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ON SOME TOXINOLOGICAL ASPECTS OF THE STARFISH *Stellaster equestris* (RETZIUS, 1805)

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ABSTRACT: Whole-body extracts in methanol were obtained from the starfish *Stellaster equestris.* The crude toxin was fractionated stepwise using diethylaminoethyl (DEAE) cellulose column chromatography. The crude toxin was lethal to male albino mice at a dose of 1.00 mL (containing 531.0 µg/mL protein) when injected intraperitoneally (IP) but the toxicity was abolished in all cases except one upon fractionation. The crude toxin and all the adsorbed fractions exhibited potent hemolytic activity on chicken, goat and human blood. However, group B human erythrocytes were resistant to lysis by all fractions and group O by most of the fractions. Paw edema in mice was caused by the crude toxin and all fractions. Pheniramine maleate and piroxicam blocked the toxicity when administered earlier than, or along with, the crude or fractionated toxins but not when administered after the envenomation. Pretreatment with either of these drugs also blocked edema formation.

KEY WORDS: starfish, toxicity, hemolysis, human blood groups, paw edema.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

The phylum Echinodermata includes a diverse group of typically slow-moving and non-aggressive marine animals, including three venomous classes, namely the Asteroidea (starfishes), Echinoidea (sea urchins), and Holothuroidea (sea cucumbers). The crown-of-thorns starfish, *Acanthaster planci*, being venomous, accounts for the majority of human envenomations (27) but poisonous starfish also affect health in countries including Taiwan where they are consumed (15, 42). A variety of toxins, which are comparable with the most toxic organophosphate chemical warfare agents (28), are reported from starfish; these include saponins (11), asterosaponins (sulfated steroidal glycosides) (40), free and sulfated sterols (17, 18, 19), and protein-like substances (39), besides tetrodotoxin (TTX) (20, 41) and paralytic shellfish poison (PSP) (2, 21). However, these toxins can also be used as guides leading to new, more effective therapies, because their targets of action are well known (9, 28). Despite the occurrence of around 350 species under 103 genera in Indian waters, echinoderms have been poorly studied in this country especially with reference to their toxicity and pharmacological potential. Hence, the present study focused on the biotoxinological aspects of the starfish *Stellaster equestris* from Cuddalore on the eastern seaboard of India.

MATERIALS AND METHODS

Specimen Collection

The starfish *Stellaster equestris* (Retzius, 1805) (Echinodermata: Asteroidea: Valvatida: Goniasteridae) was collected from the Fishing Harbor at Cuddalore (11°45'N, 79°47'E) and Mudasalodai Fish Landing Center at Parangipettai (11°32'N, 79°45'E), brought fresh to the laboratory (32 km and 2 km, respectively) and immediately air dried for subsequent use.

Preparation of Crude Toxin

Crude toxin was prepared according to previous works with certain modifications (8). Samples were air dried for ten days and, after complete drying, were fully immersed in methanol, covered and maintained for five days. The starfish were then removed after squeezing them, and the solvent was filtered through Whatman® filter paper n.

1 (0.4 µm); it was then evaporated at low pressure using an R-200 Büchi Rotavapor® at 30°C. The resultant compound was stored at 4°C for further use as crude toxin.

Partial Purification Using Column Chromatography

The crude toxin was dissolved in phosphate buffer (pH 7.4) at 5 mg/mL and eluted through a DEAE cellulose column (height 32 cm; diameter 2 cm). Five unadsorbed fractions, 15 mL each, were eluted with phosphate buffer; and were later pooled and referred to as fraction UA. Ten adsorbed fractions, each 15 mL, were eluted with a step gradient of 0.1 to 1.0 M NaCl in phosphate buffer, and designated as fractions F1 to F10. All fractions were stored at 4°C for further use.

Protein Estimation

Crude protein content and partially purified fractions were estimated according to previous works (30) using bovine serum albumin (BSA) at the rate of 1 mg/1 mL as the standard.

Mice Bioassay for Lethality

All animal bioassays were carried out following the statement of the Institute Ethical Committee. The bioassay for lethality was done using clinically healthy male albino mice weighting 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 in phosphate buffer saline (PBS); and with 1.0 mL each of the 11 fractions. A control was maintained in each case by injecting an equal volume of PBS (pH 7.4). The times of injection and death, besides behavioral changes before death, were recorded. Mice that died upon envenomation were autopsied to observe gross anatomical alterations.

Tests for Stability of the Crude Toxin

Tests were conducted in accord with previous works to assess the stability of the crude toxin upon (35):

- heating at 50°C, 60°C, 80°C and 100°C in a water bath for five minutes;
- autoclaving at 120°C for 15 minutes;
- changing of pH (3.0 – 8.0); and
- storing at –20°C for more than one year (13 months).

Hemolytic Activity

The crude toxin and the 11 fractions were assayed for their hemolytic activity on chicken, goat and human (A, B, AB and O) erythrocytes.

Samples of chicken and goat blood were obtained from a nearby slaughterhouse, while the human blood was obtained from Sheena Clinic, Andheri (W), Mumbai, using 2.7% ethylenediaminetetraacetic acid (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 by adding 99 mL normal saline to 1 mL of packed erythrocytes.

The microhemolytic test was performed in 96-well 'v' bottom microtitre plates. Serial two-fold dilutions of the crude toxin/fractions were made in 100 µL of normal saline. Then 100 µL of 1% erythrocyte was added to all the wells. For positive control, 100 µL of distilled water, and for negative control 100 µL of normal saline were added respectively to the 1% red blood cell (RBC) suspension. The plate was gently shaken and allowed to stand for two hours at room temperature. Presentation of uniform red-color suspension in the wells was considered to be positive hemolysis and a button formation in the bottom of the wells constituted a lack of hemolysis. The reciprocal of the highest dilution of the crude toxin showing the hemolytic pattern (hemolytic unit) was divided by the protein content to obtain the specific hemolytic unit.

Edema Formation

Following previous works (37), a group of two mice in each case was injected subplantarly with 0.1 µL of the crude toxin/fraction 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 with a Vernier caliper and the growth of the envenomated paw relative to the saline-injected paw was taken as the edema ratio (ER). The minimum edematous dose was defined as the dose causing 105% ER.

Antidote Experiments

Commercially available antihistaminic drug, pheniramine maleate (Avil®, Hoechst), and an antiprostaglandin analgesic, piroxicam (Dolonex®, Pfizer) in injectable form, were procured locally from qualified pharmacists. Each of the drugs was used at a concentration of 0.2 mg/20 g body weight of mice; this dose was deduced based on the dose prescribed by the manufacturers for human use, based on body weight.

These two medications were selected since they are commonly used in India for the symptomatic relief of most envenomations in humans, though there are no recorded starfish poisonings or envenomations from this country. The antidote investigations were made using the crude toxin and those fractions which were lethal to mice. Control was maintained by injecting toxins (IP) at the previously determined lethal dose for each toxin but without any antidote. All experiments were carried out in triplicate sets.

The injectable solution was diluted further with PBS to obtain sufficient injectable volume for a dose as low as 0.2 mg. Three types of blocking experiments – pretreatment, cotreatment and post-treatment – were carried out:

- Pretreatment model: the test mice were injected IP with 0.9 mL (containing 0.2 mg of the active component) of pheniramine maleate/piroxicam, and set free in the cage. After 30 minutes, the animals were injected IP with the predetermined lethal dose, and kept under observation for 24 hours.
- Cotreatment model: 0.9 mL of pheniramine maleate/piroxicam and the predetermined lethal dose of the toxin were taken together in one syringe and injected IP to mice, which were then placed under observation for 24 hours.
- Post-treatment model: the mice were first injected with the predetermined lethal dose of the toxin. Immediately after the appearance of envenomation symptoms of, 0.9 mL of pheniramine maleate/piroxicam was administered IP to the mice, after which they were observed for 24 hours.

Edema Inhibition Tests

Only the pretreatment model was used in this case; 1 hour after IP administration of pheniramine maleate and piroxicam, at 0.2 mg/20 g body weight, the assay for paw edema was conducted as described above. Control experiments were performed on mice without the administration of drugs.

RESULTS

Crude Toxin

On an average, 5 mg of crude toxin was obtained from 50 g dry weight of the starfish.

Protein Content of the Crude Toxin/Fractions (Table 1)

Protein content in the crude toxin was 531 µg/mL while the amount of protein in the purified fractions varied between 29 µg/mL (fractions F1, F5 and F7) and 68 µg/mL (fraction F4).

Table 1. Protein content of crude toxin, and its partially purified fractions, from the starfish *Stellaster equestris* (all values are means of triplicate sets)

Sample Tested	Amount of protein (µg/mL)
Crude toxin	531.0 ± 2.3
F1	29.6 ± 0.8
F2	39.1 ± 1.2
F3	53.1 ± 1.4
F4	68.8 ± 0.9
F5	28.0 ± 0.7
F6	41.0 ± 1.0
F7	21.9 ± 0.5
F8	53.0 ± 0.9
F9	65.5 ± 1.1
F10	48.9 ± 0.8
UA	Non-detectable

Toxicity in Mice Models

Both crude toxin at a dose of 1.0 mL (containing 5 mg of crude toxin) and fraction F9 at a dose of 1 mL (containing 65.5 µg/mL) protein were lethal to mice upon IP injection; however, in all cases, various toxicity symptoms were observed (Table 2).

Table 2. Toxicity of starfish crude toxin and its fractions (at 5.0 mg/mL) IP in male albino mice (20 ± 2 g)

Serial number	Extract/ fractions	Injected volume (mL)	Death time (min:s)	Symptoms	Autopsy
1	Crude toxin	1.00	7:50	Palpitation, escape reaction, dragging of hind limbs, paralysis, and coma – **lethal**	Dark discoloration of liver,fluid accumulation in visceral cavity
2	Crude toxin	0.75	–	Palpitation, excess defecation – **not lethal**	–
3	Crude toxin	0.50	–	Palpitation, excess defecation – **Not lethal**	–
4	Crude toxin	0.25	–	Palpitation, excess defecation – **not lethal**	–
5	UA	1.00	–	Palpitation, escape reaction – **not lethal**	–
6	F1	1.00	–	Palpitation, micturition, escape reaction – **not Lethal**	–
7	F2	1.00	–	Palpitation, escape reaction – **not lethal**	–
8	F3	1.00	–	Palpitation – **not lethal**	–
9	F4	1.00	–	Palpitation, escape reaction – **not lethal**	–
10	F5	1.00	–	No symptoms – **not lethal**	–
11	F6	1.00	–	Palpitation, micturition – **not lethal**	–
12	F7	1.00	–	Palpitation – **not lethal**	–
13	F8	1.00	–	Palpitation, escape reaction, excess defecation – **not lethal**	–
14	F9	1.00	0:46	Palpitation, micturition, sudden death – **lethal**	Granular appearance and dark discoloration of kidney, non-specific hemorrhage in viscera
15	F10	1.00	–	Palpitation, sniffing, escape reaction – **not lethal**	–

Stability Tests

Lethal activity of the crude extract was not affected after storage for 12 months at 20°C. The crude extracts were found to be thermolabile above 60°C. The crude toxin lost its activity in acidic pH; mice injected with it showed some symptoms of toxicity initially, but recovered within a few minutes.

Hemolytic Assay

The crude toxin as well as the fractions produced pronounced hemolytic activity on chicken, goat and human erythrocytes (Figure 1). Hemolytic factors were present in the crude toxin as well as in all the fractions except UA, but differed considerably depending on the type of blood used. Chicken blood, like groups A and AB of human blood, were the most vulnerable to lysis provoked by the starfish extracts. None of the fractions could lyse group B erythrocytes while group O erythrocytes were lysed by only two out of the ten fractions.

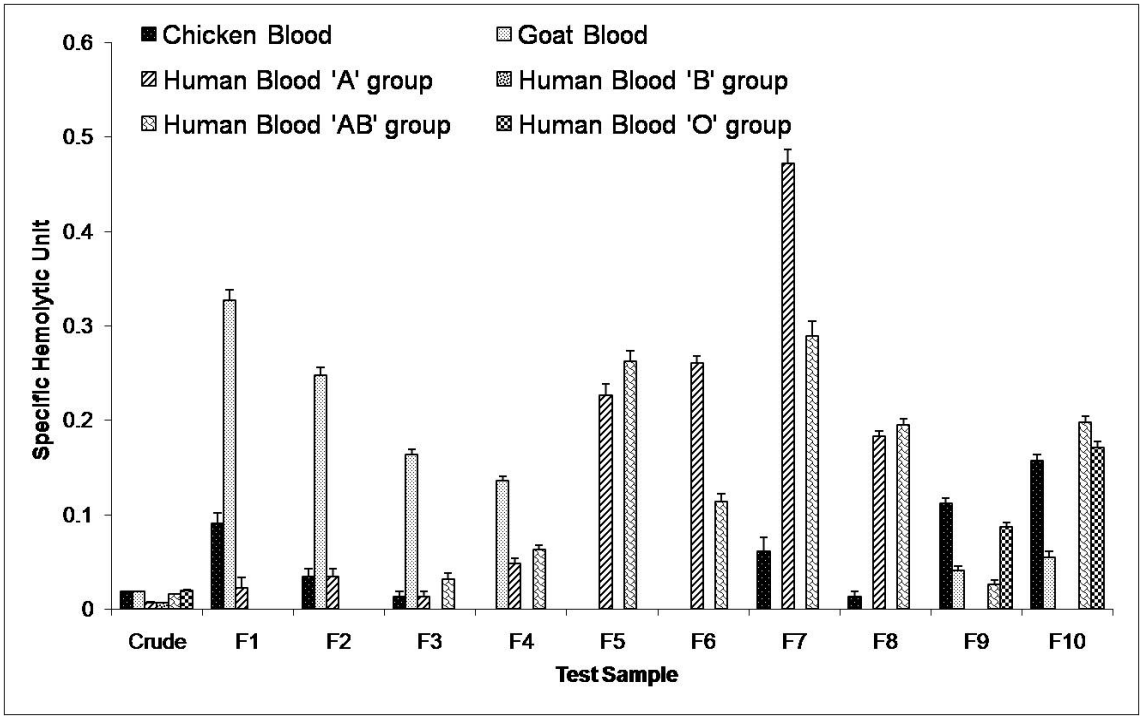

Figure 1. The extent of in vitro hemolytic activity of *Stellaster equestris* crude toxin (fractions F1 to F10) on chicken, goat and human blood.

Antidote Investigation

Both the antidotes tested – pheniramine maleate and piroxicam – negated the toxicity of the crude toxin when injected prior to envenomation or together with the toxin. Injecting each the drugs after envenomation, could only delay the onset of death and were not sufficiently effective to prevent mortality.

Edema Formation

Mice paw edema was caused invariably by the crude toxin and all the fractions (Figure 2) with ER between 157 and 334%. Neither of the drugs tested could block the edematous activity of fractions F1, F2, F8, F9, and UA but was able to variably reduce the ER in all other cases, sometimes even below the stipulated 105%.

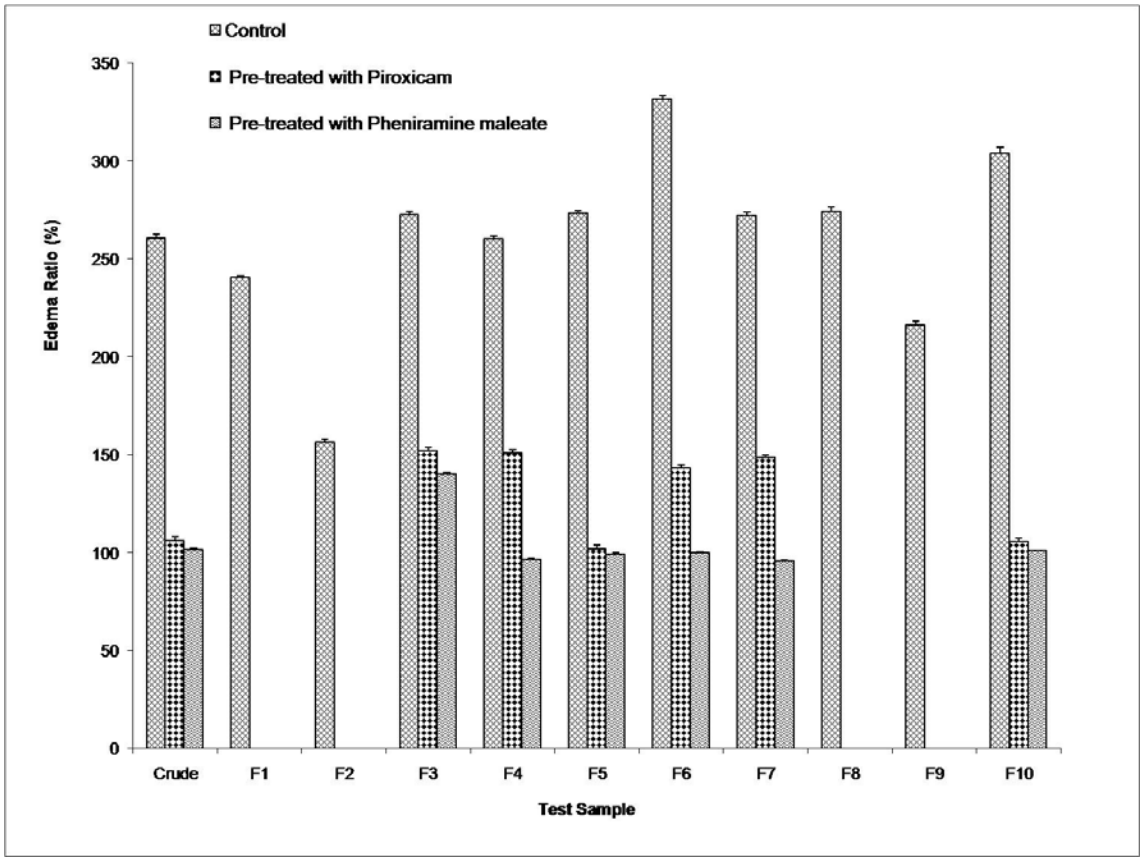

Figure 2. The extent of paw edema formation in male albino mice (20 ± 2 g) provoked by *Stellaster equestris* crude toxin (fractions F1 to F10) and the blocking of edematous activity by piroxicam and pheniramine maleate.

DISCUSSION

Methanol is considered as a universal solvent which could extract even the basic proteins without exempting any one of them; methanolic extracts of the whole body of the starfish *Anthopleura fuscoviridis* yielded two toxins AFT-I and AFT-II, that were lethal to mice (38). The protein levels observed during the present study compares well with previous results (36) that reported protein values as high as 4,820 mg/227 g of tissue from *Acanthaster planci.*

Our data further support the earlier findings that crude extracts of some echinoderms (4, 5, 24, 33, 43) and others extracted from spines of *Acanthaster planci* were lethal to mice (39). Sulfated saponins of echinoderms are toxic to fishes, annelids, arthropods etc. (10). The symptoms observed in envenomated mice in our study are indicative of central nervous system (CNS) toxicity and nephrotoxicity, concomitant with those reported for asterosaponins and saponins. Asterosaponins provoked paralysis in mice (34) while toxins of the British starfish, *Marthasterias glacialis* caused convulsions and drowsiness followed by collapse and death in mice (23).

Our data suggest that the lethal element in the present crude toxin is different from those factors exhibiting hemolytic action, as had also been observed in the case of *Acanthaster planci* (39).

Metabolites from echinoderms tend to cause cell lysis and hemolysis (29, 31, 32), as found in the present study. A partially purified product from the starfish *Asterias amurensis* was hemolytic to rabbit red blood cells, toxic to killifish, lethal to fly maggots and earthworms, and emetic to cats (43). Five of the nine polar steroids isolated from the alcoholic extract of the Far Eastern starfish *Henricia leviuscula* showed moderate hemolytic activity in the mouse erythrocyte assay (16).

It is very interesting that within the ABO system of human blood, group B resisted lysis by all the fractions while group O resisted lysis by most of them. Despite our best efforts we could not locate any previously published data on such an effect by invertebrate or vertebrate toxins. But considerable literature exists on this effect in the case of bacterial toxins. It has been shown that individuals with type O blood are more susceptible than other individuals to severe cholera (14). Compared to community controls in Bangladesh, cholera patients were twice as likely to have blood group O and one-ninth as likely to present blood group AB (12).

Similarly, it has been shown that the differential interaction of *Escherichia coli,* heat-labile toxin like cholera toxin with intestinal brush border glycoproteins of pigs (7) and rabbits (6) are dependent upon ABH and related blood group antigenic determinants.

Our data assume significance and indicate that further *in vitro* studies are essential in order to clarity possible inter-individual (and possibly inter-regional/inter-racial) variability in susceptibility to the toxic effects of various plant and animal poisons and venoms.

Present results on the toxin stability corroborate those pertaining to *A. planci* (39); this toxin was non-dialyzable, and it lost its activity at 60°C or at pH lower than 3.0 or higher than 10.0. In addition the toxin that was exposed to repetitive freezing (at –20°C) and thawing was inactivated. From these observations, it was considered that *A. planci* toxin was probably a protein-like substance (39).

Similar instances of stability, as in the present study, have been encountered by other researchers (25). The presently extracted toxins are comparatively thermostable up to 60°C, possibly due to the tropical occurrence of the starfish from which they were extracted, as had been suggested in the case of cytolysin from *Radianthus* sp (26). The phospholipase A_2 component in starfish toxin is said to be unstable at 40°C and has been reported to lose its activity completely when maintained for 25 minutes at 56°C (13). Susceptibility of the toxin to acidic pH observed is in accordance with earlier reports (22).

A number of thermolabile biotoxins of echinoderms are known to cause edema in experimental animal models and also in humans (37). Pretreatment and also cotreatment of both antihistaminic (pheniramine maleate) and antiprostaglandin analgesic (piroxicam) drugs have been effective in negating the toxicity of the crude toxin like that of the lethal fractions of the starfish species. However, on post-treatment, these compounds could only delay the onset of death, thus indicating that their use is limited only to symptomatic relief. Action of catfish mucus toxin was inhibited by pretreatment with atropine and indomethacin (1) while promoxine with hydrocorticosone, prednisone and 1% prednisone acetate with hyocine 0.25% were effective against nematocyst stings in humans (3).

Observed toxicity of the extracts may have been mediated by a prostaglandin mechanism since piroxicam has been reported (1) to act by through blocking the cyclooxygenase activity of prostaglandin synthesis. Also, in the present study,

pheniramine maleate, the antihistaminic drug, has either delayed or prevented death, indicating the involvement of histamine or histamine-like compounds in the toxins that caused death of test animals.

Between the two drugs tested, pheniramine maleate appears to be more effective than piroxicam in blocking both the lethal effect and edema formation.

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