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Research Article

BIOLOGICAL PROPERTIES OF THE ORANGE BANDED STING FISH SPINE VENOM *CHORIDACTYLUS MULTIBARBUS* (RICHARDSON,1848) FROM PARANGIPETTAI COAST, SOUTHEAST COAST OF INDIA.

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Abstract

A large number of venomous and poisonous animals are found in aquatic environments worldwide. Among these venomous fish awake special interest, since they represent more than 50% of the venomous vertebrates and are often involved in human accidents. The most dangerous venomous fish known belong to the Synanceiidae family. Enevenomation by *Choridactylus multibarbus* is relatively common among fisherman, anglers, bathers, and swimmers in which injuries may be very painful causing complication such as erythema, edema, pain, fever, nausea, vomiting. Taking in this view of frequency of accidents provoked by Orange banded sting fish *C. multibarbus* the present study was undertaken to evaluate the biological properties of *C.multibarbus* venom found in glandular epithelium which covers the spine (spine venom). A live specimen collected from Parangipettai coastal waters (Lat. 11° 29'33.62"N 79° 46'08.86"E) and the dorsal spine were cut approximately 3 - 5 mm from the base and homogenized in normal saline at pH 7.4. The partial purification of protein was carried out using DEAE cellulose. Antimicrobial properties of the fishes were tested against 5 pathogenic bacteria and 5 pathogenic fungi by using disc diffusion method. The toxicity study of sting fish was primarily done using hemolytic assay and cell line assay (Mcf 7 and Vero cell line). Further characterization was done through FT-IR. The amino acid present in the venom was screened using HPLC by comparing the Rf value of 21 standard amino acids. The antimicrobial activity shows mild activity on bacteria not on fungi. The spine venom exhibited high toxicity upon testing with erythrocytes and cell line culture assay. Arginine, Cysteine, Lysine, Threonine and Phenyl alanine are the amino acid present in spine venom (HPLC), FTIR analyze revealed the presence of bioactive compounds signals at different ranges. The estimation of protein gist were intervened by SDS –PAGE and the results showed that all the extracts showed significant number of protein bands in range from 13 KDa to 125 KDa respectively. This study reveals that Orange banded crude Sting fish *C.multibarbus* spine venom could be used to isolate structurally unique compounds of specific activities.

Keywords: Hemolytic activity, Antimicrobial activity, FT-IR and HPLC.

Introduction

A large number of venomous and poisonous animals are found in aquatic environments worldwide. Among these, venomous fish awake special interest, since they represent more than 50% of the venomous vertebrates and are often involved in human accidents (Russell *et al.,* 1965; Haddad Jr *et al*., 2003; Smith and Wheeler, 2006). The most dangerous venomous fish known belong to the Scorpaenidae family and, according to the morphology of the venom apparatus, they are classified in three main genus: Pterois (lionfish), Synanceja (stonefish) and Scorpaena (scorpionfish) (Keegan and Macfarlane *et al*., 1963; Russell *et al*., 1965). These fish are widely distributed in tropical and temperate shallow waters and they are usually slow-moving animals found disguised in the environment (near rocks, reefs or plants), which is a predispositioning factor to human accidents (Russell *et al*., 1965; Haddad Jr *et al*., 2000).

The sting fish *C.multibarbus*, a member of the Synanceiidae family, is considered one of the most

dangerous (Carvalho-Filho *et al*., 1999). The venomous secretion of this fish is mainly proteic in nature (Carrijo *et al*., 2005) and it is produced by specialized tissues located around the fin spines (Smith and Wheeler, 2006). During envenomation, the spines are erect and the mechanical pressure on the spine tears or pushes down the integumentary sheath over the spine as venom passes into the wound. *C.multibarbus* envenoming appears within 5–10 min as excruciating and persistent local pain disproportionate to the size of injury, redness, swelling and a throbbing sensation that extends to the limbs, followed by dizziness. The symptoms are variable, depending on the size of the fish and the quantity of poison injected therefore, envenomation represents a serious health hazard.

Studies on the chemical and biological properties of piscine venom have mainly focused on stonefish (Saunders *et al*., 1960; Saunders *et al*., 1962; Hopkins *et al*., 1994), lionfish (Cohen and Olek *et al*., 1989), scorpion fish (Schaeffer *et al*.,1971) and toadfish. Due to technical difficulties with obtaining and storing venom extracts and its labile nature, fish venom remains a largely untapped source of novel compounds. Current literature is mainly descriptive of *C.multibarbus* and the venom gland. The mode of action and the biochemistry of venoms are obviously complex and require better knowledge and investigation to explore the toxic action and resulting biochemical changes. A better understanding of this venom could lead us to the development of new therapeutic strategies complementary to conventional therapy. In the present study, we investigated the biological properties of sting fish *C.multibarbus* venom from Parangipettai coast.

Materials and Methods

Collection of fish sample

The live species of *C.multibarbus* were collected from Parangipettai (Lat. 11° 29’33.62”N 79° 46’08.86”E) landing center South east coast of India and were immediately taken to laboratory figure1.

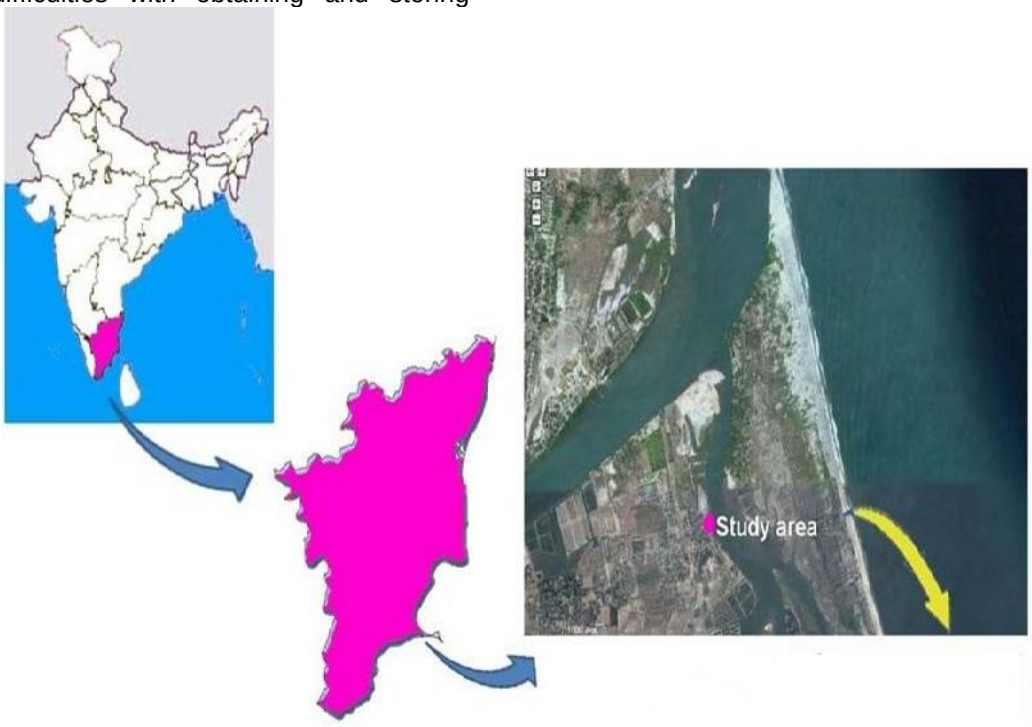


Figure.1: Description of the study area of *C.multibarbus*

Venom extraction

C.multibarbus, (150 specimens) were collected by local fishermen from Parangipettai coastal water. The collected fish were transported live or frozen to our laboratory, while this species was identified by FAO Sheets (Smith *et al*.,1957). The dorsal spines from each *C.multibarbus* specimen were cut from their base (Fig.2). The dorsal spines with the integumentary sheath containing a fusi form strand of glandular tissue were extracted with 0.15 M Nacl followed by centrifugation at 6,000 × *g* for 20 min at 4°C. The resulting supernatant was lyophilized. The lyophilized venom was stored at −80°C until use.

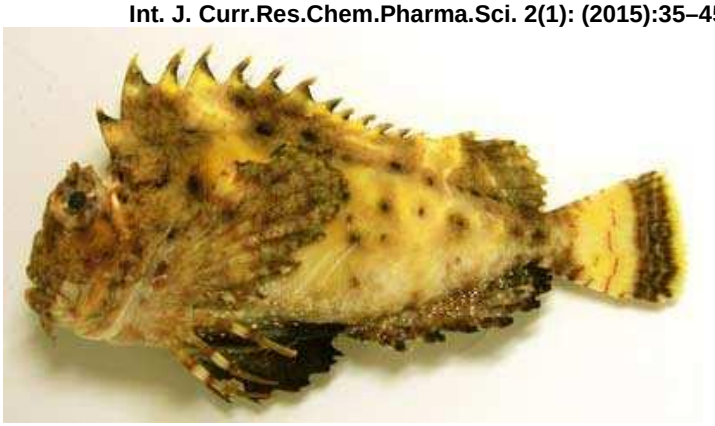


Figure.2: Shows the venomous fish of *C.multibarbus*

Protein quantification

Partial purification of crude sting fish spine protein

Partial purification of the crude extract *C.multibarbus* was carried out using DEAE Cellulose Anion Exchange chromatography according to the procedure (Stempein *et al*.,1970).

Biochemical parameters

Estimation of protein in spine

Amount of protein in the sample was estimated according to the method (Lowry *et al.*,1951) with BSA as a standard . Briefly, 20 mg of the spine sample was homogenized separately in 1 ml of 10% trichloroacetic acid (TCA) to precipitate the protein. Then the sample was centrifuged at 4000×g at4°C for 10 min. The sample was mixed with 5 mL of Bradford reagent. The samples were incubated for 10 min and the developed color was read at 595 nm using Shimadzu160 UV-VIS double beam spectrophotometer and the amount of protein was calculated.

Protein profile analysis by SDS-PAGE

The protein profile of partially purified crude proteins were qualitatively analyzed by SDS-PAGE (sodium dodecyl sulphate-poly acrylamide gel eletrophoresis) is carried out by 12% resolving gel and 5% stocking gel as described by (Laemmli *et al*., 1970).

Antimicrobial activity
***In vitro* studies**

The antimicrobial activity of *C.multibarbus* was investigated against clinical isolated bacterial strains *viz*., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E. coli*, *Bacillus*, *Lactobacillus brevis*, *Vibrio* sp. and fungal strains *viz*., *A. flavus, A. niger, Candida albicans A. oryzae* and *A.sojae* were obtained from the department of Medical Microbiology (Raja Muthiah Medical College hospital) Annamalai University, Annamalai Nagar, India. The stock cultures were maintained on nutrient agar medium at 4°C. *In vitro* antibacterial activity was determined by using Mueller Hinton agar and Mueller Hinton broth.

Preparation of inocula

Twenty four hour old culture of selected bacteria was mixed with physiological saline and turbidity was adjusted by adding sterile physiological saline until a McFarland turbidity standard of 0.5 (10^6 colony forming units (CFU) per ml) was obtained.

Disc diffusion method

The agar diffusion method (Brumfitt *et al.,*1990) was followed for antibacterial and antifungal susceptibility test. Petri plates were prepared by pouring 20 ml of Mueller Hinton Agar; Mueller Hinton Agar supplemented with 4% sodium chloride and Sabouraud's dextrose agar and allowed to solidify for the use in susceptibility test against bacteria. Plates were dried and 0.1 ml of standardized inoculum suspension was poured and uniformly spread. The excess inoculum was drained and the plates were allowed to dry for 5 minutes. After drying, the discs with extract were placed on the surface of the plate with sterile forceps and gently pressed to ensure contact with the inoculated agar surface. Finally, the inoculated plates were incubated at 37°C for 24 hours. The zone of inhibition was observed and measured in millimeters.

Toxicity studies
Hemolytic assay

The micro hemolytic test was performed as described by (Venkateshwaran *et al*.,1997) in 96 well 'V' bottom micro titer plates using EDTA solution (2.7g/ 100 ml) as anticoagulant.

Different rows were selected for chick blood. Serial two fold dilutions of the crude venom were made in 100 ml 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.

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 40 min of total volume of 20 µl. The RP-HPLC column elutes was monitored by their absorbance at 215 nm and 280 nm. 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 sting fish venom relied on a Bio-Rad FT-IR – 40 model, USA. The crude stingfish venom (10 mg) was mixed with 100mg of dried potassium bromide (KBr) and compressed to prepare as a salt disc (10 mm diameter) for reading the spectrum further.

Anticancer activity

MTT assay for cell viability and toxicity

The MTT assay is based on the ability of live but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product (Mossman,T *et al*., 1983). Cells were maintained in DMEM medium, supplemented with 10% Fetal Bovine Serum, at 37^{o}C in humidified atmosphere with 5% CO_2.The cells were plated in 96 well flat bottom tissue culture plates at a density of approximately 1.2 X 10^4 cells/well and allowed to attach overnight at 37^{o}C. The medium was then discarded and cells were incubated with different concentrations of the extract for 24 hours. After the incubation, medium was discarded and 100 µl fresh medium was added with 10µl of MTT (5mg/ml). After 4 hours, the medium was discarded and 100 µl of DMSO was added to dissolve the formazan crystals. Then, the absorbance was read at 570nm in a microtitre plate reader. Cell survival was calculated by the following formula:

Viability % = (Test OD/ Control OD) X 100

Cytotoxicity % = 100 – Viability %

Statistical Analysis

Tests were carried out in triplicates. The mean values were calculated from the triplicate values. Values are expressed as the mean ± SD and differences between groups were considered to be significant if $p<0.05$.

Results

Preparation of Crude Extracts

Crude extracts yield a total amount of 2.74 g of crude extract from 250g of spine .The protein content of the crude sting venom was estimated as 3.45 mg/ml.

Antimicrobial Activity

The effect of the spine venom of sting fish on pathogenic bacteria and fungi reveled that there samples were shows mild activity. The obtained results were represented in figure 3.

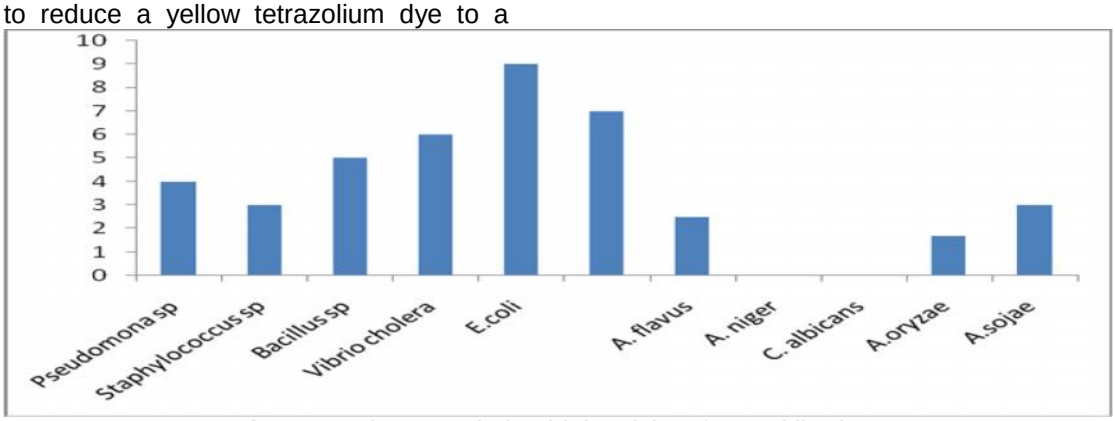


Figure 3: Shows antimicrobial activity of *C.multibarbus*

Toxicity

Hemolytic Assay

The crude extracts in spine venom induced hemolysis in chick, goat, sheep and human blood sample. The hemolytic titre in case of chick crude extract was found to be 5 and its specific hemolytic activity was estimated to be 32 HT/mg of protein. The hemolytic titre in case of goat crude extract found to be 4 and its Specific hemolytic activity was estimated to be 16 HT/mg of protein. The hemolytic titre in case of sheep crude extract was found to be 4 and its specific hemolytic activity was estimated to be 16 HT/mg of protein Similarly in human blood sample crude extract found to be 3 and its specific hemolytic activity was estimated to be 6 HT/mg of protein respectively figure 4.

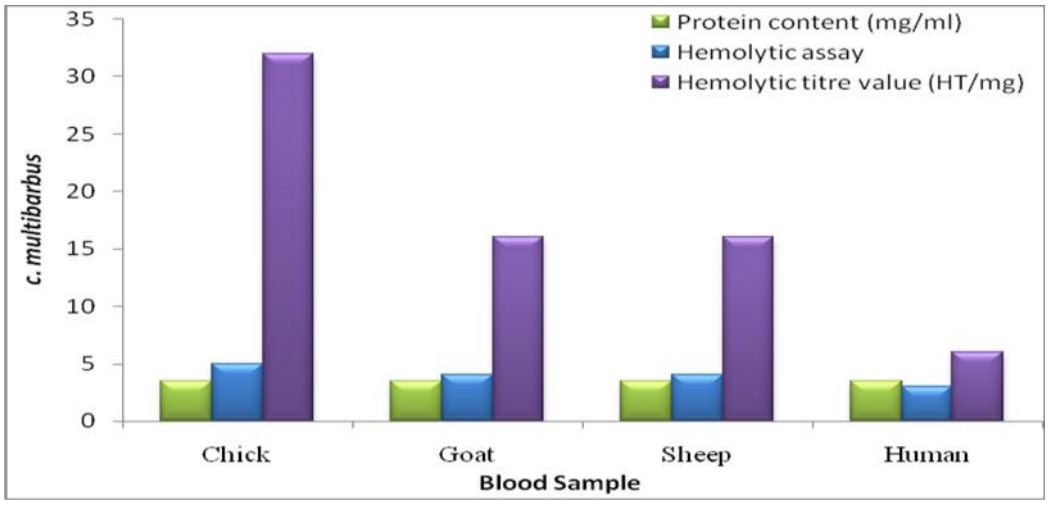


Figure. 4: Shows the hemolytic activity of *C.multibarbus*

High Performance Layer Chromatography

The free amino acid analysis was done by comparing the 21 amino acid standards with the sample. The HPLC profile found that the sting fish venom extract contains Arginine, Cysteine, Lysine, Threonine, Leucine and Phenyl alanine based on the Rf value of band formation (Figure. 5, 6) .

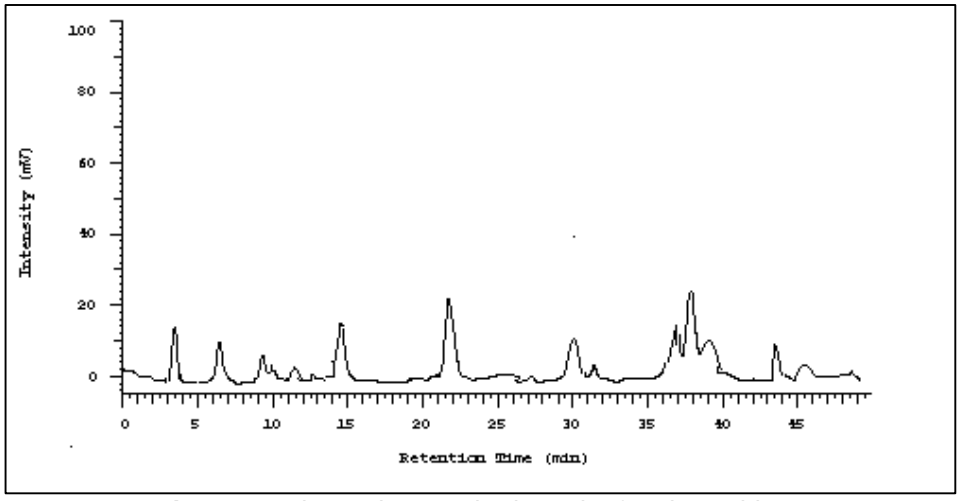


Figure 5 : Shows the standard graph of amino acid

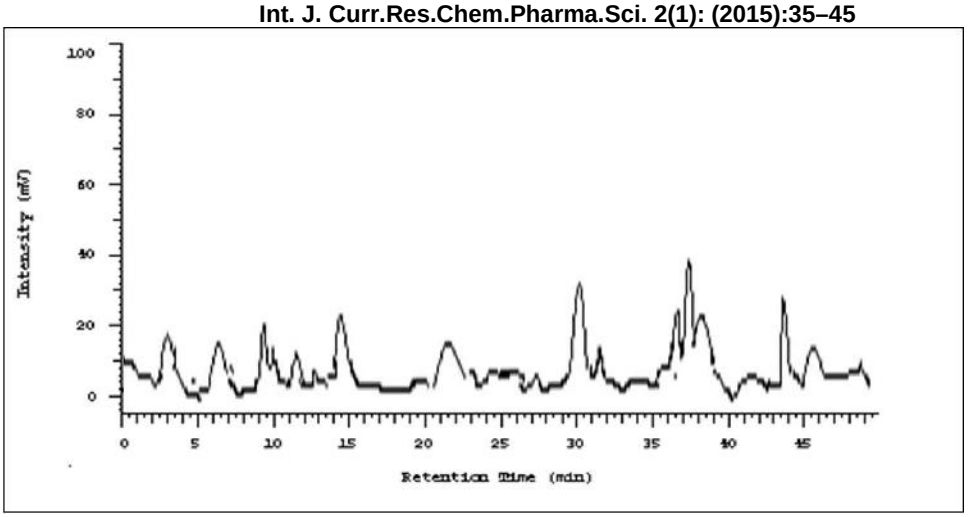


Figure 6: Shows the amino acid content of *C.multibarbus*

Fourier Transform-Infra Red spectrum analysis

The FT-IR spectrum of crude sting fish spine venom obtained reveals the characteristic functional groups showed in the (Figure 7). The FT-IR analysis of the crude sting fish sample was found to contain aromatic primary amine NH stretch at 3433cm^{-1}. It also contains isothiocyanate, aldehyde, alkenyl C=C stretch at 2081cm^{-1}, Conjugated ketone1643 cm^{-1},NH_3 group 1446 cm^{-1} and primary amine 1047 cm^{-1} respectively. Further methyl and methylene band at 1456 cm^{-1}, 1402 cm^{-1}. In addition it also contains non essential amino acid aspartic acid, glutamic acid, aspargine and proline were also present.

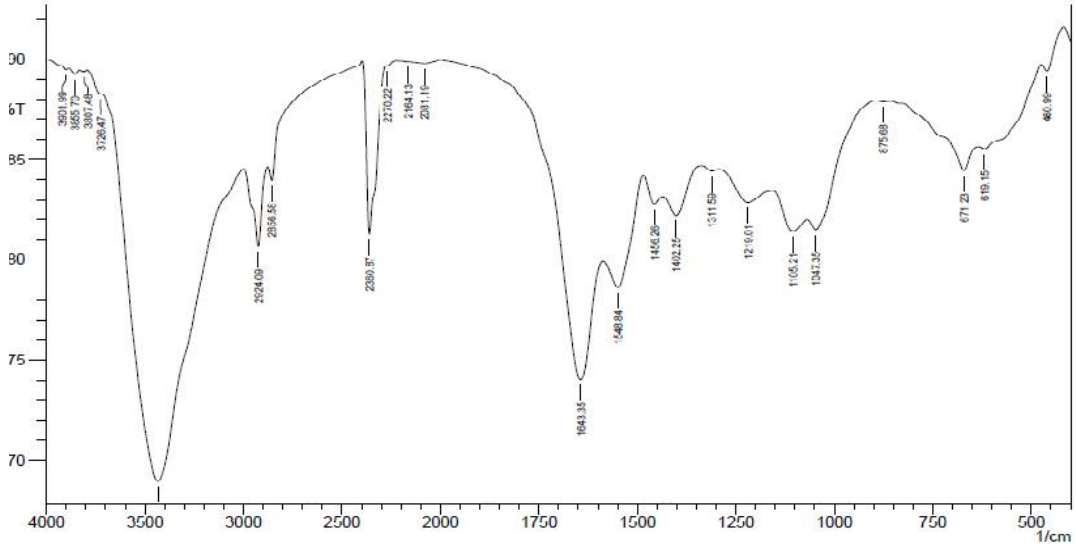


Figure 7: Shows the functional groups of *C.multibarbus*

Cell viability and toxicity for protein samples in Vero and Mcf 7 cell line.

The cytotoxicity of spine venom were studied on Vero and Mcf 7 cell line by using MTT assay at various concentrations of sample. The Viability of Vero and Mcf 7 cell line were adversely affected upon adding crude extracts. The relative cytoxicity on cells were illustrated in (figure 8, 9). The toxicity symptoms shown by the cells were lysis and detachment from substratum. Vero and Mcf 7 cell line showed venom concentration –dependent cell death. The on higher concentrations 100 µl crude spine venom on Vero and Mcf 7 cell line toxicity were more. The cells were carefully observed using inverted Microscope and photographed was shown in (figure 10,11).

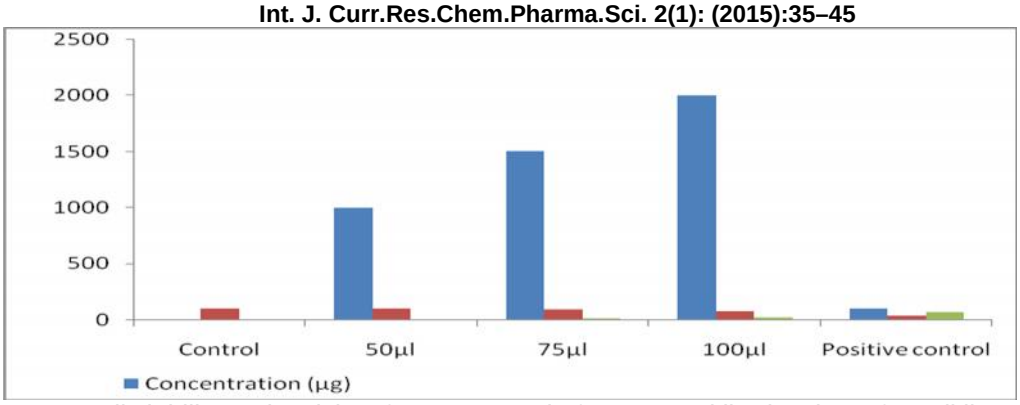


Figure: 8 Cell viability and toxicity of venom protein from *C. multibarbus* in Mcf 7 cell line

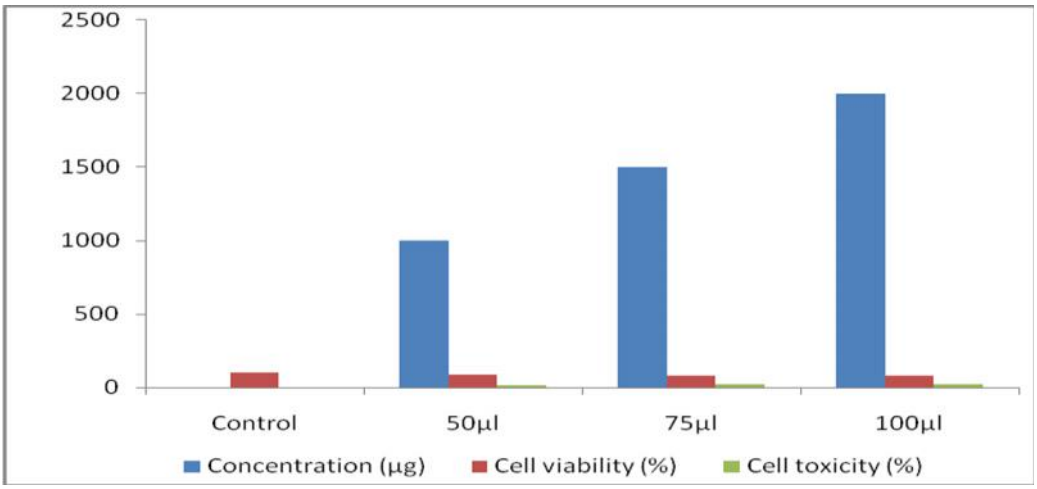


Figure: 9 Cell viability and toxicity of venom protein from *C. multibarbus* in Vero cell line

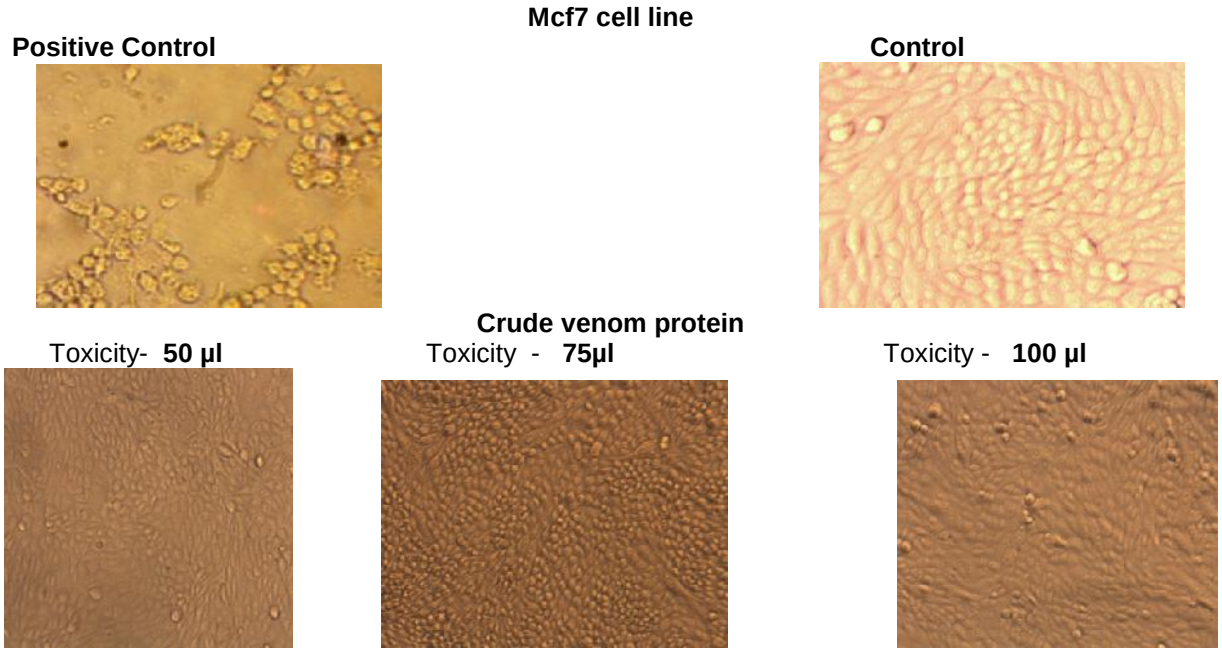


Figure 10 : Morphological changes of Mcf 7 cell line due to the effect of protein samples of *C. multibarbus*

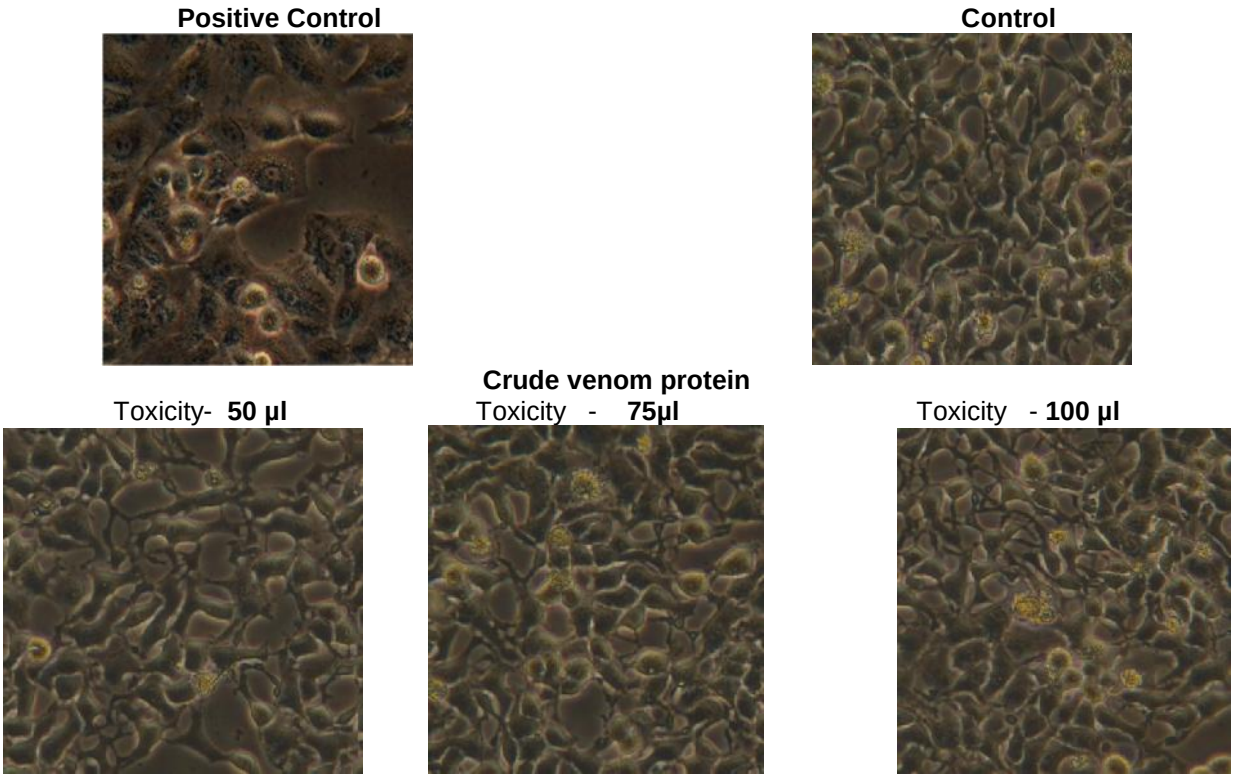
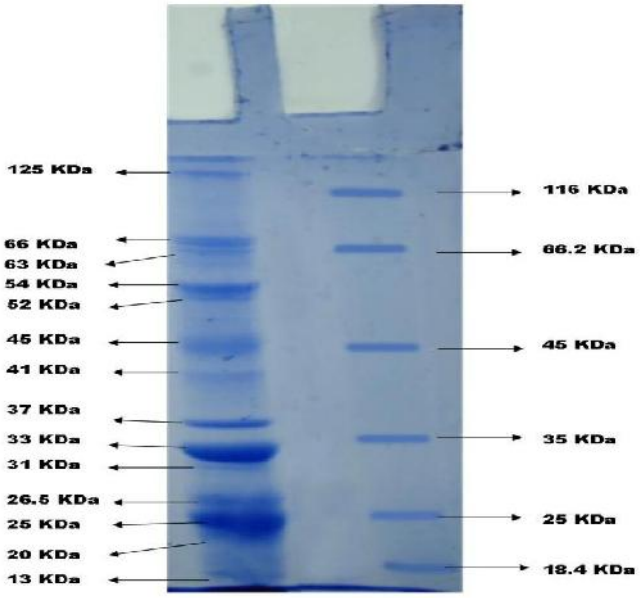


Figure 11 : Morphological changes of Vero cell line due to the effect of protein samples of *C. multibarbus*

SDS – PAGE: Electrophorectic separation of proteins were showed a significant banding patterns in 12% SDS – PAGE. The protein profiles of the crude protein samples of *C. multibarbus* on SDS-PAGE gel showed proteins ranging from 125 kDa to less than 13 kDa (Fig. 12) shows the banding pattern of spine sample. It demonstrated the presence of some toxic protein in the sample.



Lane 1: sample of *C. multibarbus*
Lane 2: Protein Marker (116, 66.2, 45, 35, 25and 18.4 KDa)
Figure 12: Protein profile of protein samples of *C. multibarbus*

Discussion

Discoveries of toxins from venoms, for the most part from marine resources that are racing ahead because of their extremely complex and notable action on various mammalian physiological systems (Sivan *et al.,* 2007). Toxic proteins serve in a number of adaptive roles such as immobilizing paralyzing, killing, liquefying competitions. Other venom proteins may act synergistically by enhancing the activity or spreading of toxins (Garnier *et al.,* 1965).

In the usual course of events, epidermal toxins, spine venom and poisonous fishes are produced by protein elaborating cells (Al –Hassan *et al.,* 1982). Thus the epidermal secretions contain a mixture of highly active biochemical and pharmacological components that are different from typical fish mucus and spine (Al-hassan *et al.,* 1985). Thus knowing importance of toxins and venom the present study was carried out on Biological Properties of The orange banded Sting Fish spine Venom *Choridactylus Multibarbus*. The present investigation of spine venom of *C. Multibarbus* were collected from Parangipettai coastal waters (Lat. 11° 29'33.62"N 79° 46'08.86"E) while this species was identified by (Richardson,1848). The extracts of spine venom were purified by DEAE - anion exchange chromatography. In purified spine venom of *C. Multibarbus* extract, 3.45 g extract whereas obtained from 250 kg fish. Poh *et al.,* (1991) had also done the same extraction in stone fish *Synanceja horrida.* SNTX was purified from crude venom by atleast two step procedure on Sephacryl S-200 high resolution gel – permeation and DEAE bio gel an anion - exchange chromatography.

The catfish has proteinaceous secretory cells in its epidermis (Al-Hassan *et al.,* 1982). In the present study, the protein content of spine venom of *C. multibarbus* was found to be 3.45 mg/ml in Similarly, the protein concentration of *Synanceja horrida* SNTX were estimated with a concentration of 1 mg/ml which showed an absorbance at 280 nm by (Chen *et al.,* 1997).

The crude extracts of spine venom *C. multibarbus* showed maximum hemolytic titre values (32 HT/mg) recorded in chick blood and minimum value (6 HT/mg) was recorded in human blood. *Carybdea marsupialis* jelly fish venom exhibits unpredictable hemolytic activities in different species, such as sheep, human and rabbit (Rottini *et al*.,1995). The jelly fish *Cassiopea xamachana* venom showed a higher hemolytic activity in human RBCs than sheep RBCs (Torres *et al*., 2001).

The amino acid composition of sting fish *C. multibarbus* spine venom consists of different amounts of essential amino acids. The skin toxins consists of all amino acids among these lysine, lecine, and aspartic acid are the major amino acids and proline tryptophan was found as lower amino acids. From the above result *C. multibarbus* spine, the amino acid compositions were rich and contain aromatic primary amine NH stretch at 3433cm^{-1}, isothiocyanate, aldehyde, alkenyl C=C stretch at 2081cm^{-1}, Conjugated ketone1643 cm^{-1}, NH_3 group 1446 cm^{-1} and primary amine 1047 cm^{-1}, methyl and methylene band at 1456 cm^{-1}, 1402 cm^{-1}respectively. Further in addition it also contains non essential amino acid, aspartic acid, glutamic acid, aspargine and proline. This suggests that the enzymic activities of spine venom differ based on amino acid composition. Previously (Al, Hassan *et al.,* 1985) amino acid analyses of both soluble and insoluble gel protein of skin secretion of Arabian gulf catfish *Arius thallasinus* yielded similar results .Further the toxin from the red sea flat fish is a hydrophobic acidic protein (paradoxin) with a molecular weight of 17,000 Da was composed of 162 amino acids (Venkateshwaran *et al.,* 1997).

The crude sting fish spine venoms showed a varied range of peaks which consists of nitrocomponds, sulphates, phosphates and methylene. As such spine venom consists of heterocyclic amine NH stretch at 3433cm, 1048cm. In addition it contain asymmetrical /symmetrical C-H stretch of methyl and methylene, methoxy, methylether, isothiocyanate of s-s stretching, aldehyde, alkenylc=c stretch, asymmetrical /symmetrical aliphatic nitro compounds of X02 stretch sulfonates, dialkyl /acyl sulfones and aliphatic nitro compounds of C-I stretch. More recently the FTIR analysis of mucus of two marine fishes *Cynoglossus Arel* and *Arius Caelatus* shows distinct spectral profile which confirms the presence of primary amine –group ,aromatic –compound, halide - group, aliphatic alkyl-group and polysaccharides (carbohydrates) (Bragadeeswaran *et al*., 2011).

MTT Assay originally developed for cell viability testing is now widely used in cancer cell cytotoxicity testing (Mossman *et al.,* 1983). In MTT Assay after incubation of Mcf 7cells and Vero cells with *C. multibarbus* venom of different concentrations, the cells were distended (a morphological feature common to necrosis) as compared to pre-exposure when examined by a phase contrast microscope. Degradation or lysis of the cell had taken place. The control group doesn't show any considerable cell death. Studies on Mcf 7cells and Vero cells have shown *C. multibarbus* venom to have potent cytolytic activity. There results infer that Mcf 7 appeared to be much susceptible to crude samples of spine venom

when comparing with Vero cells. In the Mcf 7cells and Vero cells at the susceptible concentration of cancer cells crude samples shows about 70% of viability, the higher concentration of spine venom induced more serious morphological alteration in both cell lines. Cytotoxity has also been recently reported for fish venoms of *Thalassophryne nattereri* on endothelial cell lines of capillary origin and *Gadopsis Marmoratus ,Pterois volitans and Synanceia trachynis* on cultured murine cortical cells (Lee *et al*., 2004). Earlier, the HeLa cells incubated with *S.argus* venom of different concentrations for nearly 4 hour found the cells distended (Gisha sivan *et al*., 2007).

The protein profiles of the crude protein samples of sting fish on SDS-PAGE gel showed proteins ranging from 125 kDa to less than 13 kDa . The crude protein sample showed both low and high molecular weight protein bands, and the low molecular mass proteins below 25 kDa were more prominent. The protein profile of spine extracts showed mainly high molecular weight proteins ranging from 45-125 kDa. These results were similar to the reports of (Chong *et al*.,2005). The experiment again proved from the previous study on the stone fish venom containing SNTX having proteins molecular weight (57KDa) as characterized by (Chen *et al.,*1997).

Conclusion

C. multibarbus had not been previously investigated for the presence of bioactive compounds. Hence, the present investigation has been explored the biomedical potential of this species. From the study, it is clear that this species contains a various active molecules with various biological properties. Even, this is for the first time *C. multibarbus*, a sting fish reported for having biological activities. Our data suggest that *C. multibarbus* venom is a source of pharmacologically active substances such as lead compounds and compounds that have applications as research tools.

Acknowledgments

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