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EXTRACTION OF TETRODOTOXIN FROM PUFFER FISH, DIODON LITUROSUS FROM SOUTH ANDAMAN SEA

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Abstract

High performance liquid chromatography (HPLC) using fluorescent detection following post-column alkaline degradation and a sample preparation procedure for the analysis were established to quantitatively detect tetrodotoxins (TTXs) in gastropods and puffer fishes. The analysis showed 91% recovery of TTXs using the sample preparation described in this article and a wide (10-2000 ng) linear relationship between the tetrodotoxin amount and its fluorescent response. A good correlation between the results of mouse bioassay and HPLC was also obtained in this research. Puffer fish (*Diodon liturosus*) species from the study area at Burmanala in A & N Island. In the process of tetrodotoxin extraction, methanol was used as the solvent and purified with the help of open column chromatography. The TTX accumulation mechanism in pufferfish can be explained by bioconcentration via the food chain starting from bacteria. It remains to be elucidated how TTX molecules are absorbed, transferred, and retained/eliminated after entering into the pufferfish body via the toxic food organisms.. For example, marine pufferfish and flatworms retain high toxicity in their eggs, and pufferfish and newts are equipped with TTX-secreting glands or cells in their skin , suggesting that TTX is present as a defensive substance to protect their eggs or themselves from external enemies.It was found that the methanol extracts of tetrodotoxin showed better results on *Artemia salina* larva. Concentrations resulting in 50% mortality tetrodotoxins for 16 hr, were (µg/ml).

Keywords: *Tetrodotoxins, HPLC, Puffer Fish, 50% mortality, and Artemia salina.*

Introduction

Tetrodotoxin is a heat stable neurotoxin that block sodium conductance and neuronal transmission is skeletal muscle, leading to weakness or paralysis and potentially death if ingested in sufficient quantities. It is found in various marine organisms, such as some species of gobies, octopuses, sea stars, crabs, gastropods in addition to well known puffer (Mosher and Fuhrman, 1984; Hashimoto et. al., 1992). Nevertheless, the reason for the wide distribution of these toxins in animals of such different taxa is still unclear. TTX and its derivatives have caused sporadic seafood poisonings in South Asian countries. There is always a risk of being poisoned by TTX when consuming exotic or even ordinary seafood. The minimum lethal dose in human for tetrodotoxin is estimated to be 2mg but this number can vary based on age , health and sensitivity to the toxin. In fish, teterodotoxin occur mainly in members of the family Teterodotoxin (Puffer fish) and is typically concentrated in the fish liver, followed by intestine and skin. In most of the species, teterodotoxin concentrations in the muscle are low.

Objective of Study

Well documented work has been carried out on the tetrodotoxin and its toxic effect in India. But regarding the work from the Andaman and Nicobar waters are very negligible. Thus a detailed study on the bioactive properties of tetrodotoxin belonging to the puffer fish from the Andaman Sea was undertaken. Since the occurrence of the various species has its own seasonal distribution the present study was restricted to the time frame and the species present during the study period was taken up. The present study was aimed at studying the toxic properties of bioactive components extracted from the commonly available puffer fish from the South Andaman Sea.

Review of Literature

Hasan et.val. (2008) isolated and purified tetrodotoxin from liver of puffer fish (Tetraodonpatoca) by thin layer chromatography and elucidated by IR, 1H-NMR, 13C-NMR and mass spectroscopic data. The tetrodotoxin, (2.25 mg) was administrated daily intraperitoneally for 14 days; at and control rats, it showed that all the tissues such as liver, lung, heart and kidney of rats were affected after treatment with the toxins but the changes were more pronounced in liver as compared to the other tissues. Using polymerase chain reaction techniques, they further characterized by their sensitivity to TTX-sensitive (TTX-S) subtypes are inhibited by TTX in the nanomolar range The isolated peptide had three post-translational modifications, including two hydroxyproline residues and C-terminal amidation, and 35% homology to other conotoxins. TIIIA potently displaced [3H]saxitoxin and 125I-TIIIA from rat brain (Nav1.2) and skeletal muscle (Nav1.4) membranes. Alanine and glutamine scans of TIIIA revealed several residues, including Arg14, that were critical for high-affinity binding to tetrodotoxin (TTX)-sensitive Na_ channels. It was found that [E15A]TIIIA had a 10-fold higher affinity than TIIIA for TTX-sensitive sodium channels (IC50, 15 vs. 148 pM at rat brain membrane). TIIIA was selective for Nav1.2 and -1.4 over Nav1.3, -1.5, -1.7, and -1.8 expressed in *Xenopus laevis* oocytes and had no effect on

rat dorsal root ganglion neuron. NMR studies revealed that TIIIA adopted a single conformation in solution that was similar to the major conformation described previously for conotoxin PIIIA. TIIIA and analogs provide new biochemical probes as well as insights into the structure-activity of conotoxins.

Nicole et. al. (2007) showed that Tetrodotoxin is a neurotoxin that occurs in select species of the family Tetraodontidae (puffer fish). It causes paralysis and potentially death if ingested in sufficient quantities. In 2007, two individuals developed symptoms consistent with tetrodotoxin poisoning after ingesting home-cooked puffer fish purchased in Chicago. Both the Chicago retailer and the California supplier denied having sold or imported puffer fish but claimed the product was monk fish. However, genetic analysis and visual inspection determined that the ingested fish and others from the implicated lot retrieved from the supplier belonged to the family Tetraodontidae. Tetrodotoxin was detected at high levels in both remnants of the ingested meal and fish retrieved from the implicated lot. The investigation led to a voluntary recall of monk fish distributed by the supplier in three states and placement of the supplier on the U.S. Food and Drug Administration's import alert for species misbranding. This case of tetrodotoxin poisoning highlights the need for continued stringent regulation of puffer fish importation by the U.S. Food and Drug Administration, education of the public regarding the dangers of puffer fish consumption, and raising awareness among medical providers of the diagnosis and management of food borne toxin ingestions and the need for reporting to public health agencies. Chen and Chou (1998) reported that by HPLC fluorescent detection and post-column alkaline degradation and a sample preparation procedure for the analysis were established to quantitatively detect tetrodotoxins (TTXs) in puffer fishes. Analysis showed 91% recovery of TTX. A good correlation between the result of mouse bioassay and analysed by HPLC was also obtained in this research. collected balloon puffer fish , Linnaeus trace amounts of TTX ware found in ovaries of balloon fish. Muscle tissue of balloon fish and liver, ovaries and muscle of cultured tiger puffers showed TTX (<0.2mg/g tissue) in this screening procedure.

Edmund D. B et. al. (2002) suggested that high molecular weight substance associated with TTX, Which contain 82.2% protein, where obtain from liver of puffer fish. The wide distribution of TTX form platyhelminthes to vertebrate has led scientist to assume that toxification of the above organism might be attributed to the toxin in marine bacteria or sediment .It is a matter of interest that toxic puffer and crab had a higher level of resistibility to TTXat factor of 10 to 100 compare with non toxic species. A high molecular weight substance may play an important role in the accumulation of the toxin in TTX –bearing animal. The study deals with TTX associated high molecular weight substance from toxic liver of puffer fish *Takifugu vermicularis* as part of a study to elucidate the mechanism involved in the toxification of puffer fish. Hanifin and Brodie (2001) reported that the biogenesis and biosynthesis of TTX is still poorly understood in marine taxa; but the best supported hypothesis is that TTXis produced by symbiotic bacteria. The biogenesis of TTX in *T. granulosa* is of particular interest because of the importance of TTX in the coevolutionary interaction between *T. granulosa* and a snake predator. The correlation between reduced body weight in this captive animal. The correlation between reduced body weight and reduced toxin production has been shown in *Salamndra salomndra* but toxin secreted by that Salamander is structurally dissimilar to TTX and are thought to drive from every strong molecules. They examin the steroisomer profile of individual newts to see if these profiles changed in captives. The remained (cooked) and captured (live) specimens of both gastropods were assayed for toxicity (as tetrodotoxin = TTX). Average toxicity of cooked and live specimens was 118 ± 105 and 47 ± 28 MU/specimen, respectively. In addition, another species *Natica lineata* collected from the same area was also found to be toxic. The toxin of each gastropod was partially purified from the methanolic extract of the gastropod by ultrafiltration and Bio-Gel P-2 column chromatography. HPLC and GC-MS analyses demonstrated that the toxin consisted of TTX. It was concluded that the causative agent of the above food poisoning was TTX.

Materials and Methods: Study Area

The Andaman and Nicobar Islands are present in the Bay of Bengal forming a chain of 576 Islands, islets and extending for about 850 kms between Lat. 6 º - 14 º N and Long. 92 º - 94 º E. The Andaman group is the biggest and accounts for 6340 km^2 area. For the present study the species of puffer fish was collected from the coast of Burmanala, (Lat. 22 º 03.940N, Long. 93º 09.017 E) Port Blair, Andaman and Nicobar Islands.

TTX used as a calibrant. The HPLC grade methanol, acetonitrile, and acetic acid were from Fisher (USA). Trimethylamine (25%) solutionin water and ammonium formate were from HiMEDIA (Mumbai). Formic acid was obtained from Merck (Germany). The *DIODON LITUROSUS* was purchased in an Angala market. Its harvest place was later traced back to the south coast of Andaman& Nicobar (Fig-1)

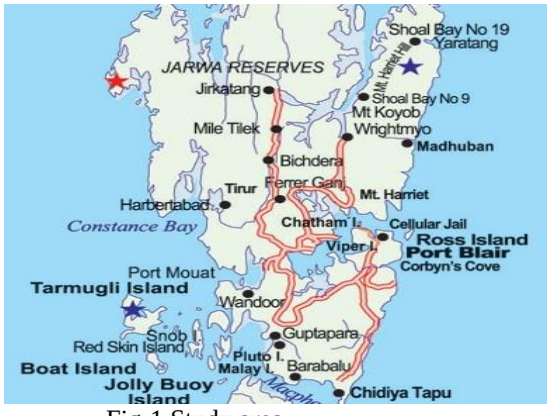


Fig-1 Study area.

Extraction Process:

- The processes for the isolation for the tetrodotoxin include the sample collection approximately the 10 gm of the fresh intestine of a normal fish without any change in the fauna of the environment.
- After the isolation of the Fresh intestine three volumes of 1% acetic acid (CH_3COOH) in chilled methanol was added carefully without damaging the tissue.
- Now keep the whole materials in the Refrigerator for 24 hours at a sterile condition, as incubation period.
- In the next step macerate the tissue in a pestle and mortar gently, it the tissues get dried up add required volume of the chilled ethanol if needed.
- Once the slurry of the tissue is ready filter the materials with the whatman filter paper.
- Now the filtrate is been transferred to a sterile centrifuge tube and the centrifugation is been started for 10000 RPM for 10 minutes at 4 degree Celsius.
- Now the Supernatant is been collected gently in a fresh sterile tube and the pellet is been discarded.
- In the last step the supernatant is been transferred to a rotary evaporator and the crude extract been obtained.(Plate –1)

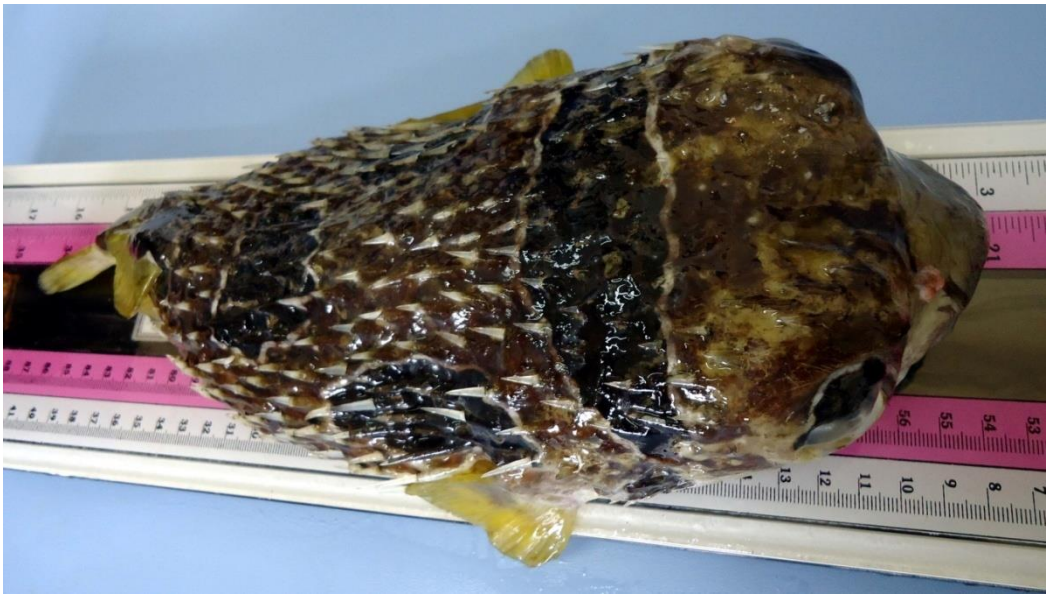


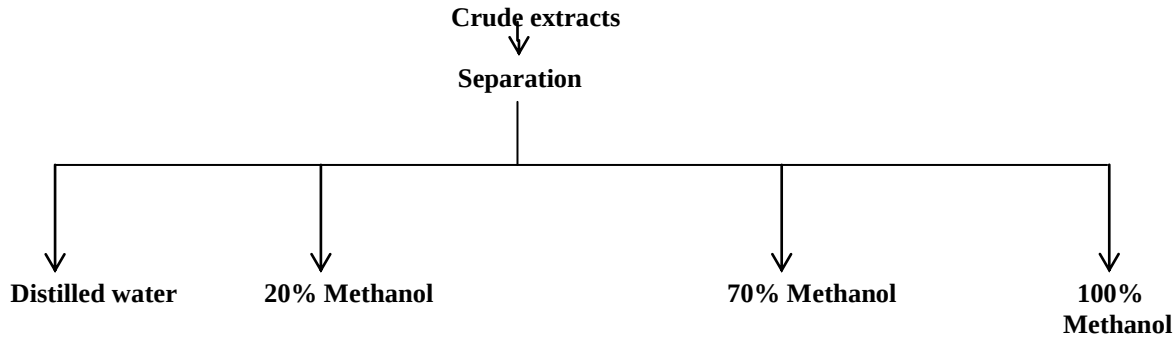
Plate –1 *Diodon liturosus*

Preparation of TTX from puffer fish

The collected puffer fish and dissect it. After that remove visceral organs like liver, intestine, muscle and skin. Then take 10gm of intestine and crushed it by piston and mortar and then add 100ml of three volume of 1% acetic acid in methanol and filter the sample by using whatman no. 1 filter paper. Then filtrate solution was centrifuged at 12000 rpm for 20 min for three times. Separate the supernatant and later the sample was concentrated by using rotary evaporator.

Partial purification of tetrodotoxin compounds from the crude methanol solvent extracts by column chromatography

Among the all solvent extracts the methanol extracts were showing active result against larva. So the tetrodotoxin compounds from the methanol extracts were further purified by using the column chromatography based on the fractions. The fractions used for purifying the crude extracts were: Distilled water; 20% Ethanol; 70%Ethanol and 100% Ethanol.



The purified compound fractions were tested for knowing the toxic properties of individual fractions.

Result t& Discussion

It can be concluded from the present data that the tetrodotoxin, TTX has high toxic effects in rats at dose and duration used in this study. It was also reported that symptoms of poisoning patients resembled partly those caused by tetrodotoxin (TTX) or paralytic shellfish poison (PSP). Puffer fish possesses paralyzing/ palytoxin (Tetrodotoxin, TTX and analogues) that are secreted upon stimulation. It was also described that an endogenous origin of tetrodotoxin in

puffer fish. Puffer fish accumulates TTX at an extremely high concentration in their tissues among with saxitoxin (STX).when they inject TTX in rat, the weight of rat is decreases after couple of time with decreasing of haemoglobin and it lead to death of rat.Richard et. al. (2006) This study reports the isolation and characterization of_-conotoxin TIIIA. The sequence of TIIIA was initially determined by PCR amplification and cloning of *C. tulipa* venom duct cDNA, and the sequence was confirmed by assay-guidedfractionation of crude *C. striatus* venom. Shiu et. al. (2000) reported that TTX caused the first food poisoning incident of the gastropod *P. didyma* that occurred in Chiayi County, western Taiwan. The highest toxicity score of the remaining cooked *P.didyma* (261 MU/specimen) was higher than that of *P.didyma* collected from Chiayi County (123 MU/specimen). Edmund D. B et. al. (2002) suggested that high molecular weight substance associated with TTX, Which contain 82.2% protein, where obtain from liver of puffer fish. The wide distribution of TTX form platyhelminthes to vertebrate has led scientist to assume that toxification of the above organism might be attributed to the toxin in marine bacteria or sediment.

Conclusion

Extraction of tetrodotoxin from Puffer fish was studied first time from the Andaman Sea for their toxicity activity. Different kinds of solvents were used for extracting the components from these puffer fish. Several studies have pointed out that many bacteria strains isolated from toxic shells or fishes are TTX producers (Noguchi et. al., 1987; Yasumoto et. al., 1986).

It was found that marine puffer fish from the coastal waters of Burmahanala, Andaman Sea, Andaman and Nicobar islands, may serve as a potential source of tetrodotoxin. It is one of the important bioactive compounds. Puffer fish accumulate TTX at an extremely high concentration in their tissue among with saxitoxin. It is also reported that TTXis one of the most potent molecule that selectively block the voltage sensitive sodium channels of excitable tissues. In recent research the TTX is used as suppressing pain in cancer patient. It is one of the important bioactive compounds.

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