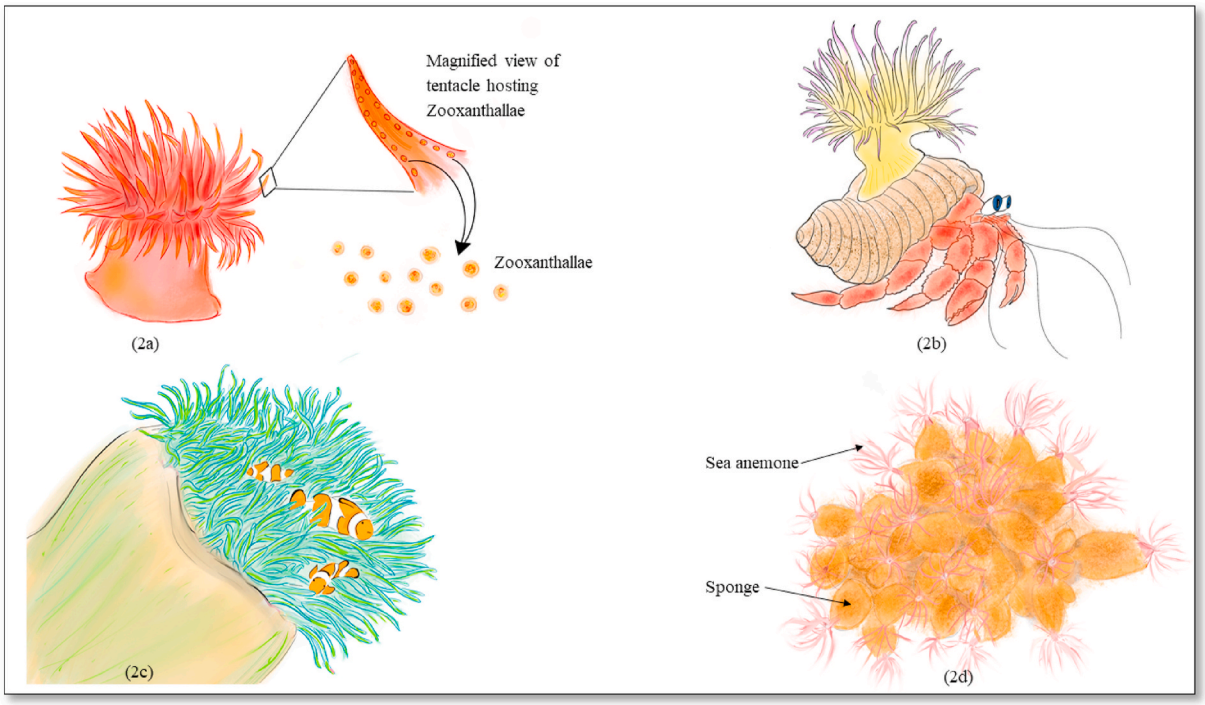


Fig. 2. aSymbiosis between Sea anemone and Zooxanthallae; Fig. 2b: Symbiosis between Sea anemone and Hermit crab; Fig. 2c: Symbiosis between Sea anemone and *Pomocentridae* (Anemone fish); Fig. 2d: Symbiosis between Sea anemone and homoscleromorph Sponge (*Oscarella* sp.).

(2018)). Despite enormous research on sea anemone venom and the discovery of several novel toxins, the number of venom-derived peptides that have successfully advanced to clinical trials is currently limited.

In 1995, Olga Castañeda and her team discovered and characterized a potassium channel toxin ShK from the Caribbean sea anemone Stichodactyla helianthus (Castañeda et al., 1995). Extensive structure-activity studies led to the development of an ShK analog, ShK-186, as a selective Kv1.3. blocker (Beeton et al., 2005, 2006; Pennington et al., 2009). ShK-186 (now known as Dalazatide) is the first drug derived from sea anemone venom that has completed Phase 1 clinical trials (Tarcha et al.,2017) and is ready to undergo Phase II clinical trials for treating several autoimmune diseases such as lupus erythematosus, psoriasis, rheumatoid arthritis, inflammatory bowel disease, type 1 diabetes, and multiple sclerosis (MS).

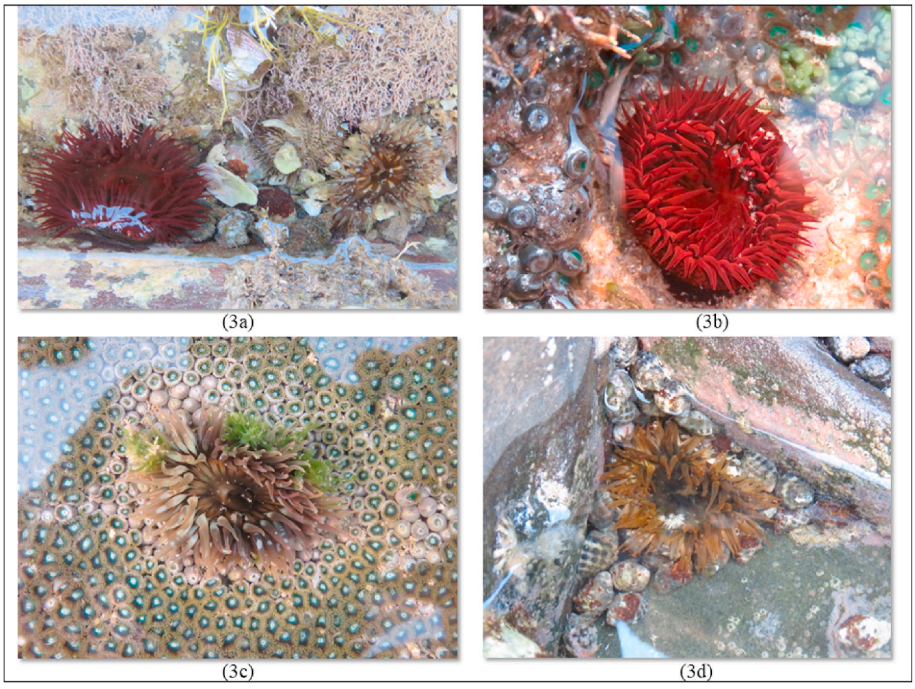


Fig. 3. Images of Sea anemones from the tidepools of the rocky shores of the West Coast of India. This is highly competitive habitat with regard to space. Sea anemones maintain their niche in this habitat by using their venom arsenal to compete with other intertidal organisms. Fig. 3a and **b:** Sea anemone in tidepools along with neighbors- Sea urchin, Macro-algae, sponge, Zoanthus. Fig. 3c & d: Sea anemone seen in space crunch with Zoanthus and Gastropods.

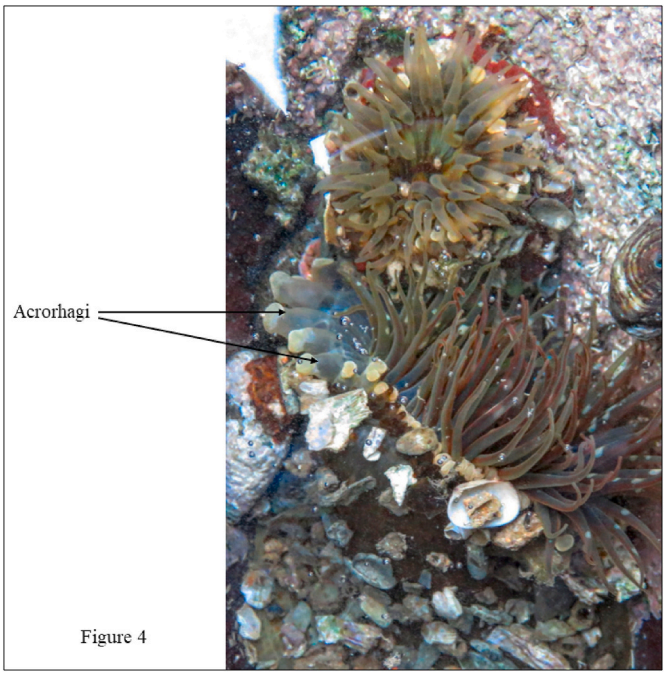


Fig. 4. *Anthopleura anjunae* displaying its acrorhagi during non-clonal intraspecific aggression.

5.2. Other sea anemone bio-products in biomedical applications

Besides medicinal drugs, there are a few other sea anemone-derived molecules that have application in the biomedical industry. Aneroin, a recombinant protein originating from sea anemone *Nematostella vectensis*, is being exploited as a hydrogel. It shows relatively stronger and stiffer properties than collagen, gelatin, and elastin, and four times stronger mechanical properties than silkworm silk. Aneroin finds its application for cell-containing biomaterials, cell-carrier patches, bio-artificial grafts, and burn dressing materials (Yang et al., 2013, 2015). A patent has been granted to the inventor for the method for manufacturing Aneroin (Patent no. US20150057435A1), Postech Foundation and Postech Academy Industry Foundation, Pohang-si, South Korea is the current assignee of the patent. Based on aneroin's durable mechanical properties and biodegradability a new 3DBP ink named aneroin ink has been developed. To improve the ink's printability and cell adhesiveness hyaluronic acid and mussel adhesive protein were respectively incorporated and a life-sized human ear, vascular graft, and rectangular multi-layered lattice were successfully bio-printed. Aneroin ink can further find an application in developing various 3D-shaped bioscaffolds in tissue and biomedical engineering fields (Park et al. (2019). Similarly, Anegen hydrogel is a Load-Bearing Hydrogel Based on Engineered *Nematostella vectensis* Sea Anemone Skin-Derived Collagen-Like Protein that can be developed as a prospective load-bearing and cell-loading scaffolding biomaterial in the tissue and biomedical engineering area (Yang et al., 2018).

HcRed is a far-red fluorescent protein from the sea anemone *Heteractis crispa*, having absorption maxima at 590 nm and emission maxima at 650 nm, showing potential as a fluorescent marker in quantitative microscopy investigations (Lessard et al., 2008).

5.3. Sea anemone as prospective bio-control agents

Research on animal venom to a great extent is being used to develop biopesticides. Equistatin is a protease inhibitor from *Actinia equina*. It is found to be active against gut cysteine and aspartic proteases in common insect pests of crops, such as Coleopteran, Dipteran, and Thysanopteran insects. The gene coding for equistatin is isolated and cloned into vectors and transformed into agricultural plants, thus reducing the insect pest infestation (Lenarcic and Turk, 1999; Lenarcic et al., 1997; Outchkourov et al., 2002, 2003; Valizadeh et al., 2013). There is a patent granted for a method of plant protection from pests using the above-mentioned technology (CA2294421A1) with Stichting Dienst Landbouwkundig Onderzoek as the current assignee. Sea anemone venom toxins, derived particularly from the acontia and acrorhagi, are being studied to develop novel anti-biofoulants. At the same time, laboratory trials on various species of sea anemones as augmentative biocontrol are being conducted to minimize biofouling on marine artificial structures, whilst this work needs to be refined before its use.

6. Future direction of this research

The discovery of novel drug-lead molecules from natural resources is the need of the hour today due to various shortcomings encountered in synthetic compounds. Marine natural products play an important role in the discovery of novel biologically active compounds. While exploring the potential of marine natural products, several bioactive secondary metabolites have been identified. However, very little attention has been given to understanding their ecological functions. Sea anemone venoms are well known as a copious source of peptides with varied pharmacological applications but most of the research on sea anemone toxins has concentrated on its medical significance, and as a result, fundamental research underlying venom constituents and the ecological function of venom toxins are very sparse. More studies should be directed towards unveiling the venom constituents and their ecological role, which can lead to the discovery of unique peptide and bioactive molecules with novel and unexpected pharmacological and industrial potentials.

In the quest to find, develop and market novel and potent biomolecules from nature, it is of utmost importance to note and eliminate/reduce the environmental risk associated with it. The major risks involved are overharvesting of species and habitat damage. After the discovery of a prospective bio-product from any organism, the researchers then ought to find or develop alternate methods to acquire the bio-product in such a manner that over-harvesting and overexploitation of the species can be avoided and damage to its environment can be prevented. To resolve this problem, researchers can take the help of recombinant DNA technology, wherein the gene encoding the bio-product is cloned into a vector and transformed into microorganisms which then express the gene and produce the bio-product on a larger scale. Another approach to producing the bio-product on a large scale can be checked by developing particular cell lines by cell/tissue culture technology.

7. Summary

This review presents an understanding of the chemical ecology of sea anemones and their bio-prospective applications. Sea anemones have very impressive life strategies, especially for feeding and self-defence. A thorough study on the ecology of sea anemones and their chemical interactions in their habitat will allow the discovery of novel and potent drug molecules and other industrially important compounds. To maintain their niche in their habitat, sea anemones interact with other inhabitants essentially by chemical cues. Predation, spatial competition, prey capture, defence, symbiosis, and other ecological interactions are mediated by chemical cues. Understanding the ecological roles of these molecules will unravel potentially new compounds with interesting applications.

Sea anemone venom consists of a mixture of various proteinaceous and non-proteinaceous molecules. From an ecological point of view, these molecules aid the sea anemone in prey capture, protection against predators, or deter competitors. But from a bio-prospecting perspective, these molecules have high potential in pharmacological applications. For example, *Stichodactyla helianthus* produces ShK toxin which is a neurotoxin that paralyzes prey. ShK, being a Potassium channel blocker,

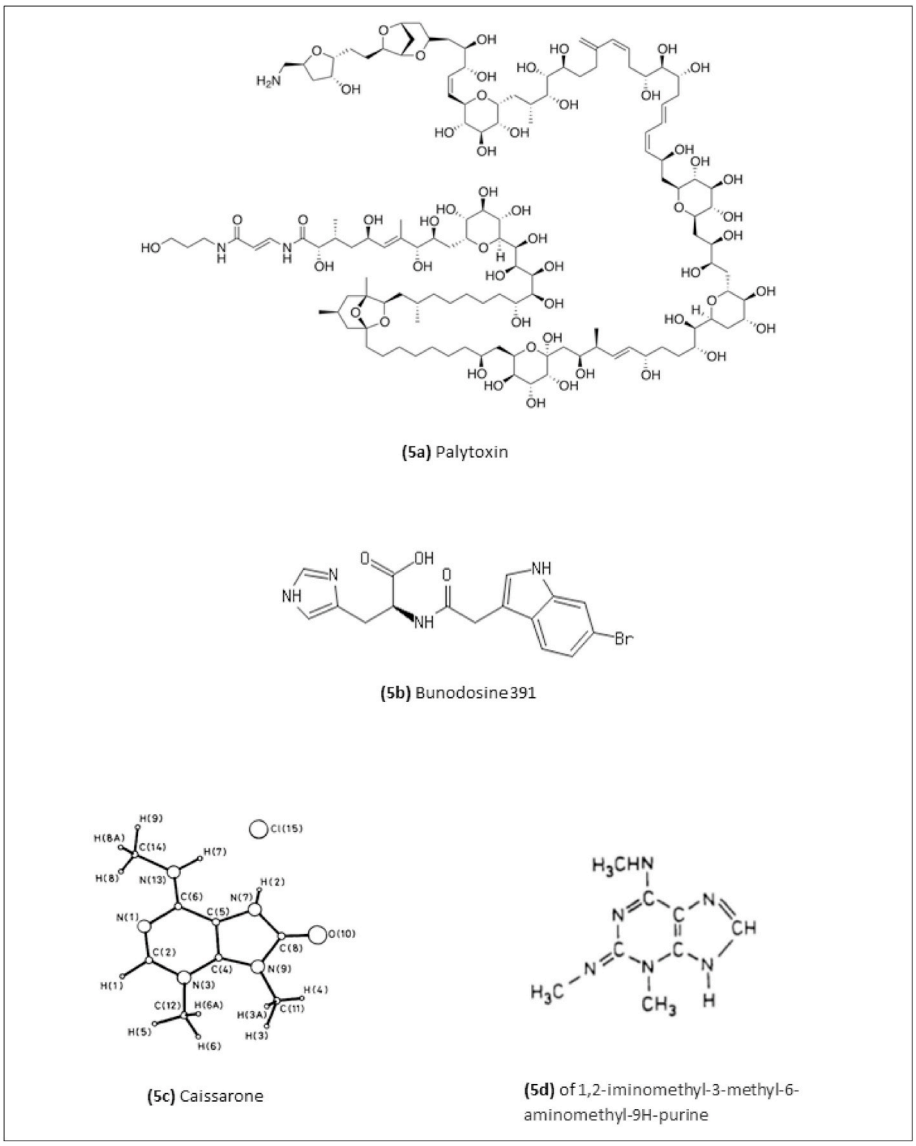


Fig. 5. a Molecular structure of Palytoxin (Ref: Pubchem); **Fig. 5b:** Molecular structure of Bunodosine 391 (Zaharenko et al., 2011); Fig. 5c: Molecular structure of Caissarone (Cooper et al., 1995); **Fig. 5d:** Molecular structure of 1,2-iminomethyl-3-methyl-6-aminomethyl-9H-purine (de Rosa *et al.*, 1987).

showed an excellent ability to treat many autoimmune diseases. Dalazatide (ShK-186) is an ShK derivative that is undergoing Phase II clinical trial for the treatment of auto-immune diseases. Other non-venomous proteinaceous molecules such as Aneroin, Anegen, HcRed show potential in the biomedical industry. Besides in medicine, sea anemone natural products are promising as antifoulants and pest control agents.

Exploration of novel molecules must be done sustainably. Over-exploitation of marine resources leads to overharvesting of species and habitat damage. Therefore, alternate methods like recombinant DNA technology and cell/tissue culture techniques can be employed to obtain these molecules on a large.

Ethical statement

I testify on behalf of all authors that there are no animal subjects involved in this article.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Observations of the likelihood of asexual reproduction in sea anemone *Bunodosoma goanense* den Hartog & Vennam, 1993

Cecelia Menezes [1] · Narsinh L. Thakur [1]

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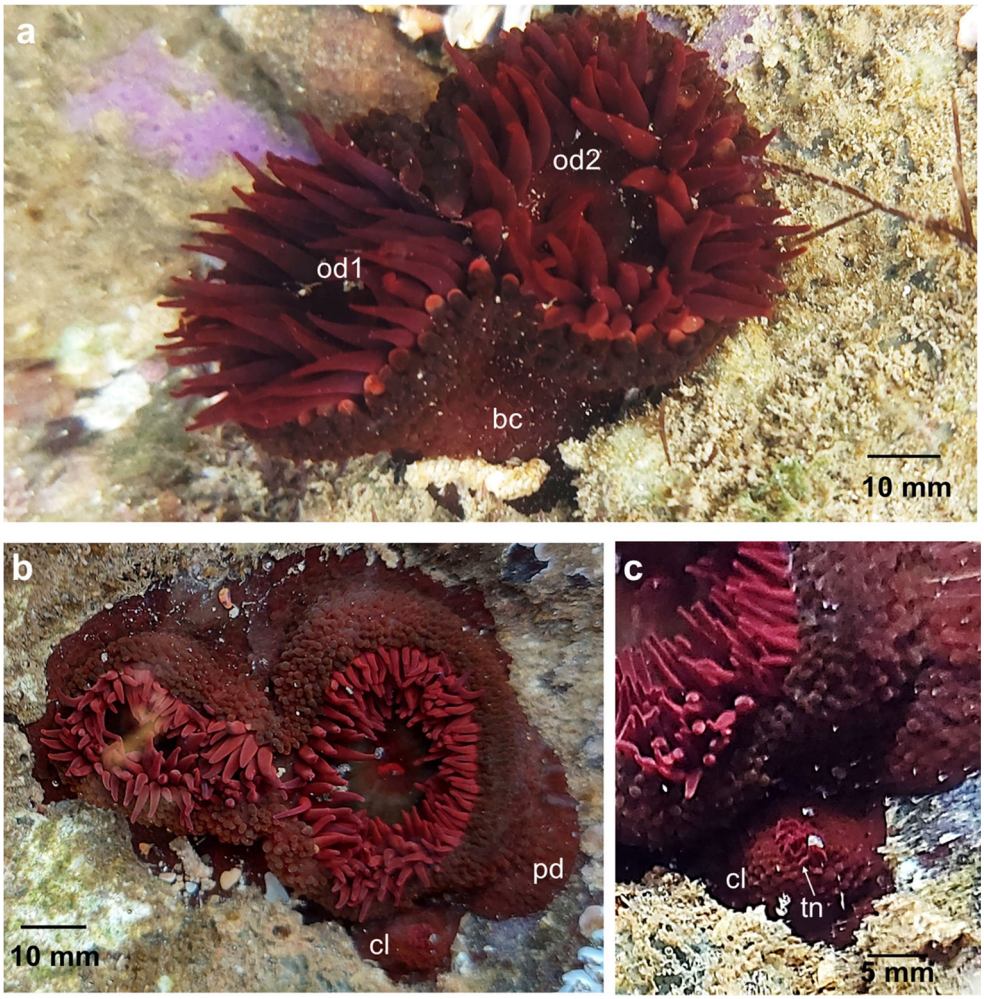


Fig. 1 **a** *Bunodosoma goanense* possibly undergoing longitudinal fission. The two separate oral discs (od1 and od2) with a common body column (bc) indicate that the separation began at the oral disc; **b** The likelihood of pedal laceration observed in *B. goanense*. With the stretching and extension of the pedal tissue of the parental individual, a new tiny clonal individual (cl) developed and separated from the parent. Another region of pedal disc tissue (pd) stretched and extended to possibly form a new clone; **c** A magnified image of the clone (cl) formed by pedal laceration showing its separation from the parental tissue. The tiny tentacles (tn) of the clone are clearly visible

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✉ Narsinh L. Thakur
thakurn@nio.org

Cecelia Menezes
cmenezes@nio.org

[1] CSIR-National Institute of Oceanography, Dona Paula, Panaji, Goa 403004, India

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Sea anemones are marine benthic invertebrates belonging to the class Anthozoa within the phylum Cnidaria that are known to reproduce sexually, asexually, or both (Chia 1976). Sexual reproduction involves fertilization, during which the male and female gametes fuse and form genetically unique offspring. Some sea anemones exhibit hermaphroditism which is mutually exclusive from asexual reproduction (Shick 1991). During asexual reproduction, according to Chia (1976), sea anemones can regenerate by cloning themselves through binary fission where an individual separates longitudinally or transversely to form two equal halves, pedal laceration during which small pieces of tissues of sea anemone separate from the base and form new individuals. Budding involves the formation of new individuals, without division of the parent, from primordial tissue by growth and cellular differentiation (Hughes 1989) and has been reported in very few species. According to Shick (1991), sea anemones rely upon asexual reproduction rather than sexual reproduction in harsh environmental conditions. Shick (1991) also noted that pedal laceration is predominantly found in littoral sea anemones, whereas binary fission is more common in intertidal sea anemones.

The genus *Bunodosoma* comprises 14 species of sea anemones, none of which had been described to reproduce asexually, until recently when Da Silva Junior et al. (2022) reported evidence for longitudinal fission in *B. cangicum* on the shores of Brazil. den Hartog (1987) mentioned that there were so far no reports of longitudinal or transverse fission within the genus *Bunodosoma* Verrill, 1899. *Bunodosoma caissarum* reproduces exclusively sexually (Belem 1987). The ability of *Bunodosoma californicum* to undergo fission is unknown (Daly 2004).

Bunodosoma goanense den Hartog and Vennam 1993, a shallow water intertidal sea anemone was first discovered on the rocky shores of Anjuna, Goa, on the West coast of India (den Hartog and Vennam 1993). In their survey, den Hartog and Vennam (1993) reported that *B. goanense* showed separate sexes and no indications of asexual reproduction. In the present study, we provide evidence of possible asexual reproduction by longitudinal fission and pedal laceration in the sea anemone *Bunodosoma goanense*.

The findings reported herein were made in the mid intertidal zone of the rocky shores of Anjuna in Goa, India (15°34′02.7″N, 73°44′29.2″E) during low tide. The animals were observed and photographed in situ using a Samsung Galaxy M51 phone camera. Longitudinal fission of *Bunodosoma goanense* (Fig. 1a) was observed on 21 November 2021. It is interesting to note two separate oral discs with a common body column and pedal disc (mid-fission) indicating that the separation began at the oral disc, but the completion of the separation is not confirmed. Da Silva Junior et al. (2022) reported a similar observation in the case of *B. cangicum*. Although in *B. cangicum* and *B. goanense* the conclusion of fission was not visualized, in both, the process started at the oral end, keeping two completely individualized oral discs while the lower part of the column continued together. This mechanism of longitudinal fission is opposite of the documented method for several species where the process begins in the aboral part. On 19 December 2021, the possibility of cloning through pedal laceration was observed (Fig. 1b, c). From this observation, we suspect that stretching and extension of the pedal tissue of the parental individual were seen, from which a new tiny clonal individual developed and separated from the parent. The photographs are observations and we, therefore, hypothesize the possibility of longitudinal fission in *B. goanense* and the likelihood of pedal laceration in the genus *Bunodosoma*.

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Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval No animal testing was performed during this study.

Sampling and field studies No permits were needed for the present observational field study.

Data availability Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Author contribution CM conducted field observation, photography, and wrote the manuscript. NLT is responsible for supervision, reviewing the manuscript, project administration, and fund acquisition.

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OCEANARIUM

Facultative association of *Purpura panama* with *Bunodosoma goanense*

Cecelia Menezes[1] · **Narsinh L. Thakur**[2]

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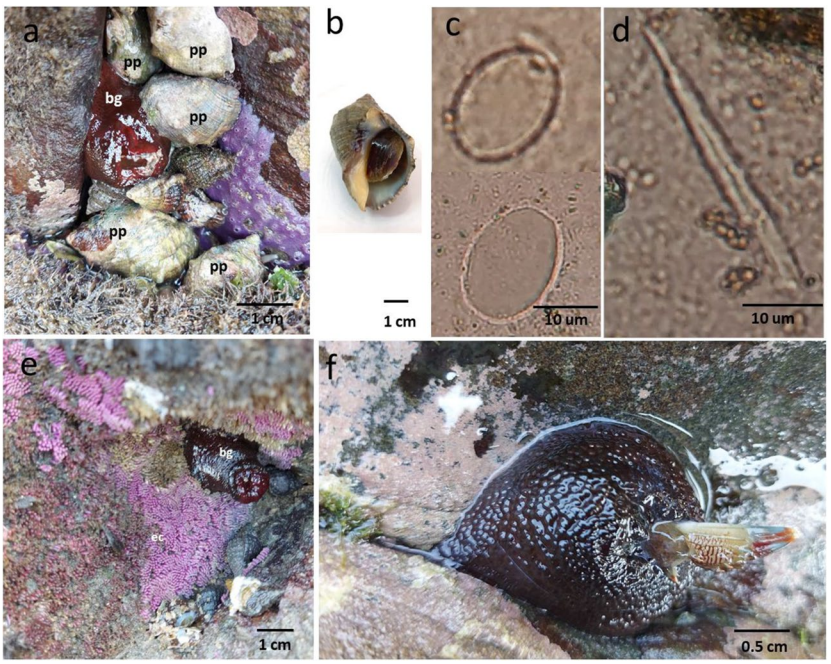


Fig. 1 **a** Three marine snails *Purpura panama* (pp) attached to the body column of *Bunodosoma goanense* (bg) and others in close proximity; **b** Shell of *P. panama* with live snail inside; **c** Discharged nematocysts in gut content of *P. panama*; **d** A nematocyst in the gut content of *P. panama*; **e** A mat of *P. panama* egg-capsules (ec) deposited around *B. goanense* to protect their egg capsules from predators; **f** A *B. goanense* sea anemone feeding on a crab

The rocky intertidal zone is known for its high diversity of species, which shows high densities of individuals compressed into the narrow intertidal area. Consequently, there is competition for limited space and food (Dayton 1971). Its species employ various ecological strategies to maintain their niches by coping with environmental disturbances and interactions with other species (Southwood 1977). In such ecosystems with physical disturbance, stress, and predation, positive symbiotic interactions like mutualism and commensalism seem prevalent, where species can boost the growth and survival of other species that are unable to exist in isolation (Hacker and Gaines 1997).

The rocky coast of Anjuna in Goa, India, (15° 34′ 02.7″ N,73° 44′ 29.2″ E), features basalt and granite rocks, which are exposed during low tide, harboring a diverse array of organisms in tidepools. This paper reports observations of an association between a marine snail and a sea anemone, conducted monthly during low spring tide from June 2021 to March 2023. The snails (Mollusca: Gastropoda) were identified as *Purpura panama* (Röding, 1798) and the sea anemone (Cnidaria: Actiniaria) as *Bunodosoma goanense* (den Hartog & Vennam, 1993) by CSIR-NIO taxonomists. This snail was not associated with other actiniarians in the study area. Also, this association was seen only with *B. goanense* in the mid-littoral zone. The snails firmly adhered to the body column or close to the pedal disc (Fig. 1a). The snail (Fig. 1b) gut-content analysis highlighted the presence of both discharged and undischarged nematocysts (Fig. 1c, d). The undischarged nematocysts (Fig. 1d) resembled

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✉ Narsinh L. Thakur
thakurn@nio.org

[1] School of Earth Ocean and Atmospheric Sciences (SEOAS), Goa University, Taleigão, Goa, India

[2] CSIR-National Institute of Oceanography, Dona Paula, Goa, India

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those present in *B. goanense*. Besides nematocysts, there were no other actinarian tissues in the snail's gut. So far, there have been no reports of nematocysts in snails except for the report by Ohkawa (1975), who observed that eggs of the parasitic snail *Alexania inazawai* (Kuroda, 1943) were coated with nematocysts of *Diadumene lineata* (Verrill, 1869)

Purpura panama belongs to the carnivorous snail family Muricidae (Rafinesque, 1815). When carrion is present, some predator species act opportunistically as facultative scavengers (Morton 2006; Moleón and Sánchez-Zapata 2015). Some muricids exhibit opportunistic behavior by scavenging leftovers of other predators (Morton 2006; de Carvalho-Souza and González-Ortegón 2022). We observed *B. goanense* feed on mussels and crabs. It ejects out and discards the empty mussel shells with tiny bits of mussel tissue still stuck onto them. *Purpura panama* opportunistically feeds on these leftovers ejected by *B. goanense*.

The snails did not adhere to the sea anemone during monsoons (June–October 2021). From August to September 2021, the snails mated and deposited egg capsules as mats on rocky surfaces (Fig. 1e). Interestingly, we observed three *P. panama* individuals start to deposit egg capsules on rocky surfaces around the pedal disc of a *B. goanense*, eventually proceeding outwards to form a mat of egg-capsules around it. Along a stretch of approximately 150 m of this rocky shore, we observed patches of egg-capsule mats deposited around ~20 individual sea anemones. This egg-capsule deposition event was not observed in succeeding monsoons.

Predatory crabs on the rocky shores of Anjuna are predators of gastropod eggs. These crabs are one of the prey species of *B. goanense* (Fig. 1f). Therefore, we hypothesize that *P. panama* uses *B. goanense* as a "babysitter" to protect its egg capsules from predatory crabs. Any potential predators of snail eggs would be preyed upon by the sea anemone. Shimek (1981) reported a similar observation in Alaska about the snail *Neptunea pribiloffensis* (Dall, 1919) that deposited its corncob-shaped egg-capsules near a "babysitter" sea anemone of *Urticina grebelnyi* Sanamyan and Sanamyan, 2006, to protect eggs from predatory sea urchins.

In conclusion, this study emphasizes the multifaceted benefits of the association between *Purpura panama* and *Bunodosoma goanense* in the rocky intertidal zone of Anjuna. The snail's eggs gain protection against predators through the "babysitter" role played by the sea anemone, which, in turn, prey on these predators. Additionally, the snails opportunistically feed on anemone leftovers. We saw no evidence that the association between *P. panama* and *B. goanense* other than it was like a form of facultative association. The presence of nematocysts in the snail's gut implies the possibility of kleptocnidism, where the snail may be acquiring and utilizing nematocysts from the sea anemone. Very little is known about the biology and ecology between these gastropods and sea anemones. Our findings, although preliminary, may contribute to filling the ecological knowledge gaps and unveil the complexity of such partnerships and their broader implications.

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Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval All applicable guidelines for animal testing, animal care, and use of animals were followed by the authors.

Sampling and field studies All necessary permits for sampling have been obtained by the authors.

Data availability No datasets were generated during the current study.

Author contribution All authors contributed to the conceptualization of the study. CM conducted field observation and photography and wrote the manuscript. NLT is responsible for reviewing and funding acquisition.

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Identification and functional characterization of a Nav-targeting peptide NavTx-Bg1 from sea anemone *Bunodosoma goanense* (Vennam & den Hartog, 1993) transcriptome

Cecelia Menezes [a,b,c], Narsinh Thakur [a,b,*], Kathleen Carleer [c], Steve Peigneur [c,d], Jan Tytgat [c]

[a] CSIR-National Institute of Oceanography, Dona Paula, Goa, India
[b] School of Earth, Ocean and Atmospheric Sciences, Goa University, Taleigao, Goa, India
[c] Toxicology and Pharmacology, KU Leuven, Campus Gasthuisberg, Leuven, Belgium
[d] University of Vienna, Faculty of Chemistry, Institute of Biological Chemistry, Währinger Straße 38, 1090, Vienna, Austria

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ABSTRACT

Sea anemones are a rich source of venom peptides, many of which target voltage-gated sodium channels (Nav), having immense potential for therapeutic development. In this study, we employed a transcriptomics-guided approach to predict and functionally characterize a Nav-targeting peptide from the sea anemone *Bunodosoma goanense*. A single putative peptide transcript with homology to known sodium channel modulators was discovered. This transcript-derived peptide was chemically synthesized and tested for activity. Electrophysiological assays employing two-electrode voltage clamp (TEVC) on *Xenopus laevis* oocytes overexpressing Nav1.4 and Nav1.5 channels, along with contraction paralysis assay, confirmed its functional activity. Subsequent tentacle venom purification isolated a native peptide with the same bioactivity. Our work demonstrates the power of predictive transcriptomics for sustainable venom peptide discovery.

1. Introduction

The function of excitable cells relies heavily on voltage-gated sodium channels (Nav), which govern action potential initiation and conduction tissues such as nerves, muscles, and cardiac cells. The Nav channel activity is involved in a variety of human disorders such as epilepsy, chronic pain, arrhythmias, and myotonia. Consequently, the pharmacological modulation of specific Nav channel isoforms has become an attractive strategy for therapeutic intervention (Catterall, 2012; King and Vetter, 2014; King, 2011). A rich and underutilized source of Nav modulators lies within animal venoms, particularly those derived from marine organisms. Sea anemones (Cnidaria: Anthozoa) produce peptide toxins that are known to interact with Nav channels with high specificity and affinity. These peptides, typically 3–5 kDa in size, are often highly stable due to multiple disulfide bridges and act either by delaying channel inactivation or by shifting the voltage dependence of activation (Bosmans and Tytgat, 2007). Several of these peptides have proven valuable as pharmacological tools and as lead compounds for drug development.

The genus *Bunodosoma* is a known source of such toxins. Several studies have highlighted the pharmacological richness of sea anemone venoms, particularly within the genus *Bunodosoma*, known for producing peptides that modulate voltage-gated ion channels. From the sea anemone *Bunodosoma granulifera*, BgII and BgIII, both sodium channel toxins, were obtained in purified form (Goudet et al., 2001). Zaharenko et al. (2008a,b) characterized the cangitoxin, a sodium channel modulating toxin initially described from *Bunodosoma cangicum* and reported the purification and functional validation of two new isoforms cangitoxin II and III. BcIV is *Bunodosoma caissarum* venom peptide exhibiting paralyzing effects was reported by Oliveira et al. (2006). Salceda et al. (2010) toxin BcIII, isolated from *Bunodosoma caissarum*, was shown to influence sodium current kinetics in rat neurons. Despite this, other species within the genus remain virtually unstudied from a toxinological perspective.

Bunodosoma goanense (Vennam & den Hartog, 1993), (Fig. 1) endemic to the intertidal rocky shores of India's west coast and Singapore (Vennam and den Hartog, 1993; Fautin et al., 2015), represents a potentially rich but unexplored resource for peptide-based bioactives. In the present study, we employed a transcriptomics-guided approach to identify and functionally characterize Nav-active peptides

* Corresponding author. CSIR-National Institute of Oceanography, Dona Paula, Goa, India.
E-mail address: thakurn.nio@csir.res.in (N. Thakur).

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Fig. 1. Sea anemone *Bunodosoma goanense* on the rocky shores of Anjuna, Goa.

from *B. goanense*. Using a previously assembled transcriptome from this species (Menezes and Thakur, 2026) Accession no. GKWR01000000), we mined the dataset for putative toxin sequences and identified a peptide with high similarity to known sea anemone sodium channel toxins. This predicted peptide was synthesized and functionally validated through two-electrode voltage clamp (TEVC) recordings and a fish paralysis bioassay, confirming its activity on Nav1.4 and Nav1.5 isoforms. In parallel, we extracted native venom from *B. goanense*, fractionated it using chromatographic techniques, and isolated an active peptide that matched the functional profile of the transcriptome-derived candidate.

On similar lines, proteomic and transcriptomic investigations identified several distinct groups of expressed peptide toxins, among them neurotoxins and protease inhibitors. A Kunitz-type peptide, ZoaKuz1, was predicted to act as a blocker of voltage-gated potassium channels. Functional bioassays confirmed its neuroprotective activity in a zebrafish model of Parkinson's disease (Liao et al., 2019). A multi-omic approach integrating transcriptomics, proteomics, and peptidomics was used to investigate the venom composition. This led to the identification of various toxins, including those with neurotoxic and hemostatic activities. The study highlighted the complexity of the venom and the effectiveness of integrated omics in uncovering novel bioactive compounds (Mazzi et al., 2023). Transcriptomic studies led to the discovery of novel peptides, such as U-AITx-Ate1, which was structurally characterized and tested in functional assays. This approach highlights the potential of transcriptomics in identifying peptides with unique structures and bioactivities (Elnahriry et al., 2019). These studies demonstrate the efficacy of combining transcriptomic data with functional validation to discover and characterize novel peptides from venoms. Our findings contribute to the growing body of evidence supporting marine venoms as a promising source of pharmacologically active peptides and offer new understanding into the venom composition of *Bunodosoma goanense*, a previously uncharacterized Indian sea anemone species.

2. Materials and methods

2.1. Transcriptome resource and venom peptide prediction

The annotated transcriptome used in this study was previously assembled by our group as part of a separate investigation into the spatial competition between *Bunodosoma goanense* and zoanthid corals (Menezes and Thakur, 2026). The Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GKWR00000000. The version described and used in this study is the first release, GKWR01000000. This transcriptome assembly served as the foundational dataset for the subsequent annotation and characterization of venom-related transcripts. A database specifically for cnidarian was obtained from the UniProt database https://www.uniprot.org.This database included peptide/protein sequences from various cnidarian. The assembled *B. goanense* transcriptome was queried against the custom cnidarian database using BLASTx and BLASTp tools using the galaxy platform. An E-value cut-off of 1e-5 was employed to ensure the selection of high-confidence matches. Only the Transcripts associated with the GO term for toxin activity GO: 0090729 were extracted. Annotation of these putative venom peptide toxin transcripts was performed through the ID mapping tool available on the UniProt website https://www.uniprot.org/id-mapping. Accession numbers obtained from the BLAST results were input into the tool to retrieve detailed annotations, including peptide toxin names, toxin families, and sequences which provided information about the nature and classification of the venom peptides present in *Bunodosoma goanense.*

2.2. Protein modelling and synthesis

To model the three-dimensional structure of venom peptide, amino acid sequences obtained through the transcriptomic analysis of *Bunodosoma goanense* were used. This sequence was input into the SWISS-MODEL online platform (https://swissmodel.expasy.org), which performed an automated search for homologous templates in its database using BLAST. Suitable templates with high sequence similarity and adequate resolution were selected, and the platform aligned these templates with the input sequence. Using the selected template, SWISS-MODEL generated 3D structure of the venom peptide through homology modelling. The finalized 3D structure was visualized to examine overall structural integrity. Based on the *in-silico* prediction from the transcriptome of *B. goanense*, the putative sodium channel toxin was synthesized using Solid-phase peptide synthesis (Service provided by Priveel Peptide, Tamil Nadu, India). The candidate sodium channel toxin peptide was synthesized using a Prelude automated peptide synthesizer on a 0.1 mmol scale with chlorotrityl chloride (CTC) resin. A 20 % piperidine solution in N,N-dimethylformamide (DMF) was used for deprotection. Coupling was performed using 3 equivalents of Fmoc-protected amino acids activated by 3 equivalents of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) in the presence of 70 % N,N-diisopropylethylamine (DIPEA) in DMF for 50 min per cycle. Cleavage of the peptide from the resin was done using a cleavage cocktail comprising trifluoroacetic acid (TFA), 1,3-dimethoxybenzene (DMB), triisopropylsilane (TIPS), and 3,6-dioxa-1,8-octanedithiol (DODT) in a ratio of 90:5:2.5:2.5 (v/v), incubated for 3 h at room temperature. The cleavage solution was concentrated under nitrogen gas for 45 min to remove excess TFA. Ice-cold diethyl ether was used to precipitate the crude peptide, followed by three washing steps. The resulting peptide pellet was dissolved in 50 % acetonitrile in Milli-Q water. The peptide was purified using reverse-phase HPLC on a Vydac C18 column (250 × 10 mm, 10 μm). Elution was achieved with a linear gradient of 5–50 % buffer B (0.1 % TFA in 90 % acetonitrile) over 50 min at 5 mL/min flow rate. Buffer A was 0.1 % TFA in Milli-Q water. Absorbance was monitored at 214 nm. The bioactive peptide eluted as a sharp peak at approximately 35 min. Fractions corresponding to this peak were pooled and analyzed via LC-MS. Only fractions with >95 % purity were collected and lyophilized.

2.3. In vivo *paralysis assay*

To assess the bioactivity of the synthesized peptide, we conducted in vivo toxicity assays using juvenile *Gambusia* sp. fish (n = 3 per condition, in triplicates). Each fish was exposed to 0.5 mg/mL of the synthesized peptide and native crude tentacle venom (positive control) in water. Fish were continuously observed for up to 120 min post-exposure for signs of neuromuscular effects, including paralysis, erratic movement, or loss of equilibrium or death. Control fish were maintained in identical conditions without venom exposure. The contraction paralysis assay was performed following the zebrafish protocol described by Sachkova et al.

(2020), using a concentration of 0.5 mg/mL as a standard qualitative screening dose to assess neurotoxic activity.This concentration was selected for qualitative assessment and does not represent a molar-equivalent dose relative to electrophysiological assays.

2.4. Voltage-gated ion channel activity of synthesized peptide

2.4.1. Isolation of Xenopus laevis oocytes by partial ovariectomy

The use of *Xenopus laevis* frogs was authorized by the KU Leuven Ethical Committee for Animal Research, with all experimental procedures adhering to EU Directive 2010/63/EU on laboratory animal care. Adult females were immersed in aqueous solution containing 0.1 % buffered tricaine (ethyl 3-aminobenzoate methanesulfonate, 1 g/L, Sigma-Aldrich, Rockville, MD, USA) and NaHCO3 (1 g/L; Sigma-Aldrich, Rockville, MD, USA) in aquarium water (pH 7.5) for 15 min. Following recovery, the frogs were observed daily and subsequently returned to their tanks in the KU Leuven Aquatic Facility. Ovarian lobes were surgically excised and enzymatically defolliculated in a Ca^{2+}-free ND96 solution (96 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, and 5 mM HEPES) containing collagenase from *Clostridium histolyticum* type IA (1.5 mg/mL; Sigma-Aldrich, Rockville, MD, USA). The tissue was incubated on a rocking platform at 16 °C for 2 h 30 min. Defolliculated oocytes were then transferred into a calcium-containing ND96 buffer (96 mM NaCl, 2 mM $MgCl_2$, 2 mM KCl, 5 mM HEPES, and 1.8 mM $CaCl_2$, pH 7.5) supplemented with geomycine (100 mg/L; Schering-Plough, Heist-op-den-Berg, Belgium) and theophylline (90 mg/L; ABC Chemicals, Nazareth, Belgium) and maintained at 16 °C. (Peigneur et al., 2012, 2019).

2.4.2. Heterologous expression of ion channels in oocytes of Xenopus laevis

To express ion channels in *Xenopus laevis* oocytes, rat Nav1.4 (rNav1.4) and human Nav1.5 (hNav1.5) channels were used. Linearized plasmids carrying the respective channel genes were transcribed in vitro with the SP6 mMESSAGE mMACHINE transcription kit (Ambion, Austin, TX, USA). Stage V–VI oocytes were selected for microinjection, which was performed with a Drummond Scientific® microinjector (Broomall, PA, USA), delivering 10–20 nL of cRNA depending on the channel subtype. After injection, oocytes were maintained at 16 °C for 1–3 days in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) supplemented with 50 mg/L gentamicin sulfate to ensure sterility and support optimal channel expression.

2.4.3. Electrophysiological recordings

Electrophysiological recordings were performed at room temperature (18–22 °C) using the two-electrode voltage-clamp (TEVC) method. Data were acquired with a GeneClamp 500 amplifier (Axon Instruments®, Burlingame, CA, USA) and stored using Clampex 9 software (Axon Instruments®, USA). Borosilicate glass capillaries (WPI 1B120-6) were pulled into micropipettes with a manual puller (World Precision Instruments®, Sarasota, FL, USA). Whole-cell currents were measured in oocytes 1–3 days after RNA injection. Both current and voltage electrodes were filled with 3 M KCl and had resistances of 1.0–2.5 MΩ. Currents were sampled at 20 kHz for Nav channels and low-pass filtered at 1 kHz using a Bessel filter. Sodium currents were elicited by 100 ms depolarizations to 0 mV from a holding potential of −90 mV. For current–voltage (I–V) relationships, 50 ms depolarizing steps from −90 to +65 mV were applied in 5–10 mV increments, starting from a −90 mV holding potential. All experiments were carried out in triplicate using oocytes.

2.5. Native peptide validation

2.5.1. Crude venom extraction from Bunodosoma goanense

Specimens of the sea anemone *Bunodosoma goanense* were collected from the rocky intertidal zone at Anjuna, Goa, India (15°34′04.8″N, 73°44′27.5″E). The collected animals were maintained in an aquarium for four days, with fresh seawater changed daily to ensure a clean environment and to remove any mucus discharge and excretory waste. During this period, the anemones were not fed to prevent contamination from food residues and to ensure venom production in high concentration. The sea anemones venom was extracted from tentacles by subjecting it to sonication in distilled water to disrupt the cells and release the venom components (Carrette and Seymour, 2004). The resulting sonicated tissue suspension was centrifuged at 15,000 rpm for 5 min to separate the cell debris from the proteinaceous supernatant. The supernatant was then lyophilized to obtain freeze-dried crude venom extract, which was subsequently stored for further analysis.

Table 1
Summary of venom peptide families identified from the *Bunodosoma goanense* transcriptome.

Toxin Peptide family	No. of transcripts
Actinoporin family, Sea anemone subfamily	8
Cnidaria small cysteine-rich protein (SCRiP) family	4
EGF domain peptide family	22
Jellyfish toxin family, Type II subfamily	4
NEP3 family	24
Phospholipase A2 family	9
Sea anemone 8 toxin family	3
Sea anemone short toxin (type III) family	1
Sea anemone sodium channel inhibitory toxin family	**1**
Sea anemone structural class 9a family	2
Sea anemone type 1 potassium channel toxin family, Type 1a subfamily	2
Sea anemone type 1 potassium channel toxin family, Type 1b subfamily	7
Sea anemone type 3 (BDS) potassium channel toxin family	3
Sea anemone type 5 potassium channel toxin family	2
Sea anemone type 6 potassium channel toxin family	2
Venom Kunitz-type family, Sea anemone type 2 potassium channel toxin subfamily	19

2.5.2. Peptide fractionation

Crude venom fractionation was performed using size-exclusion HPLC (Superdex 30 column, 10/30 GL). During the analysis, peptide fractions were eluted with a 30 % acetonitrile solution) over 60 min at a flow rate of 0.5 mL/min. The fractions were freeze-dried and tested for bioactivities. This was followed by further separation of the active fractions by gradient HPLC (C-18 column) using a gradient of water and acetonitrile over 120 min at a flow rate of 1 mL/min (Orts et al., 2013). The final purified fractions were tested for activity against rNav1.4 and hNav1.5 channels using two-electrode voltage clamp (TEVC) assays.

2.5.3. Peptide mass determination

For peptide mass determination, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) was employed using a timsTOF Flex instrument operating in positive ion mode. For the mass determination of venom peptides, samples were resuspended in a solution of 50 % acetonitrile (ACN) and 0.1 % trifluoroacetic acid (TFA). Each sample (1 μL) was combined with 1 μL of the matrix solution, consisting of α-Cyano-4-hydroxycinnamic acid (CHCA), specifically prepared for MALDI-TOF MS analysis. The mixture was then dispensed onto a 384 Anchorchip target plate. Mass spectrometric analysis was performed using a timsTOF Flex instrument operating in MALDI positive mode.

3. Results

3.1. Transcriptomic discovery of a Nav peptide candidate

A transcriptomic approach was employed to identify putative venom peptides from *B. goanense*. The annotation of venom-related transcripts

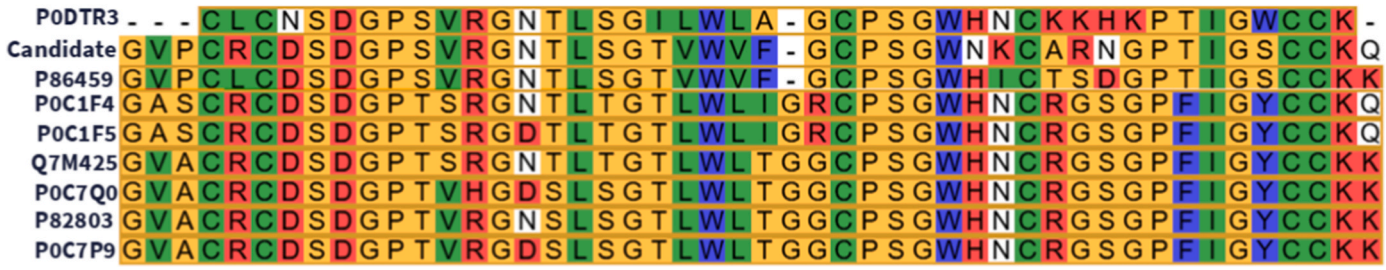


Fig. 2. Multiple sequence alignment of the candidate Nav peptide (Query) with sodium channel toxins from other *Bunodosoma* sp. Functionally important residues implicated in sodium channel interaction (e.g., R14) are boxed in red. The candidate peptide shares high sequence identity and cysteine framework conservation with known Nav toxins, indicating a conserved structural and functional profile. Refer to Table 2 for more information about the other *Bunodosoma* sp. venom peptides similar to the candidate peptide. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
BLAST results of the candidate peptide against known *Bunodosoma* sodium channel toxins.

Sequence ID	Species	Percentage identity	Alignment score	E value
Candidate (Query)	*Bunodosoma goanense*	–	–	–
P86459	*Bunodosoma cangicum*	87 %	270	4.6e-31
P82803	*Bunodosoma cangicum*	70 %	203	3.2e-20
P0C1F4	*Bunodosoma granuliferum*	66.7 %	201	6.7e-20
P0C7P9	*Bunodosoma cangicum*	68.1 %	200	9.7e-20
P0C1F5	*Bunodosoma granuliferum*	64.6 %	198	2e-19
P0DTR3	*Bunodosoma capense*	69.8 %	196	3e-19
Q7M425	*Bunodosoma caissarum*	68.1 %	195	6.2e-19
P0C7Q0	*Bunodosoma cangicum*	66 %	192	1.9e-18

revealed 16 different venom peptide families (Table 1 and Supplementary Information), including neurotoxins and cytotoxins, which are characteristic of sea anemone venoms. This includes a candidate peptide with significant homology to sea anemone Nav toxins. The peptide contained six conserved cysteine residues, forming the Beta defensin like motif (King, 2019; Madio et al., 2019). The amino acid sequence is GVPCRCDSDGPSVRGNTLSGTVWVFGCPSGWNKCARNGPTIGSCCKQ

A multiple sequence alignment with sodium channel toxins from other *Bunodosoma* species (e.g., *B. cangicum, B. granuliferum, B. caissarum*) demonstrated high sequence conservation, particularly at functionally important sites. Conserved cysteine residues (forming the ICK motif) were consistently aligned across sequences, reinforcing the structural and functional conservation of this peptide family (Fig. 2). The candidate peptide showed up to 87 % identity with known sodium channel toxins from *B. cangicum* (Accession: P86459), with an alignment score of 270 and a highly significant e-value of 4.6e-31 (Table 2). All BLAST hits fall well below the 1e-5 threshold, confirming that the observed similarities are unlikely due to chance. This high sequence identity, cysteine framework conservation, and alignment of functionally important residues (e.g., R14 in the predicted β-turn) support the classification of this peptide as a putative Nav-targeting neurotoxin.

3.2. Structural prediction of the candidate Nav toxin

To validate the identity and function of the putative sodium channel toxin, in silico structural modelling was performed using SwissModel. The predicted structure revealed the canonical fold of Type I/II sea anemone sodium channel toxins, comprising of four antiparallel

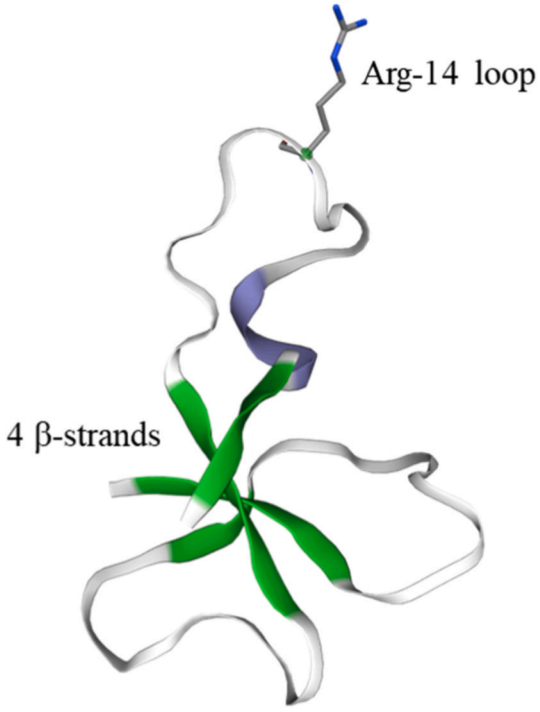


Fig. 3. Predicted 3D structure of the *Bunodosoma goanense* Nav toxin candidate showing the antiparallel β-sheet core (green), and functionally important Arg-14 loop. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

β-strands, forming a compact β-sheet core, a flexible β-turn loop (Arg-14), positioned prominently and predicted to interact with the voltage sensor domain (VSD) of Nav channels, Six conserved cysteines, forming three disulfide bonds of the Beta defensin like motif (Fig. 3).

3.3. Functional validation of synthetic candidate peptide

3.3.1. Activity of synthesized peptide against voltage-gated sodium ion channels

Electrophysiological assays revealed that the synthetic peptide induced a clear modulation of sodium current waveforms of voltage-gated sodium channels rNav1.4 and hNav1.5, producing current traces characteristic of sea anemone Nav-targeting toxins, including an apparent alteration of inactivation kinetics. Two-electrode voltage clamp recordings demonstrated a typical slowing of the inactivation of the Na currents (Fig. 4).

3.3.2. Paralytic effect in juvenile fish

The crude tentacle venom induced paralysis within approximately 3

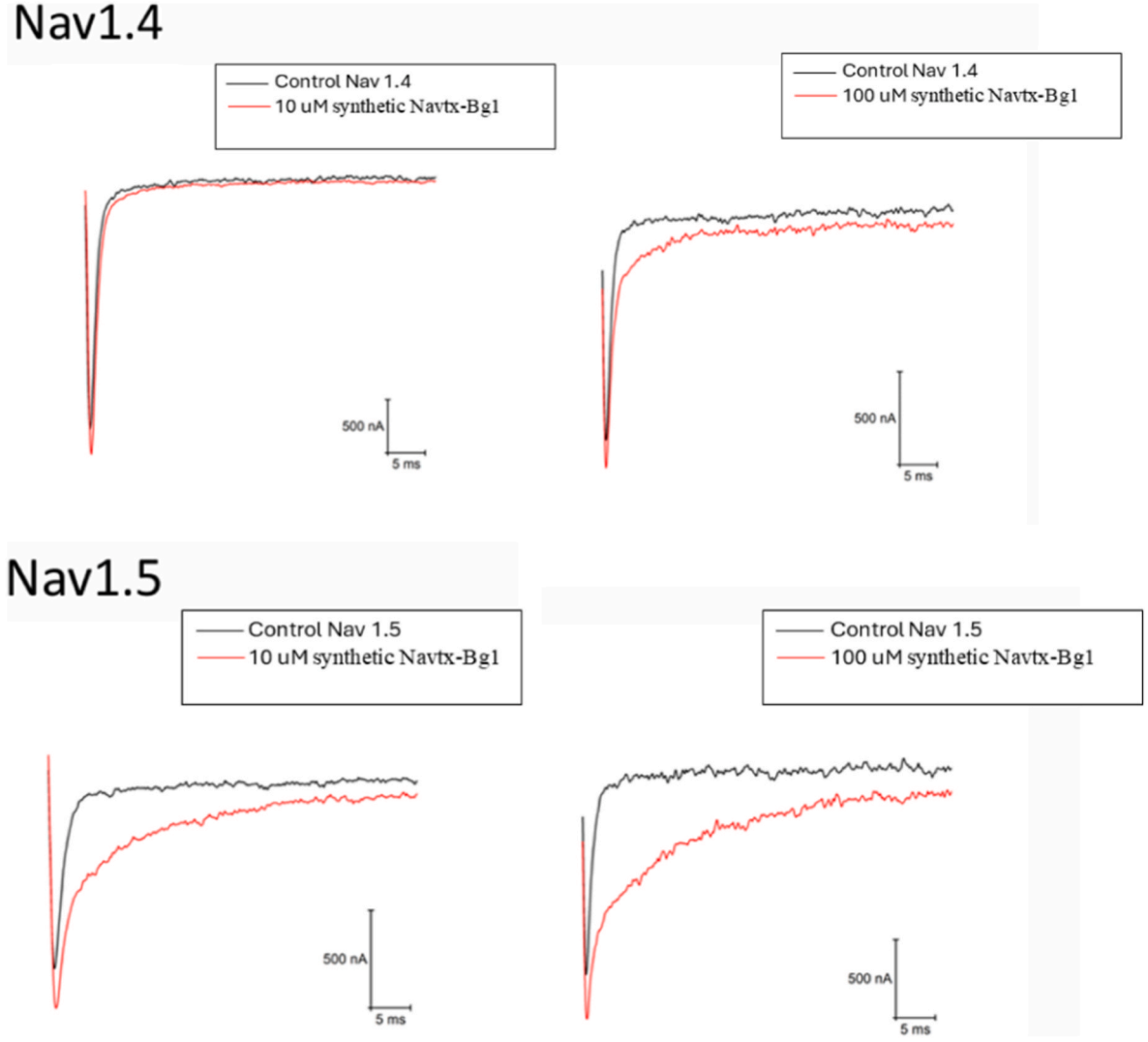


Fig. 4. Representative Na current traces illustrating qualitative modulation of Nav channel currents in the presence of synthetic NavTx-Bg1. Data represent recordings from n = no. of oocytes tested for activity of the synthetic peptide against ion channels: Nav1.4: 10 μM→ n = 3; 100 μM → n = 4; Nav1.5: 10 μM → n = 6; 100 μM → n = 3.

min when administered via water exposure. The synthesized peptide did not induce observable effects under water exposure but caused rapid contraction paralysis when directly injected into the bloodstream of juvenile Gambusia fish. The contraction paralysis assay demonstrated that venom extracted from the tentacle of *B. goanense* exhibits potent neurotoxic activity. Interestingly, the synthesized sodium channel toxin peptide did not induce any observable effect when administered through water exposure, but it caused instant contraction paralysis when injected into the bloodstream of juvenile Gambusia fish. The observed discrepancy in the efficacy of the synthesized sodium channel toxin peptide between water exposure and direct bloodstream injection in Gambusia fish can be attributed to the route of administration. Peptide toxins often require direct access to the circulatory system to effectively reach their molecular targets.

3.4. Isolation of native peptide

To isolate the native sodium channel-modulating peptide from tentacle venom, a two-step chromatographic purification strategy was employed: gel filtration chromatography (GFC) followed by reverse-phase HPLC. The crude tentacle venom was initially fractionated using a gel filtration column. The elution profile revealed distinct protein peaks (Fig. 5), with one fraction (Fraction T2) corresponding to an estimated molecular weight between 4 and 6 kDa, consistent with the predicted size of the synthetic Nav peptide. This fraction demonstrated preliminary Nav toxin bioactivity and was therefore selected for further purification. Following gel filtration and RP-HPLC separation, collected fractions were initially screened for bioactivity using in vivo paralysis assays in juvenile Gambusia fish and by preliminary electrophysiological observation to identify fractions producing Nav channel modulation. Only fractions showing reproducible bioactivity were subsequently subjected to detailed TEVC recordings on *Xenopus laevis* oocytes expressing rNav1.4 and hNav1.5 channels.

Subsequent purification using C18 RP-HPLC yielded a major peak (native Navtx-Bg1) at 35 min (Fig. 6). This peak corresponded to a single, symmetrical peak, which was the only fraction that demonstrated activity against Nav 1.4 and 1.5 channels. MALDI-TOF MS analysis of this fraction confirmed the molecular mass matching the synthetic Nav peptide.

3.5. Functional validation of the native tentacle venom peptide

3.5.1. Activity of native peptide against voltage-gated sodium ion channels

To validate the functional activity of the native tentacle venom

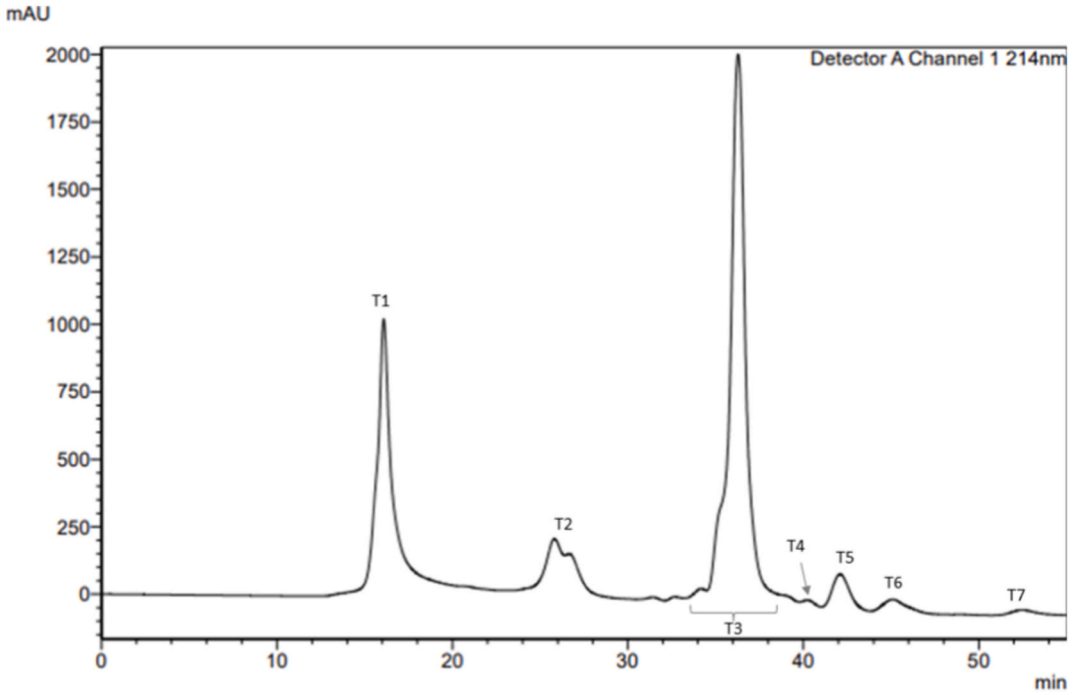


Fig. 5. Gel filtration HPLC profile of Crude Acontia venom separated into 8 distinct fraction peaks.

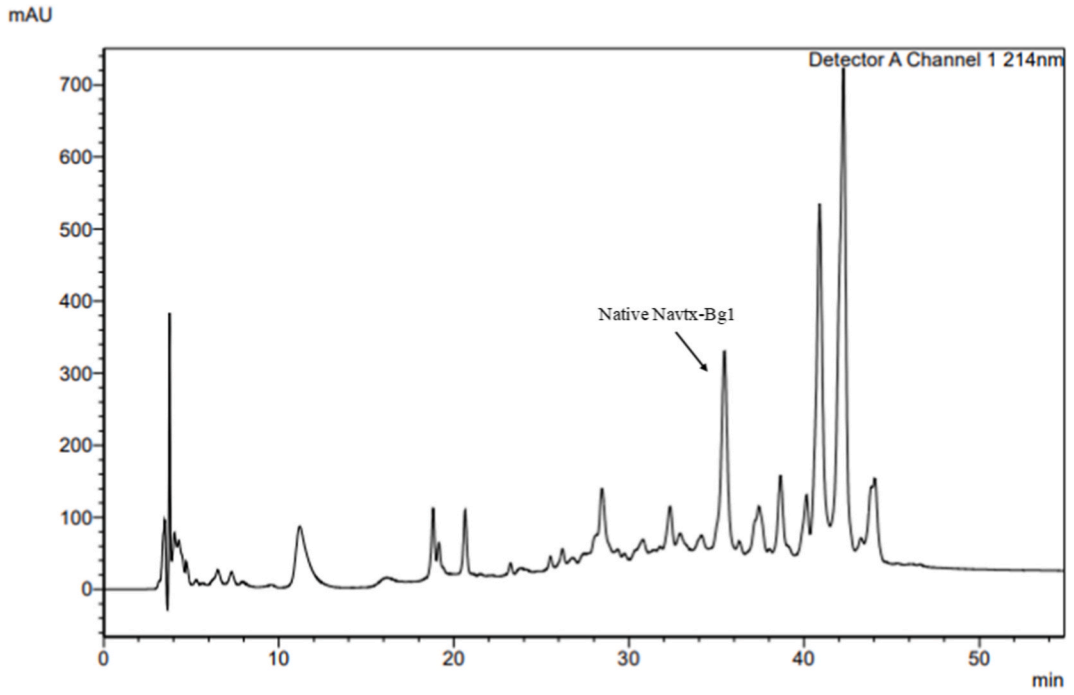


Fig. 6. Reverse phase (HPLC) profile of T2 Tentacles venom fraction with arrow indicating the peak obtained for the candidate Nav toxin peptide (native Navtx-Bg1).

peptide, electrophysiological assays were conducted on *Xenopus laevis* oocytes expressing rNav1.4 and hNav1.5 channels. The purified peptide (native Navtx-Bg1), isolated via gel filtration and RP-HPLC, produced a marked modulation of sodium channel function. Electrophysiological recordings demonstrated a typical slowing of the inactivation of the Na currents, consistent with known Nav-targeting toxins (Fig. 7). These alterations were consistent across both channel isoforms and mirrored the effects seen with the synthesized candidate peptide.

3.5.2. Mass validation from native venom

Mass spectrometric analysis of the native tentacle venom fraction (native Navtx-Bg1) revealed a molecular mass of 4973.1925 Da (folded peptide mass) (Fig. 8). These values indicate the successful isolation of a bioactive peptide from the tentacle venom of *Bunodosoma goanense*. The molecular mass closely matches the expected range for sodium channel-modulating sea anemone toxins. The chemically synthesized peptide is 4859.47 Da (linear peptide mass).

The electrophysiological recordings presented here are intended as qualitative functional validation to confirm transcriptome-guided

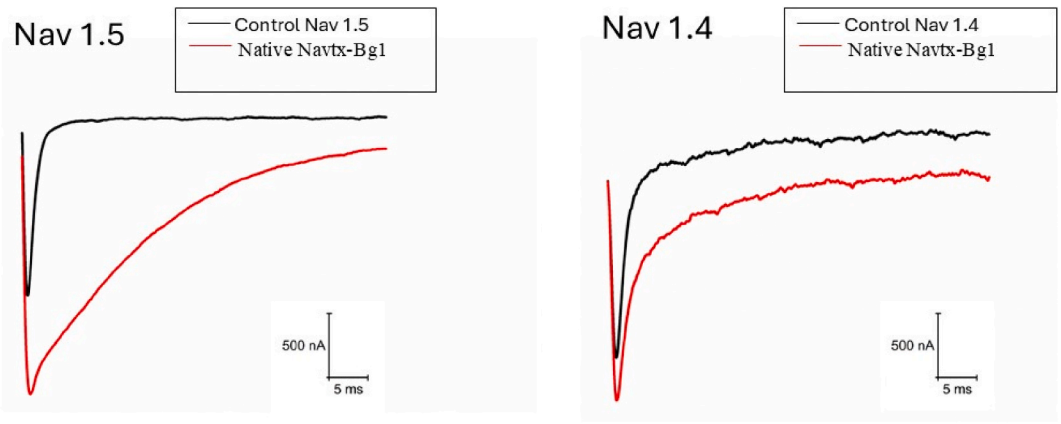


Fig. 7. Representative current traces illustrating qualitative modulation of rNav1.4 and hNav1.5 channels by native NavTx-Bg1 on currents of the cloned rNav1.4 and hNav1.5 channels overexpressed in *X. laevis* oocytes. ((x-axis: time (ms), y-axis: current (nA) n = no. of oocytes tested Nav1.4 n = 6; Nav1.5 n = 6.

prediction of Nav activity; detailed quantitative kinetic analysis was beyond the scope of the present study.

4. Discussion

We present the first evidence for a sodium channel-targeting peptide derived from the venom of sea anemone *Bunodosoma goanense*, along with its functional characterization, guided primarily by transcriptomic analysis. Through evolution, venoms have developed into intricate mixtures of peptides and proteins functioning as advanced chemical arsenals, often targeting key physiological systems such as ion channels (Fry et al., 2009; Menezes and Thakur, 2022; Madio et al., 2019). Sea anemone venoms, in particular, are known for their potent effects on voltage-gated sodium (Nav) and potassium (Kv) channels, with peptide toxins acting as modulators or blockers (Bosmans and Tytgat, 2007; Moran et al., 2009). Using a transcriptome-wide mining approach, we identified 16 toxin peptide families, including several with conserved domains implicated in ion channel modulation. Among these, a transcript encoding a putative Nav toxin was found to possess features of sodium channel-modulating peptides, including the conserved six-cysteine scaffold forming the beta defensin like motif. BLASTp analysis showed high similarity of this candidate sequence with known sodium channel toxins from related *Bunodosoma* species, particularly *B. cangicum* and *B. granuliferum*, with percent identities ranging from 64 % to 87 % and E-values as low as 4.6e–31, indicating strong homology. Structural modelling further supported the annotation of the transcript as a functional Nav toxin. The predicted tertiary structure showed the canonical sea anemone toxin fold: β-stranded scaffold with an exposed, flexible loop containing Arg-14, known to be critical for interaction with the voltage-sensor domain. This overall topology is highly consistent with known sea anemone Nav toxins such as Delta-AITX-Bca1a from *Bunodosoma capense* (van Losenoord et al., 2019) and Delta-actitoxin-Bcg1d from *Bunodosoma cangicum* (Zaharenko et al., 2012), all the peptides isolated from *Bunodosoma* sp. listed in Table 2, supporting the classification of this peptide as a Nav-targeting neurotoxin. The Arg-14 residue, located in a solvent-exposed loop, is conserved across all aligned sequences and has been previously shown to mediate high-affinity binding to the voltage-sensing domain of sodium channels (Khera and Blumenthal, 1994).

Functional validation of the synthetic peptide expressed from this transcript confirmed its ability to modulate rNav1.4 and hNav1.5 channels in *Xenopus laevis* oocytes. Electrophysiological recordings demonstrated a qualitative modulation of Nav channel current profiles, producing current traces consistent with the action of known sea anemone sodium channel toxins, effects that align with those previously described for type I and II sea anemone Nav toxins. (Moran et al., 2009; Wanke et al., 2009; Peigneur et al., 2014). This confirms the peptide's identity as a Nav-modulating toxin. Moreover, while initial exposure of juvenile Gambusia fish to the peptide in water did not produce any effect, intravascular administration triggered instant contraction paralysis. This phenomenon aligns with findings in peptide drug delivery research, where the administration route significantly influences the bioavailability and therapeutic efficacy of peptides (Reis et al., 2006). Conversely, direct injection into the bloodstream circumvents these barriers, allowing the peptide to maintain its structural integrity and promptly interact with its intended targets, thereby eliciting the desired physiological response (Ibraheem et al., 2014). The crude venom of *Bunodosoma goanense* consists of a complex blend of bioactive peptides and proteins that likely act synergistically to produce physiological effects. This synergism may explain why, despite the lack of an instant response in the juvenile *Gambusia* assay, the venom still exhibited notable contraction and paralysis without the need for invasive delivery or bloodstream access. The difference in paralytic effects between crude venom and the isolated synthetic peptide is likely attributable to venom complexity and route of administration. Crude venom contains multiple components that may act synergistically or facilitate tissue penetration, whereas the isolated peptide likely requires direct systemic access to exert its neurotoxic effects. The observed bioactivity suggests that some venom components are capable of penetrating epithelial barriers or acting locally at neuromuscular junctions. Such slow-onset but effective paralysis is consistent with the mode of action reported for certain sea anemone toxins, which modulate voltage-gated sodium channels at peripheral targets. Interestingly, the native peptide fraction (native Navtx-Bg1) isolated from tentacle venom also demonstrated potent activity against Nav1.4 and Nav1.5 channels, mimicking the effect observed with the synthetic candidate. Mass spectrometry of native Navtx-Bg1 revealed a molecular weight of 4973.1925 Da, closely matching the expected size of the transcript-predicted peptide. The observed molecular mass of the native peptide (4973.19 Da) is higher than that of the synthetic linear peptide (4859.47 Da) and cannot be explained by disulfide bond formation, which would instead result in a net loss of 6 Da. A more plausible explanation is the presence of one or more post-translational modifications (PTMs) in the native peptide, a phenomenon that is well documented in venom peptides (Buczek et al., 2005). Such modifications may include N-terminal processing or other oxidative or chemical modifications that contribute to increased molecular mass. In addition to other potential post-translational modifications, differences in the oxidation state of cysteine residues or other oxidative modifications may also contribute to the observed mass difference between the native and synthetic forms. Although MS/MS sequencing or direct comparison with an oxidized synthetic peptide would be required for definitive confirmation. Importantly, despite this mass difference, both the native and synthetic peptides produced highly similar qualitative modulation of Nav1.4 and Nav1.5 channels,

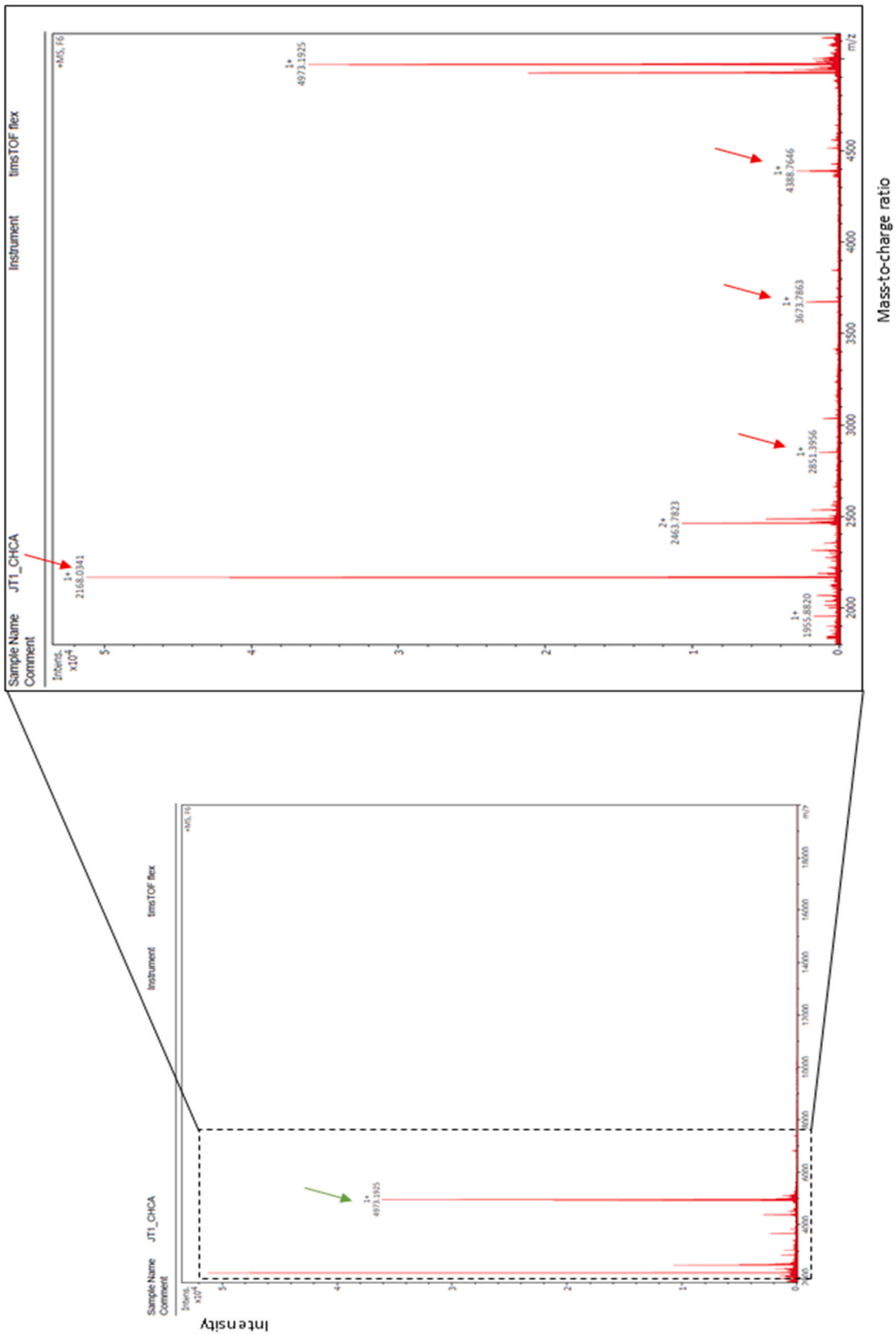


Fig. 8. Mass spectrometry profile of Native peptide native Navtx-Bg1 with an observed mass of 4973.1925 Da, which was confirmed by the presence of a double-charged species at 2463.78 Da, corresponding to the bioactive peptide isolated from the tentacle venom of *Bunodosoma goanense*. ((x-axis: (m/z), y-axis: intensity x10^4).

supporting their close functional relatedness. It can be hypothesized from our structural and functional findings that native Navtx-Bg1 and synthetic Navtx-Bg1 are likely the same peptide. We therefore have designated our peptide NavTx-Bg1, being the first characterized peptide from the venom of *Bunodosoma goanense*. The electrophysiological analyses presented here were designed for qualitative functional validation of transcriptome-guided toxin prediction. The study was not intended to provide detailed biophysical characterization, such as I–V relationships, kinetic parameter extraction, or statistical comparison across oocytes. These quantitative analyses will be addressed in future studies focused on pharmacological characterization.

The key takeaway is that transcriptomic prediction provided an accurate foundation to identify, model, synthesize, and validate a functional sodium channel-modulating toxin. Even though minor variations in mass and delivery method affected physiological outcomes, the conservation of bioactivity between native and synthetic forms underscores the predictive power of transcriptomic datasets in venom research. This strategy not only accelerates the discovery of new pharmacological tools but also enhances our understanding of venom composition and ecological function in understudied species such as *B. goanense*. The ability of the identified sea anemone-derived peptide to modulate Nav1.4 and Nav1.5 sodium channels highlights its promising potential as a lead compound for therapeutic development.

5. Conclusion

This study presents the first discovery and functional characterization of a sodium channel-targeting peptide from the sea anemone *Bunodosoma goanense*. By mining an annotated transcriptome, we identified a novel peptide sequence, confirmed its rNav1.4 and hNav1.5 activity through synthesis and electrophysiology. Our work demonstrates that transcriptome-guided venom exploration is a powerful strategy for discovering ion channel modulators and can contribute to the development of new pharmacological tools and therapeutic leads. It can be surmised that native Navtx-Bg1 and synthetic Navtx-Bg1 are the same peptide, based on our structural and functional results. Whether the native and synthetic peptides are chemically identical or functional analogues, the ability to predict and experimentally validate venom peptides opens new avenues in toxinology, neuropharmacology, and drug discovery. Moreover, the characterization of the first venom peptide NavTx-Bg1, establishes *B. goanense* as a new species of interest in the field of toxinology and venom-based drug discovery. From an ecological perspective, the identification of NavTx-Bg1 contributes to our understanding of how *Bunodosoma goanense* employs venom peptides for prey capture and defense, while from a pharmacological standpoint, this peptide expands the repertoire of sea anemone Nav modulators with potential utility as tools for studying sodium channel function.

CRediT authorship contribution statement

Cecelia Menezes: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Narsinh Thakur:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Kathleen Carleer:** Formal analysis, Data curation. **Steve Peigneur:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Jan Tytgat:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Ethical statement

The use of *Xenopus laevis* frogs was authorized by the KU Leuven Ethical Committee for Animal Research, with all experimental procedures adhering to EU Directive 2010/63/EU on laboratory animal care.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the first author CM used ChatGPT in order to improve the English language and correct grammar of the manuscript. After using this tool/service, the author reviewed and edited the content as needed and takes full responsibility for the content of the published article.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Cecelia Menezes reports financial support was provided by European Molecular Biology Organization. Cecelia Menezes reports financial support was provided by India Ministry of Science & Technology Department of Science and Technology. Steve Peigneur reports financial support was provided by European Commission. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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PRIMARY RESEARCH PAPER

Natural co-habitation with soft corals influences gene expression in *Bunodosoma goanense* den Hartog & Vennam, 1993

Cecelia Menezes · Narsinh L. Thakur

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Abstract This study explores the spatial associations between the sea anemone *Bunodosoma goanense* (Cnidaria: Actiniaria) and two soft corals, *Zoanthus sp.* and *Palythoa sp.* (Cnidaria: Zoantharia), on the rocky shores of Kunkeshwar, India, using field observations and transcriptomic analysis. Field observations revealed that *B. goanense* frequently exhibits a visible clearance zone when occurring within *Zoanthus* mats, while it coexists more closely with *Palythoa*. Although these spatial patterns suggest possible interaction differences, they remain correlative. We report the first transcriptomic dataset capturing gene expression variation in *B. goanense* across these distinct ecological contexts. Transcriptomic analysis revealed distinct gene expression profiles in the sea anemone depending on its immediate benthic neighbours. Association with *Zoanthus* was linked to upregulation of genes related to energy metabolism, cell proliferation, and protein synthesis, suggestive of an active physiological response. In contrast, presence within *Palythoa* mats was associated with a downregulation of growth-related genes and upregulation of inflammatory and stress-related pathways, indicating a distinct physiological response consistent with acclimatization. While our results do not directly test competition, they provide valuable molecular understanding of how *B. goanense* may adjust its physiology in response to its local biological environment in the intertidal zone.

Keywords Ecological interaction · Sea anemone · Soft coral · Transcriptomics · Gene expression

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C. Menezes · N. L. Thakur (✉)
CSIR-National Institute of Oceanography, Dona Paula, Goa, India
e-mail: thakurn.nio@csir.res.in

C. Menezes
e-mail: cecelia1994colvale@gmail.com

C. Menezes · N. L. Thakur
School of Earth, Ocean and Atmospheric Sciences, Goa University, Taleigao, Goa, India

Introduction

Tropical intertidal rocky shore habitats exemplify ecosystems subjected to extreme environmental fluctuations (Helmuth & Hofmann, 2001; Tomanek & Helmuth, 2002). It presents a stark contrast to the relatively stable conditions of the open ocean. While the ocean maintains consistent temperature, salinity, and other environmental factors, intertidal organisms endure dramatic and often unpredictable fluctuations in temperature, salinity, water availability, and organic content. These challenges include desiccation stress, thermal extremes, limited feeding times, and

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variable salinity levels (Newell, 1969). Despite the harsh and dynamic conditions, this region attracts a diverse array of organisms due to its abundant nutrient influx and thus high food availability. This richness in resources supports a high diversity of life, even though the space is limited (Satyam & Thiruchitrambalam, 2018). Consequently, they have adapted to evade environmental stressors in this region by selecting suitable microhabitats and by relying on specialized physiological and structural adaptations. However, the scarcity of space amidst the abundance of nutrients fosters ecological interactions among the inhabitants. (Connell, 1972; Newell, 1976; Petraitis et al., 2008). Mutualistic associations and nutrient cycling mechanisms highlight species' interconnectedness, contributing to these ecosystems' resilience and sustainability (Toby Kiers, 2010). Predation, a cornerstone of these ecosystems, shapes community structure and influences population dynamics (Dunning et al., 1992). Within the rocky tidepools, competition among species for space, food, and shelter is intense. Organisms have evolved diverse strategies to minimize direct competition (Southwood, 1977). Intense spatial competition can lead to competitive exclusion, where one species outcompetes others for a particular niche or space. However, coexistence also occurs through niche differentiation, where species partition resources or adapt to slightly different microenvironments, allowing them to thrive alongside competitors without directly competing (Tokeshi, 1997).

With limited space resource, sessile organisms like sea anemones and soft corals are particularly vulnerable to spatial competition (Chadwick & Morrow, 2011), which presents an intriguing ecological dynamic within marine ecosystems, particularly in rocky intertidal environments where these organisms often exist. Both sea anemones and soft corals belong to the class Anthozoa and share similarities in their ecological niches, leading to competition for space and resources. Sea anemones and soft corals often compete for suitable substrate and attachment sites within the rocky shore. This competition for attachment points can be intense, especially in areas with favourable conditions for both species. To gain an edge in the competition, sea anemones and soft corals may employ chemical warfare, which influences the settlement, growth, and survival rates of both organisms (Suchanek & Green, 1981; Quinn, 1982; Branch,1984; Harvell, 1990; Brace & Santer, 1991; Rabelo et al., 2013; Singh & Thakur, 2016; Avila, 2020; Singh & Thakur, 2021; Menezes & Thakur, 2022). Soft corals produce and release chemical compounds into the surrounding water, a strategy known as allelopathy (Sammarco et al., 1983). These compounds can inhibit the growth or settlement of nearby competitors, including other soft corals or encrusting organisms (Suchanek & Green, 1981; Rabelo et al., 2013; Singh & Thakur, 2021). They also possess a remarkable ability for rapid growth and encrustation, spreading across surfaces to outcompete neighbouring organisms. They use specialized structures like stolons or creeping mats to encrust available substrate efficiently. This growth strategy allows them to occupy space and shade out competitors, reducing their access to light and resources (Sebens, 1982; Doucette, 2022). Some soft corals have physical defences that deter competitors. By dominating available substrate, soft corals effectively reduce the chances of other organisms establishing themselves in the same area (Suchanek & Green, 1981; Wood, 1995; Pouryousef et al., 2020). They might have spiny structures, rough textures, or produce mucus coatings that discourage other organisms from settling or growing nearby. Some species possess specialized nematocysts or stinging cells that defend against predators and competitors (Dyrynda, 1986; Hines & Pawlik, 2012; Rabelo et al., 2013; Schmidt et al., 2019). Similarly, sea anemones possess defensive mechanisms against competitors. The tentacles of sea anemones are armed with nematocytes, which, when triggered by physical contact, explosively discharge, releasing a barbed thread that injects venom into the target organism (Jenner & Undheim, 2017). This venom contains a mix of peptide toxins, which can paralyse or deter competitors (Madio et al., 2019; Menezes & Thakur, 2022). Besides the nematocysts, sea anemones also produce venomous ectodermal mucus secretions. The mucus contains toxins that serve as an additional defence (Moran et al., 2012). Acrorhagi are specialized structures found in some sea anemones, particularly employed during non-clonal intraspecific spatial competitions (Jenner & Undheim, 2017; Bigger, 1980). When in contact with neighbouring competitor sea anemones, acrorhagi can be inflated and used in aggressive warring interactions (Francis, 1973; Ottaway, 1978; Brace et al., 1979; Brace, 1981; Daly, 2003).

Several studies have investigated the agonistic and competitive behaviours of sea anemones in intertidal and reef ecosystems which include reports about interspecific and intraspecific non-clonal competition. Sebens (1976) reported a competitive interaction between the Caribbean sea anemone and scleractinian corals. Sebens (1984) studied the agonist behaviour of the intertidal sea anemone *Anthopleura xanthogrammica* (Brandt, 1835) and reported that it exhibits acrorhagial inflation primarily as a communication mechanism rather than severe intraspecific competition, allowing neighbours to adjust positioning based on space availability. Sebens (1983) studied the population dynamics of *Anthopleura xanthogrammica* and *A. elegantissima* (Brandt, 1835) in Washington state. The growth and reproductive success of both the sea anemones are influenced by both non-interference competition for prey and direct interference from adult tentacle crowns blocking prey capture by smaller individuals. Francis (1973) studied the intraspecific interaction of *A. elegantissima* and reported that it exhibits aggressive behaviour, involving damage or threats between genetically distinct individuals, influencing their field distribution and isolation. Ottaway (1978) studied the intraspecific competition in *Anthopleura tenebrosa* (Brandt, 1835) that involved acrorhagi among adults, prompting non-fatal conflicts, wounding and movement away. Palaoro et al., (2017) reported that environmental cycles, such as tidal flow, influence fighting behaviour in *Actinia equina* (Linnaeus, 1758) by increasing resource value and persistence, while reducing escalation likelihood due to altered costs. Purcell & Kitting (1982) studied the intraspecific aggression in *Metridium senile* (Linnaeus, 1758). Mixed clonal aggregations of *M. senile* persist due to habituation to non-clonemate contact, with occasional loss of habituation reigniting aggression. Brante et al., (2019) reported that the range expansion of invasive anemone *Anemonia alicemartinae* Häussermann & Försterra, 2001, along the Chilean coast, involves weak interspecific competition with native *Phymactis papillosa* (Lesson, 1830). Zoanthid species (*Palythoa caribaeorum* Duchassaing & Michelotti, 1860*, Zoanthus sociatus* (Ellis, 1768), and *Palythoa variabilis* (Duerden, 1898)) in the intertidal zone of Northeastern Brazil exhibit distinct competitive strategies, with *P. caribaeorum* and *Z. sociatus* (Ellis, 1768) outgrowing and often displacing *P. variabilis*, while coexisting without aggression in intertidal zones (Rabelo et al., 2013). Our study investigates the interaction of sea anemone *Bunodosoma goanense* den Hartog & Vennam, 1993, with soft corals *Zoanthus* sp. and *Palythoa* sp., at the molecular level using the transcriptomic approach and it aligns with recent investigations into sponge competition along the West coast of India, the work of Deshpande et al. (2025), highlighting the emergence of transcriptomic approaches in understanding the complex molecular interplay in sessile organism interactions.

The rocky shores along the West Coast of India are home to diverse benthic communities, including sea anemones and soft corals (Harkantra & Parulekar, 1989; Den Hartog & Vennam, 1993; Balasaheb et al., 2017). Among these, the sea anemone *Bunodosoma goanense* (Cnidaria: Actiniaria) and the soft corals *Zoanthus sansibaricus* Carlgren, 1900 (Cnidaria: Zoantharia) and *Palythoa* sp. (Cnidaria: Zoantharia) have been observed co-occurring within this habitat (Fig. 1a–c). This study aims to investigate the molecular underpinnings of the effects of these co-habitation on *B. goanense* by combining field observations with transcriptomic analysis. Based on field observations, we hypothesized that spatial association of *B. goanense* with *Zoanthus sp.* and *Palythoa sp.* be associated with distinct transcriptional responses in *B. goanense*.

Our work presents a novel examination of the physiological response of *B. goanense*, making it the first study to explore both the *in-situ* spatial dynamics between sea anemones and soft corals, as well as the associated gene expression patterns. Through differential gene expression analysis of *B. goanense* during these interactions, we aim to uncover the transcriptional response of this sea anemone as a result to these co-habitations. This approach offers valuable insights into the molecular mechanisms of adaptation and resilience in an organism navigating competitive pressures, extending beyond traditional ecological observations.

Materials and methods

Study site and field study

Field observations and sampling were conducted during low tide in April 2023 along the mid-littoral

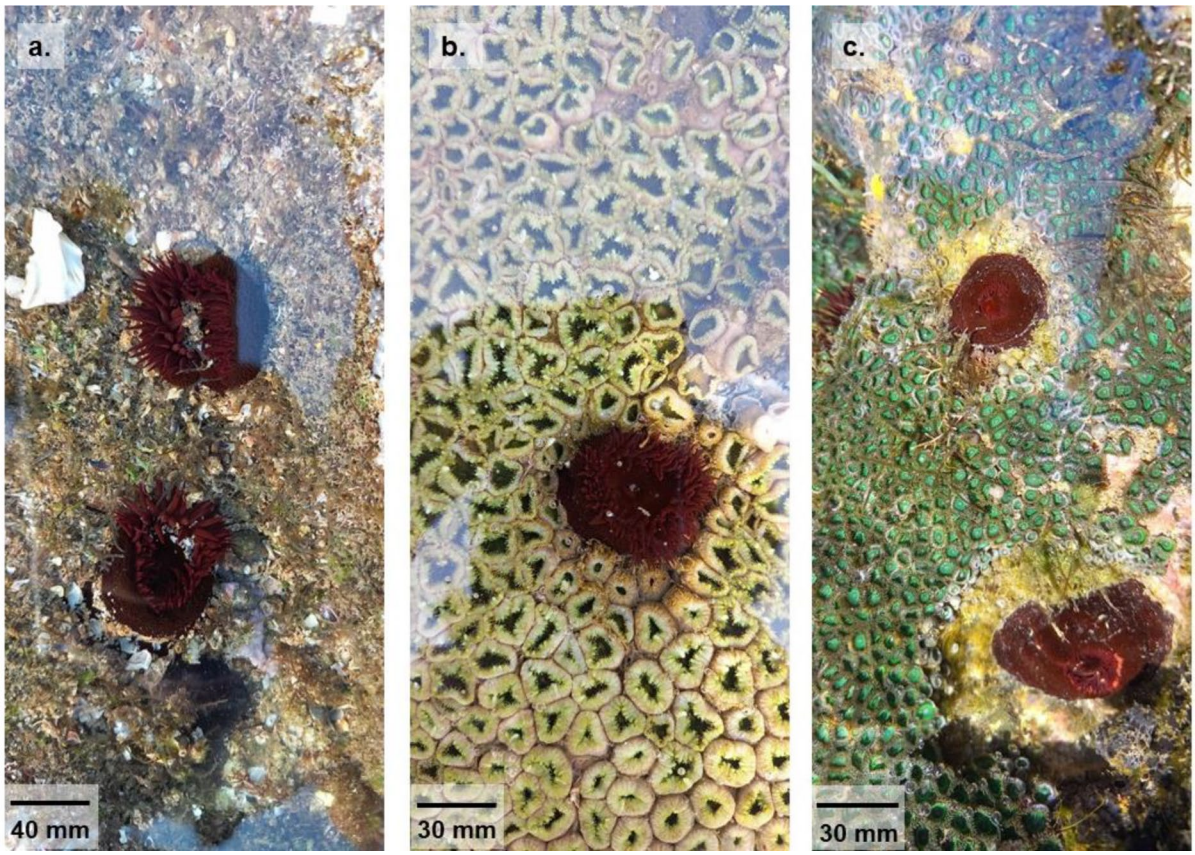


Fig. 1 **a** *B. goanense* without cnidarian neighbours (control) **b** *B. goanense* individual co-habitating with the *Palythoa sp*. **c** *B. goanense* individuals co-habitating with the zoanthid *Zoanthus* sp. with a presence of a clearance zone around the sea anemone on the rocky shores of Kunkeshwar

rocky shore of Kunkeshwar, Maharashtra, India. This intertidal zone, characterized by a heterogeneous rocky substrate, hosts extensive mats of *Zoanthus* and *Palythoa* colonies interspersed with solitary individuals of the sea anemone *Bunodosoma goanense*. These natural assemblages provided an ideal setting for investigating the spatial associations and potential competitive interactions between sea anemones and soft corals. To explore whether different spatial contexts might be associated with distinct molecular responses in *B. goanense*, we categorized sea anemone samples into three groups based on their proximity to soft corals: (i) individuals without nearby soft corals (hereafter referred to as Control, C) (Fig. 1a), (ii) individuals found in proximity to *Palythoa sp*. colonies (P) Fig. 1b, and (iii) individuals located within or adjacent to *Zoanthus* sp. mats (Z) (Fig. 1c). Two parallel transects, each 35 m long, were laid across the mid-littoral zone on gently sloped rock platforms with tide pools where *B. goanense, Zoanthus* sp., and *Palythoa* sp. co-occurred. A total of 24 quadrats (1 m × 1 m) were placed at intervals of 1 m along these transects. Within each quadrat, the presence, abundance, and contracted diameter of all adult *B. goanense* individuals were recorded, along with the distance between each anemone and the nearest soft coral colonies. Differences in the frequency of spatial associations between *Bunodosoma goanense* and *Zoanthus* sp. or *Palythoa* sp. were tested using a Chi-square (χ^2) test of independence. Observed frequencies of associations were compared against expected frequencies under the null hypothesis of random association. Statistical significance was assessed at $\alpha = 0.05$.

Triplicate samples of *B. goanense* individuals from each condition were collected (whole body),

immediately flash-frozen in liquid nitrogen, and stored at − 80 °C until RNA extraction.

RNA extraction, library preparation, and sequencing

Total RNA was extracted from the whole bodies of nine sea anemone samples using the TRIzol reagent (Invitrogen, USA) following a standard protocol. Tissue samples were placed in centrifuge tubes with 1 ml TRIzol reagent, homogenized thoroughly, and incubated at room temperature for 5 min. The homogenate was centrifuged at 10,000×*g* for 10 min at 4 °C, and the supernatant was collected. To the supernatant, 0.2 ml chloroform was added, mixed vigorously, and incubated at room temperature for 3 min. The mixture was centrifuged at 12,000×*g* for 10 min at 4 °C, and the upper aqueous phase was collected. To the aqueous phase, 0.5 ml isopropanol was added, gently mixed, and incubated at room temperature for 10 min. The solution was centrifuged at 12,000×*g* for 10 min at 4 °C, and the RNA precipitate was retained. The pellet was washed with 1 ml of 75% pre-cooled ethanol and vortexed gently. After centrifugation at 12,000×*g* for 5 min at 4 °C, the ethanol was removed, and the RNA pellet was air-dried. The RNA pellet was dissolved in 20 µl of DEPC-treated water and incubated at 60 °C for 5 min. RNA integrity was assessed using an Agilent 2100 instrument, while RNA purity and concentration were evaluated using a NanoDrop spectrophotometer (Stefanik et al., 2013; Mansfield et al., 2017; Shum et al., 2022). RNA sequencing libraries were prepared using the Illumina TruSeq Stranded mRNA Sample Prep Kit and were analysed on the 4200 TapeStation system. The libraries were sequenced on an Illumina NovaSeq 6000 platform to generate paired-end reads of 150 bp.

De novo transcriptome assembly

The analysis was performed using the tools available on the Galaxy platform following the tutorial Galaxy training tutorial on De novo transcriptome assembly, annotation, and differential expression analysis (Batut et al., 2018; Bretaudeau et al., 2023; Hiltemann et al., 2023). This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GKWR00000000. The version described in this paper is the first version, GKWR01000000. Raw sequencing reads were subjected to quality control using FastQC (Galaxy Version 0.74 + galaxy0) (Andrews, 2010) and preprocessing using Trimmomatic (Galaxy Version 0.38.1) (Bolger et al., 2014) to remove low-quality bases (Phred score < 20) and adapters. Paired-end reads were used, and the raw FASTQ dataset was processed with the following settings. Adapter sequences were removed using the ILLUMINACLIP step with the TruSeq3 configuration optimized for paired-end sequencing on MiSeq and HiSeq platforms. Several quality trimming operations were applied sequentially: TRAILING to trim low-quality bases from the ends of reads, LEADING to remove low-quality bases from the start of reads, and SLIDINGWINDOW to eliminate regions with low average quality score below 20. Additionally, reads with an average quality score below a set threshold were removed using the AVGQUAL operation. A two-step length filter was applied, retaining reads with a minimum length of 25 bases during the trimming process and further filtering out reads shorter than 50 bases in the final step. Log messages were generated to document the trimming process, ensuring the reproducibility of the preprocessing workflow. Quality assessment of cleaned reads after trimming was conducted to ensure the removal of low-quality sequences and adapters, utilizing FastQC (Galaxy Version 0.74 + galaxy0) (Andrews, 2010) for evaluation. De novo transcriptome assembly was performed using Trinity (Galaxy Version 2.15.1 + galaxy1) (Grabherr, 2011). Reads were remapped to the raw transcriptome to validate the assembly and assess its compatibility with the original data using the Align reads and estimate abundance tool (Galaxy Version 2.15.1 + galaxy0) (Grabherr, 2011). The abundance estimation was performed using Salmon, a highly efficient tool for quantifying transcript abundance. Using the tool Build expression matrix (Galaxy Version 2.15.1 + galaxy0), mapping tables were merged, and read counts were normalized. To assess the completeness of the transcriptome assembly, we employed the BUSCO (Benchmarking Universal Single-Copy Orthologs) software (metazoa odb9) (Galaxy Version 5.5.0 + galaxy0) (Simão et al., 2015). BUSCO analyses the presence and integrity of a set of highly conserved genes expected to be found in a complete transcriptome. Transcripts with low expression levels were filtered out using Filter low expression transcripts (Galaxy Version 2.15.1 + galaxy0) (Grabherr, 2011) to ensure the accuracy and

reliability of downstream analyses, retaining only transcripts with significant expression levels for further evaluation.

Annotation

A gene-to-transcript map was generated to establish relationships between genes and their corresponding transcripts using the tool Generate gene–to-transcript map (Galaxy Version 2.15.1 + galaxy0) (Grabherr, 2011). Protein-coding sequences were predicted from transcripts using TransDecoder (Galaxy Version 5.5.0 + galaxy2) (Haas et al., 2013). Predicted peptides were subjected to a similarity search against Uniprot database using NCBI BLAST + BLASTp (Galaxy Version 2.14.1 + galaxy1) (Altschul, 1997; Camacho et al., 2009; Cock et al., 2015). Annotation of transcripts was carried out using Trinotate (Galaxy Version 3.2.2 + galaxy0) (Grabherr et al., 2011). The analysis utilized the filtered transcriptome file as input for transcript sequences and the predicted peptide sequences for protein-level analysis.

Differential expression analysis

Reads were remapped to the filtered set of transcripts to focus on the most relevant transcripts using Align reads and estimate abundance (Galaxy Version 2.15.1 + galaxy0) (Grabherr et al., 2011). Mapping tables were merged, and read counts were normalized using the TMM method using Build expression matrix (Galaxy Version 2.15.1 + galaxy0) (Grabherr et al., 2011). Differential expression analysis was performed using Differential expression analysis (Galaxy Version 2.15.1 + galaxy0) (Grabherr et al., 2011) tool using the DESeq2 method (Love et al., 2014) using negative binomial distribution model. Differentially expressed transcripts were extracted and clustered based on expression patterns using Extract and cluster differentially expressed transcripts (Galaxy Version 2.15.1 + galaxy0) (Grabherr et al., 2011). Genes were grouped into expression clusters using tool Partition genes into expression clusters (Galaxy Version 2.8.4 + galaxy1) (Grabherr et al., 2011).

The accession numbers corresponding to transcripts were extracted from the Trinotate output, and a comprehensive search was conducted utilizing the UniProt ID mapping tool (https://www.uniprot.org/id-mapping) to retrieve the corresponding Gene Ontology (GO) terms associated with these accession numbers. Predicted peptide sequences obtained from Trinotate were mapped to UniProt accessions using the UniProt ID mapping tool to retrieve associated GO terms. The acquired GO terms were then systematically amalgamated with their respective transcripts.

Gene ontology enrichment analysis

Gene Ontology enrichment analysis was executed employing Panther 18.0 software (accessed online on 08 January 2024). Protein Analysis THrough Evolutionary Relationships (PANTHER, 2024; http://pantherdb.org) is a database that classifies proteins into families and functionally related subfamilies based on evolutionary relationships, enabling precise functional annotations linked to Gene Ontology terms and pathways (Thomas et al., 2003a, 2003b; Mi et al., 2005; Zhou et al., 2017; Thomas et al., 2022). PANTHER uses the complete set of genes of the organism under study as the background for enrichment analysis. The statistical assessment of enrichment was accomplished through the application of Fisher's exact test (Azizpour et al., 2020; Liu et al., 2020; Mohammadi et al., 2023). The enrichment results were filtered using Fisher's exact test ($P < 0.05$).

Results

The sea anemone, *Bunodosoma goanense*, displayed a zone of clearance around its base when inhabiting mats of the zoanthid, *Zoanthus sp*. This clearance zone, devoid of *Zoanthus* polyps, formed a distinct halo around the sea anemone. Interestingly, this phenomenon was not observed when *B. goanense* co-occurred with mats of the *Palythoa* sp.

In the field study, a total of 24 quadrats along 2 transects were examined, resulting in a count of 258 sea anemones across different interaction conditions: 142 in the *Zoanthus* mats, 95 in the *Palythoa* mats, and 21 control with no immediate neighbours. For the contracted diameter measurements, the average diameter of 20 sea anemones was recorded in each condition: 3.25 cm for those interacting with *Zoanthus* (Z), 2.05 cm for those interacting with *Palythoa* (P), and 3.37 cm for control individuals (C). Additionally, the distance of the clear zone (halo) between the sea anemone and the nearest *Zoanthus* polyp was

measured for 20 individuals, yielding an average halo distance of 3.065 ± 0.920 cm across the samples. It is important to note that in the entire area surveyed, there were no sea anemones in the *Zoanthus* mat that did not have a halo around it. To determine if interaction with *Zoanthus* or *Palythoa* is more frequent than expected by chance, Chi-Square test was performed. Chi-Square test ($\chi^2 = 86.53$, $P < 0.0001$) revealed significant differences between the observed frequencies of sea anemone interactions with *Zoanthus, Palythoa*, and control areas. The interactions are not random, and the observed frequencies significantly differ from the expected frequencies. This suggests non-random spatial associations between the sea anemone and *Zoanthus* as well as *Palythoa*. This supports the idea of potential competitive interactions; the observed patterns are correlative and do not represent a formal test of competitive exclusion. This initial field observation sparked a deeper investigation into the potential mechanisms underlying these contrasting interactions.

Triplicates of *B. goanense* individuals co-habitating with the zoanthid *Zoanthus* sp. (Z), *B. goanense* individuals co-habitating with the *Palythoa sp*. (P), and control samples of *B. goanense* individuals (C) were collected from areas devoid of any immediate soft corals. The collected RNA samples from each group were then subjected to RNA sequencing. This process generated millions of short sequence reads representing the transcripts present in the samples. The sequencing data were processed on the Galaxy platform. The initial read count was approximately 25,000,000. Following trimming, 90% of reads remained, indicating minimal data loss. The average base quality score after trimming was Phred 33, which corresponds to a base call accuracy of 99.9%. This high score suggests that the trimmed reads are of excellent quality and suitable for downstream analyses. The trimmed reads from each RNA-seq sample were assembled into a de novo transcriptome. The resulting transcriptome comprised a total of 309,954 assembled transcripts. The N50 length of the contigs was determined to be 1 kb. The BUSCO analysis for our transcriptome assembly yielded a score of 93.3%, indicating a high degree of completeness. This score signifies that a large proportion of the expected universal single-copy genes were successfully identified within the assembled transcripts, suggesting a high-quality transcriptome suitable for further analyses. Transdecoder predicted 82,146 peptide sequences. Among these sequences, 29,916 exhibited significant matches to proteins within the Swiss-Prot database. Predicted transcripts are 26.50%, and annotated peptides are 9.65% of the total assembled transcripts.

The differential expression analysis included 3 biological replicates each distributed across 3 conditions (control, sea anemone co-habitating with *Zoanthus* and sea anemone co-habitating with *Palythoa*). 759 transcripts were identified as significantly differentially expressed, of which 316 were annotated. During interaction with *Zoanthus sp*, 21 (annotated) genes were upregulated, and 27 (annotated) genes were downregulated in *B. goanense*, whereas during interaction with *Palythoa sp.* 87 (annotated) genes were downregulated and 89 (annotated) genes were upregulated. The differentially expressed genes (DEG) can be visualized in the heatmaps, where Fig. 2 shows all the genes (annotated and unannotated gene) that are differentially expressed, and Fig. 3 represents only the annotated DEGs.

We filtered the dataset to include only those genes that were upregulated in the *Zoanthus* interaction and simultaneously downregulated in the *Palythoa* interaction and vice versa. A total of 121 genes were identified as upregulated during the interaction with *Palythoa sp.* and downregulated during the interaction with *Zoanthus sp.*, whereas 112 genes were found to be upregulated during the interaction with *Zoanthus sp.* but downregulated during the interaction with *Palythoa sp*. These differentially expressed genes highlight distinct adaptive responses employed by the sea anemone *Bunodosoma goanense* during its interactions with these two competing soft coral species. Also, a subset of genes was consistently upregulated during interactions with both *Zoanthus* and *Palythoa*, and another group of genes was found to be consistently downregulated in both interaction scenarios. The presence of both interaction-specific and shared gene expression patterns highlights the complexity of these interactions. (Online Resource 1).

Analysing individual genes alone can be limiting, as it overlooks the complex biological context in which genes operate and may miss important interactions or processes. Therefore, we performed a Gene Ontology (GO) enrichment analysis that provides a more comprehensive approach. The number of enriched GO terms that are upregulated and downregulated in both these interactions differs and is listed

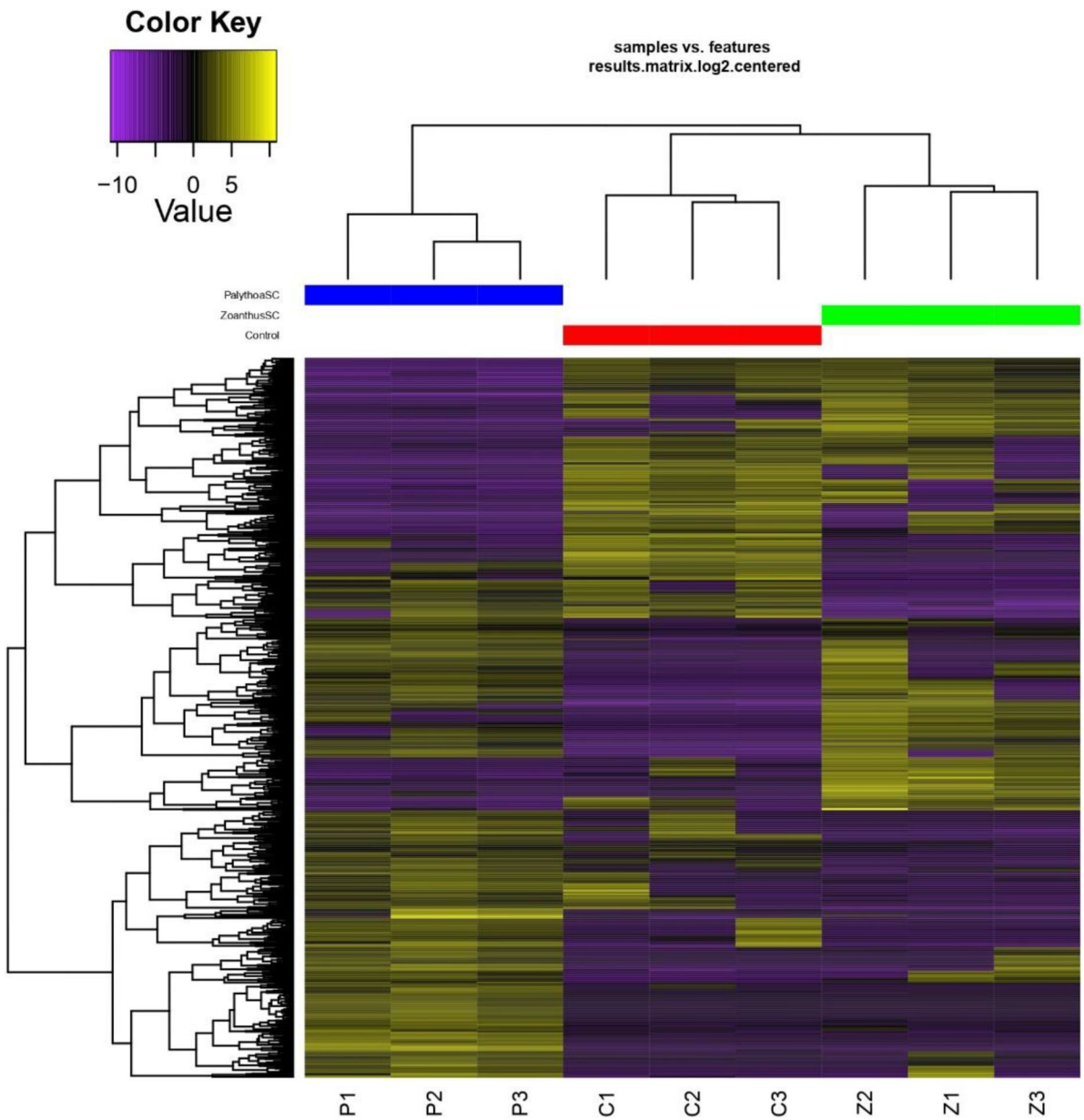


Fig. 2 The heatmap shows the results of a differential gene expression analysis (includes annotated and unannotated genes) comparing the gene expression profiles of the sea anemone *Bunodosoma goanense* under three conditions, that are sea anemone co-habitating with *Palythoa* (P1, P2, P3), sea anemone co-habitating with *Zoanthus* (Z1, Z2, Z3) and Sea anemone without any immediate neighbours (Control C1, C2, C3). Each row in the heatmap represents a gene, and each column represents a sample. The colour intensity in each square represents the level of gene expression, with yellow indicating upregulation and purple indicating downregulation. The higher the intensity of the colour, the greater the change in gene expression. The heatmap shows clear differences in gene expression patterns between the three groups, indicating that *B. goanense* modulates its gene expression depending on the soft coral neighbour it encounters

in Table 1. The List of the number of distinct GO categories (CC/MF/BP) that are upregulated and downregulated in both these interactions is listed in Online Resource 2.

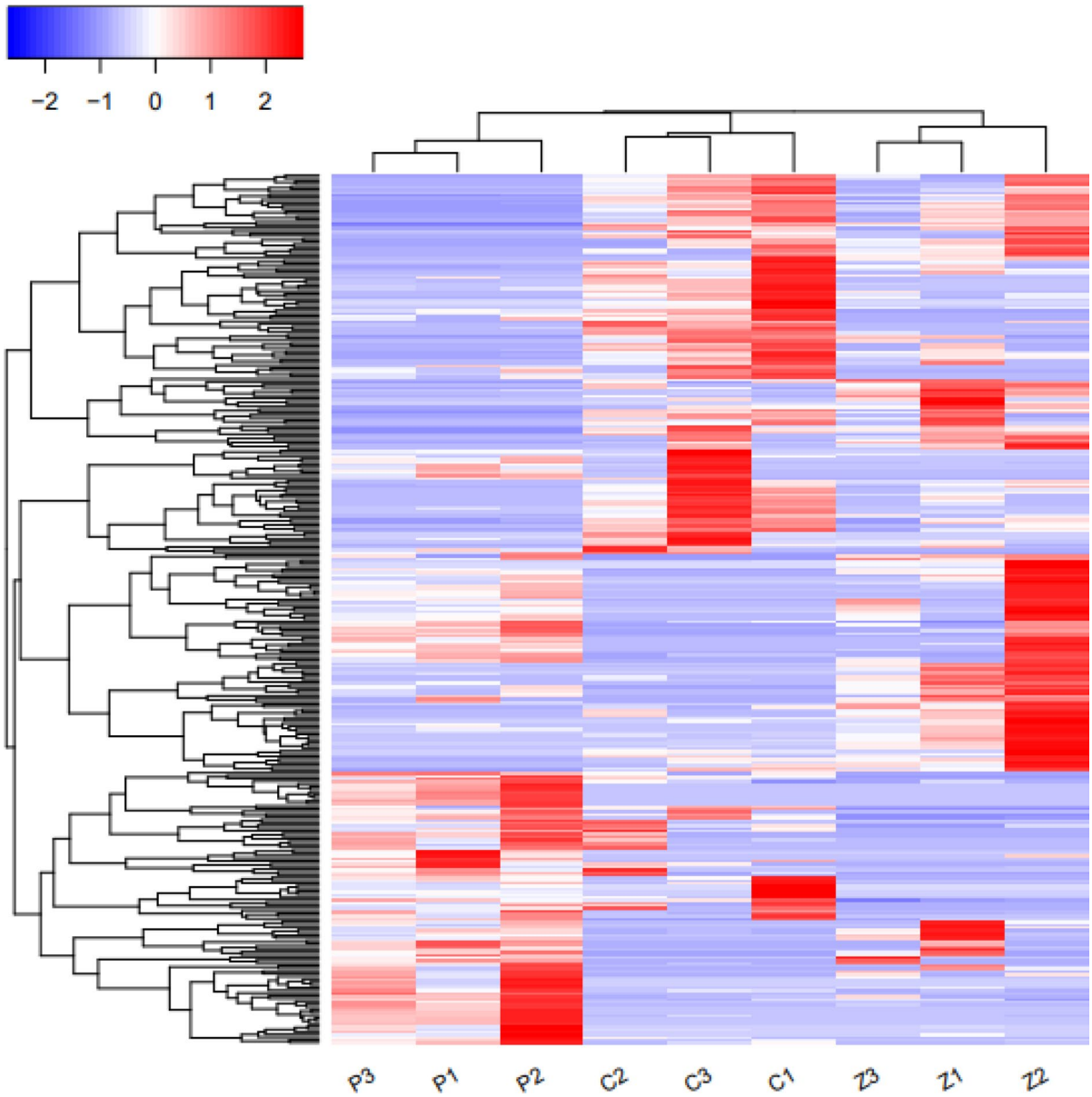


Fig. 3 The heatmap indicating differential gene expression profiles (only the annotated 316 genes) of the sea anemone *Bunodosoma goanense* competing with *Palythoa* and *Zoanthus* compared to a control sample

In the case of sea anemone interacting with *Zoanthus sp*., the significant enriched upregulated MFs were associated with galactokinase activity, galactose binding, carbohydrate kinase activity, monosaccharide binding, and carbohydrate binding. The enriched BP were associated with galactose catabolic process, protein localization to nucleoplasm, carbohydrate catabolic process, and DNA replication, whereas the significant downregulated BP were associated with glutamate metabolic process, establishment of protein localization to plasma membrane, heterochromatin formation, ribosomal large subunit biogenesis, dicarboxylic acid metabolic process, amide metabolic process. While in the case of sea anemone interacting with *Palythoa sp*., the significantly enriched upregulated

Table 1 List of the number of distinct GO categories upregulated and downregulated genes in sea anemone *Bunodosoma goanense* interactions with *Zoanthus sp*. and *Palythoa sp*. The GO terms are grouped into Cellular Component (CC), Molecular Function (MF), and Biological Process (BP), showing the differences in gene expression profiles under varying ecological interactions

Gene ontology categories	Sea anemone vs *Zoanthus* sp.		Sea anemone vs *Palythoa* sp.	
	No. of distinct GO categories from the upregulated genes	No. of distinct GO categories from the downregulated genes	No. of distinct GO categories from the upregulated genes	No. of distinct GO categories from the downregulated genes
Cellular component CC	0	10	17	25
Molecular function MF	5	12	31	20
Biological Process BP	24	9	268	182

BP were associated with positive regulation of inflammatory response to wounding, regulation of defence response to bacterium, and inflammatory response. The MFs were associated with Neuropeptide Y receptor activity, neuropeptide binding, and Protein kinase activity. However, the enriched downregulated BP were associated with regulation of growth, gamete generation, cellular processes, and developmental processes. Figure 4 shows the GO terms upregulated and downregulated by *B. goanense* during interaction with *Zoanthus sp.* and *Palythoa sp.* The relatively few genes that were

	Sea anemone interacting with *Zoanthus* sp.				Sea anemone interacting with *Palythoa* sp.			
GO term Category	Upregulated GO terms	Inference	Downregulated GO terms	Inference	Upregulated GO terms	Inference	Downregulated GO terms	Inference
Biological process	Galactose catabolic process (GO:0019388) Carbohydrate catabolic process (GO:0016052) DNA replication (GO:0006260) Protein localization to nucleoplasm (GO:1990173)	Increase energy demand for increased cell growth/division in the sea anemone	Glutamate metabolic process (GO:0006536) Dicarboxylic acid metabolic process (GO:0043648) Amide metabolic process (GO:0043603)	Focus on venom production	Positive regulation of defense response to bacterium (GO:1900426) Regulation of inflammatory response to wounding (GO:0106014) Inflammatory response (GO:0006954) Regulation of lysosome organization (GO:1905671)	Suggest the sea anemone is threatened by *Palythoa* and is actively defending itself	Gamete generation (GO:0007276) Cellular developmental process (GO:0048869) Developmental process (GO:0032502)	Indicate a decrease in activities related to growth and reproduction
Cellular component	No significantly Upregulated Cellular component	No significantly Upregulated Cellular component	Nuclear inner membrane (GO:0005637) Heterochromatin (GO:0000792) Euchromatin (GO:0000791) Cytosolic large ribosomal subunit (GO:0022625) Site of DNA damage (GO:0090734) Golgi lumen (GO:0005796)	Suggest less need for active gene expression changes	Signal recognition particle (GO:0048500) Semaphorin receptor complex (GO:0002116) Vacuolar lumen (GO:0005775) Primary lysosome (GO:0005766)	Increased protein synthesis and targeting, and potential changes in cell signaling	Cell cortex (GO:0005938) Extracellular matrix (GO:0031012)	Suggest a decrease in structural components and potentially reduced movement/adhesion
Molecular function	Galactokinase activity (GO:0004335) Galactose binding (GO:0005534) Carbohydrate kinase activity (GO:0019200) Monosaccharide binding (GO:0048029) Carbohydrate binding (GO:0030246)	Suggest the sea anemone's increase energy demand	D-glutamate cyclase activity (GO:0047820) Extracellular matrix constituent, lubricant activity (GO:0030197) Methylated histone binding (GO:0035064)	Suggest a decrease in glutamate production, extracellular matrix maintenance, and potentially chromatin remodeling	Neuropeptide Y receptor activity (GO:0004983) Neuropeptide binding (GO:0042923) Protein kinase activity (GO:0004672)	Suggest the sea anemone might be using neuropeptides for defense against *Palythoa*	G protein activity (GO:0003925) Mitogen-activated protein kinase p38 binding (GO:0048273)	Associated with stress signaling pathways

Fig. 4 GO terms upregulated and downregulated by *B. goanense* during interaction with *Zoanthus sp.* and *Palythoa sp*

annotated and had GO terms are only a very small fraction of the total transcripts assembled in this analysis.

Discussion

The observed spatial patterns and gene expression differences may be indicative of competitive dynamics between the target species. Our study at the Kunkeshwar rocky shores revealed intriguing insights into the potential competitive physiological responses employed by the sea anemone, *Bunodosoma goanense*. We observed a distinct difference in its interactions with two co-occurring cnidarians: *Zoanthus sp.* and *Palythoa sp.* A striking observation was the consistent presence of a clearance zone around every *B. goanense* when inhabiting *Zoanthus* mats. This halo, devoid of *Zoanthus* polyps, suggested a potential competitive edge held by the sea anemone. On the other hand, when co-habitating with *Palythoa*, *B. goanense* lacked a clearance zone, and the two cnidarians appeared to coexist in closer proximity. This observation aligns with the known aggressive spatial competition exhibited by *Palythoa sp*. (Bastidas & Bone, 1996; Rabelo et al., 2013; Rosa et al., 2016; Singh & Thakur, 2016).

To gain deeper understanding into the underlying mechanisms driving these contrasting behaviours, we analysed the transcriptome of *B. goanense* co-habitating with each of the two soft coral species. Several studies have employed heatmap visualizations of transcriptomic data to effectively illustrate differences in gene expression across various experimental conditions, providing a clear and intuitive representation of the molecular variations between competing species or different environmental contexts. These heatmaps help to highlight the upregulation or downregulation of specific genes, facilitating the identification of key molecular responses associated with ecological interactions and competition (He et al., 2018; Chang et al., 2020; Deshpande et al., 2025). In our study, the heatmap reveals a diversity in gene expression patterns across the three groups. This suggests that *B. goanense* exhibits adaptability, tailoring its gene expression profile to the specific soft coral species it encounters, highlighting its sophisticated physiological response for navigating the possible competitive interaction.

Both the control and *Zoanthus* co-habitating groups might share some similar expression patterns compared to the *Palythoa* co-habitating group. This could be because they represent scenarios where the anemone might not actively competing for space, unlike the *Palythoa* interaction. However, the heatmap also suggests distinct differences between the control and *Zoanthus* co-habitating groups. The *Zoanthus* co-habitating group appears to have more pronounced changes in gene expression (higher colour intensity) compared to the control. This suggests that even though they share some similar patterns, the anemone still exhibits a unique gene expression response when facing *Zoanthus* compared to the absence of any soft coral neighbour. The *Palythoa* interaction appears to trigger a more distinct response at the gene expression level. In our transcriptomic analysis, the HSP90 gene was significantly upregulated in the sea anemone (*Bunodosoma goanense*) surrounded by *Palythoa* (P) with a log2 fold change value of 4.4, suggesting that the anemone may be experiencing heightened stress or altered physiological conditions in this environment. This finding aligns with previous studies that have identified HSP genes as a key genes involved in stress responses including competitive interactions (Rossi & Snyder, 2001; Snyder & Rossi, 2004; Hoepner et al., 2019).

The GO term analysis reveals distinct responses the sea anemone employs when interacting with *Zoanthus sp*. and *Palythoa sp*. During the interaction with *Zoanthus sp*., the enriched upregulated Molecular Functions (MFs) like carbohydrate and galactose metabolism terms suggest the sea anemone's increased energy demand (Mattila and Hietakangas, 2017; Maughan, 2009). Biological Processes (BP) like galactose catabolism, carbohydrate metabolism, protein localization to the nucleoplasm, and DNA replication support the increased energy demand for increased cell growth/division (Mazia and Prescott, 1955). It is well established that changes in metabolism occur in sea anemones under various stress conditions and are critical for enabling the organism to adapt to and mitigate the effects of stressors (Ellington, 1982; Farag et al., 2024; Molina et al., 2017). Downregulated BP, like Glutamate metabolism, could free up these amino acids for promoting venom peptide production. In the case where the sea anemone interacts with *Palythoa sp*., the enriched upregulated BP such as inflammatory response, defence response

to bacteria, and positive regulation of inflammatory response suggest the sea anemone is threatened by *Palythoa* and is actively defending itself. MFs such as Neuropeptide Y receptor activity and neuropeptide binding suggest the sea anemone might be using neuropeptides for defence against *Palythoa*. The downregulated BP like regulation of growth, gamete generation, cellular processes, and developmental processes indicate a decrease in activities related to growth and reproduction. Previous studies have reported that sea anemones often exhibit reduced growth, development, and gamete production as a trade-off to cope with stress conditions. This adaptive response allows them to allocate energy and resources towards stress mitigation and survival, rather than reproduction or growth (Bucklin, 1987; Chadwick & Morrow, 2011; Howe et al., 2014; Glass et al., 2023; Surm et al., 2024).

In summary, the distinct molecular and physiological responses observed in our study suggest that *Bunodosoma goanense* may adjust its biological processes depending on the surrounding benthic context. Our observations lay the groundwork for exploring how sea anemones might modulate their responses to adjacent soft coral species and provide a basis for generating hypotheses about these potential competitive interactions.

Conclusion

Our study uniquely sheds light on the intricate relationship between sea anemones and soft corals by being the first to report on both the field observation of their potential spatial competition and the transcriptomic analysis of the sea anemone's response at the gene expression level. This paves the way for a deeper understanding of the molecular mechanisms underlying these interspecies interactions in their natural environment. Through this study we understand how spatial context can influence the physiological responses of a sessile marine invertebrate in a competitive benthic environment. By integrating field observations with transcriptomic analyses, our results suggest that the association of *Bunodosoma goanense* with different neighbouring taxa is accompanied by distinct patterns of gene expression, reflecting context-dependent physiological responses rather than fixed adaptive states. From an ecological perspective, these findings highlight the importance of local biotic interactions in shaping organismal responses at the molecular level, even in systems where direct behavioural avoidance or displacement is limited. The observed transcriptional shifts associated with different spatial associations may represent short-term acclimatory mechanisms that allow persistence in heterogeneous and competitive rocky shore habitats. Nevertheless, this study also raises several unresolved questions. Transcriptomic data alone cannot disentangle whether the observed expression patterns are driven directly by biotic interactions, underlying environmental heterogeneity, or a combination of both. Moreover, the correlative nature of the field-based associations limits inference about causality. Future research should therefore aim to combine controlled experimental manipulations with time-resolved transcriptomic or physiological measurements to explicitly test the mechanisms underlying competitive interactions in benthic cnidarians. Expanding such approaches to additional taxa and interaction types will further improve our understanding of how molecular responses scale up to influence ecological dynamics in space-limited marine communities.

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Author contributions Both the authors contributed to the conceptualization of the study. CM is responsible for sample collection, photography, data analysis, and wrote the manuscript. NLT is responsible for sample collection, reviewing, and fund acquisition.

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Data availability This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GKWR00000000. The version described in this paper is the first version, GKWR01000000.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the use of animals were followed by the authors. All necessary permits for sampling and observational field studies have been obtained by the authors from the competent authorities. The study is compliant with CBD and Nagoya protocols.

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Certificate of attendance

This certificate is proudly presented to

Cecelia Menezes



For participating in the 20th MBA postgraduate conference 2024

On the *23rd-25th April 2024* Signed *Rob Moore (social media secretary)*

Marine Biological Association

PML Plymouth Marine Laboratory

UNIVERSITY OF PLYMOUTH

NMA NATIONAL MARINE AQUARIUM

WOSC

World Ocean Science Congress 2024

"Sustainable Utilization of Oceans in Blue Economy"

Certificate

This is to certify that **Ms. Cecelia P.C. Menezes** has participated and presented a **Oral Presentation** at the **World Ocean Science Congress 2024** on the theme of **"Sustainable Utilization of Oceans in Blue Economy"** jointly organized by NIOT, IIT - Madras, and VIBHA at IIT-Madras Research Park, Chennai held from 27th to 29th February 2024.

Chairman
Dr. G. A. Ramadass
Director - NIOT

Co-Chairman
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Professor-IIT Madras

Current Trends in Drug Discovery Research
9th CTDDR 2025

9th International Conference on

Current Trends in Drug Discovery Research

February 19-22, 2025

Certificate

This is to certify that Dr./Mr./Ms. Cecelia Menezes has presented a poster entitled Therapeutic Potential of Marine Venom in the conference held at CSIR-Central Drug Research Institute, Lucknow

(Dr. Kumaravelu J.)
Organizing Secratary

(Dr. Kishor Mohanan)
Co-Organizing Secratary

(Director, CSIR-CDRI)
Chairperson

CSIR-Central Drug Research Institute, Lucknow, India
Sector 10, Jankipuram Extension, Sitapur Road, Lucknow- 226031 (India)

International Conference
on
Emerging Trends in Translational Bioinformatics

2024-2025
Platinum Jubilee Year Celebration

ET^{2}B-2024

December 5th-7th, 2024

Department of Bioengineering and Biotechnology,
Birla Institute of Technology, Mesra, Ranchi, India

This certificate is awarded to :

Cecelia Perpetua Celsa Menezes

CSIR-National Institute of Oceanography, Goa

for achieving the third position in **Poster Presentation**
at the International Conference on Emerging Trends in Translational Bioinformatics
Organised by Birla Institute of Technology Mesra, Ranchi, Jharkhand, India.

. Koel Mukherjee	Dr. Raju Poddar	Dr. Alok Jain	Dr. Kunal Mukhopadhyay
Organising Secretaries		Convenor	Chairman

Delegate No.: DELG000337

9th National Conference of Ocean Society of India

OSICON-25

CSIR-National Institute of Oceanography, Dona Paula, Goa

5-7 February 2025

C E R T I F I C A T E

This is to certify that

CECELIA PERPETUA CELSA MENEZES

of CSIR-National Institute of Oceanography, Goa
has participated and presented Poster in THEME 5 - Biological Oceanography
at the Ninth National Conference of Ocean Society of India, OSICON-25.

Dr. N. P. Kurian
President
Ocean Society of India

Dr. Mani Murali R
General Secretary
Ocean Society of India

Prof. Sunil Kumar Singh
Chairman
OSICON-25

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BHARATHIDASAN COLLEGE OF ARTS AND SCIENCE

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NATIONAL WEBINAR ON " MARINE BIORESOURCES AND BIOPROSPECTING"

Organized by

PG and Research Department of Biochemistry

CERTIFICATE

This is to certify that Ms. Cecelia P.C. Menezes, DST-Inspire JRF, CSIR-National Institute of Oceanography, Dona Paula, Goa. has participated in the National webinar on 14th June 2021.

Convener	Principal	Secretary and Correspondent
Dr.S.Selvi	Dr.R.Shunmughan	Thiru.N.K.K.Periasamy

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प्रतिभागिता प्रमाणपत्र
CERTIFICATE OF PARTICIPATION

प्रमाणित किया जाता है कि डॉ. / सु. / श्री / श्रीमती
This is to certify that Dr./ Ms./ Mr./ Mrs.

Cecelia Perpetua Celsa Menezes

ने 30-31 मार्च 2021 के दौरान डोना पौला, गोवा स्थित सीएसआईआर-एनआईओ, द्वारा आयोजित "समुद्री जैव-संसाधनों से प्राकृतिक उत्पाद" विषयक कार्यशाला सह प्रशिक्षण (वर्चुअल मोड) में भाग लिया
has participated in the Workshop cum Training "Natural Products from Marine Bioresources" (Virtual Mode), organized by CSIR-NIO, Dona Paula, Goa, during 30-31 March 2021.

R.A. Sreepada
कौशल प्रमुख, सीएसआईआर-एनआईओ
Skill Nodal, CSIR-NIO

Prof. Sunil Kumar Singh
निदेशक, सीएसआईआर-एनआईओ, गोवा
Director, CSIR-NIO, Goa

C-CAMP

Centre for Cellular and Molecular Platforms

CERTIFICATE OF PARTICIPATION

This is to certify that **Miss. Cecelia Menezes**

has attended and successfully completed the

'An Online Course in Untargeted Metabolomics using High Resolution Mass Spectrometry'

Organized by Centre for Cellular and Molecular Platforms (C-CAMP), held during 28th to 29th Oct, 2021

The course included Basics of Mass Spectrometry and Liquid Chromatography, Metabolite Profiling and Comparative Metabolomics using High-Resolution Mass Spectrometry, and Data Analysis of Untargeted Data Sets covering Peak Detection, Identification and Basic Multivariate Analysis. This course will help the participant to understand the basic work flow of Untargeted Metabolomics using High Resolution Mass Spectrometry and how this can be used in the context of addressing his/her own specific research problems.

Dr. Taslimarif Saiyed
CEO & Director, C-CAMP

Digital Signature for Course Certification
October 2021

Ms. Padma Ramakrishnan
Course Instructor

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OF PARTICIPATION

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Cecelia Perpetua Celsa Menezes

for attending 3 Day Online Technical Hands-on Online Training in
Next Generation Sequencing | RNASeq | Transcriptome Analysis
from **29 - 31 March 2023**

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Number: 1572570383

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of European Molecular Biology Organization

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Good research conduct	80%
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Planning your research	100%
Managing and recording your research	100%
Data selection, analysis and presentation	80%

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Certificate

Number: 1572570383

This is to certify that

Cecelia Menezes

of European Molecular Biology Organization

Successfully completed the course

Research Integrity: Core

Scholarly publication	80%
Professional responsibilities	90%
Communication, social responsibility and impact	80%

as part of the Epigeum Online Course System with a score of 86%.

Dated: 19 May 2023

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C-CAMP
Centre for Cellular and Molecular Platforms

CERTIFICATE OF PARTICIPATION

BLiSC
Bangalore
Life Science
Cluster

This is to certify that Ms. Cecelia . P. C. Menezes

has attended and successfully completed the

Hands-on Workshop on 'Proteomics'

held from 26th to 28th September 2022, organized by the Centre for Cellular and Molecular Platforms (C-CAMP).

The course covered

Basics Of Mass Spectrometry & Liquid Chromatography, Sample Preparation Strategies For Protein Identification & Quantitation, Phospho-peptide Enrichment & Analysis, Protein Quantitation Strategies, and Data Acquisition & Analysis.

This course will help the participant to understand the basic workflow of 'MS-proteomics' using 'High-resolution Mass Spectrometry' and how this can be used in the context of addressing their specific research-related problems.

Dr. Taslimarif Saiyed
CEO & Director
C-CAMP

Nirpendra Singh

Dr. Nirpendra Singh
Scientist D, inStem
Head, Mass Spectrometry Facility

Dr. Shadab Ahmad
Sr. Technology Scientist, C-CAMP
Mass Spectrometry Facility

No: DST/INSPIRE Fellowship/[IF190436]
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE and TECHNOLOGY
Department of Science and Technology
Technology Bhawan, New Mehrauli Road
New Delhi-110016

Date: 15 February, 2021

Subject: Award of INSPIRE Fellowship to the Research Students [IF190436]

Dear Cecelia Perpetua Celsa Menezes ,

The Government of India has launched a unique Scheme **"Innovation in Science Pursuit for Inspired Research (INSPIRE)"** with several components. INSPIRE Fellowship provides fellowship in Basic and Applied Sciences. I am pleased to inform you that you have been Selected for the award of INSPIRE Fellowship to host the same at the University/Institute/College/National Laboratory as indicated in the application form of your subsequent admission.

The value of the Fellowship will be at Par with the Junior Research Fellowship (JRF)/ Senior Research Fellowship (SRF) of Government of India along with a Contingency grant. The Fellowship shall be available to you from INSPIRE Fellowship Effective Date (which will be communicated through 1st Sanction Order) for a period of five years or completion of your doctoral (PhD) program, whichever is earlier.

If you are willing to join or switching over from earlier fellowship to INSPIRE Fellowship, you will require to upload the scan copy of Joining-cum-Acceptance Letter (JCA) (available at http://online-inspire.gov.in, and at template in your online dashboard), and also submit Bank details of your Host Institute along with the scan copy of cancelled blank cheque of given account details **within one month from the date of this letter, in your online portal only**, for taking necessary actions at INSPIRE Program Secretariat for releasing of your fellowship amount. Please also note that in the JCA Letter the Host Institution, Research Supervisor and Research Topic shall not be change or modification from initially submitted documents/ information for Final Offer.The Terms & Conditions for implementation of INSPIRE Fellowship are enclosed herewith.

Docuents submitted in any other modes like email attachment or by post or in-person shall not be acceptable.

In the event of your having being found ineligible at any state in future for the award/eligibility for INSPIRE Fellowship due to any reason(including unintentional computer error or printer's devil etc) this offer will be deemed withdrawn.

Ms.Tamanna Arora
Scientist 'C'

Cecelia Perpetua Celsa Menezes
C/O : Menezes Seraphino Joseph
Address : H.no. 55/3,St. Roque Vaddo, Colvale, Bardez, Goa
City : North Goa
State/UT: GOA - 403513

This is a Computer Generated Offer Letter, No Signature is required

Date: 15 February, 2021

GENERAL INSTRUCTIONS

Dear Cecelia Perpetua Celsa Menezes ,

Please note that these documents are necessary to upload within one month from the date of this letter at INSPIRE Online Webportal only for financial release after final selection

1. Joining-cum-Acceptance (JCA) Letter for INSPIRE Fellowship (template available in your dashboard in online portal))
2. Relieving Order from previous Fellowship/Job (if availing).
3. Copy of Cancelled Blank Cheque of the Host University/ College/ Institute Account for transferring the fund (Account should be registered in Public Financial Management System: https://pfms.nic.in).
4. PFMS registration certificate, or Unique Agency Code in case of University/College/Institute is newly registered in Public Financial Management System.
5. Since the fund would be transferred under Science & Technology Institutional and Human Capacity Building (1817), Hence bank account number of Host University/College/Institute should be registered under this scheme in PFMS.
6. **Please note that these documents are necessary to upload within one month from the date of this letter at INSPIRE Online Webportal only, otherwise it will be treated as the candidate has not intrested in accepting the INSPIRE Fellowship.**

Thanking you,

Ms.Tamanna Arora
Scientist 'C'

INSPIRE

Karin Dumstrei I EMBO I Meyerhofstr. 1 I 69117 Heidelberg I Germany

Ms. Cecelia Menezes
Chemical Oceanography Division
CSIR- National Institute of Oceanography
Raj Bhavan Road
Dona Paula, Goa 403004
India

FELLOWSHIP PROGRAMME

Dr. Karin Dumstrei
Head, Fellowship Programme

phone +49-6221-8891-122
fax +49-6221-8891-215

fellowships@embo.org

7 June 2024

Certificate for EMBO Scientific Exchange Grant Number 10178

To whom it may concern,

The purpose of this document is to certify that Ms. Cecelia Menezes has received an EMBO Scientific Exchange Grant (number 10178) to conduct research in the lab of Dr. Steve Peigneur (Department of Pharmaceutical and Pharmacological Sciences, Onderwijs & Navorsing 2 Herestraat 49 - box 424, Institue: Katholieke Universiteit Leuven, KU Leuven Oude Markt 13, 3000 Leuven, Belgium). The topic of the research project is "Analyzing the tissue-specific venom peptide profiles of the sea anemone Bunodosoma goanense and the pharmacological characterization of the peptides.".

The fellowship started on 17 July 2023, and EMBO funding was granted for a total period of 90 days.

If you need any further confirmation or information, please contact me.

Yours sincerely,

Karin Dumstrei

BP-2024-25-9001

FILE NO. ITS/2024/000406

Anusandhan National Research Foundation (ANRF)

(A statutory body created by an Act of Parliament - ANRF Act, 2023)

Science & Engineering Research Board (SERB) **International Travel Support (ITS) Scheme**

ANRF
3rd & 4th Floor, Block II
Technology Bhavan, New Mehrauli Road
New Delhi - 110016

Dated: 27 September, 2024

ORDER

Subject: Reimbursement of travel grant to **Ms. Cecelia P.C. Menezes (Young Scientist), Chemical Oceanography Division, CSIR National Institute of Oceanography , Raj bhavan road, Dona paula, Goa-403004** for attending "**Marine Biological Association Postgraduate Conference, Plymouth, United Kingdom (23 April, 2024 to 25 April, 2024)**" held in "**Plymouth, United Kingdom** ".

1. Sanction of the **Anusandhan National Research Foundation (ANRF)** is here by accorded to the payment of a sum of **Rs. 107675/- (Rs.** One Lakh Seven Thousand Six Hundred and Seventy Five **Only)** Under Grant-in-aid General for meeting the expenses incurred towards participating in the above International event.

2. Sanction of the grant is subject to the condition as detailed in Terms & Conditions available at website (www.serb.gov.in and www.serbonline.in).

3. The expenditure involved is debitable to "**Science & Engineering Research Fund**".

4. **This grant is being reimbursed** under the **ITS scheme.**

5. The Sanction has been issued to CSIR National Institute Of Oceanography, Raj Bhavan Road, Dona Paula with the approval of the competent authority under delegated powers on **11 July, 2024** and vide Diary No. **ANRF/F/4869/2024-2025** dated **12 July, 2024**.

6. The release amount of **Rs. 107675/-** (Rupees One Lakh Seven Thousand Six Hundred and Seventy Five only) will be drawn by the DDO of the ANRF and will be disbursed by means of RTGS transaction as per their Bank details given below:

Agency Name	CSIR-National Institute of Oceanography
PFMS Unique Code	DOD8PCNIO1
Account Name	CSIR National institute of Oceanography
Account Number	30267368692
Bank Name & Branch	State Bank of India Dona Paula, NIO Campus, Dona Paula, Goa, 403004
IFSC/RTGS Code	SBIN0003566
Email id of A/C Holder	sanil@nio.org
Email id of PI	cecelia1994colvale@gmail.com

7. It is certified that original boarding passes have been received along with other documents and retained in ANRF.

8. In the eventuality of any excess payment arising on account of typographical errors, etc., the excess amount should be refunded immediately to the ANRF by way of an a/c payee cheque in favour of the "Science & Engineering Research Fund". Non-compliance would lead to the ANRF initiating recovery procedures which would also attract applicable penal interest which would be decided by the ANRF.

9. In case of any discrepancy you may contact **ITS Section** at **ms.its@anrf.gov.in.**

I.K.

(Dr. Magesh K K)
Scientist E
ms.its@anrf.gov.in