Lethal, oedema, haemorrhagic activity of spotted butterfish (Scatophagus argus, Linn) sting extract and its neutralization by antiserum and pharmacological antagonists

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Received 16 June 2004; revised 8 February 2005

An attempt has been made in this communication to develop antiserum in rabbit against Scatophagus argus sting extract. Antiserum did not neutralized the sting extract induced proinflammatory and haemorrhagic activity but successfully neutralized lethality up to 2LD50. Cyproheptadine, indomethacin and BW 755C pretreatment significantly reduced sting extract induced proinflammatory activity. The haemorrhagic activity of sting extract was significantly inhibited by temperature, UV-exposure, EDTA, cyproheptadine, indomethacin and BW 755C pretreatment. The results conclude that the local effects of S.argus venom is likely to be mediated through release of mediators and may be encountered by pharmacological antagonists better than the antiserum.

Keywords: Butterfish. Fish venom, Haemorrhage, Oedema. Scatophagus argus

The spotted butterfish (Scatophagus argus Linn), locally named as ‘pyrachanda’ is a common venomous fish, inhabiting the waters of India, Sri Lanka, through the East Indies; to China, Taiwan, Philippines, Melanesia, Polynesia and Queensland (Australia). The dorsal and the anal fin of S. argus contain 11 and 4 strong spines respectively. The spines are equipped along the sides with venom glands. The sting of this fish is very common among swimmers, fish eater and fisherman. The clinical characteristics of envenomation are characterized by intense pain, severe swelling, redness, fever, and throbbing sensation. In addition to the pathophysiological changes, the sting of this fish keeps the poor fisherman out of the work for several days, as there is no specific antidote, except some symptomatic treatment using antipyretics, analgesic, anti-inflammatory and antibiotics. Muhuri et al.1,2 showed that the crude sting extract was pharmacologically active. The present study has been carried out to establish the neutralization of lethal, oedema and haemorrhagic activities of S. argus sting extract in experimental animals through antiserum and pharmacological antagonists.

Materials and Methods
The following chemicals were used: agarose (Sigma); anaesthetic ether (Kabra Drugs Ltd.); bovine serum albumin (Sigma); BW 755C (Burrows Wellcome); cyproheptadine hydrochloride (Merck Sharp Dhome); ethylene diamine tetraacetic acid, disodium salt (Qualigen); Folin phenol reagent (SRL); Freund’s complete adjuvant (Sigma); indomethacin (Sigma), and merthiolate (Sigma).

Collection of fish and sting extract preparation—The live adult fish Scatophagus argus (pyrachanda) of either sexes was collected from Canning local fish market (West Bengal, India).The dorsal pectoral and anal spines were cut from live fish, stored at 0°C, immediately transferred to the laboratory and finally stored at -20°C. The pooled spines were ground, homogenized with 0.9 % NaCl or 0.1 M phosphate buffer (pH 7.2) and centrifuged at 10,000 rpm at 4°C for 30 min. The supernatant was used as sting extract (expressed in terms of protein), stored at 4°C until further use. Protein content was
determined as per Lowry et al., using bovine serum albumin as standard protein.

**Paw oedema—**Swiss albino male mice (18-20 g, n=6) were used for the measurement of paw oedema. Paw oedema was induced by a subplantar injection of 10 µl of sting extract (5, 10, 25, 40, 50, 60 µg/paw), in 0.02 M phosphate buffer (pH 7.4), into the right hind-paw. An equal volume of phosphate buffer was injected into the left hind-paw as control. The diameter (mm) of both hind-paw of each mouse was measured by screw-gauge at 0, 0.5, 1, 2, 3, 4 and 6 hr after induction of paw oedema. Percent hind-paw swelling was calculated as follows:

$$\% = \frac{\text{Right paw diameter} - \text{initial diameter}}{\text{Right paw initial diameter}} \times 100$$

$$\frac{\text{Left paw diameter} - \text{initial diameter}}{\text{Left paw initial diameter}} \times 100$$

In case of antagonists treatment, cyproheptadine (10 mg/kg, sc), indomethacin (10 mg/kg, sc) and BW 755C (15 mg/kg, sc) were given to treated group. The control group of animals received vehicle only. After one hour of antagonists treatment, sting extract (50 µg in 10 µl) was injected into the right hind paw of treated and control animals, same volume of phosphate buffer was injected into the left hind paw.

Assessment of haemorrhage activity—+(Model-I) This was performed according to the method of Kondo et al. Sting extract (50-500 µg in 0.1 ml in 0.02 M phosphate buffer, pH 7.4) was injected on the shaved dorsal flank of the Swiss albino mice (n=6). Same amount of phosphate buffer was injected in control animal. After 24 hr, the mice were sacrificed, skin was opened and observed for haemorrhagic lesion.

(Model-II)—This experiment was performed in male albino Charles Foster rats (180-200g, n=7). Rats were deprived of food, for 18-24 hr before the experiments. The animals were anaesthetized with anesthetic ether, abdomen was opened and sting extract (200 µg in 10 µl in 0.02 M phosphate buffer) was injected on the stomach wall (experimental group). In the control group, same volume of phosphate buffer was injected in the stomach wall. After 2 hr, all the rats were sacrificed and the haemorrhagic spot was measured.

The haemorrhagic activity of sting extract was antagonized by using physical and chemical agents. Temperature variation (37°, 40°, 50°, 60°, 80°C for 30 min) and UV-ray exposure (2.90 x 10^15 quanta/s second strength for 30 min treatment of sting extract) were used as physical agents. In pharmacological antagonism, sting extract was incubated with 10 mM EDTA at 37°C for 30 min, tested and compared with EDTA untreated sting extract. In other antagonisms study, the overnight fasted rats were divided into control and treated group (n=7). The treated group of rats were pretreated with cyproheptadine (10 mg/kg, sc) and BW 755C (15 mg/kg, sc), the control group received vehicle only. After one hour, the abdomen of the animals of both control and treated group was opened under mild ether anaesthesia. The sting extract (200 µg) was injected into the stomach wall of all the rats. After 2 hr of injection of sting extract, all the animals were sacrificed and the haemorrhagic spot was measured and minimum haemorrhagic dose (MHD) was calculated after Kondo et al.

Hyperimmunization of rabbit—Antiserum was prepared against the sting extract by hyperimmunizing rabbits (1.5 kg, n=6). A total of 1 mg of sting extract was used per kg of body weight. Sting extract was mixed with Freund’s complete adjuvant (v/v) and administered subcutaneously at different sites on the back of the rabbits in first, second and third week. After one week rest, booster doses of sting extract (without adjuvant) were injected intravenously, for another three weeks (one injection per week). After the schedule, the rabbits were bled from marginal ear vein and serum was separated and stored at 4°C with 0.01% merthiolate for further testing.

Immunogel diffusion and immunogel electrophoresis—Immunogel diffusion was carried out by the method of Ouchterlony and immunogel electrophoresis was done following Graber and William.

Indirect haemagglutination—The antiserum developed against sting extract was monitored by the indirect haemagglutination test according to the method of Stavitsky to observe the antibody titre expressed in terms of number of wells.

Neutralization studies—Neutralizing ability of the antiserum was tested by incubating a fixed amount of antisera with varying doses of sting extract at 37°C for 30 min as per Ipsen. The solutions were then centrifuged at 5000 rpm for 5 min and the lethal, oedema and haemorrhagic activity were tested in experimental animals.
Values are shown as mean ± SE and the significance of the differences between means were determined by Student’s t test. P < 0.05 were considered significant. Institutional animal ethical clearance was obtained before the experiments.

Results

Effect of sting extract on mice paw oedema—S. argus sting extract produced dose and time dependent oedema in mice hind paw. In dose dependent studies, sting extract at different doses (5, 10, 25, 40, 50, 60 μg/paw) showed 15, 25, 75, 90, 100, and 100% swelling in test animal respectively. The ED50 of sting extract for oedema was found to be 20.5± 0.8 μg. The oedema producing action of the sting extract was characterized by rapid onset of swelling and reached peak level after 3 hr of injection, after which oedema started declining and after 6 hr, 31.2 ± 0.61% of the oedema was observed which was found to be maintained for 72 hr of observation (Table 1).

Effect of pharmacological antagonists on sting extract induced mice paw oedema—Cyproheptadine pretreatment significantly reduced the mice hind paw oedema by about 44.1 ± 0.21%, indomethacin pretreatment reduced the oedema by 22.9 ± 0.36%, whereas BW 755C pretreatment reduced the oedema by 36.2 ± 0.7% induced by the sting extract (40 μg) (Table 2).

### Table 1—Time dependent changes in oedema with S. argus sting extract

<table>
<thead>
<tr>
<th>Sting extract dose (μg/paw)</th>
<th>% oedema (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>45.04 ± 0.08</td>
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<tr>
<td></td>
<td>56.86 ± 0.36</td>
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<tr>
<td></td>
<td>82.96 ± 0.24</td>
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<tr>
<td></td>
<td>99.78 ± 0.31</td>
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<tr>
<td></td>
<td>92.90 ± 0.33</td>
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<tr>
<td></td>
<td>61.54 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>31.28 ± 0.61</td>
</tr>
</tbody>
</table>

Sting extract applied 40 μg = 2 ED50 dose.

### Table 2—Effect of pharmacological antagonists on oedema forming activity of S. argus sting extract

<table>
<thead>
<tr>
<th>Group of animals</th>
<th>Sting extract (μg)</th>
<th>Dose of antagonists (mg/kg, sc)</th>
<th>Increase in paw diameter at 3 hr (%)</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (sting extract only)</td>
<td>40</td>
<td>-</td>
<td>99.71 ± 0.77</td>
<td>-</td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Cyproheptadine</td>
<td>40</td>
<td>10</td>
<td>55.71 ± 0.64*</td>
<td>44.12 ± 0.21</td>
</tr>
<tr>
<td>(b) Indomethacin</td>
<td>40</td>
<td>10</td>
<td>76.85 ± 0.59*</td>
<td>22.92 ± 0.36</td>
</tr>
<tr>
<td>(c) BW 755C</td>
<td>40</td>
<td>15</td>
<td>63.57 ± 0.36*</td>
<td>36.24 ± 0.78</td>
</tr>
</tbody>
</table>

*P<0.001

Effect of sting extract on haemorrhagic activity—In model I the sting extract 50 and 500 μg, id (in 0.1 ml 0.02 M phosphate buffer, pH 7.4) did not produce any significant haemorrhagic spot in skin in mice, up to 24 hr. In model II the sting extract (200 μg) produced haemorrhagic spot of about 10 ± 2 mm on the serosal surface of the stomach and profuse bleeding on the mucosal surface of the stomach. The minimum haemorrhagic dose (MHD), was calculated to be 200 ± 8.5 μg.

Effect of physical agents (temperature, UV rays and EDTA)—The untreated(temperature) sting extract (200 μg) showed mean diameter of haemorrhagic spot 10.12 ± 0.16 mm (n=7). Sting extract treated at 37°C showed activity of 40 ± 5%, at 50°C 45 ± 4%, and at 80°C 5.5 ± 0.5% haemorrhagic activity.

The untreated (UV) sting extract (200 μg) produced mean diameter of haemorrhagic spot of 10.70 ± 0.28 mm whereas the same dose of the sting extract treated under UV-exposure for 30 min produced haemorrhagic spot of about 5.71 ± 0.28 mm, showed significant inhibition of haemorrhagic activity 46.68 ± 0.5 (n=7, P<0.001).

It was observed that the untreated (EDTA) sting extract (200 μg) produced mean diameter of haemorrhagic spot of 10.86 ± 0.33 mm, whereas same dose of the sting extract treated with 10 mM EDTA at 37°C for 30 min, produced 6.86 ± 0.30 mm of mean
diameter of haemorrhagic spot and caused a significant inhibition of the haemorrhagic activity. (35.92 ± 2.8 % n=7, P<0.001).

Effect of pharmacological antagonists (cyproheptadine, indomethacine and BW788C)—Cyproheptadine (10 mg/kg, sc) pretreatment reduced the haemorrhagic activity of the sting extract by about 29.80 ± 0.35 % (mean diameter of haemorrhagic spot (mm), untreated animals: 10.57 ± 0.29, cyproheptadine treated animals: 7.42 ± 0.20, n=7, P < 0.001). BW 755C (15 mg/kg, sc) treatment of animals reduced the haemorrhagic activity of the sting extract by about 58.08 ± 0.9% (mean diameter of haemorrhagic spot (mm), untreated animals: 10.33 ± 0.33, BW 755C treated animals: 4.33 ± 0.21, n=7, P < 0.001). Similarly indomethacine (10 mg) pretreatment reduced the haemorrhagic activity of the sting extract (40 μg) by 44.80 ± 0.22%.

Hyperimmunization of rabbits and neutralization studies—The antiserum developed in rabbit against sting extract (1 mg) produced multiple prominent bands in immunodiffusion and immunoelectrophoresis. The IHA titre of the antiserum was found to be 1:4096. The lethal action of the sting extract was neutralized by the antiserum up to 2LD50 dose in male albino mice (n=6), but the antiserum failed to neutralize the proinflammatory and haemorrhagic activity of the sting extract.

Discussion

Fish envenomation is a common major health hazard among fish eater and fisherman. Several pathophysiological conditions like severe pain, intense oedema, haemorrhage and necrosis may occur due to envenomation. Uses of anti-inflammatory, analgesic, antipyretic drugs together with antibiotics are very common but proved ineffective in many cases. In Australia, fish envenoming has been successfully treated by immunotherapy and an antivenom against Synanceja trachynis (stonefish) venom is commercially available and used for accidents where the identity of this fish is assured11,12. The venomous edible fish S. argus envenomation manifested by intense pain, severe oedema and haemorrhages. Due to lack of knowledge about the pharmacological actions and active constituents, it is very difficult to encounter envenomation consequences and this contribute towards a major occupational health hazard among the fishermen. In this communication, the local effects of S. argus sting extract and its antagonism have been tried to establish with a view to develop effective therapeutic measure.

One of the major clinical signs of S. argus sting was oedema, redness and intense pain at the site of sting. Similarly, oedema produced in mice paw by the sting extract was found to be dose dependent i.e. the intensity of oedema varied with time and the amount of venom injected. This is the reason why the victims, immediately try to squeeze out blood from the site of sting, thereby reducing the amount of venom from the site.

In order to develop antagonist against the oedema, an attempt was made to develop antiserum in rabbit, keeping in view that most of the venom (sting) constituents are protein in nature. The antiserum raised showed precipitating bands in immunodiffusion and immunoelectrophoresis but failed to encounter oedema produced in mice, indicating that the oedema inducing factors present in venom (sting) are unlikely to be protein or oedema effects are due to the release of inflammatory mediators (like histamine, serotonin, prostaglandin, leukotriene etc.). In fact, further experiments with pharmacological antagonists proved the above proposition. Cyproheptadine, indomethacine and BW 785C significantly reduced the S. argus sting extract induced oedema in mice. So, from the present study, it is very likely that pharmacological antagonists are a better choice to encounter oedema than the antiserum.

Similarly, responses to the venom of stonefish (Synanceja trachynis) were significantly inhibited by the cyclooxygenase inhibitor indomethacin, the leukotriene D4 receptor antagonist FPL55712, the thromboxane A2 receptor antagonist GR 32191B, the muscarinic receptor antagonist atropine and the neurokinin-1 receptor antagonist CP96345. These results suggest that stonefish venom may cause the release of acetylcholine, substance P and cyclooxygenase products, or contain components which act at these receptors13. In another study, evidence was provided which suggests that venom of the soldierfish, Gymnapistes marmoratus also promote production of cyclooxygenase metabolites, such as prostaglandins, which may play a role in enhancing the pain-producing activity of a range of inflammatory mediators to exert their effects14. Church and Hodgson15 studied the cardiovascular neutralization activity of G. marmoratus venom which was very similar with the venom of stonefish Synanceia sp.
Local cutaneous haemorrhage was one of the major clinical signs observed due to the *S. argus* sting. Therefore experiments on haemorrhagic models in rat and mice were done to elucidate the pathophysiology of haemorrhage. The minimum haemorrhagic dose was found to be 200 μg. It was observed that the haemorrhagic action was not antagonized by rabbit antiserum, once again confirming that the haemorrhage inducing factor present in the *S. argus* sting extract is unlikely protein or may be protein having low immunogenic response. Karmakar et al. identified a haemorrhagic protein toxin (SA-HT) from the *S. argus* sting extract having low immunogenic activity. The haemorrhagic activity of SA-HT was antagonized by pharmacological antagonist such as cyproheptadine, indomethacin and BW577C. The same pharmacological antagonist was found to be effective in the present study also, indicating that pharmacological antagonist are a better choice that the antiserum, at least in the management of local effects induced by the *S. argus* sting.

The only exception regarding the neutralization of lethal effects of *S. argus* sting extract by the antiserum where it was found successful. It is likely that the lethal factor present in sting extract are protein in nature, against which neutralizing antibodies were raised. There is not a single report of human mortality due to *S. argus* sting. Therefore at the present situation, more serious are the local effects which could be well managed by the pharmacological antagonists.

This study needs further experimentation for the development of more effective antagonists to encounter the local effects against *S. argus* sting. Till then cyproheptadine and indomethacin may be an alternate choice for the treatment and relief for pain, oedema, redness against *S. argus* sting.

Acknowledgement

This paper is dedicated in the memory of Late Prof. A K Nagchoudhury, Department of Pharmaceutical Technology, Jadavpur University, Calcutta, who expired on 19th March, 2004.

References