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Characterization and anti microbial properties from the sea anemones [*Heteractis magnifica* and *Stichodactyla mertensii*] toxins

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ABSTRACT

Sea anemones are rich sources of biologically active substances and the present finding describes the toxic proteins obtained from the sea anemones, *Heteractis magnifica* and *Stichodactyla mertensii*. The toxic activities were analyzed by SDS-PAGE and the results showed protein bands with an apparent molecular weight of both the species. To assess the toxic activity of the venom, bioassays were carried out and to evaluate the effect of toxin, toxicity assay was also determined for in-vivo study using Ocypoda crab, *Ocypoda macrocera*. The hemolytic activities were also tested from the two crude extracts for chick, goat and human blood erythrocytes respectively. Further, the percentage inhibition of sea anemones extracts on the growth of the human pathogens, *Staphylococcus aureus* and *Salmonella typhi* were also studied. The results obtained from the above experiment were discussed in this paper.

Keywords Sea anemone, protein, bio-assay, hemolytic activity, antimicrobial activity, characterization.

INTRODUCTION

Marine organisms are known to have rich source of interesting organic molecules. Several compounds with diverse structural features and interesting biological activities have been found in invertebrates like Cnidarians. The marine ecosystem provides a variety of novel substances which control bacterial, fungal and viral diseases and also for cancer chemotherapy. Many soft bodied marine organisms have a sedentary life style necessitating chemical means of defense. Therefore, they have evolved the ability to synthesize toxic compounds obtain from marine micro organisms. These compounds help them deter predators, keep competitor at bay or paralyze their prey [1, 2].

The sea anemones belong to the class Anthozoa, phylum Cnidaria have got much attention in recent years. The members of this class are solitary and ocean dwelling. More than 32 sea anemones have been reported to produce cytolytic peptides and the proteins exhibits a wide diversity of biological activities such as haemolysis, cytotoxicity, cardio tropic activity and membrane depolarization [3-5]. The sea anemone cytolsins are water soluble polypeptides, exhibiting the unique property of inserting and accommodating spontaneously in to membranes due to their lytic capacity and possibility to address them to specific tissues, and have been evaluated as promising antitumor agents [6]. They attract more attention for potential use, particularly treating tumors and killing parasites [7]. Considering the immense use of the bioactive peptides, present study has been carried out with a view of isolating the bioactive peptides from the sea anemones, *H. magnifica* and *S. mertensii*. The nematocysts present on the edges of the tentacles of the anemone expel specific toxins [8]. Considering the immense use of these bioactive peptides, the present study has been planned with view to antimicrobial activities against *Staphylococcus aureus* and *Salmonella typhi* from the sea anemones, *H. magnifica* and *S. mertensii*.

MATERIALS AND METHODS

Sample preparation

Sea anemones, *H. magnifica* and *S. mertensii* were procured from the ornamental fish traders at Mandapam, Tamilnadu. They were transported using polythene bags and maintained alive in the laboratory condition. The healthy sea anemones were selected and subjected to stress applying 40°C hot water. The tentacle and the sucker region of the anemones were removed and the toxin was extracted by adopting two different methods.

One kg each of both the sea anemones were homogenized separately in one liter of ethanol with pH 7.4 and centrifuged at 15000 rpm for 30 min at 4°C. The supernatant was collected and added with 10 volumes of acetone and followed the precipitate was centrifuged. The pellet was dissolved in distilled water and lyophilized. Likewise, one kg each of both the sea anemones were homogenized with phosphate buffer saline [pH 7.4] and centrifuged at 15000 rpm for 30 min at 4°C. The supernatant was collected and lyophilized. Prior to lyophilisation, the amount of protein in both the samples were estimated by the method described by Lowry *et al.*[9], using bovine serum albumin (BSA) as the standard ranging from 0.1 to 1 mg/ml. 5ml of alkaline Copper reagent was added, mixed well and incubated at room temperature for 10 min. Followed, 0.5 ml of folin's reagent was added to all the test tubes of mixed well and the mixture was incubated for 30 minutes at room temperature. The absorbance at 650 nm was read using spectrophotometer [10].

Characterization

Molecular weight determination of the crude protein extracted from the sea anemone species viz. *H. magnifica* and *S. mertensii* was done with SDS-PAGE [Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis]. Crude protein was subjected to electrophoresis using 12% Poly acrylamide slab gels [11]. SDS-PAGE is a technique often employed for separation of large number of protein in heterogeneous samples largely on the basis of the molecular weight.

Toxicity assay

The lethal and paralytic activities of the sea anemones extracts were studied in the crab, *O. macrocera*. The crabs weighing about 5 ± 2 gm were collected from the shore of Parangipettai coast. The crude toxin of *H. magnifica* and *S. mertensii* were injected to the crabs at the junction of body and chelate leg. Four groups [Group-I, carpet anemone with acetone precipitation extract, Group-II, tentacle anemone with acetone precipitation extract, Group-III, carpet anemone with PBS extract and Group-IV, tentacle anemone with PBS extract] of 10 animals each were challenged with various doses [1.25, 2.50, 3.75 and 5.00 mg/ml] of crude toxins and the mortality was observed. The effects of these concentrations on colour change, foaming, paralytic effect and restlessness of the crabs were also observed.

Hemolytic assay

The hemolytic activity was carried out with the crude toxins extracted from the two anemone species with chicken, goat and human blood erythrocytes by micro hemolytic method [12].

Chicken and goat blood was collected in the vicinity of slaughter houses and the human blood was obtained from the hospital, using 2.7% EDTA solution as an anticoagulant at 5% of the volume of blood and brought to the laboratory. After collecting the blood with the anticoagulant, they were centrifuged at 5000 rpm for 5 minutes and the supernatant were discarded and the pellets suspended in normal saline [pH 7.4]. The mixture was centrifuged again at 5000 rpm for 5 minutes, the supernatant were discarded and the pellet was resuspended in normal saline [pH-7.4]. This procedure was repeated thrice. 1% erythrocyte suspension was prepared by adding 99 ml normal saline to 1 ml of packed erythrocytes.

Serial two fold dilutions of the crude toxin were made at 100 μ l of normal saline. This process was repeated up to the last well and 100 μ l was discarded from the last well. Later, 100 μ l of 1% RBC erythrocytes was added to all the wells and appropriate control was included in the test. 100 μ l of distilled water was added with the 1% RBC suspension which served as a positive control and 100 μ l of normal saline served as a negative control. The plate was gently shaken and allowed to stand for two hours at room temperature and the results were recorded. Uniformed colour suspension in the wells was considered as positive hemolysis and a button formation in the bottom of the wells was considered as lack of hemolysis. Reciprocal of the highest dilution of the crude toxin showing the hemolytic pattern divided by the protein content was taken as 1 hemolytic unit [HU].

Antimicrobial activity

The two different crude extracts obtained from the anemones using acetone precipitation and PBS extracts were evaluated against two bacterial stains namely *Salmonella typhi* and *Staphylococcus aureus*. The extracts were evaluated at concentration of 1000, 500, 250, 125, 62.5, 31, 3, 15.6, 7.8, 3.9 and 2.0 μ g/ml. The final concentrations were cultured in 5ml of Brain Heart Infusion [BHI] Broth [Hi-Media, P^H 7.4 $\pm$ 0.2 at 25°C]. The initial bacterial concentration were evaluated at concentrations were adjusted to about 0.05 OD or approximately 10² bacterial cells /ml concentration and the OD value of each of the concentration was noted at 0 hrs before incubation. The bacterial cells in presence of the test concentration and also the control tubes were incubated at 37°C for 24 hrs. Appropriated controls with solvent and with out solvents were also maintained simultaneously along with the test concentrations at the same bacterial count.

After incubation at 24 hrs the OD value at 620 nm was measured again for both control and test. The difference in the OD between control and the tested samples were used to calculate the percentage of growth inhibition.

RESULTS AND DISCUSSION

In the acetone precipitation method, the amount of crude protein obtained from the 500g fresh weight in each case was 1247 μ g/ml *H. magnifica*, and 1016 μ g/ml *S. mertensii*. In the PBS extraction method the amount of crude protein obtained from the 500g fresh weight in each case was 716 μ g/ml *H. magnifica*, and 603 μ g/ml *S. mertensii*.

SDS-PAGE on 12% gel crude extracts of *H. magnifica*, *S. mertensii* yielded bands ranging from 11-170KDa. Well distinct bands were observed in the case of *H. magnifica* at 14.0, 24.0, 40.0, 45.0, 66.0, 103.4, and 130.0 KDa in acetone precipitation extraction methods. Prominent bands were also observed in the case of *H. magnifica* between 24.0, 42.0 and 72.0 KDa. In the case of *S. mertensii*, prominent bands could be observed at 14.1, 40.0, 55.0, 72.0 and 130.0 KDa. In acetone precipitation and In the case of *S. mertensii*, prominent bands could be observed at 40.0 and 72.0 KDa. In PBS extract the SDS-PAGE on 12% gel crude extracts of *H. magnifica*, and *S. mertensii* yielded bands ranging from 11-170 KDa. Prominent bands were also observed in the case of *H. magnifica* band was not showed. In the case of *S. mertensii*, prominent bands were observed at 38.0 KDa [Fig.1 a-d].

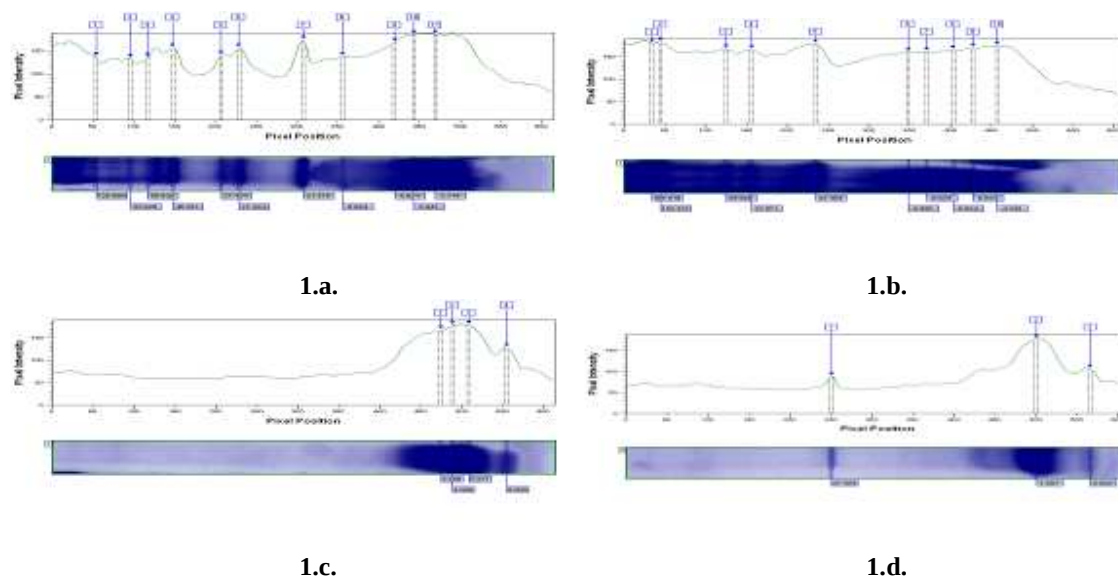


Fig: 1. The SDS-PAGE on 12% gel, crude extracts of *H. magnifica*, and *S. mertensii* yielded bands (lab.version 2.01).

The toxicity of the venom of both the sea anemones increased with increasing concentrations in all the four groups and the extracts caused the death of the test animals within 5 minutes at a dosage of 5 mg/ml. The mortality rate of the group II and group III were similar. Mortality was more pronounced in crabs challenged with the *H. magnifica* with acetone extract. The changes like foaming, paralytic effect, colour changes and restlessness are shown in table.1. [a-d].

Table.1. (a). Toxicity assay for toxicological studies of PBS extracts method in *S. mertensii*

Sl no.	Concentration (5mg/ml)	Observation			
		Colour changes	Foaming	Paralytic effect	Restlessness
1	0.25	-	-	-	+
2	0.50	-	-	-	+
3	0.75	-	+	+	+
4	1.00	-	+	+	+

Table.1. (b). Toxicity assay for toxicological studies of acetone precipitation extraction method in *S. mertensii*

Sl no.	Concentration (5mg/ml)	Observation			
		Colour changes	Foaming	Paralytic effect	Restlessness
1	0.25	-	+	+	+
2	0.50	-	+	+	+
3	0.75	+	+	+	+
4	1.00	+	+	+	+

Table.1.(c). Toxicity assay for toxicological studies of PBS extracts method in *H. magnifica*.

Sl no.	Concentration (5mg/ml)	Observation			
		Colour changes	Foaming	Paralytic effect	Restlessness
1	0.25	-	-	+	+
2	0.50	-	-	+	+
3	0.75	+	+	+	+
4	1.00	+	+	+	+

Table.1. (d) Toxicity assay for toxicological studies of acetone precipitation extraction method in *H.magnifica*.

Sl no.	Concentration (5mg/ml)	Observation			
		Colour changes	Foaming	Paralytic effect	Restlessness
1	0.25	-	+	+	+
2	0.50	+	+	+	+
3	0.75	+	+	+	+
4	1.00	+	+	+	+

Lethal doses of venom extracted from both sea anemones were found to be 0.5 ml per 5 ± 2 gm crab [*O. macrocera*]. Out of 10 animals of each test, the deaths were observed in between 30 minutes.

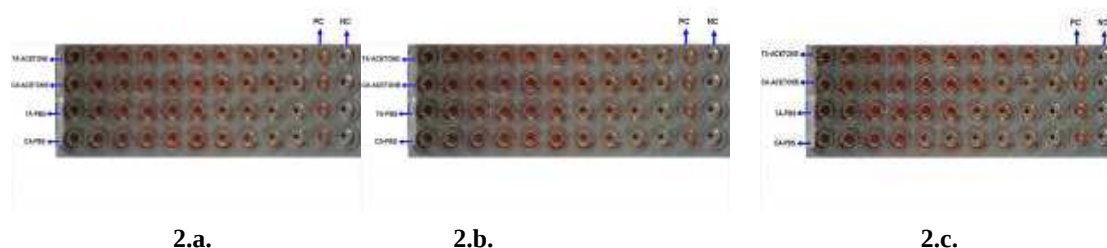


Fig: 2. Acetone precipitation extraction method and PBS extracts in (2.a) chicken, (2.b) goat and (2.c) human erythrocytes was shown that the haemolytic activity of both the sea anemones *H. magnifica* and *S. mertensii* toxins

The crude protein of the anemones produced pronounced hemolytic activity on chick, goat and human erythrocytes [Fig.2. a, b & c]. In acetone precipitation extraction method the crude protein (1.247 mg/ml) of *H. magnifica* elicited 2048, 512 and 1024 HU in chicken, goat and human erythrocytes respectively. The crude protein [1.016 mg/ml] of *S. mertensii* exhibited hemolytic activity of 512, 128 and 256 HU in chicken, goat and human erythrocytes respectively. Similarly PBS extraction method the crude protein [0.716 mg/ml] of *H. magnifica* elicited 2048, 256 and 2048 HU in chicken; goat and human erythrocytes respectively. The crude protein [0.603 mg/ml] of *S. mertensii* exhibited hemolytic activity of 512, 128 and 256 HU in chicken, goat and human erythrocytes respectively.

The percentage inhibition of sea anemone extracts on the growth on *Salmonella typhi* [Fig. 3. a-b] and *Staphylococcus aureus* [Fig. 4. a-b] is measured. An acetone extract showed highest inhibition against *S. aureus* [69.23%] and *S. typhi* [63.16%]. Among the four extracts, *H. magnifica* acetone precipitation extracts showed maximum inhibition followed by *H. magnifica* PBS extracts and *S. mertensii* acetone precipitation extracts and PBS extracts. *S. mertensii* acetone precipitation extracts and PBS extracts up to 30% inhibition against *S. aureus*, however poor inhibition was observed against *S. typhi*.

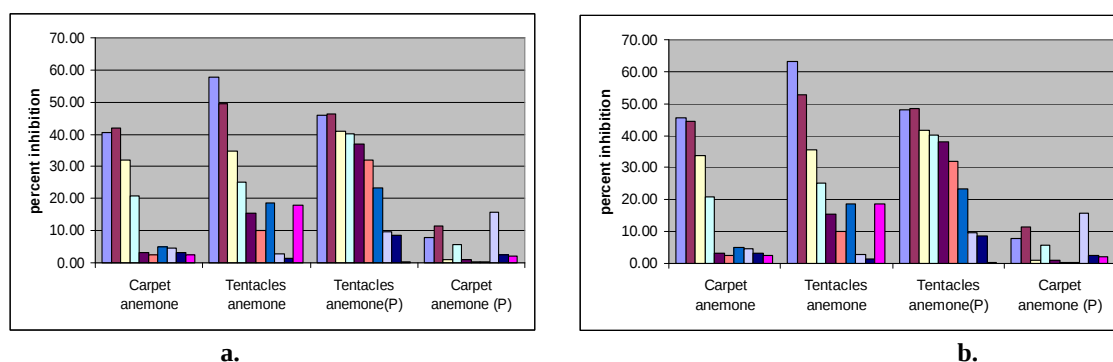


Fig. 3. Percent bacterial growth inhibition of sea anemones extracts on *Salmonella typhi* (a) after inoculation and (b) after 24 hours.

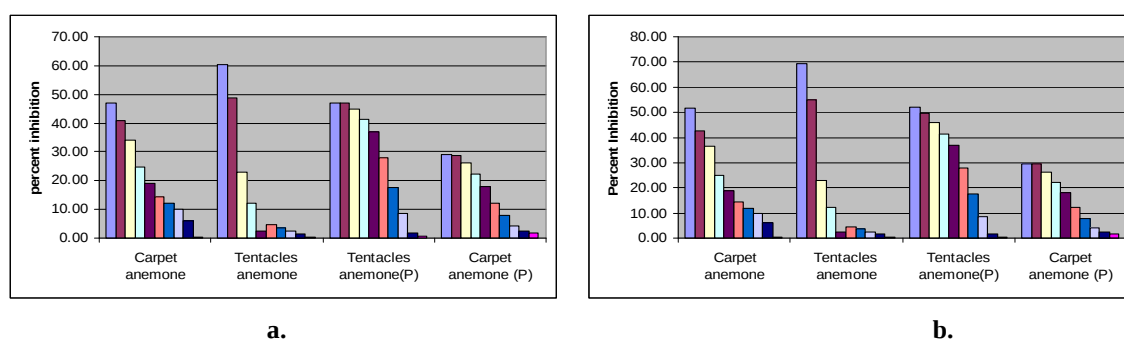


Fig. 4. Percent bacterial growth inhibition of sea anemone extracts on *Staphylococcus aureus* (a) after inoculation and (b) after 24 hrs

In the present study, toxic proteins have been isolated from the body of the sea anemones viz. *H. magnifica* and *S. mertensii* using acetone precipitation and the phosphate buffer solution as two different extraction methods, so as to identify the better extraction procedure. The earlier reports on the extraction of toxic proteins from the body of sea anemones have been made using

different organic solvent systems such as ethanol [13], methanol [14], chloroform [15] and acetone precipitation methods [16]. These results are supportive to the extraction of toxic proteins from the body of the anemones using acetone precipitations and PBS as extraction media and that the toxic proteins are not only restricted to the nematocysts but also the body of the anemones.

In the present study, crude protein level was found to be maximum in *H. magnifica* [1247 µg/ml] followed by *S. mertensii* [1016 µg/ml] in acetone precipitation method and in PBS methodology the crude protein level was found to be maximum in *H. magnifica* [716 µg/ml] followed by *S. mertensii* [603 µg/ml]. The protein content obtained by these methods are better as compared to the extraction procedure [17], where 200 µg/ml was obtained from the *H. magnifica*.

The toxic proteins have been isolated from the body of the sea anemones using acetone precipitation and the PBS extraction medium, as it is considered as a universal solvent which could extract even basic proteins without exempting any one of them. Such experiments of extraction from body tissue using both medium and also with different organic solvents have been conducted earlier by various researchers [13,16].

In order to evaluate the biological effect of both the sea anemones venom with two different toxins extraction methodology, a preliminary study of toxicity assay was carried out with the crab, *O. macrocera*. The toxicity of the venom was increased with decreasing concentrations. The toxicity was more pronounced in crabs treated with *H. magnifica* with acetone precipitation extracts as compared to other extracts. The different doses of venom revealed different effects but in all the cases restlessness was noticed. The higher dose of 1ml resulted in immediate paralytic effect and sudden death in crabs. This is in accordance with the reports of several researchers. However, the toxin Magnificalyins - III from *H. magnifica* has been found to be least active in crabs [18]. Beress [19] was purified two crab-paralyzing Polypeptides from the sea anemone, *Bolocera tuediae*.

The crude protein of both anemone species viz. *H. magnifica* and *S. mertensii* exhibited pronounced hemolytic activity on chicken, goat and human erythrocytes. The crude protein of *H. magnifica* had shown maximum hemolysis in chicken and human erythrocytes. Moderately hemolysis has occurred in the case of goat erythrocytes and comparatively low hemolysis has occurred in human erythrocytes. The crude protein of *S. mertensii* has shown maximum haemolysis in human erythrocytes followed by chicken and goat erythrocytes [16]. The hemolytic activity reported by [16] was certainly lower when compared to that of the present investigation.

The values obtained in the previous studies were higher than the values obtained in the present investigation [16]. This may be due to the different extraction procedures from various species of anemones and could be due to the different types of erythrocytes used.

The anemone toxins have been evaluated for determined their molecular weights by several workers. Based on this, it has been proposed that the anemone polypeptides ranged between 3000 and 30,000 Da [19], while the cytolytic toxins range between 10 and 20KDa [20]. In the present study, SDS-PAGE analysis revealed the presence of medium sized proteins in the crude

extracts of both the anemone species. Peptides corresponding to 20.1 and 97.4K Da were found to be common in the extracts of both the species.

Proteins with molecular weight of 29.0 KDa have been recorded from *H.magnifica* and *S.mertensii*. In addition, the crude extract of *H. magnifica* has recorded a protein of molecular weight between 43.0 and 66.0 KDa. Anderluh and Macek [5], have designated the 20KDa proteins as pore forming proteins. In this context, the 20.1KDa peptide of the crude extract of the both anemone species might be pore forming cytolytic proteins. The proteins, at the range of ~20.1 KDa have been reported as cytolytins from various other sea anemones and their molecular weight fall in a similar range as in the present investigation, thus it is suggesting that the protein with the molecular weight of 20.1 KDa obtained in the present study might be a cytolytic toxin.

In addition, in the present study, protein with a molecular weight of 29.0KDa has been obtained. Anderluh and Macek [5], have classified 30-40KDa proteins as cytolytins with or without phospholipase activity. Proteins with molecular masses of 43.0 and slightly above obtained during the present study can also be classified under this group. Further, the toxin was extracted with a molecular weight of 31.8KDa from the nematocysts of *Aiptasia Pallida* with a molecular weight of 45.0KDa [7]. These toxins are similar to the 43.0 KDa proteins obtained in the present study. High molecular weight proteins (97.4KDa) evidenced in the present study can be compared with that of the co-lytic factor (CLF) of the venom of *A. pallida*, having a molecular weight of 89.5 KDa and cytolytin from *Metridium senile* with a molecular weight of 80 KDa [5].

In the present study, toxic proteins have been isolated from the sea anemones viz. *H. magnifica* and *S. mertensii* using acetone precipitation and the phosphate buffer solution medium adopting two different extraction methods, so as to identify the better extraction procedure. Reports on the extraction of toxic proteins from the body of sea anemones have been made using different organic solvent systems such as ethanol [13], methanol [5], chloroform [6], and acetone precipitations [16]. These results are supporting to the extraction of toxic proteins from the body of the two sea anemone species using, acetone precipitations and PBS as extraction media and that the toxic proteins are not only restricted to the nematocysts but also present in the sea anemones.

In the present study, crude protein level was found to be maximum in *H. magnifica* [1247µg/ml]. Followed *S. mertensii* [1016 µg/ml] in acetone precipitation extraction method and PBS methodology crude protein level was found to be maximum in *H. magnifica* [716 µg/ml] followed by *S. mertensii* [603 µg/ml]. The two different crude extracts were evaluated against two bacterial stains namely *S. typhi* and *S. aureus*. The extracts were evaluated at concentration of 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9 and 2.0 µg/ml final concentrations in 5ml of Brain Heart Infusion broth. The bacterial growth inhibition of sea anemones of both species and the both extracts were observed in acetone precipitation extracts and PBS extracts on *S. typhi* and *S. aureus*.

According to Burkholder and Burkholder [14], screened for antimicrobial activity in gorgonians and scleractinian corals provided fruitful results in several species, but the authors were unable to identify whether the tissue is responsible the symbiotic zooxanthellae. The discovery of a potent

remedy from sea anemones will be a great advancement in bacterial infection therapies and showed an interesting activity against the Gram-positive bacteria *S. aureus* and *S. typhi*.

The present study on the toxins of the sea anemones viz. *H. magnifica* and *S. mertensii* exhibits that the toxins of these anemone species are having higher potential against microbial pathogens and present preliminary characterization studies indicate that two are possibilities of existence of compounds like cytolytic actinoporins, cardio stimulatory proteins and cytolysins. Further purification and structural elucidation of compounds are required to confirm the designation of toxins in the proposed groups.

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