Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 1, 2014

Research Article

BIO-PROSPECTING OF CATFISH STING VENOM *ARIUS MACULATUS* AVAILABLE ALONG SOUTH EAST COAST OF INDIA

P. ABIRAMI*, M. ARUMUGAM, S. GIJI, S. NAGARAJAN

Centre of Advanced study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai 608502, Tamil Nadu, India. Email*: mamnplab@gmail.com

Received: 21 July 2013, Revised and Accepted: 5 Oct 2013

ABSTRACT

Objective: To study the biochemical and toxicological properties of crude catfish sting venom (*Arius maculatus*).

Methods: The crude catfish sting venom molecular weight was determined using SDS-PAGE. The gelatinolytic activity was determined to ascertain the activity of protease in the sting venom. The toxicity study of catfish venom was primarily done using hemolytic assay and brine shrimp lethality assay (LC_{50}). Further characterization was done through HPLC and FT-IR. The amino acids present in the venom were screened using HPTLC by comparing the Rf value of 21 standard amino acids.

Result: The molecular weight was determined as bands ranging from 93- 18 kDa. It also possessed gelatinolytic activity between 97- 43 kDa. The sting venom exhibited high toxicity upon testing with erythrocytes and brine shrimp napulii. Arginine, Glutamine, Cysteine, Methionine, Glutamic acid, Lysine, Threonine, Tyrosine and Phenyl alanine are the amino acid present in sting venom (HPTLC), further characterized using HPLC and FT-IR.

Conclusion: This study reveals that crude catfish sting venom could be used to isolate structurally unique compounds of specific activites.

Keywords: Catfish, Venom, Proteolytic, Hemolytic, Brine Shrimp lethality, HPTLC.

INTRODUCTION

There are more than 200 species of marine fish including Stingrays, Scorpionfish, Zebrafish, Stonefish, Weeverfish, Toadfish, Stargazers and some species of Shark, Ratfish, Catfish, Surgeonfish and Blenny are known or suspected to be venomous [1]. The production of toxins by aquatic animals is an important strategy that guarantees its survival in a highly competitive ecosystem. In this way, these animals defend themselves or their territories and produce a significant number of metabolites, which in combination results in a great variety of chemical structures and complex molecules, such as alkaloids, steroids, peptides and proteins with chemical and pharmacological properties, different from those venoms of terrestrial animals [2]. While there has been much work in characterization and the biological activity of the most terrestrial animals (e.g. snakes, spiders and scorpions), less research work has been undertaken on venomous fish. Since fish toxins represent a vast source of novel pharmacological compounds, they may prove to be useful as both research tool and therapeutic agent [3].

Wright reported that possibly over 1600 species of catfishes may be venomous, whose number is far greater than any previous estimate of venomous catfish diversity [4]. This envenomations caused by catfsh sting are common injuries in both freshwater and saltwater. Such injuries are complex puncture wounds, often complicated by severe infection where symptoms range from simple local pain and bleeding to systemic manifestations with hemodynamic compromise [5].

One such venomous fish of medical importance found in south east coast of India is Catfish (*Arius maculatus*). The catfish have economic importance. Its wide distribution and importance as food provoke proximity to human resulting catfish envenomation. The venom glands are found along serrated and sharp bony stings on the edges of the dorsal and pectoral fins and these stings can be locked into place when the catfish is threatened [4]. As the sting penetrates the skin, the integumentary sheath is torn and the venom glandular tissue is broken, releasing venom into the wound. The wound is immediately painful, described as stinging, throbbing, or scalding, with rapid central radiation from the involved extremity. Although a mild envenomation may cause pain for 30-60 mins, multiple stings from a large tropical animal may cause severe discomfort for up to 48 hours [6]. The soft tissue surrounding the puncture wound generally becomes ischemic, with initial pallor that progress from cyanosis to reactive hyperemia and edema. The latter can be quite severe. Secondary infections are common which includes local muscle spasm, sweating and less frequently causes lymph edema, lymphadenopathy, weakness, hypotension [7]. In such envenomations the patients present extreme pain. Thus the treatment of these injuries should include cleansing of the wound and surrounding area by immersing the wounded extremity in hot water (45°C) for the pain control [8]. This envenomation may be sever due to the presence of hydrolytic enzymes such as proteases hydrolysing proteins of the extracellular matrix and induce coagulopathies by hydrolyzing fibrinogen and fibrin [9,10,11,12].

Thus by knowing the importance of catfish venom, here we have made an attempt to explore the biological and biochemical activity of the catfish sting extract (Catfish venom) of the Catfish *Arius maculatus* which is a common inhabitant of the inshore of South East coast of India, Parangipettai. The ability of the catfish to inflict extremely painful wounds with their pectoral and dorsal stings has been well known for many decades.

MATERIALS AND METHODS

Collection of fish sample

The live species of *Arius maculatus* were collected from Parangipettai- Annakovil landing center South east coast of India and were immediately taken to laboratory.

Preparation of the Crude Extract

Fishes were chilled at -20°C for 10-20 minutes and then decapitated; the dorsal and pectoral stings were cut approximately 3-5mm from the base and homogenized in phosphate buffer at pH 7.4. The supernatant was centrifuged at 6000 x g for 15 minutes at 4°C to remove insoluble material. The pellet was discarded and the supernatant was collected and lyophilized. The extract (crude sting venom) was stored at -20°C for further analysis. All the steps were carried out in cold room at 4-10°C.

Estimation of Protein

Amount of protein in the sample was estimated according to the method of Lowry *et al.* [13] with BSA as a standard.

Estimation of total sugars

Content of total sugars present in Catfish venom was quantified using the method of Ashwell [14] using D-glucose as standard.

Determination of molecular weight

SDS-PAGE was performed to estimate the molecular weight of the crude catfish venom using 5% stacking gel and 12% resolving gel following Laemmli [15].Molecular weight was estimated by comparing the relative mobility of proteins of different standard molecular weight marker (97.4, 66, 43, 29, 20 and 14.3 KDa) (Genei: Bangalore, India).

Determination of Gelatinolytic activity

Gelatin is used as substrates and the method was adapted from Heussen and Dowdle [16]. Briefly, 2 mg/ml (w/v) substrate was incorporated in the 10% resolving gel with a 4% stacking gel. The sample (10 mg) was loaded in non-reducing sample buffer. After electrophoresis the SDS was removed by washing the gel twice for 20 min in 2.5% Triton X-100 before incubation in 20 mM Tris, 0.4 mM calcium chloride pH 7.4 at 37°C for 16 h and stained with 0.125% Coomassie blue. The clear zone in the gel indicates the regions of enzyme activity and compared with pre-stained molecular weight marker (Genei).

Toxicity studies

Hemolytic assay

The micro hemolytic test was performed as described by Venkateshwaran [17] in 96 well 'V' bottom micro titer plates using EDTA solution (2.7g/ 100 ml) as anticoagulant. Different rows were selected for chicken, sheep and human erythrocytes. Serial two fold dilutions of the crude venom were made in 100ml of normal saline. This process was repeated upto the last well. Then 100 µl of RBC was added to all the wells. Appropriate controls were included in the test. To the 1% RBC suspension 100 µl was added normal saline, which served as negative control. The plate was gently shaken and then allowed to stand for two hours at room temperature and the results were recorded. Uniform red colour suspension in the wells was considered as positive hemolysis and a button formation in the bottom of these wells was considered as lack of hemolysis. Reciprocal of the highest dilution of the crude venom showing pattern was taken as 1 Hemolytic Unit (HU) was divided by the protein content to obtain the specific hemolytic unit.

Brine Shrimp lethality assay

The Brine Shrimp lethality assay was performed using the method of Meyer *et al.* [18]with *Artemia Salina* nauplii (24hr post hatching). Fifty organisms were used for each concentration of crude catfish venom, which were 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg/ml and were subjected to 24 h exposures. Triplicates of each concentration were used. To calculate the LC_{50} (mean lethal concentration) the results were plotted as % mortality V_s concentration of crude venom extract.

High Performance Liquid Chromatography

The crude spine venom was fractioned by analytical HPLC using Shimadzu C-18 column with two solvent systems: a. 0.1% TFA solution b. 0.1% TFA in 90% Acetonitrile. The column was eluted at a flow rate of 1ml/min with 10-90% gradient solution B over 40min of total volume of 20µl. The RP-HPLC column elutes was monitored by their absorbance at 215nm and 280nm.

High Performance Thin Layer Chromatography

Qualitative analysis of amino acids were performed on TLC aluminum plates silica gel 60 F254 (Merck), 20×20cm. Standards of 21 amino acids were prepared by dissolving 1mg in 1ml distilled water. The crude catfish venom was precipitated using cold ethanol at -4^{0}C and centrifuged, the pellet was subsequently dissolved in water and used for further analysis. Samples and standards (2µl) were injected using Hamilton syringe. Initial zones were allowed to air dry and plates were developed in TLC chamber saturated with n-Butanol: acetic acid: water (4:1:5). Detection of the zones was achieved by spraying the developed and air-dried plates with Ninhydrin reagent, briefly, air drying for 30 mins and heating for 10 mins at 110ºC in oven. The plates were then scanned using Camag, TLC scanner3 at 290nm. The amino acids present in the sample were determined by comparing the Rf value of band formation sample with that of 21 standard amino acids.

Fourier Transform-Infra Red spectrum analysis

FT-IR spectroscopy of solid samples of crude catfish venom relied on a Bio-Rad FT-IR – 40 model, USA. The crude catfish venom (10mg) was mixed with 100mg of dried potassium bromide (KBr) and compressed to prepare as a salt disc (10mm diameter) for reading the spectrum further.

RESULTS

Estimation of protein

The protein content of the crude sting venom was estimated as 748µg/mg according to Lowry *et al.* [13] method.

Estimation of total sugars

The amount of total sugars present in crude catfish sting venom was estimated as 0.025µg/mg.

Determination of molecular weight

The crude catfish sting venom of *A. maculatus* was subjected to 12% SDS-PAGE analysis and after running; several protein bands were visualized (Fig. 1). Totally 9 bands were observed at 93kDa, 84kDa, 72kDa, 61kDa, 43kDa, 33kDa, 30kDa, 25kDa and 18kDa respectively.

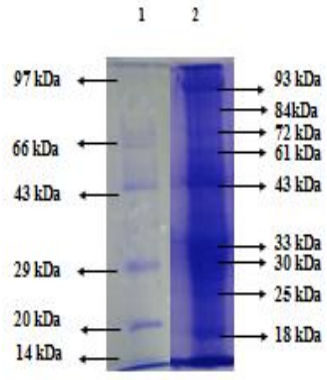

Fig. 1: Molecular weight determination of catfish crude sting venom on SDS gel.

Lane 1: Molecular marker (GeNei, medium marker). Lane 2: Crude sting venom.

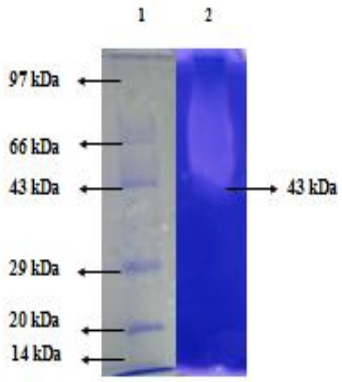

Fig. 2: Showing the gelatinolytic activity of catfish crude sting venom.

Lane 1: Molecular marker (GeNei, medium marker). Lane 2: Gelatinolytic activity of crude sting venom.

Determination of Gelatinolytic activity

Proteolytic activity was identified by a zymogramy experiment. The crude catfish sting venom sample catalyzed the hydrolysis of protein substrate gelatin. The Proteolysis was detected as colorless bands in an otherwise blue gel (Fig. 2). The gelatinolytic activity of crude venom was found between 97kDa to 43kDa.

Toxicity

Hemolytic activity

The hemolytic activity of crude catfish sting venom against chicken, sheep and human blood were detected. The crude extracts induced moderate level of hemolysis on these bloods which shows that the venom consists of hemolytic factors inducing hemolysis. The maximum hemolytic effect was observed for chicken blood of about 32HU/mg with the specific actiivty of 42.78 (HT/mg) whereas for both sheep and human blood minimum of 8HU/mg with the specific actiivty of 10.69 (HT/mg) was observed (Table. 1).

Brine Shrimp Lethality assay

The crude catfish sting venom was screened for probable cytotoxicity using brine shrimp lethality bioassay. The crude venom extract showed moderate toxicity against brine shrimp nauplii and calculated the LC_{50} value. The lower LC_{50} values indicate higher toxicity and higher LC_{50} values are suggestive of lower toxicity. The sample was found to exhibit the minimum LC_{50} value of 2.244µg/ml for *Artemia salina*.

High Performance Liquid Chromatography

The HPLC analysis of crude catfish sting venom was done at 215nm and 280nm. Totally 14 & 13 peaks were observed in 215nm & 280nm respectively with differing retention time. The major peak found in 215nm was 11th peak of 6.797 retention time and in 280nm it was 8th peak with the retention time of 6.770. Several other peaks found were tableted in Table. 2 & 3 with respect to retention time of respective nm.

Table 1: Hemolytic activity of the catfish crude sting venom on chicken, sheep and human erythrocytes

S. No.	Type of blood	Amount of protein (mg)	Total hemolysis	Hemolytic Titre	Specific hemolytic activity (HT/mg)
1.	Chicken blood	0.748	5	32	42.78
2.	Sheep Blood	0.748	3	8	10.69
3.	Human Blood	0.748	3	8	10.69

Table 2: Showing the Area, Height and Retention time of every individual peak at 215nm of catfish sting venom

Peak	Retention Time	Area	Height	Area %	Height %
1	2.639	499393	72896	2.146	4.436
2	2.953	590116	90370	2.536	5.499
3	3.246	272379	43393	1.171	2.640
4	3.669	103554	15732	0.445	0.957
5	3.947	465344	34346	2.000	2.090
6	4.311	589172	77890	2.532	4.740
7	4.940	338144	40637	1.453	2.473
8	5.447	19962	2112	0.086	0.129
9	6.049	1063460	69612	4.570	4.236
10	6.393	619489	55253	2.662	3.362
11	6.797	17936838	1019750	77.087	62.051
12	7.779	496759	77755	2.135	4.731
13	8.083	41364	8815	0.178	0.536
14	8.223	232288	34844	0.998	2.120
Total		23268261	1643405	100.00	100.00

Table 3: Showing the Area, Height and Retention time of every individual peak at 280nm of catfish sting venom

Peak	Retention Time	Area	Height	Area %	Height %
1	2.706	17027	2376	1.118	1.185
2	2.910	33612	4392	2.207	2.190
3	3.950	750185	56902	49.251	28.378
4	4.354	4264	882	0.280	0.440
5	4.943	25525	5098	1.676	2.543
6	6.135	7534	528	0.495	0.263
7	6.442	224	26	0.015	0.013
8	6.770	550822	108339	36.162	54.032
9	7.002	31914	7113	2.095	3.547
10	7.416	8106	1855	0.532	0.925
11	7.506	20494	4319	1.345	2.154
12	7.838	3247	301	0.213	0.150
13	8.227	70243	8381	4.612	4.180
Total		1523197	200511	100.00	100.00

High Performance Thin Layer Chromatography

The free amino acid analysis was done by comparing the 21 amino acid standards with the sample. The HPTLC profile found that the catfish venom extract contains Arginine, Glutamine, Cysteine, Methionine, Glutamic acid, Lysine, Threonine, Tyrosine and Phenyl alanine based on the Rf value of band formation (Fig. 3)

Fourier Transform-Infra Red spectrum analysis

The FT-IR spectrum of crude catfish sting venom obtained reveals the characteristic functional groups showed in the (Figure 4 & Table 4). The FT-IR analysis of the crude catfish sample was found to contain aromatic primary amine NH stretch at 3460cm^{-1}. It also contains isothiocyanate, aldehyde, alkenyl C=C stretch at 2066 cm^{-1},

1733 cm^{-1}, 1637 cm^{-1} respectively. Further methyl and methylene band at 1474 cm^{-1}, 1458 cm^{-1} and 1437 cm^{-1}.

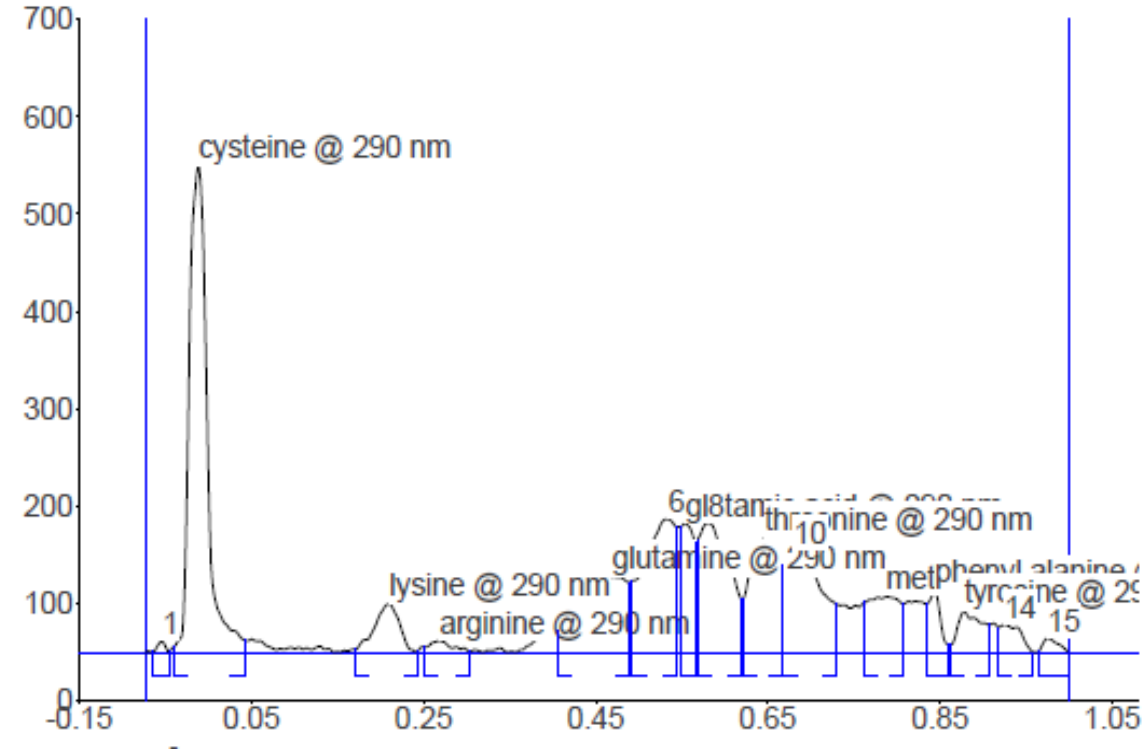

Fig. 3: HPTLC digital scanning profiles showing the amino acids present in the crude catfish sting venom *A. maculatus* at 290nm.

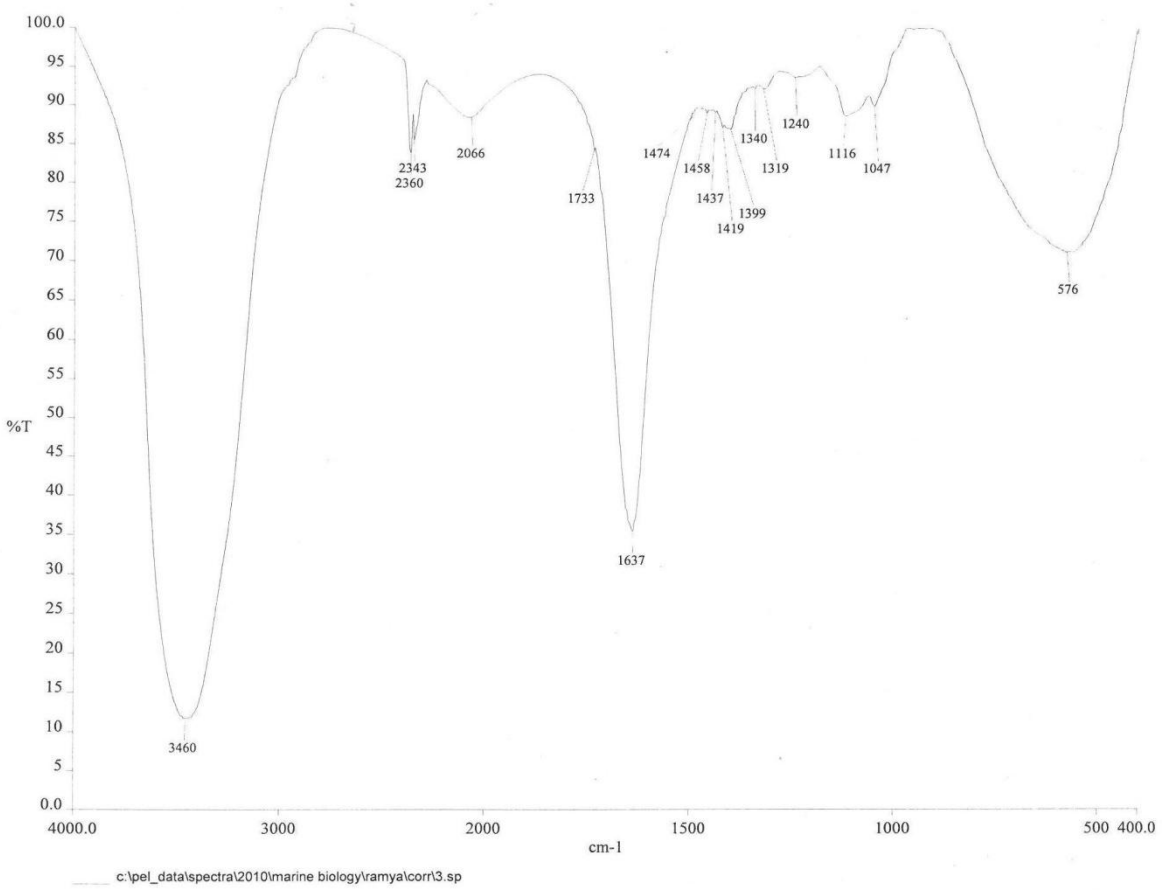

Fig. 4: Showing the FT-IR spectrum analysis of Catfish crude sting venom *A. maculatus*.

DISCUSSION

Venomous creatures have long been studied as a potential source of pharmacological studies. The discovery of toxins from venom, especially from marine resources, are racing ahead because of their extremely complex and unique action on various mammalian physiological systems. Animal venoms are usually complex mixtures of bioactive molecules, including proteins and peptides. These combinations confer a formidable array of toxic properties on the venom, the peptides, and polypeptides being responsible for a variety of toxic properties [19]. Venom complexity and demonstrated isoform selectivity in ion channel targeting makes these extracts a natural pharmacopoeia for biological testing and drug lead discovery programs [20]. After all, the venom secretion is part of an organism mainly for their defense or predatory mechanism, whose specificity is honed over a million years of evolution [21]. Envenomations caused by these catfish are accompanied by intense pain, numbness of the site, dizziness, local oedema and erythema [8].

With the knowledge of knowing the importance of these catfish sting venom, this present study is concentrated to explore the biological and biochemical properties of the catfish *Arius maculatus*. The protein content is found to be the major part of the catfish *Arius maculatus* sting venom and molecular weight visualizing as 9 bands ranging between 93kDa to 18kDa. Earlier, Al-Hassan *et al*. [22]reported more than thirty proteins in venom and fifty proteins in epidermal secretions in marine catfish *A. thalassinus* on the SDS gels. Whereas our study is further supported by, Ramos et al.[5] that the the electrophoretic analysis of sting venom of *Cathorops spixii* revealed 9 bands, with intensity of some of the bands.

Many proteases from various snake venoms, especially crotalid venoms, have been isolated and well-studied. They are responsible for some pathological activities triggered by these venoms. In this respect, the venom of fishes is no different, containing various enzymatic activities. The crude sting venom (*A. maculatus*) shows gelatinolytic activity at the detectable range from 97kDa to 43kDa by hydrolyzing the protein substrate gelatin. In a similar way, a gelatinolytic protease (Sp-GP) was purified to homogeneity from *Scorpaena plumieri* venom [23] and mild proteolytic activity has been observed in bullrout, *Notesthes robusta* venom [24]. The proteases enzyme may also contribute to the local and systemic effects observed in envenomations and point to the venom spreading in the body of bite victims, an event that may be dependent on venom proteases [23]. Generally, venom proteases act directly on extra cellular matrix components causing local tissue necrosis and hemorrhage [10]and increase the envenomation

Preliminarily, biological characterization was carried out by employing hemolytic activity and brine shrimp lethality assay. Earlier, it was found that the F2 fraction of spine venom of scorpionfish *Scorpaena plumieri* showed hemolytic activity [25,26]. In similar way, the hemolytic activity of crude sting venom was recorded as 32HU/mg, 8HU/mg and 8HU/mg in Chicken, Sheep and Human blood respectively. It indicates that the crude sting venom have cytotoxicity effect and lyses the erythrocytes leading to homogenous appearance of the blood for positive reaction, where as other wells with button formation because the erythrocytes in them are not lysed indicating the absence of toxic effect. This hemolytic activity is reported to be the most common biological activity [27]. Although hemolysis, may be of some significance in experimental situations which has not been observed in clinical situations. However, no systematic clinical study of hemolysis has been conducted [28].

The crude catfish sting venom exhibited toxicity against brine shrimp nauplii, *Artemia salina*. The LC_{50} value of catfish venom was found to be 2.244µg/ml for *Artemia salina*. Earlier, a lethal protein toxin (toxin-PC) has been isolated from the Indian catfish (*Plotosus canius*, Hamilton). This Toxin-PC produced cardiac arrest on isolated toad and guinea pig hearts [29]. Similarly, injection of the purified skin toxin of Arabian Gulf catfish (epidermal secretions) into rabbits caused agitation, convulsions and death within 5 min [30].

The analytical analysis of the crude catfish sting venom was done by using HPLC, C-18 column. The peaks were detected in two wave lengths; at 215nm, 14 peaks were observed whereas at 280nm, 13 peaks were observed. Every individual peaks refer to a specific compound present in the extract. The role of every peak can be studied by further fractionation of individual peak. Earlier, RP-HPLC was done for the mucus and the sting venom from the catfish *Cathorops spixii,* where seventeen RP-HPLC fractions of the venom were obtained and the mucus contained only four major peaks while the sting presents eight peaks [5].

The amino acid present in the crude sting venom was analyzed using HPTLC. The band formed in crude venom extract was confirmed by comparing with standard band formation of 21 amino acids and found to contain Arginine, Glutamine, Cysteine, Methionine, Glutamic acid, Lysine, Threonine, Tyrosine and Phenyl alanine. Earlier, Manivasagan *et al*. [31] reported that the proteinaceous gel secretion from the skin of the catfish, *A. maculatus* found to contain Leucine in high quantity (9.8 mole %) in the soluble fraction and aspartic acid (9.0 mole %) in high in the insoluble fraction.

The FT-IR analysis of crude catfish venom infer the presence of aromatic primary amine, aromatic tertiary amine CN stretch, primary amine CN stretch at 3460 cm^{-1},1319 $cm^{-1}$and 1047 cm^{-1}. It also contains alcohol group at 576 cm^{-1}.Further the sample contains aldehyde group, alkenyl C=C stretch, methyl C-H bend, methylene groups C-H bend, cyanate at 1733 cm^{-1}, 1637 cm^{-1}, 1474 cm^{-1}, 1458 cm^{-1} and 1437 cm^{-1}, 1116 cm^{-1} respectively.

CONCLUSION

The crude catfish sting venom was found to have a significant hemolytic activity and effective protesase activity which act as key factor for tissue necrosis and altered hemostasis which enhances the envenomation effect. These promising results suggest the presence of structurally unique active components. This venom will also pave a new way for the development of isolation of new lead or structurally unique and complex compounds for the treatment of disease that do not respond to currently available therapies. Further, a study in the patho-physiological mechanism and characterization of sting venom is necessary to understand the toxic effects.

ACKNOWLEDGEMENT

The authors are thankful to Prof. T. Balasubramanian, Former Dean & Director, Faculty of Marine Sciences, CAS in Marine Biology and higher authorities Annamalai University, Parangipettai for the facilities provided in carrying out this work. We also thank the Ministry of Earth Sciences, Govt. of India (MOES) for financial support of this work and for funding under the project 'Drugs from the Sea'.

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