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OSTRACITOXIN – A POTENT NATURAL FISH POISON

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ABSTRACT

Ichthyocrinotoxic fishes are those which produce their own toxins through glandular secretions without any venom apparatus. Sixty species of fishes under eighteen families fall in this category among which the family Ostracidae is one. Ostracidae is a family of squared, box shaped fishes very much related to the puffers and filefishes under the order Tetraodontiformes. Skin mucous secretions of the members of family Ostracidae are found to possess a potent ichthyocide known as Ostracitoxin. The toxin is also known as Pahutoxin. Suspected to be produced by the club cells of the epidermis of boxfishes, Ostracitoxin is secreted only when the boxfishes are under stress. The toxin is found to possess various effects on the biological systems. The notable among them are its toxicity to various marine organisms and hemolytic activities. Chemically, the toxin is a choline chloride ester of 3-acetoxy palmitic acid which is relatively similar to red tides and sea cucumber toxins in its basic properties. RP-HPLC separation of the crude toxin yields an ichthyotoxic protein fraction termed “Boxin”. This review summarizes the origin of the ostracitoxin, its toxicity characteristics, properties, mode of action and poisoning effects.

KEYWORDS: **Marine biotoxins, Boxfishes, Ostracitoxin, Boxin, Vibrindole A**

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INTRODUCTION

Toxins are substances produced endogenously within the cells of living organisms and few exogenously. Toxins can be small molecules of proteins or non proteins that are capable of interacting with biological macromolecules of the host such as enzymes or cellular receptors and cause diseases and in some worst cases cause death[1]. Marine biotoxins have drawn worldwide attention due to their involvement in human intoxications and socio-economic impacts brought by them. They pass through food chains and when ingested by humans, cause illnesses. Several marine toxins are identified and studied in detail till date [2, 3]. The most popularly known and discussed marine phycotoxins fall into two categories namely shellfish toxins and ciguatoxins [3, 4].

Apart from the shellfish toxins and ciguatoxins, there is one particular toxin which is least concentrated and less studied. Even though few studies have been carried out globally, in India it is still not touched upon. The toxin is called the Pahutoxin or Ostracitoxin that originates from the boxfishes, cowfishes, trunkfishes and turretfishes of family Ostracidae. The toxin has caused some food poisoning incidents in and around Japan [5, 6]. But as there is no existing widespread knowledge about the toxin produced by these fishes, people still ingest them as a part of their diet worldwide.

Members of Ostracidae exude an ichthyotoxic, hemolytic, substance in their skin's mucous secretions when they are stressed or disturbed [7, 8]. It is a heat-stable, non-dialyzable toxin which foams abundantly in aqueous solutions when agitated and is toxic to various biological systems. The most notable feature being its high toxicity to marine fishes and its hemoagglutinating action on fish erythrocytes [7]. Decades back, they are recognised to be non-proteinaceous, but later proved that there are minute fractions of proteins found [9]. Its uniqueness is that it is toxic even to the boxfishes that produce them. The toxin is very much similar to red tides and sea cucumber toxins in its basic characteristics [9, 10]. The toxic principle in crude form is termed as "Ostracitoxin" in accordance with its family name of the species. It is also called Pahutoxin in its pure crystalline form as "pahu" is the Hawaiian name for the boxfish. Among the three distinct gland cells of the epidermis of boxfishes namely, mucous cells, club cells and labial cells, it is suspected that the club cells are responsible for synthesis of ostracitoxin. Toxicity occurs only upon application of the toxin into the surrounding water, which suggests the presence of externally located receptors. The toxin chemically is a quaternary ammonium salt surfactant. It is a choline chloride ester of 3-acetoxypalmitic acid that behaves similarly to steroidal saponins found in echinoderms [10, 11].

SOURCES OF OSTRACITOXIN

The family Ostracidae is distributed through the tropical and subtropical seas of the world, found in shallow waters. The fishes of the family Ostracidae are classified under the order Tetraodontiformes and suborder Balistoidei. Members of Ostracidae family are identified by the absence of spiny dorsal fin and pelvic fin of any kind and presence of conical teeth. Scales are in the form of enlarged, exceptionally thick, usually hexagonal plates sutured together to form a box-like encasement of the body. And that is why Ostracidae are limited to slow rowing movements. The most notable feature of the family is the characteristic Honeycomb patterns on their skin. Found in various colours especially the juveniles which generally used to fade on growth. Adult fishes are generally square shaped, but seems to be rounded when they are young [12]. These fishes are confined to the Indo-Pacific and Atlantic oceans generally at middle latitudes. Thirteen genera and thirty species are reported to be found worldwide [6]. Only four genera and six species occur in Indian waters[13] and these are Longhorn cowfish, *Lactoria cornuta* (Linnaeus, 1758), Thornback cowfish, *L. fornasini* (Bianconi, 1846), Yellow boxfish, *Ostracion cubicus* (Linnaeus, 1758), Whitespotted boxfish, *Ostracion meleagris* Shaw, 1796, Shortnose boxfish, *Rhyncostracion nasus* (Bloch, 1785) and

Humpback turretfish, *Tetrosomus gibbosus* (Linnaeus, 1758).

STRESS SECRETIONS OF THE BOXFISHES

Boxfishes that are freshly captured used to be immediately placed in beakers or containers with distilled water of around 10 – 50 ml, mostly less than 50 ml. When the containers are agitated to create a stress environment to the fishes, they are stimulated to release copious amounts of mucus secretion that contains the toxin [7, 14]. These used fishes may be released to the sea, since prolonged contact with the toxic solution will lead to the death of the fishes. It is observed that the toxic aqueous solutions gradually become less toxic when left to remain in room temperature for a longer time. So it is reported that immediate heating of the solution to drive off all water without any loss in toxicity followed by cold storage prevents this reduction of toxicity [15, 16]. It is estimated that around 50 – 100 mg of crude toxin can be obtained from a single adult boxfish, with an average of 60 mg [15]. Few others have reported that even 100 – 300 mg of crude toxin can be obtained from a single adult boxfish [10]. A notable feature is that the aqueous and ethanolic extracts of the skin, viscera and muscles of freshly killed boxfish are surprisingly nontoxic [15]. Fishes caught in wire-cage traps are consistently found less toxic than the freshly captured ones, possibly due to the prolonged stress stimulated toxin secretion [17]. Also the fishes maintained in captivity are reported to be less toxic [17. 18]. But the surprising feature of the fishes kept in captivity is that its toxicity increases with the increase in days of captivity. This shows that the toxicologists can be able to milk the toxic secretions repeatedly from the captivated fishes for their uninterrupted work [18].

EXTRACTION METHODS OF OSTRACITOXIN

Several methods have been carried out to obtain the crude or semi-pure forms of toxin [7, 10]. Repeated extraction of the dried residue with acetone or chloroform and diethyl ether results in a particulate substance. The semi-pure form of toxin is used for bioassay. Even though the adsorption of the aqueous solution on a column packed with powdered polyethylene and elution of the toxin with aqueous methanol yielded a stable product, excellent results used to be obtained when the toxin extraction are done with 1-butanol which achieves a 20-fold purification [7]. Chromatography of the toxic butanol solution carried out on a column of silicic acid which uses a combination of eluents namely chloroform and methanol used to produce the toxic fraction. Once the solvent is removed, a toxic white amorphous solid is obtained. A single passage through an anion-exchange column (Dowex 1-X4) treated with picric acid yields a product which is crystallized from acetone in the form of long colourless needles carrying the toxic properties and it is named pahutoxin or ostracitoxin [7]. These methods practiced years back are more tedious and time consuming. So a relatively simple method is followed in recent days. The toxic skin secretions are now-a-days lyophilized into a powdered form and stored at -20^0 C. Pahutoxin can be obtained from the dry crude secretion by an acetonic extract and RP-HPLC separation [8].

PHYSICOCHEMICAL PROPERTIES

The physicochemical properties of the toxin are analysed and reported [7, 14]. From acetone, ostracitoxin can be crystallized as fine white needles which are very much soluble in water, alcohol, hot acetone, chloroform, hot ethyl acetate, and slightly soluble in benzene and ether [15, 16]. The presence of chlorine and nitrogen in the toxin is confirmed by sodium fusion tests. The presence of tertiary and quaternary nitrogen is proved by the toxin's strong positive response to Dragendorff test and silicotungstic acid tests [7, 15]. Melting point of the toxin is reported to be between 74^0 C – 75^0 C. The optical rotation is proved to be $+3.05^0$. The presence of quaternary nitrogen, saturated hydrocarbon and ester functions are determined through the infrared spectral studies in chloroform solution. The nuclear magnetic resonance in deuteriochloroform confirmed the presence of a large aliphatic portion and a choline moiety. The critical micelle concentrations are observed to be 69 µM (30 µg ml^{-1}) in sea water and 900 µM (391

µg ml^{-1}) in distilled water [19]. The electrical surface potential (Δψ) values of the toxin micelles are shown to be 67.2 and 60.2 mV in distilled water and sea water respectively [19]. The partition coefficient (Kp) between liposomes and sea water of the toxin is known to be 62 ± 22 (n=3) for neutral liposomes and 84 ± 7 (n=3) for negatively charged liposomes [19]. Liposome permeability assessed by monitoring the release of fluorescence from preloaded liposomes with carboxyflourescein, indicated that the crude secretion is not active in releasing the liposomal contents whereas natural pahutoxin has the ability to release 20.0 µg/ml (±4.n=3) and synthetic liposomes released 10.0 µg/ml (±3,n=3) of the liposomic contents [19].

CHEMICAL STRUCTURE

Chemically, ostracitoxin is a choline chloride ester of 3 – acetoxy palmitic acid also known as 2-{[(3s)-3-(acetyloxy) hexadecanoyl] oxy}-n,n,n-trimethylethanaminium chloride with a molecular formula $C_{23}H_{46}N0_4Cl$. The molecular mass of ostracitoxin is reported to be 435.3115 Da and a molecular weight of 436.06864 g/mol [20, 21].

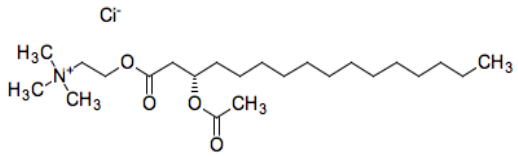

Figure 1
Chemical Structure of Ostracitoxin

PROTEIN CONSTITUENTS OF OSTRACITOXIN

Ostracitoxin is earlier believed and reported to be non-proteinaceous in nature until it is proved that there is a protein fraction found in it [9, 22]. The protein is named "Boxin" in accordance with boxfishes. Boxin is first isolated from the skin secretion of the Yellow boxfish *Ostracion cubicus* (Linnaeus, 1758) [9]. On addition of cold acetone to the lyophilized powdered toxin, an acetonic precipitate possessing water extractable ichthyotoxicity is obtained along with the lipophilic ichthyotoxic fraction. RP-HPLC separation needs to be done to separate the ichthyotoxic hemolytic fraction through gel filtration which yields two fractions, in which the first is polar and identified to be a protein and the second is a hydrophobic fraction and identified to be pahutoxin (the purified form of ostracitoxin) [9, 10]. It is the acetonitrile phase in the RP-HPLC separation, like the hexane-isopropanol treatment, that the proteins are separated from the organic surfactant pahutoxin. Proteins are estimated quantitatively by the Folin phenol assay [10]. The protein nature of the final fraction is suggested by the UV absorbance pattern [10]. Treatment of the final fraction with proteolytic enzymes is necessary to verify that the protein factor is responsible for the toxicity of this fraction.

Boxin is reported to be heat stable and resistant to trypsin but destroyed by the potent proteolytic mixture pronase. It is also shown to possess an LC_{50} of around 1.5 µg ml^{-1} using *Sparus aurata* fries. The entire crude secretion of ostracitoxin contains of about 3.5% (by weight) of boxin and is responsible for 3% ichthyotoxicity [10]. The molecular weight of the Boxin is known to be 15-18 kDa by laser desorption induced time of flight mass spectrometry (LD+TOF-MS) [10, 11]. Boxin has an amydated N-terminus as revealed by its resistance to the conventional phenyl isothiocyanate cleavage and presence of methionine is revealed by the preliminary amino acid analysis [23]. The profound difference in the composition of boxin and pahutoxin is compared and reported. The ichthyotoxicity of boxin is around 1.57 µg ml^{-1} and pahutoxin was 1.25 µg ml^{-1} which means that boxin is 30 times more potent that pahutoxin [10]. Both of them are not effective by injections whereas different concentrations of both yield different results on ichthyotoxicity. Boxin is shown to be non-hemolytic and pahutoxin is weakly hemolytic. There is no effect on liposomal permeability by boxin and

effective liposomal permeability by pahutoxin. Spectrophotometry reveals that the absorbance of proteins is seen at 280 nm and 254 nm and pahutoxin only at 254 nm [9]. Further presence of proteins can be confirmed by Flourescamine and folin phenol assays whereas dragendorff assay is meant for pahutoxin which detects the presence of quaternary amines [10].

EFFECTS OF OSTRACITOXIN ON MARINE ORGANISMS

Initially the toxicity of ostracitoxin is tested using various marine organisms at dosages that could kill the assay organisms in 10 minutes. Number of marine organisms are exposed to ostracitoxin - sea water solutions. The reactions observed are grouped viz (1) not affected (2) slowly affected and (3) rapidly killed [17]. The Polychaete worms, most of the crustaceans, a single bivalve mollusc and the ascidian tunicate are apparently not affected even after several hours of exposure to this toxin. The coelenterates and the sea urchins appeared to be narcotized. Tentacles of the anemones and hydroids showed insentivity to probing but responded to stimuli once retuned to fresh sea water. Tube feet of the sea urchins retracted and the animals lost their locomotion. Injection of the toxin in the muscle base of a spine of the echinoderm *Echinothrix diadema* caused rigidity of the spine. The echinoderms, coelenterates, platyhelminthes, and two species of crustaceans, fall into the category of the slowly affected ones. Fertilized eggs of the sea urchin *Tripneustes gratilla* exhibited marked cleavage inhibition, whereas fertilized eggs of the sea cucumber *Holothuria fuscorubra* are rapidly killed but unaffected by holothurin.

All marine fishes that are assayed are rapidly killed by the toxin either followed by injection or immersion in the toxin sea water, but the pearlfish *Carapus homei*, boxfishes *Ostracion lentiginosus* and *O. meleagris* are very slowly affected [17]. Stress signs shown by these fishes are relatively similar to that of poisoning out of red tide toxins. The effects of ostracitoxin on fishes are irreversible which means that the assay fish could not survive even after it has been returned to fresh sea water. *Tilapia mossambica* immersed in 5 ppm ostracitoxin for varying periods of time and returned to fresh sea water showed a mean survival time of 15 minutes in the toxin. Whereas when they are exposed to the toxin for 30 - 120 seconds, they produced no clear distress signs but agitated after 180 seconds and quiescent after 300 seconds. Only two marine fish species surprisingly are most resistant to the toxin and they are *Ostracion meleagris* (Whitespotted boxfish) and *Carapus homei* (Pearlfish) [17]. Boxfishes are moribund and if they were left in their mucous secretions during toxin collection longer than five minutes, they would not survive even after returning to fresh sea water. The thornback cowfish *Lactoria fornasini* is found to be very sensitive to ostracitoxin. But boxfishes showed more resistance to ostracitoxin than cowfishes. However, an intraperitoneal injection of about 1 ml of non-diluted fresh mucous secretions equivalent to 20 - 30 mg of semi-pure toxin is reported to kill 80 – 110 mm boxfish in 5 – 20 minutes [17].

A standard bioassay has been developed and the toxicity of ostracitoxin is tested only on the sailfin molly *Mollienesia latipinna* [15]. A portion of the toxin is added to 100 ml of sea water which contains the brackish-water mollies. The mean survival time is used as an index of toxicity. This test is then replaced by the Dragendorff test for tertiary or quaternary amines. The toxin responded to the test producing a light orange coloured turbidity characteristic of a quaternary nitrogen function. In recent practise, the toxicity of the toxin is tested with the commercially bred marine bony fish *Sparus aurata*, the gild-head sea bream [10]. Fries of 150 - 200 mg body weight are employed in fish lethality assays. When each fry is placed in a separate beaker containing the test toxin in 10 ml of filtered sea water. Lethality is expressed by loss of balance and the arrest of opercular movements.

EFFECTS ON ALBINO MICE AND RATS

Fresh boxfish secretions and semi-purified ostracitoxin are injected intraperitoneally to the albino mice of around 10 - 30 g. The mice are found to be very sleepy and took deep breaths, which are followed by ataxia (loss of the grasping and righting reflexes) and

labored breathing. Soon the mice appeared to be gasping for breath and became comatose and appeared to be dead even though the heart continued beating. Post-mortem examination showed that the lungs are collapsed and the peritoneum showed no visible vascular damage and no convulsions are seen. On sublethal doses, even though similar behaviour is observed, recovery completed within few hours. It is reported that an intraperitoneal injection with 1.0 ml of a highly toxic rinse is needed to kill a 20 g mouse. The minimal lethal dose of the semi-purified ostracitoxin is noted to be of around 0.20 mg/g of mouse, which means 4 mg of the toxic rinse is needed to kill 20 g mice, which is fairly a very large amount [7, 14]. Injections with 200 µg toxin/g of mouse intraperitoneally caused constriction of the pupils, prostration and death in 3 hours. In rats, intravenous injections with toxin of 15 µg/g of rat produced an immediate drop in blood pressure accompanied by a slight increase in the frequency and amplitude of respiration, followed by death [14].

EFFECTS ON HUMANS

Members of the family Ostracidae are generally considered to be non-toxic and so regarded as a table fare in few places of Japan. However, since 1990, there are several reports about boxfish poisoning in thirteen humans due to ingestion of the cooked ones. The major symptoms appeared were severe muscle pains arising from rhabdomyolysis, usually accompanied by discharge of black urine and abnormal elevation of serum creatinine phosphokinase. Twelve out of thirteen victims recovered in few days to two months, and one case reported death. A 40 year old man in Goto, Japan who after several hours of consumption of the liver of the cowfish Umisuzume, *Lactoria diaphana*, developed weakness and myalgia of the shoulders and brachia [6]. Rhabdomyolysis is reported which is a destructive skeletal muscle disease. And the serum creatinine phosphokinase is elevated to 180,000 IU/L on day 3 which is fairly a very large amount. Hemodiafiltration is performed due to the cardiopulmonary arrest and acute renal failure after the 59th hour. On day 9, the patient was found in coma and an EEG performed diagnosed cerebral death, followed by the death of the patient on day 16. This case is reported to have very similar characteristic clinical symptoms as seen in palytoxin poisoning.

The case report from Taiwan where a 51 year old man who on ingestion of three specimens of boxfish *Ostracion meleagris* developed general discomfort, diaphoresis and dyspnea after 30 - 40 minutes [5]. This is followed by respiratory failure and ventricular fibrillation for which Cardio Pulmonary Resuscitation (CPR) for 50 minutes is performed. The patient received 10 electrical countershocks at a total of 3220 J leading to myoglobinuric acute renal failure. After successful CPR, an electrocardiogram revealed sinus rhythym without myocardial infarction [5]. The serum creatine phosphokinase after CPR is around 375,705 IU/L along with proteinuria. Following remarkable fluid accumulation and exacerbated azotemia, hemodialysis sessions were started which the patient received for 9 times. On the 25th day of admission, the patient was discharged who had a serum Cr level of 2.8 mg/dl after 3 months. Even though the patient complained of weakness, no muscle disability was noted.

HEMOLYTIC ACTIVITY

Ostracitoxin is reported to have notable hemolytic activity on most red blood cells. Its potential to agglutinate and lyse the RBC's from the hearts of several species of bony fishes, mouse, rabbit, toad and human and stored in sodium citrate solution is noted [7, 14]. They are then washed several times with citrate-saline followed by cold storage. Semi-pure ostracitoxin dissolved in citrate-saline was drawn into a capillary tube into which the washed RBC cells are drawn and the tube is set vertically. Readings are noted after the RBC passed through the toxin and settled at the tube's bottom. Haemoglobin from the lysed RBC would colour the suspension into uniform red if hemolysis occurred. And RBCs would clump at the bottom if agglutinated.It is reported that the toxin at a dilution of 1:1000 caused complete hemolysis of all RBCs tested. At this concentration, strong RBC

agglutination preceded hemolysis in all fish blood and rabbit but incase of human or mouse RBCs, no agglutination is seen. Boxfishes and cowfishes RBC are also reported to be agglutinated and hemolysed. When the dilutions are as high as 1:20,000, agglutinations occurred and at 1:1,000,000 dilution, hemolysis is observed. These reactions are reported to be altered or destroyed if the protein impurities in the toxin are denatured. Even the size of the agglutinated and hemolysed RBCs are reduced which was observed through microscopy. Interestingly, a decrease in chain length from C_{16} to C_{12} produced a marked decrease in the toxin's haemolytic activity and toxicity as well. So it was suggested that hemolysis may be the cause of toxicity. In recent practice, haemolytic assays are performed by using 20% v/v suspensions of washed human and fish (*Sparus aurata*) red blood cells in phosphate-buffered saline (PBS, pH 7.4). When phosphate buffered saline is added to the reaction mixture (washed blood cells and toxin) and centrifuged, hemolysis is reported to occur which is determined by the measurement of the supernatant's absorbance at 540 nm [9]. Hemolysis in double distilled water serves as the 100% hemolysis reference. One hemolytic unit (H.U.) of a given substance is the concentration that causes 50% hemolysis.

CONCEPT OF RECEPTOR MEDIATED TOXICITY

The detergent or the surfactant nature of the toxin is not responsible for its toxicity which is suggested by its properties, especially by the toxin's lethal concentration which is found to be 30 times lower than its critical micelle concentration values and its very low affinity for biological membranes proved by liposome/sea water coefficients which is 50 times lower on comparison with Sodium dodecyl sulphate. Hence the toxin neither possesses a defensive role as a solubilizer nor the pore forming ability. The concept of receptor hypothesis is an excellent alternative suggested as a reason for the toxin's toxicity also well supported by certain experiments [19, 24].

If the toxin plays an allomonal role in the stress secretion, it needs some mechanism to differentiate the fish that produces them from its potential enemies. So the differences in membrane composition of the fishes might be of great importance. Ostracitoxin on application to the surrounding water with experimental fishes is reported to show its toxicity is due to the receptors found on the fish gill membranes. The toxin particularly targets the gill membranes and this is supported by fish gill binding assays [19]. Added to that the occurrence of more than two classes of ostracitoxin binding sites on the gills is evident by Statchard analysis of the equilibrium saturation binding assay [19]. These binding sites are termed receptors not only because of their specificity but also for receiving the toxicity effect. So it is strongly believed that the toxicity characteristic of ostracitoxin is solely receptor mediated. Even though the boxfishes are devoid of receptors to its own toxin, prolonged exposure of the boxfishes to the toxin render them moribund or even cause death.

PROBABLE ORIGIN OF OSTRACITOXIN

The presence of symbiotic microorganisms in many marine organisms is of vital importance in the biosynthesis of biologically active natural products within those organisms. It is reported once that antimicrobial compounds can be isolated from the toxic stress secretions of the boxfishes, especially *Ostracion cubicus* [25]. *Vibrio parahaemolyticus* is isolated from the white foamy mucus of the boxfishes when diluted using sterile sea water and inoculated onto petridishes with SLB agar. The batch cultured strain in liquid SLB medium is cooled and maintained in cold storage at -70^0 C and is extracted with ethyl acetate, dried and evaporated. This crude material is then packed into a vaccum column and eluted with petroleum ether. Several components are reported to be obtained after elution. The major component and the least polar one is identified to be indole which is determined on red colour production during thin layer chromatography with vanillin [25]. The second non-polar component to be identified as indole -3-carboxaldehyde. Two other components with anti-bacterial properties and

medium polarity are also observed due to the production of red colour upon spraying of vanillin on TLC plates.

Purification of these components on a reverse-phase (C_{18}) HPLC column affords two pure compounds which is named as Vibrindole A and 2,2-di(3-indolyl)-3-indolone on the basis of the NMR spectral characteristics. Purification of the polar fraction using reverse phase HPLC afforded seven diketopiperazine derivatives. Based on the NMR data Vibrindole A was identified to be 1,1-di(3-indolyl) ethane [25]. Those two pure compounds are tested for antibacterial properties using *Bacillus subtilis, Staphylococcus aureus* and *Staphylococcus albus*. It is reported that Vibrindole A exhibited an 11 mm zone of inhibition against *Staphylococcus aureus* and *Staphylococcus albus* and a 7 mm inhibition zone against *Bacillus subtilis* at 100 µg/disk. Compound 2 gave an 11 mm inhibition zone against *Staphylococcus aureus* at 100 µg [25]. This is then compared with the standard gentamycin inhibition of zones. The presence of the bacterium *Vibrio parahemolyticus* in the toxic mucus may be suspected to be the origin of the ostracitoxin in the boxfishes which is yet to be confirmed with further studies.

DISCUSSION AND CONCLUSION

Members of the family Ostracidae under stress tend to secrete a potent substance known as Ostracitoxin perhaps for chemical defense mechanism [11]. Among the eight extant genera and twenty-four species, there are only six species under four genera in India [13]. From a single adult boxfish, it is approximately estimated that around 60 – 100 mg of the toxin can be procured. Active toxin could be extracted from neither the dead fishes nor the dissected tissues. Freshly captured boxfishes placed in a container with less than 50 ml of sterile distilled water or sterilized sea water, should be agitated for few minutes, during which they secrete a white foamy mucus which can be lyophilized to powdered form and maintained under cold storage of around -20^0 C. It is suspected that the club cells present in the epidermis of boxfishes are responsible for the production of the toxin [17]. These stress secretions are assumed to be a defense mechanism against predations. The pure form of toxin is named pahutoxin[15]. The toxin is found to be highly toxic to several marine organisms especially fishes in less concentrations but 4 mg of toxin was required to exhibit toxicity in white mice that is of 20 g. The toxin indeed is poisonous to boxfishes when they are exposed in the toxic rinse for a prolonged duration [17].

The toxin has a notable characteristic of exhibiting hemolysis by agglutinating erythrocytes of different organisms [7,14]. An antibacterial substance named Vibrindole A is obtained from the symbiotic microorganism *Vibrio parahemolyticus* which is seen on the body of boxfishes. It is reported to possess a good antibacterial property against bacteria *Staphylococcus albus*, *Staphylococcus aureus* and *Bacillus subtilis* [25]. Acetone precipitation and RP-HPLC separation of the lyophilized crude toxin produced various fractions, through which the protein named Boxin of molecular weight 15 - 18 kDa is obtained. Further studies are still needed to explore the characteristics of the toxin to a more complete extent. The effects of Ostracitoxin on marine organisms and humans are very deadly. Because Ostracitoxin is identified from marine fishes of Ostracidae, it may be classified under seafood toxins. Even though the boxfishes are noted to be nontoxic due to lack of awareness, ingestion of the cooked fishes produced sporadic food poisoning in thirteen patients overseas in which one case reported death [5, 6]. All the patients who reported of boxfish poisoning are noted to show symptoms of rhabdomyolysis, myalgia and myoglobinuria [5]. Existing knowledge about the toxic boxfishes are very much inadequate to educate the people about the cases of food poisoning incidents. Prompt diagnosis and treatments are highly essential for management of such patients. While Ostracitoxin is seen as a dangerous substance, its bioactivities can be further studied for other functions. Very few researches have been carried out about the boxfishes elsewhere. Lack of knowledge about them still prevails in India due to inadequate or absence of studies.

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