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Original Paper

Assessment of biomedical and pharmacological activities of sea anemones *Stichodactyla mertensii* and *Stichodactyla gigantea* from Gulf of Mannar Biosphere Reserve, southeast coast of India

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Abstract: Cnidarians comprise an old and diverse animal phylum, and possess a wide variety of biologically active substances. Sea anemones contain a diversity of interesting biologically active compounds including some potent toxins. In the present work, the sea anemones *Stichodactyla mertensii* and *Stichodactyla gigantea*, collected from the Mandapam coast, are characterized biomedically and pharmacologically. The crude protein was obtained by using methanol and aqueous extracts. The respective protein contents of *S. mertensii* and *S. gigantea* were found to be 2.10 µg/mL and 1.87 µg/mL. The methanol and aqueous extracts of *S. mertensii* and *S. gigantea* yielded six and nine bands by SDS-PAGE on 12% gel. In the hemolytic assay, both extracts exhibited hemolytic effect on chicken, goat, cow and human erythrocytes ('A', 'B' and 'O'). The neurotoxic effects of these crude extracts were determined *in vivo* using the sea shore crab *Ocypode macrocera* and mortality was observed. The mouse bioassay for lethality was performed on male albino mice. The crude extract of *S. mertensii* showed higher lethality (58 seconds at 1 mL-dose) than that of *S. gigantea* (2 minutes and 10 seconds at 0.75 mL-dose). The analgesic activity test was also carried out on albino mice by Eddy's hot plate and tail-flick methods. The extracts showed moderate analgesic effect by both hot-plate and tail-flick methods. These characteristics emphasize the need for the isolation and molecular characterization of new active toxins in *S. mertensii* and *S. gigantea*.

Key words: aqueous extract, neurotoxicity, mouse bioassay, analgesic activity.

INTRODUCTION

The study of marine organisms for their bioactive potential and importance in the marine ecosystem has accelerated in recent years along with the growing recognition of their importance in human life. Sea anemones, like other coelenterates, produce many biologically active polypeptides and proteins, including neurotoxins, pore-forming toxins (or cytolysins), phospholipases and proteinase inhibitors. Anemone neurotoxins (polypeptides with relative low molecular weight (3000 to 5000 kDa) are very important tools in neurophysiological and pharmacological research (1, 2). Nematocysts possess high concentrations of polypeptides and proteins that act as neurotoxins, hemolysins and enzymes, which are responsible for a variety of harmful effects such as cardiotoxicity, dermatitis, local itching, swelling, erythema, paralysis, pain and necrosis (3).

Sea anemones contain a variety of interesting organic compounds including some potent toxins (4). For this reason many investigators have focused attention on the biological activities of the protein molecules of several species of sea anemones. New trends in drug discovery from natural sources have emphasized investigation of the marine ecosystem to explore numerous complex and novel chemical entities. These entities are the source of a new lead for treatment of many diseases such as cancer, AIDS,

inflammatory condition, arthritis, malaria and a large variety of viral, bacterial and fungal diseases (5, 6). A majority of the natural marine products have been isolated from sponges, coelenterates (sea whips, sea anemones, sea pens and soft corals) tunicates, opisthisbranch mollusks, echinoderms, sea grass and bryozoans (5). Therefore, the present study aimed to investigate the biomedical and pharmacological activity of the tropical sea anemones *Stichodactyla mertensii* and *Stichodactyla gigantea* from the Mandapam coast of southeast India for their biomedical applications.

MATERIALS AND METHODS

Sea Anemone

Specimens of *S. mertensii* Brandt, 1835 and *S. gigantea* Forsskal, 1775 were captured at Mandapam (lat. 09^0 16' N and long. 72^0 12' E), southeast coast of India. They were transported alive in sea water to our laboratory and maintained in the culture tank for extraction.

Animals

Male albino mice weighing 20 ± 2 g were housed under standard laboratory condition. The animals had free access to food and water. All animal bioassays were carried out according to the statement of the Institutional Ethics Committee of Rajah Muthiah Medical College, Annamalai University, Annamalai Nagar, India.

Preparation of the Crude Extracts

Methanolic extract

Crude protein was prepared according to the method of Sunahara *et al.* (7). The sea anemone *S. mertensii* was fully immersed in methanol and maintained for five days, then the material was removed by squeezing the animal, and the solvent was filtered through Whatman® n. 1 filter paper (0.4 μm) (England); it was then evaporated at low pressure using a rotary evaporator (VC 100A, Lark Innovative, India) at 30°C. The resultant compound was stored at 4°C for further screening.

Aqueous extract

A typical extraction is described below. One frozen specimen of *S. gigantea* was thawed and extracted twice with two volumes of distilled water. The aqueous extract was centrifuged at 5000 rpm for 15 minutes. The supernatant was collected for lyophilization. The lyophilizing powder was used as a crude toxin and stored at 4°C for further use.

Protein estimation

The protein was determined using the method of Bradford (8) with bovine serum albumin (BSA) as the standard.

SDS-PAGE

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to estimate the molecular weight of the hemolytic toxin according to the method of Laemmli (9). The protein was analyzed by SDS-PAGE, which utilized 5% stacking gel and 10% resolving gels. Five molecular weight markers (20, 40, 60, 80, and 120 kDa) were used. Ten microliters of the marker was loaded in the right well as marker and the crude proteins were loaded subsequently wells. Upon completion of electrophoresis, the gel was washed gently with distilled water to remove excess SDS, stained in Coomassie Brilliant Blue R-250 (Coomassie Brilliant Blue R-250, 1.25 g; methanol, 227 mL; glacial acetic acid, 46 mL; distilled water to complete a volume of 500 mL) for two hours at room temperature and destained (methanol, 7 mL; glacial acetic acid, 7 mL; and distilled water to reach 100 mL) for 48 hours. Protein bands were visualized as dark blue bands on a light blue background. Samples were denatured by boiling in loading buffer containing SDS and β-mercaptoethanol prior to loading onto the gel. Following electrophoresis at 30 mA for four hours, gels were stained by Coomassie blue staining.

Hemolytic Study

Preparation of erythrocyte suspension

Methanolic and aqueous extracts of the sea anemones *S. mertensii* and *S. gigantea* were assayed on chicken, goat, cow and human erythrocytes ('A', 'B' and 'O' blood) according to the method of Pani Prasad and Venkateshvaran (10). The chicken, goat and cow blood were obtained from the nearby slaughterhouse in Parangipettai, while clinically healthy human blood samples were obtained from local hospital using 2.7% ethylenediaminetetraacetic (EDTA) solution as an anticoagulant at 5% of the blood

volume and brought to the laboratory. The blood was centrifuged thrice at 5,000 rpm for five minutes. A 1% erythrocyte suspension was prepared for hemolysis study.

Hemolytic assay

The hemolytic assay was performed on a 'V' shaped sterile Laxbro microtitre plate (India). Serial two-fold dilutions of the venom extract (100 μL; 1 mg crude in 1 mL PBS) were made in PBS (pH 7.2) starting from 1: 2. An equal volume of 1% human RBC was added to each well. The plate was shaken to mix the RBC and venom extract. The plates were incubated at room temperature for two hours before reading the results. Appropriate control was included in the tests. Erythrocyte suspensions to which distilled water was added (100 μL respectively) served as blanks for negative control. Button formation at the bottom of the wells was taken as negative. The reciprocal of the highest dilution of the venom extracted showing the hemolysis was defined as one hemolytic unit.

Acute Toxicity Test

Crab toxicity

The acute toxicity study was performed on the isolated crude extract using adult *Ocypode macrocera* sea shore crabs (20 ± 30 g total body weight). This assay was carried out by injecting crude extract into the third walking leg of the crab at concentrations of 0.2, 0.4, 0.6, 0.8 and/or 1.0 mg/mL with subsequent symptoms being observed for two hours. Triplicates of each concentration were used. The LD_{50} was obtained by the Lehman method. The crude extract, which displayed sodium-channel activity, can be already detected by this test. Positive reactions were observed as tetanic concentrations in the extremities of the sea shore crab.

Mice bioassay

The mice bioassay was carried out according to the method of Gouiffes *et al.* (11). The lethality bioassay was done by using healthy male albino mice 20 ± 2 g that were maintained in a healthy condition in the laboratory. Mice in triplicate sets were challenged intraperitoneally with 0.25, 0.50, 0.75 and 1.0 mL of the crude toxin, dissolved at 5 mg/mL. A control was maintained in each case by injecting an equal volume of PBS (pH 7.4). The times of injection and death, in addition to behavioral changes before death, were recorded.

Evaluation of analgesic activity

Tail-flick method

The central analgesic activity was tested by the tail-flick method in male albino mice as described by D'Amour and Smith (12). Healthy male albino mice weighing 5 mg/kg having fasted overnight were divided into eight groups with six animals in each group. The crude extracts were dissolved in PBS solution and administered intramuscularly into the root of the tail at 100 μL/mouse. Control mice were maintained without administration of any toxin. For this tail-flick test, the mice were retrained in a soft tissue pocket and the distal half of the tail was immersed in water heated up to 50°C. Latency for tail-flick was measured with a 10-second cutoff time to avoid animal injury. Tail withdrawal from the heat (flicking response) was taken as the endpoint. The tail flick latencies were recorded pre-drug and then at 15, 30, 60, 90 and 120 min after administration of drugs.

Hot-plate test

The hot-plate was used to measure response latencies according to the method described by Eddy and Leimbach (13). The paws of mice are very sensitive to heat even at temperatures not damaging the skin. The response is in the form of jumping, paw withdrawal or licking of the paws. The animals were placed on Eddy's hot plate kept at a temperature of 55 ± 0.5°C. A cut off period of 15 seconds was observed to avoid damage of the paw. Reaction times and the type of response were noted using a stopwatch. The latency was recorded before and at 15, 30, 60 and 120 minutes after both test and standard.

Central Nervous System Depressant Activity

The CNS depressant activity was measured according to the method of Kulkarni and Dandiya (14) using an actophotometer (Medicraft, model n. 600 M-6 D, serial n. PA-0135). Male albino mice (20± 2 g) were housed under a 12:12 hour dark-light cycle. The extract concentrations were 2 mg/kg of body weight. A mouse without administration of any toxin or known painkiller was used as control while those injected intraperitonealy (IP) with paracetamaol (Crocin® at 0.25 mL) served as reference standard. The

basal reaction times and administrations of crude extract after 15, 30, 45, 60, 120 minutes were noted and percentage decrease of motor activity represented.

Anti-Inflammatory Activity

Anti-inflammatory activity was measured according to Smith (15). A group of two mice in each case was injected subplantarly with 0.1 µL of the crude toxin in the right footpad and with 0.1 mL of buffered saline in the left footpad. Two hours after injection, percentage of size increase was measured and the growth of the envenomoted paw relative to the saline-injected paw was defined as the edema ratio (ER). The minimum edematous dose was defined as the dose causing 105% ER.

RESULTS

Extraction

The aqueous extract of the sea anemone *S. mertensii* was filtered through Whatman n.1 filter paper and was then transformed into a lyophilized powder form by using a lyophilizer (Penquin Classic plug 4 kg, freeze dryer, Lark Innovative). The methanol extract of the sea anemone *S. gigantea* was concentrated under reduced pressure in a rota evaporator (model Lark Innovative, VC 100A). Finally, these crude extracts were stored for further studies.

Protein Content of the Crude Extracts

The respective protein contents in *S. mertensii* and *S. gigantea* were found to be 2.10 µg/g and 1.87 µg/g.

Molecular Weight Determination-SDS-PAGE

Utilizing SDS-PAGE on 12% gel, crude protein of *S. mertensii* yielded four bands ranging from 45 to 65 kDa with well-defined bands at 45 kDa, 58 kDa, 61 kDa, 65 kDa, whereas *S. gigantea* contained seven bands ranging from 42 to 95 kDa ranging from 42, 65, 70, 75, 78, 85 and 95 kDa (Figure 1) respectively. From the above results, it is clearly indicated that these two samples of sea anemones possess some protein bands in common.

Hemolytic Assay

The features of hemolysis were present in the crude extracts of *S. mertensii* and *S. gigantea*,

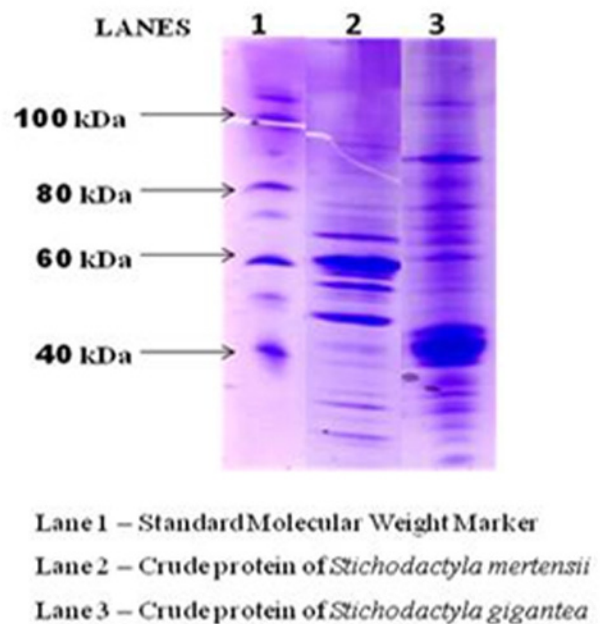

Figure 1. SDS-PAGE analysis of crude venom from *S. gigantea* and *S. mertensii*.

but activities differed slightly depending on the type of blood (Figure 2). The chicken, goat, cow and human blood groups, including erythrocyte types 'B' and 'O', were vulnerable to lysis provoked by either *S. mertensii* or *S. gigantea* extracts. The crude protein of *S. mertensii* extract showed a maximum of 32 HU in chicken blood and a minimum of 4 HU in cow blood. *S. gigantea* presented a peak of 16 HU in chicken, goat and human 'B' and 'O' blood groups and a minimum of 8 HU in cow blood.

Crab Toxicity Assay

The results showed that the crude extract of both species *S. mertensii* and *S. gigantea* evidenced biological activity on crab *O. macrocera* at the doses of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. After injection of crude extracts (1 mg/mL) into their third walking legs, strong contraction of the walking appendages was observed followed by intense spasmodic movement. The legs became tremulous with involuntary lateral movement; appendages shivered and presented stiffness; the carapace changed color; and there was complete loss of control and paralysis. In the case of *S. mertensii* mortality was observed within 30 seconds at the dose of 1.0 mg/mL; the crabs had died within four minutes. *S. gigantea* crude toxin injections with 0.8 mg/mL produced crab fatality

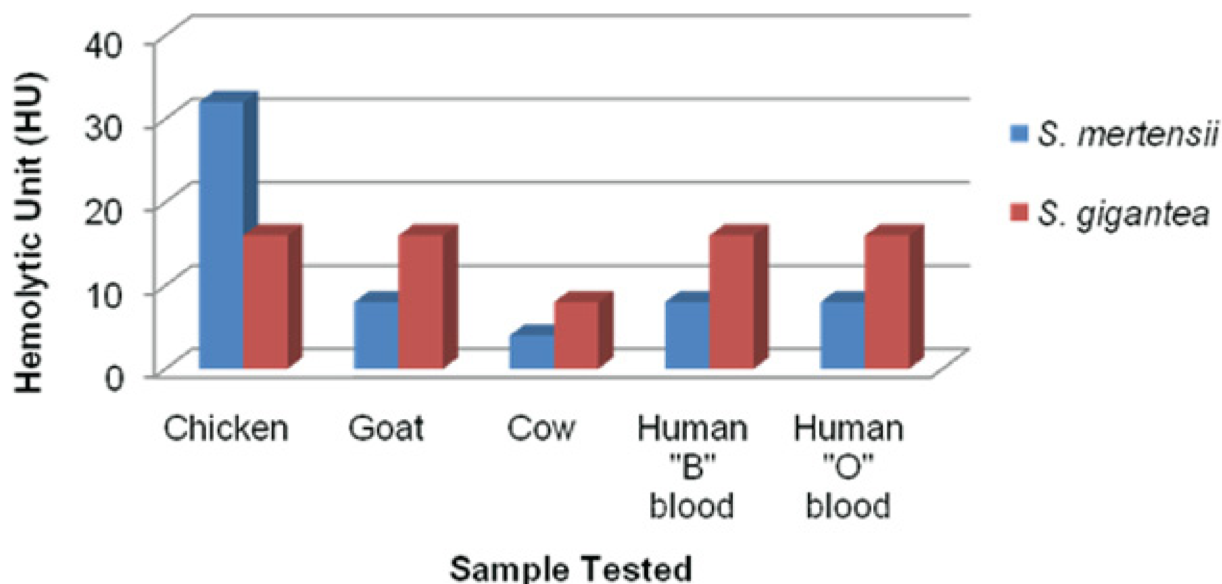

Figure 2. Hemolytic activity of the sea anemones *S. mertensii* and *S. gigantea*.

at 6 minutes and 48 seconds, and at 1.0 mg/mL crab death occurred within 5 minutes and 20 seconds, respectively. No lethality was observed from the doses 0.2 mg/mL and 0.4 mg/mL. Across the dose range from 0.2 to 1.0 mg/mL all crabs showed spasmodic movement.

Mice Bioassay for Lethality

Crude protein of *S. mertensii* and *S. gigantea*, when injected IP into male albino mice (20 ± 2 g) at doses of 0.25, 0.50, 0.75, and 1.0 mL, showed toxicity symptoms and mortality. In the case of *S. mertensii* lethality was observed at the dose of 1.0 mL with a death time of 58 seconds. The crude extract of *S. gigantea* showed lethality at a dose of 0.75 mL in 2 minutes and 10 seconds (Table 1).

Analgesic Activity

Tail-flick method

Employing the tail-flick method, the maximum analgesic ratio (AR) was found to be 10 in the crude extract of *S. mertensii* and a 5 AR minimum was noted after 15 minutes. In the case

Table 1. Mouse toxicity of sea anemones *S. mertensii* and *S. gigantea* samples at 5.0 mg/mL intraperitoneally injected into male albino mice (20 ± 2 g)

Sample number	Extracts	Injected volume (mL)	Symptoms
1	*S. mertensii*	0.25	Widespread fore limbs, prolonged palpitation, closed eyes, grooming, shivering of fore limbs – **not lethal**
		0.50	Foaming from mouth, tonic convulsions – **not lethal**
		0.75	Rolling of tail, paralysis – **not lethal**
		1.00	Palpitation, urination, suddenly death – **lethal**
2	*S. gigantea*	0.25	Widespread fore limbs, prolonged palpitation, grooming, shivering of fore limbs – **not lethal**
		0.50	Escape reaction, tonic convulsions – **not lethal**
		0.75	Excess defection, dragging of hind limbs, paralysis – **lethal**
		1.00	Urination, Paralysis and coma – **lethal**

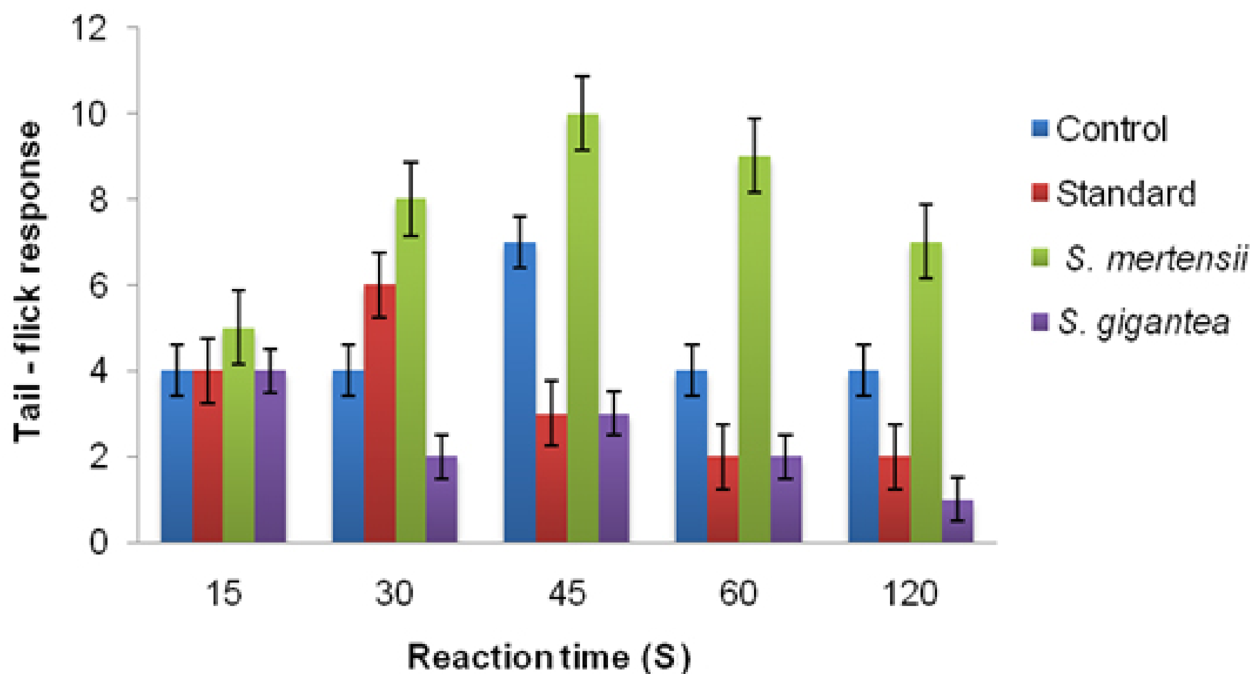

Figure 3. Analgesic activities by tail-flick response of male albino mice to *S. mertensii* and *S. gigantea* extract at 2 mg.kg^{-1} of 20 ± 2 g.

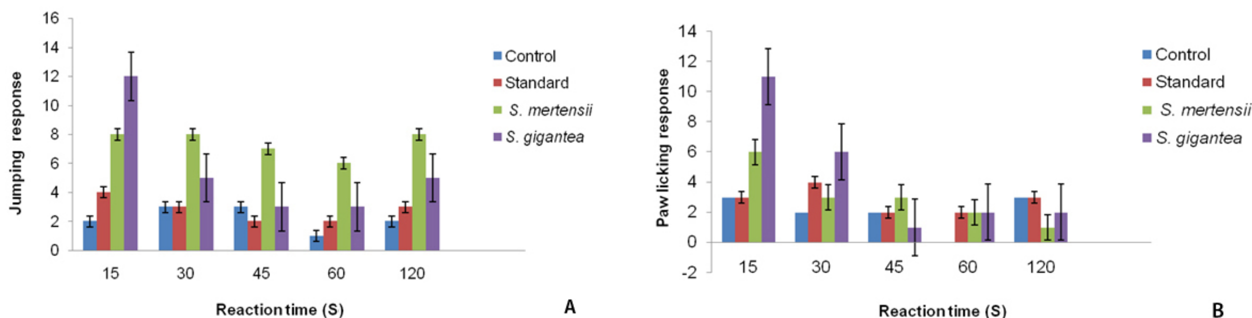

Figure 4. Analgesic activities of 20 ± 2 g male albino mice produced by *S. mertensii* and *S. gigantea* extract at 2 mg.kg^{-1} under the hot plate method.

of *S. gigantea* the maximum analgesic ratio was 4 AR after 15 minutes and minimum 1 AR after 120 minutes (Figure 3).

Hot-plate method

In the hot-plate method, the analgesic ratio (AR) was determined by registering paw licking and jumping response of mice after drug administration. The paw licking peaked at 6 AR 15 minutes after *S. mertensii* extract administration, whereas the *S. gigantea* showed a maximum of 11 AR after 15 minutes. The jumping response presented an 8 AR maximum in *S. mertensii* at 15, 30, 120 minutes and minimum of 6 AR at 60 minutes, while *S. gigantea* peaked at 12 AR in 15 minutes and reached its minimum of 3 AR at 45 and 60 minutes (Figure 4).

Central Nervous System Depressant Activity

Respective maximum decreases of depressant activity of 69.6% and 35.5% were recorded in crude extracts of *S. mertensii* and *S. gigantea* (Table 2). It was clearly shown that the percentage of motor activity has been calculated from the

Table 2. Central nervous system activity (CNS) of *S. mertensii* and *S. gigantea* extract at 2 mg.kg^{-1} of 20 ± 2 g male albino mice

Sample number	Treatment (5 mg.kg^{-1})	Body weight (g)	Locomotor activity (scores) in 10 min		
			Before treatment	After treatment	% Decrease of motor activity
1	Control (saline)	20.8	534	485	9.1
2	Standard (paracetamol)	22.02	382	345	9.6
3	Crude extract of *S. mertensii*	29.98	462	140	69.6
4	Crude extract of *S. gigantea*	27.18	414	267	35.5

Table 3. Anti-inflammatory formation effect of *S. mertensii* and *S. gigantea* extract on at 2 mg.kg^{-1} of 20 ± 2 g male albino mice

Sample number	Treatment (5 mg.kg^{-1})	Paw edema response (cm)	
		Before injection	After injection
1	Control (saline)	1.2	1.3
2	Crude extract of *S. mertensii*	1.4	1.6
3	Crude extract of *S. gigantea*	1.2	1.5

basal score and after ten minutes of injection of crude extract.

Anti-Inflammatory Activity

The effects obtained by 100 mg/kg of the aqueous and methanol extracts of *S. mertensii* and *S. gigantea* on mice hind paw edema are shown in Table 3. The both extracts significantly inhibited the inflammatory action *in vivo* in male albino mice.

DISCUSSION

The present investigation found the respective protein contents of *S. mertensii* and *S.gigantea* extracts to be 2.10 μg/mL and 1.87 μg/mL. Previously, Sánchez-Rodríguez and Cruz-Vazquez (16) reported the protein content of the sea anemone *L. danae* as 0.122 mg in 1 mg of crude extract and also a high protein concentration of 79.6 μg/mg from the box jellyfish *Carybdea marsupialis*. Adhikari *et al.* (17) have showed 50-400 μg/mL from the tentacle extract of sea anemone *P. indicus*.

The toxic compounds from sea anemones are proteins whose structural properties are determinable. In the present study, *S. mertensii* and *S. gigantea* presented three types of neurotoxins with molecular weights between 45 kDa and 95 kDa. Subsequent results were from the sea anemone *L. danae* found molecular weights of 62.5 and 58 kDa (16). Uechi *et al.* (18) isolated 19 kDa proteins from the globular vesicles of the sea anemone *A. villosa*. Bernheimer and Avigad (19) have isolated 80 kDa protein from sea anemone *M. senile*. Monastyrnaya *et al.* (20) have isolated 20 kDa protein from the sea anemone *R. macrodactylus*.

Sea anemones contain a variety of bioactive compounds including some toxins that are known to possess potent hemolytic properties (21). In the present research the *S. mertensii* and *S. gigantea* extracts showed 32, 16 HU in chicken blood and 4, 8 HU in cow blood. These hemolytic activities agreed with an earlier report of Vinoth S. Ravindran (22), who reported the hemolytic activity of three anemone species *H. magnifica, S. haddoni* and *S. helianthus* to be 20, 23 and 25

HU, respectively, in chicken, goat and human erythrocytes. Similar results have been shown from the sea anemone *B. annulata* in mouse erythrocytes by Santamaría *et al.* (23). Shiomi *et al.* (24) found specific hemolytic activities of 106,500 Hu/mg from the sea anemone *A. japonica*.

The neurotoxicity of the sea anemone toxins to the sea shore crab *Ocypode quadrata* was documented for the first time by Beress and Zwick (25), who characterized biological activity and physiological effects of neurotoxin on the organism followed by neurotoxic effects, convulsions, paralysis and death. The present study has isolated a neurotoxin that was extremely active in the sea shore crab *Ocypode macrocera.* From the extract of *S. mertensii* at a dose of 1.0 mg/mL mortality was observed 30 seconds and the crabs had died within four minutes, while in the case of *S. gigantea* at 1.0 mg/mL mortality occurred at 6 minutes and 48 seconds following spasmodic movement, shivering, change in carapace color and paralysis. Similar symptoms were also reported as being caused by whole extracts of two sciaenids (26, 27).

The *S. mertensii* and *S. gigantea* extracts were found to have both biomedical and pharmacological potential. The analgesic potential of these sea anemone extracts produced good results at all time intervals (15, 30, 45, 60 and 120 minutes) in tail-flick and hot-plate methods, these finding differ slightly from those of previous studies on analgesic effects of fruit extract of *M. parvifolia* by Saneja *et al.* (28). In the previous studies the fruit extract of the plant was found to be highly active in both hot plate and acetic acid-induced writhing methods at the doses of (100, 250 and 500 mg/kg) on leaf extract. Andreev *et al.* (29) have studied the analgesic effect from sea anemone *Heteractis crispa*. Sakthivel (30) has reported extracts of *Conus lentiginosus* and *C. metabilis* as possessing 128 times more analgesic effect than paracetamol. Marwick (31) has shown the venom of *Conus magnus* to have 1000 times more analgesic activity than morphine. Malarvannan (27) has reported that the ootoxins from fish possess analgesic activity and exhibited an analgesic ratio above 1.0. But in the present investigation, the two sea anemone toxins, *S. mertensii* and *S. gigantea* have exhibited much higher analgesic ratios (AR) than fish.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the statement of the Institutional Ethics Committee of Rajah Muthiah Medical College, Annamalai University, Annamalai Nagar, India (registration number 160/1999/CPCSEA/11.01.2008).

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