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In vitro Screening of Toxin from Puffer Fish *Lagocephalus scleratus* for Antimicrobial, Hemolytic, *Acetylcholinesterase* and Cytotoxicity Activities

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The present study reports the toxicity of a marine puffer fish, *Lagocephalus scleratus* collected from South Indian coastal region (Chennai, India). The toxin from the fish was purified in water-washed activated charcoal and passed through a column of Bio Gel P 2. Toxicity was analyzed by mouse bioassay. Preliminary characterization was performed using UV-vis, TLC and fluorescence spectrophotometry which detected the presence of tetrodotoxin. *In vitro* antimicrobial activity of this toxin was performed by disc diffusion method against six pathogenic bacterial and fungal strains. Hemolytic, acetylcholinesterase and cytotoxicity tests were also carried out to study the potential bioactivity of the toxin. The results of this study indicate that the toxin from *L. scleratus* has bioactive properties which can be explored and utilized for therapeutic purposes.

Key words: *Lagocephalus scleratus*, Tetrodotoxin, Antimicrobial activity, Hemolytic activity, Acetylcholinesterase activity, Cytotoxicity test.

Human beings frequently come across infections caused by microorganisms. It is important to screen these pathogenic classes of microorganisms towards new drugs due to the increased indiscriminative use of common antibiotics for treatment and prominent increase in multi-drug resistant strains. This nudges scientists to explore novel forms from newer sources. Not only are these bioactive compounds studied for their pharmaceutical properties, but they are also exploited for their application in food and chemical industries. This necessitates screening of bioactive compounds even from extreme environments such as marine biota.

Marine ecosystem is a mixture of complex living organisms and is practically an unlimited source of bio-active compounds with unique biological properties. Marine bio-active compounds are significant due to their tremendous therapeutic and industrial applications. Moreover it is still one of the most underutilized biological sources having much scope for potential compounds. Faunae from oceanic community are commonly used as models for studies of diseases like anemia (Shafizadeh et al., 2004), glaucoma (McMahon et al., 2004) and also in toxicological studies like, response to pollutants such as dioxins (Yamauchi et al., 2005) and heavy metals (Bielmyer et al., 2005), embryos of teleost fish are used as model to study vertebrate development (Berman et al., 2005; Sherwood et al., 2005).

Even the toxins from marine sources have promising potential as bio-active compounds

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(Bialojan and Takai, 1988; Kao and Levinson 1986). Though these toxins are of severe health threat in their original form and at high concentrations, they are of high significance owing to their properties like binding to cell surfaces, penetrating into cytosol, etc. which are exploited for diagnostic and therapeutic uses like treatment of diseases like cancer (Johannes et al., 2010). The chemistry of these toxins are imperative because they help in understanding the mechanism of action of the toxin, counter measures like detection and determination of therapeutic methods. The structural modification and structure activity relationships of such toxins are of great interest for chemist and biologists (Bhakuni 2005). Marine toxins have found vast scope in recent years. Further investigations on these developmental toxins have become a topic of major interest among the scientific community.

Tetrodotoxin (TTX) is a deadly neurotoxin produced by many marine, freshwater and terrestrial vertebrates. This toxin was first isolated from puffer fish. TTX got its name from the order name Tetraodontiformes. As it is reported from different species and habitats, the possible origin and source of TTX remains unknown. Because of its chemical structure, TTX has become a popular pharmacological probe in a laboratory study for neurophysiology (Narahashi, 2001). It acts specifically by blocking the passage of ions through the sodium channel, because of which it is used to understand the mechanism of action of drugs interacting with the sodium channels.

In the present study, toxicity of the toxin isolated from marine puffer *Lagocephalus scleratus* was assessed. The toxicity of the fish toxin was determined by mouse bioassay which is the standard conventional method to determine the toxicity of the puffers. Characterization of TTX was carried out using UV/visible, TLC and fluorescence spectrophotometry. The extracted toxin was studied for its bioactive potentials.

MATERIALS AND METHODS

Puffer fish specimen collection

Samples of *Lagocephalus scleratus* (Gmelin, 1789) was collected from Kasimedu fish harbor 13°7'N 80°17'E (Chennai, India) during May-June, 2011 (Figure 1). The fish samples were immediately transferred on ice to the laboratory and stored at -20 °C until further assayed.

Extraction and purification of toxin

The specimens were thawed to room temperature before dissecting various organs. 10 g of each organ was weighed and separately homogenized with 0.1% acetic acid and boiled for 10 minutes followed by centrifugation at 5000 rpm for 15 minutes. The above step was carried out thrice to extract the toxin. The known volume of supernatant of various organs was injected intraperitoneally into the mice to check the toxicity of various organs of the puffer. Remaining extracts were concentrated in rotary vacuum evaporator and defatted by shaking with dichloromethane. Dichloromethane from the extracts was evaporated *in vacuo* and passed through water washed activated charcoal to remove any impurities. Extract was partially purified by filtering through a whatmann filter paper no. 2 through a funnel. Toxin adsorbed was eluted with 3 volumes of 1% acetic acid in 20% ethanol and lyophilized for further use. It was further purified by passing it through a column of Bio Gel P2 and eluted with 0.03 M acetic acid. The eluate was lyophilized for further use (Khora, 1991).

Assay for toxicity

The length and weight of the collected puffers along with the toxicity of its organs is presented in table 1. Mouse bioassay for each tissue was carried out by employing standard mouse bioassay method for determination of toxicity of TTX by Kawabata (1978). The toxicity of the tissues was expressed in Mouse units (MU), where 1MU is the amount of toxin required to kill 20g male ddY mouse within 30 minutes of intraperitoneal administration of TTX. Due to the unavailability of ddY strain in India, Swiss albino male mice are generally used for conducting experiments on puffer fish (Ghosh *et al*., 2004; Bragadeeswaran *et al*., 2010). Adult male Swiss albino mice weighing approximately 20±2 grams were used in the study. They were housed at 22 ± 3 °C temperature with consecutive 12 hrs light and dark periods. Mouse bioassay was conducted as per the guidelines by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The protocol for conducting the experiment was approved by Institutional Animal Ethical Committee (IAEC), VIT University (Permission no. VIT/IAEC/IV/013/2011). 1 ml of the

toxin was injected intraperitoneally into the mice. Time of injection and death of mice were recorded along with their behavioral changes. The experiment was conducted taking six mice in each group.

UV spectrophotometry

A known volume of sample was dissolved in 2 ml of 2 N NaOH and heated in a boiling water bath for 45 min. Upon cooling, the solution was analyzed by UV spectrophotometer (Narita et al., 1987; Wu et al., 2005) for the presence of TTX.

Thin layer chromatography

Thin Layer Chromatography was performed on a pre-coated silica gel 60 F_{254} plate with the solvent system of pyridine:ethyl-acetate:acetic acid:water (15:5:3:4). Samples were spotted onto the plate and placed into the solvent system. After solvent development, the plate was sprayed with 10% KOH followed by 1% H_2O_2 and heated for 10 minutes at 110 °C. The plate was visualized under UV light (365 nm) and the presence of tetrodotoxin was detected as fluorescent spot (Tanu and Noguchi 1999).

Fluorescence spectrophotometry

For fluorescence spectrophotometry small amount of toxin was dissolved in 0.5 ml of 4N NaOH and heated in boiling water bath for 45 minutes. Upon cooling, the sample was analyzed for the characteristic peak for TTX at 495 nm (Nunez et al., 1976; Wu et al., 2005).

Based on Mouse bioassay, UV Spectrophotometry, TLC and Fluorescence spectrophotometry the toxin extracted and isolated from *Lagocephalus scleratus* was identified as Tetrodotoxin. This toxin was further studied for its bioactive potentials.

Antibacterial activity

Diverse bacterial groups were selected to test the activity of the toxic extracts obtained from the puffer fish. *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus* from Gram-positive and *Escherichia coli, Proteus vulgaris and Vibrio chloraeare* from Gram-negative strains were selected. Agar disc diffusion method was performed *in vitro* following the method by Kirby, with small modifications (Bauer, 1966). 100 µl of actively growing log phase bacterial culture were swabbed on to Muller Hinton agar plates and incubated at 37° C for 24 hours. Sterile discs prepared from Whatman No.1 filter paper of 5 mm diameter were impregnated with 20 µl of tissue extracts. Disc containing ampicillin (100 µg/ml) was used as a positive control. After incubation, antibacterial activity of the toxin extracts was assessed by measuring the diameter around the disc.

Antifungal activity

Antifungal activity was performed based on modified Agar disc diffusion method by Mederios *et al.,* 2011. The antifungal activity was measured based on the diameter of zone of inhibition. Plates were swabbed with fungal culture on to Muller Hinton agar (MHA) obtained from Himedia (Mumbai). MHA plates were prepared by adding 15 ml of media into sterile petri plates and were allowed to solidify for 5 minutes. 0.1 % inoculum suspension was swabbed uniformly and allowed to dry for 5 minutes. Sterile discs of 5 mm diameter were impregnated with 20 µl of tissue extracts. Plates were incubated for 72 hours at 37° C. The dimension of the inhibition zones were measured in millimeters and Fluconazole was used as a positive control.

Hemolytic activity

Micro dilution method was employed to evaluate hemolytic activity of the toxin extracts. 5 ml of freshly collected heparinized human blood was washed with phosphate buffered saline (PBS) by centrifuging at 2500 rpm for 15 minutes. Washed red blood cells were suspended to a final concentration of 2.0 %. 50 µl of red blood cells were added into each well containing serially diluted (in PBS) toxin extract. Plates were incubated at room temperature for 3 hrs. Uniform red color suspension in the wells confirmed the occurrence of hemolytic activity while button formation at the bottom indicated lack of hemolysis (Prasad et al., 1997).

Acetylcholinesterase (AChE) activity

Male Swiss albino mice weighing 20±2 gms were used to conduct the experiment. The test mice were administered with single dose, following the OECD Acute oral toxicity procedure. The mice used as positive control was sacrificed after anesthetizing it with intraperitoeal injection of chloral hydrate. Brain was dissected immediately and washed in phosphate buffer and stored at -30°C. The weight of the brain was noted and it was homogenized with two volumes of phosphate buffer, pH 7.7. The frozen brain samples were homogenized with 0.5 w/v of ice cold phosphate

buffer, pH 7.7 at low temperature. The homogenate was centrifuged at 5000g for 30 minutes at 4°C. The supernatant was frozen at -30°C.

The esterase activity of AChE for mouse brain was determined in microtitre plates according to Ellman et al., 1961 with slight modifications. The total assay mixture consisted of 12.5 µL potassium phosphate buffer (pH 7.0), 25 µL DTNB, 5 µL ATCI and an aliquot of enzyme made up to a volume of 225 µL with distilled water. The reaction mixture was incubated for 10 min at 37 °C. For the termination of the reaction 125 ml of 0.5 mM eserine hemisulfate was added to the mixture. The yellow color developed was measured at 412 nm in an automated µQuant ELISA reader. The experiment was conducted in duplicates. One unit of esterase activity is defined as quantity of enzyme required to liberate 1 mM of acetylcholine per min under the standard assay conditions.

Cytotoxicity assay

HEL92.1.7 cell lines were used for studying the cell line toxicity test. 70-80% confluent cell lines were trypsinized and checked for viability. Seed 60000 cells/well of HEL92.1.7 in a 96 well plate and incubate for 24 hours at 37 °C in CO_2 (5%) incubator. When cells reached 80% confluency, they were used for cytotoxicity test. The sample was diluted with 2 fold variations in RPMI media without FBS & antibiotics and was incubated for 24 hours. MTT stock solution was prepared with 5 mg/10ml of MTT in 1X PBS. The solution was filtered through a 0.22 µm filter and stored at 2-8 °C for frequent use or frozen for extended periods. After incubation, the media was removed from the wells and 100 ml/well (50 µg/well) of MTT was added and incubated for 3-4 hours. After incubation with MTT reagent, the reagent was removed from the wells and 100 µl of DMSO was added to rapidly solubilize the formazan crystals. The plates were read at 590 nm (Mosmann 1983). The inhibition percentage was calculated according to the formula-

% Inhibition= 100-(Sample/Control) x100

RESULTS

Toxicity of Lagocephalus scleratus

In this study, the toxicity of *Lagocephalus scleratus* extract was evaluated by mouse bioassay, UV spectrum, TLC and Fluorescence spectrophotometry.

The extracts from fish were initially tested by mouse bioassay. Mouse Bioassay is considered as the gold standard in the identification of TTX. The extracts could kill mice with typical signs of TTX toxicity.

The result of mouse bioassay is shown in Table 1. Toxicity was detected in the muscle and skin extract of the fish. The highest toxicity was found in skin i.e. 78±0.1 MU whereas muscle i.e. 60.5±0.1 was comparatively less toxic. There was no symptom of toxicity found in other organs like intestine, liver and gonad. The toxic extracts from skin and muscle were studied for determining the bioactive potentials of tetrodotoxin. Toxicity in MU/g for the toxic organs was expressed in Mean ± SE.

The result of UV absorption spectrum is shown in Fig 2. The toxin from *L. scleratus* contains TTX which is visible as a shoulder like peak in the spectrum after heating the extract with NaOH. The peak was visible at 275nm, indicating the formation of C_9 base which is specific to TTX.

On TLC plate, TTX was observed as a fluorescent spot with an R_f value of 0.6 which was similar to that of purified TTX.

The alkaline derivative of TTX which emits fluorescence was monitored on a

Table 1. Anatomical distribution of toxicity in the puffer fish *Lagocephalus scleratus* collected from Chennai coast

Collection			Body size		Toxicity (MU/g)*				
Date & Location	Specimen no.	Sex	Body weight (g)	Body length (cm)	Skin	Muscle	Intestine	Liver	Gonad
June - Aug.	1.	F	18	14.9	78±0.1	66.5±0.3	ND	ND	ND
2012.	2.	F	16.60	12.6	76.5±0.4	62±0.2	ND	ND	ND
Chennai	3.	M	13.24	12.4	67±0.4	60.5±0.1	ND	ND	ND

*= toxicity in MU/g expressed in mean ±SE; ND: toxicity not detected

Table 2. Screening of antibacterial activity of toxin from puffer fish against 6 human pathogenic bacteria

Organism	Std. Ampicillin	Skin	Muscle
Bacillus cereus	12.6 ± 0.2	12.1 ± 0.3	11.0 ± 0.2
Bacillus subtilis	11.0 ± 0.1	10.8 ± 0.1	9.8 ± 0.3
Staphylococcus aureus	9.9 ± 0.4	9.7 ± 0.2	9.2 ± 0.1
Proteus vulgaris	8.2 ± 0.1	8.2 ± 0.3	8.0 ± 0.1
Vibrio cholerae	8.2 ± 0.1	8.1 ± 0.4	7.9 ± 0.2
Escherichia coli	10.1 ± 0.2	9.4 ± 0.1	9.2 ± 0.2

Table 3. Screening of antifungal activity of toxin from puffer fish against 6 human pathogenic fungi

Fungi	Std. Fluconazole	Skin	Muscle
Aspergillus niger	9.1 ± 0.4	9.3 ± 0.3	9.0 ± 0.4
Aspergillus flavus	9.0 ± 0.6	8.7 ± 0.4	8.6 ± 0.7
Aspergillus fumigatus	8.4 ± 0.2	8.2 ± 0.4	8.0 ± 0.3
Candida albican	8.1 ± 0.7	8.0 ± 0.2	8.0 ± 0.4
Trichoderma viridae	8.0 ± 1.2	7.8 ± 0.5	7.4 ± 0.6
Trichophyton rubrum	7.6 ± 0.2	7.8 ± 0.7	7.7 ± 0.6

Table 4. Inhibition of growth of HEL 92.1.7 cell lines at various concentrations of toxin

Conc. µg/ml	% Inhibition
1.56	6.22
3.125	8.42
6.25	14.00
12.5	22.38
25	26.06
50	47.51

fluorescence spectrophotometer. The intensity of the fluorescence was determined at 370nm and 495nm as the excitation and emission wavelengths respectively. Fluorescence peak for TTX was observed at a wavelength of 502 nm (Nunez *et al*., 1976) (Fig. 3).

Bioactive potentials of TTX extracted from Lagocephalus scleratus

Antibacterial activity

The toxic extracts from the muscle and skin of the toxic puffer were screened against six

Fig. 1. Puffer fish *Lagocephalus scleratus* collected from Chennai coast

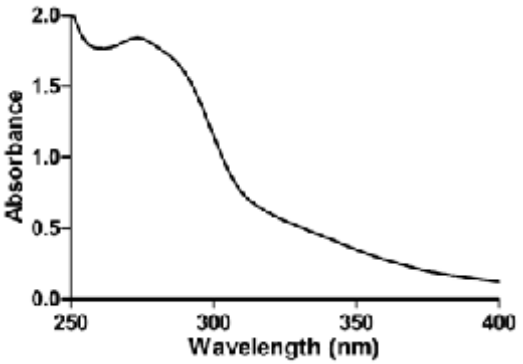

Fig. 2. UV absorption spectra of toxin from Lagocephalus scleratus collected from Chennai

human pathogenic strains. The inhibition zone of standard Ampicillin was used for comparison. The extracts displayed inhibition towards the growth of both gram positive and negative bacteria. The maximum zone of inhibition was seen in skin extract i.e. 12.1 ± 0.3, against *Bacillus cereus*. Minimum inhibition was seen by muscle extract i.e. 7.9 ± 0.2, against *V. cholera.*

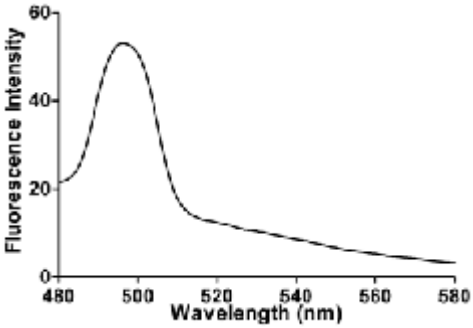

Fig. 3. Fluorescence emission spectra of toxin of puffer Lagocephalus scleratus collected from Chennai

Antifungal activity

The extracts from the toxic organs of puffers were screened for their anti-fungal activity against five human pathogenic fungi. The zone of inhibition was compared with the standard fluconazole. Maximum zone of inhibition was seen in skin extract against *A. niger* and minimum inhibition was seen in case of muscle against *T. viridae*.

Hemolytic activity

In vitro hemolysis of human erythrocytes was seen in the presence of toxic extract. Pronounced hemolytic activity was noticed in the presence of both skin and muscle extract.

Acetylcholinesterase activity

In our experiment, the TTX treated mouse brain cells showed a decrease in Acetylcholinesterase activity for upto 23.67% when compared to positive control.

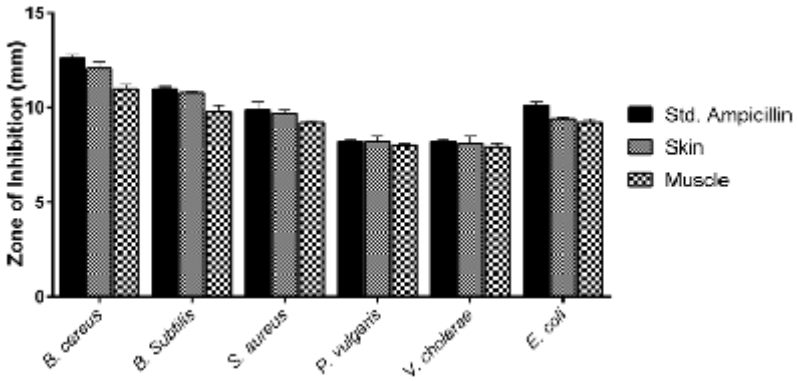

Fig. 4. Antibacterial activity of TTX extracted from *L. scleratus*

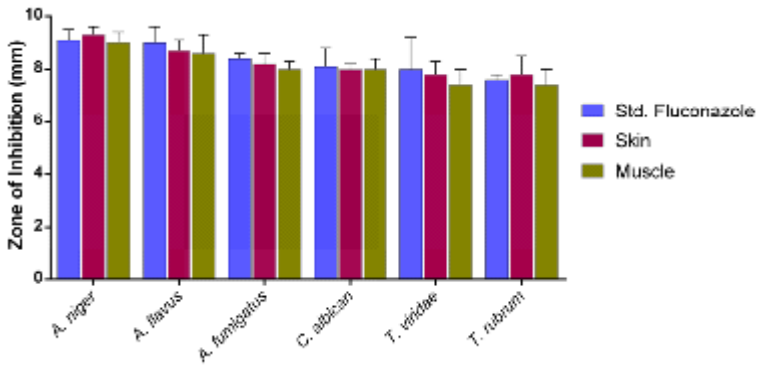

Fig. 5. Antibacterial activity of TTX extracted from *L. scleratus*

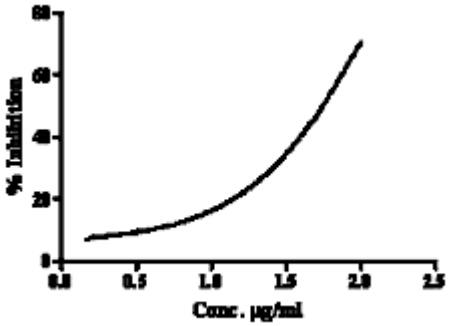

Fig. 6. Cytotoxic effect of toxin extracted from L. scleratus on human leukemia cell lines (HEL 92.1.7)

Cytotoxicity assay

Cells exposed to the extract resulted in dose dependent cytotoxicity. With increase in concentration of extract, the viability of cells decreased. The lowest mortality was obtained at a concentration of 1.56 µg/ml and at 50 µg/ml concentration the highest mortality was observed. At 1.56 µg/ml concentration the inhibition of cells at 3-4 hours was 6.22 %, with increase in concentration to 50 µg/ml the inhibition of cells was 47.51 %. The IC_{50} value was calculated to be 141.7 µg/ml.

DISCUSSION

Several biologically active compounds with varying degrees of action have been isolated from marine sources. Still the marine ecosystem represents a largely unexplored domain for isolation of novel compounds. Widespread research is being done to uncover the bioactive potentials of marine compounds and some of these bioactive compounds with potential antibacterial and antifungal activities are being intensely used as antibiotics against large number of diseases. Research is also being conducted on characterization of biophysical and biochemical properties of the marine compounds. Large numbers of marine organisms produce toxins or poisons as defense mechanism or as secondary metabolites, which have also shown effectiveness in treating many diseases. In this study, the toxicological and bioactivity profile of the toxin isolated from the puffer fish, *Lagocephalus scleratus* was assayed. Preliminary spectroscopic investigations provided indications of existence of TTX. Toxin extract showed positive antibacterial, antifungal and hemolytic activities. Toxin extract also showed positive neuro-modulatory and cytotoxicity effects. It could be understood that tetrodotoxin and related substances extracted from the puffer fish have multiple drug potentials if developed systematically. Further, enzymatic and drug interaction studies on this toxic extract along with the purification and structure determination gives precise understanding of the mechanism of toxin action. The development of more automated and advance techniques for isolating and characterizing marine compounds would definitely make marine compounds more available for the human utilization.

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