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Purification, Characterization and Toxic Profile of Two Toxins Isolated from Puffer Fish *Tetraodon patoca*, Available in Bangladesh

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Abstract: Among the marine toxins related to human intoxication, tetrodotoxin has been known as one of the most prejudicial. Two tetrodotoxins, namely PFT-1 and PFT-2 were isolated and purified from liver of puffer fish by thin layer chromatography. The structure of both the toxins was elucidated by means of IR, ^{1}H-NMR and ^{13}C-NMR and mass spectroscopy. Sub acute toxicity study showed that both the toxins had pronounced effects on total RBC, WBC, platelet and ESR. Further serum levels of SGPT, SGOT, SALP, bilirubin, creatinine and urea are also affected by the toxins. The histopathological examinations showed that all the tissues such as liver, lung, heart and kidney of rat were severely changed after treatment with the toxins. The toxicity of the purified compounds, PFT-1 and PFT-2 were also performed by brine shrimp lethality bioassay.

Key words: PFP, TTX, puffer fish, NMR, histopathology

INTRODUCTION

Poisoning, including fatality, attributed to ingestion of freshwater puffer, has occurred occasionally in Bangladesh. For example on 16 November, 1998 a food poisoning incident due to ingesition of a puffer *Takifugu oblongus* occurred, affecting 8 people died among 15 victims (Mahmud *et al*., 2000). Puffer fish possess paralyzing toxin (Tetrodotoxin, TTX and analogues) that are secreted upon stimulation (Nakamura and Yasumoto, 1985). Matsumuraka also describes an endogenous origin of tetrodotoxin in puffer fish. TTX is known to selectively block the voltage sensitive sodium channels of excitable tissues and as a result, it inhibits or reduces the chances of an action potential to be produced (Mosher, 1986).

It has already been demonstrated that a considerable number of marine bacteria, including one's from puffer fish intestine, can produce tetrodotoxin. Zaman *et al*. (1998) detected a group of toxins from Bangladeshi puffer fish, composed of saxitoxin, decarbamoylsaxitoxin, gonyautoxins 2 and 3, decarbamoylgonyautoxins 2 and 3 and three unidentified components, which are possibly related to paralytic shellfish poison. Zaman *et al*. (1998) also reported occurrence of a methyl derivative of saxitoxin in Bangladeshi freshwater puffers.

Recently, Taniyamaa *et al*. (2000) identified a palytoxin (PTX) or PTX-like substance from Bangladeshi puffer fish based on the delayed haemolytic activity, which was inhibited, by an anti-PTX antibody and ouabain (g-strophanthin). Still, there is very little information available on the freshwater puffers responsible for the poisoning, their toxicity scores and toxin composition. In order to clarify this situation, freshwater puffer specimens of *Tetraodon* sp. were collected from Bangladesh during November 2003 and their toxicity was assayed. This paper describes the purification, characterization and toxicity of two toxins from the liver of the puffer fish *Tetraodon patoca*.

MATERIALS AND METHODS

Collection and identification: The fish was collected after catching from Rupsha river surrounding Shundarban area which is located in southwest part of Bangladesh in the month of November 2003. The puffer fish was identified by Fisheries lab, Zoology Department, Rajshahi University, Bangladesh.

Chemicals: All organic solvents used in extraction and isolation was analytical grade supplied by Marck, Germany. The PTLC Silica gel-60 plates were collected from Fluka, Switzerland. IR-spectrum was measured by KBr using Shimadzu FTIR 8400 spectrophotometer. ^{1}H NMR and ^{13}C-NMR spectra were recorded using Bruker 400 MH_Z spectrophotometer and CD_3 $COOD/D_2O$ as solvent. Normal saline, haematoxylin and eosin (H and E) were all analytical grade.

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Animals: Long Evan's rats (110-113 g) were collected from the Animal Resources Branch of The International Center for Diarrhoeal Research, Dhaka, Bangladesh. The experiment was performed at the Department of Pathology, Rajshahi Medical College. The rats were kept in numbered iron cages for two weeks before treatment. They were fed a balanced diet and tap water, under standard conditions of a 12 h dark-light cycle, 60±10% humidity and a temperature of 21.5±1.0°C. These protocols were approved by the Institutional Animal Care and Use Committee of UNICAMP which follows the recommendations of the Canadian Council on Animal Care.

Isolation and purification: The liver (100 g) was taken in a container containing cold distilled water (300 mL) and mixed uniformly with slow stirring for 2 h at room temperature. After centrifugation at 8000 rpm for 8 min at 10°C, the supernatant (aq.) extract was washed with different organic solvents such as n-hexane, chloroform and ethyl acetate successively to remove organic solvents soluble materials and the aqueous fraction was freezed dried. Then it was developed with PTLC on Silica gel-60 (Fluka) using 1-butanol: acetic acid: water (8:1:1) as developing solvent. Then the corresponding bands (R_f 0.76 and 0.65) were scrap off under UV at 254 nm and eluted with water followed by freeze-dried to afford two desired compound, PFT-1 (8 mg) and PFT-2 (5.2 mg).

Administration: Compound, PFT-1 and PFT-2 (0.25 mg) was dissolved in 3.3 mL water to get a stock solution and administered 300 μL intraperitoneally at a dose of 2.25 μg day for 14 consecutive days to experimental group and control group received only water. Four rats were injected in each group.

Experimental procedure: A measured amount of fresh food was supplied daily at 10.00 am and the general well-being and behavior of the animals were observed daily, throughout the study. For the haematological study, blood was drawn from the tail vein of both the groups before administration of the compound and after the experimental period, to estimate the total and differential blood count, platelet count and percent haemoglobin. For the biochemical study, blood was collected from each rat sacrificed at day 14 from the jugular veins of each of the animals. Serum glutamic-oxaloacetic transaminase, serum glutamate pyruvate transaminase, serum alkaline phosphatase, urea, uric acid and creatinine were determined using standard procedures and reagents supplied by Boehringer Mannheim GmbH Diagnostica. Histopathological studies of the liver, kidney, heart and lung were performed using haematoxylin, eosin stain and D. P. X mounting fluid. The samples were observed under a microscope at the Department of Pathology, Rajshahi Medical College, Rajshahi, Bangladesh.

Statistical analysis: Results are presented as the mean±SD Student's t-test was used for comparison between the experimental and control groups. $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Characterization of PFT-1 and PFT-2: The IR spectra of PFT-1 showed characteristic absorption at 3405 cm^{-1} (OH), 1623 cm^{-1} (guanidium), 1559 cm^{-1} (COO^-) and at 1120 cm^{-1} due to C-O stretching bond which were similar with the reported data for TTX (Noguchi and Mahmud, 2001). It also gave a identical peck at 279 nm similar to TTX (Yamashita, 2001). In the ^{13}C-NMR data 11 signals were observed including the carbonyl group at δ_C 156.6 and the ^{1}H-NMR data showed characteristic peaks at δ_H 5.42 (d, J = 9.4 Hz) and δ_H 2.65 (d, J = 9.6 Hz) for H-4 and H-4a of a guanidium ring system, that is the typical spin-spin coupling constant between these two protons. All other ^{13}C-NMR and ^{1}H-NMR data were very close agreement with the published data of TTX, previously isolated from puffer fishes which also gave a molecular ion peak $(M+H)^+$ at m/z 320 and at m/z 302 due to $(M+H-H_2O)^+$ ion that corresponding to the molecular formula of TTX ($C_{11}H_{17}N_3O_8$) (Nakamura and Yasumoto, 1985). To best of our knowledge, this is the first occurrence of tetrodotoxin (TTX) from freshwater puffer fish available in Bangladesh.

Compound PFT-2 showed very similar IR spectra of PTF-1. The ^{13}C-NMR of PFT-2 gave 13 absorption signals indicating that the compound contained 13-carbon atoms. The chemical shift of different carbon atoms were found at 156.55, 110.6, 82.9, 79.7, 76.04, 75.10, 73.96, 72.82, 72.02, 70.21, 65.11, 59.61 and 40.8 ppm. ^{1}H-NMR spectra for PFT-2 and it gave absorption signals at (δc in ppm are 5.51, 5.23, 4.52, 4.42, 4.3, 3.90, 3.79, 3.64, 3.49, 2.29 and 1.35. The signals obtained for PFT-1 and PFT-2 at different ppm were interpreted more clearly in the Table 1. These also gave identical peck at 279 nm similar to TTX (Yamashita *et al*., 2001). On the basis of IR-spectra, ^{13}C-NMR and ^{1}H-NMR, a tentative structure of PFT-1 and PFT-2 are given in Fig. 1 (A and B).

Table 1: ^{1}H-NMR spectral data of the compounds, PFT-1 and PFT-2

PFT-1				PFT-2			
Chemical shift (ppm)	Splitting pattern	Integration	Assignment	Chemical shift (ppm)	Splitting pattern	Integration	Assignment
5.42	Doublet	2H	One-OH and a proton	5.51	Doublet	2H	One-OH and a proton
4.52	Doublet	2H	One-OH and a proton	5.23	Doublet	2H	Two proton
4.46	Singlet	1H	One proton	4.52	Doublet	2H	One-OH and a proton
4.32	Triplet	3H	Three proton	4.42	Singlet	1H	One proton
4.07	Singlet	1H	One-OH	4.32	Triplet	3H	Three proton
4.13	Triplet	2H	Two proton and One-OH	3.91	Singlet	1H	One OH
3.91	Doublet	2H	One-OH and one proton	3.79	Triplet	3H	One-OH and two proton
2.65	Doublet	2H	Two proton	3.64	Doublet	2H	One proton and one-OH
1.86	Singlet	1H	One proton	3.49	Doublet	2H	One proton and one-OH
				2.29	Doublet	2H	Two proton
				1.35	Singlet	1H	One proton

Table 2: Haematological profile of control and toxins treated rats

		Treated with toxic compound-1		Treated with toxic compound-2	
Haematological parameters	Normal rat 1st day M±SD	7th day M±SD	14th day M±SD	7th day M±SD	14th day M±SD
Total RBC count (million/cu mm)	5.050±0.05	3.60±0.05	2.40±0.25	3.65±0.25	3.075±0.50
Total WBC count (th/cu mm)	6.525±0.51	5.80±0.10	5.07±0.005	6.02±1.20	5.275±0.025
Differential count of WBC in %					
a. Neutrophil	63.500±2.29	60.20±0.05	57.50±0.50	61.50±1.25	59.75±0.25
b. Lymphocyte	33.000±2.121	31.20±0.05	25.05±0.20	30.75±1.25	26.00±0.50
c. Monocyte	0.750±0.433	0.55±0.25	0.52±0.025	0.50±0.025	0.51±0.025
d. Eosinophil	2.750±0.829	2.12±0.005	2.05±0.025	2.325±0.125	2.08±0.075
Platelet count no/cu mm	335.000±1.25	352.00±0.52	367.00±0.05	350.00±0.52	363.25±1.25
Hemoglobin %	65.000±3.24	61.75±3.344	50.20±4.898	64.30±0.25	54.00±0.25
ESR (mm/1st h)	11.250±1.29	13.50±1.25	16.50±1.200	12.75±0.25	14.16±0.50

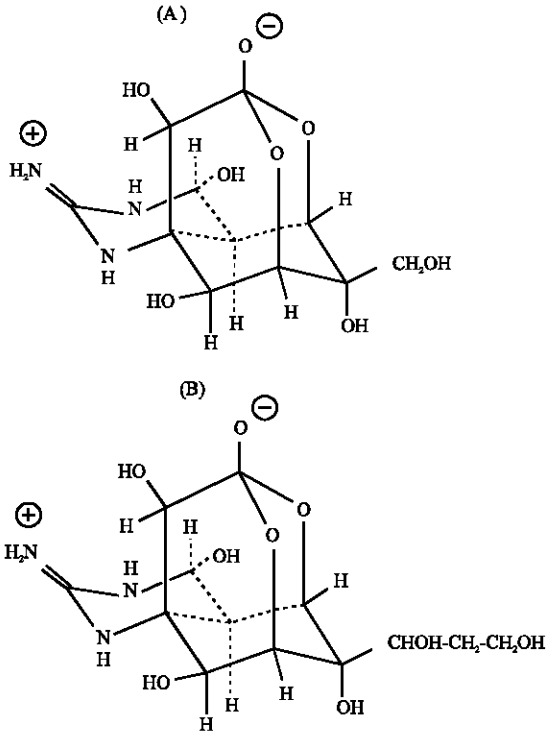

Fig. 1: Tentative structure of the purified toxins, PFT-1 (A) and PFT-2 (B)

Gross general observation: The control group rats did not show any abnormalities and their food intake was also observed to be normal. On the other hand, the experimental group (Toxin treated) rats showed some noticeable signs, such as tremor, convulsions, reflex abnormalities, muscular paralysis, muscular numbness of the hind and four legs as well as salivation. Further the food intake per-day was also found to be much less than that of control rats.

Haematological profiles: As given in Table 2, the haematological profiles such as total RBC, total WBC and differential count of WBC and haemoglobin were decreased in PFT-1 and PFT-2 treated rats as compared to those of control group. Remarkably, the platelet count and ESR were increased in toxin treated rats after the experimental period. Interestingly PTF-1 showed more haematological toxicity than PFT-2.

Biochemical parameters of blood: Table 3 shows the biochemical parameters of blood. All the parameters such as SGPT (serum glutamic-pyruvate transaminase), SGOT(serum glutamic-oxaloacetic transaminase), SALP(serum alkaline phosphatase), bilirubin, creatinine and urea levels of serum were increased significantly in toxin treated group in comparison to that of control group indicating that the both PFT-1 and PFT-2 had toxic effects mainly on liver and kidney functions.

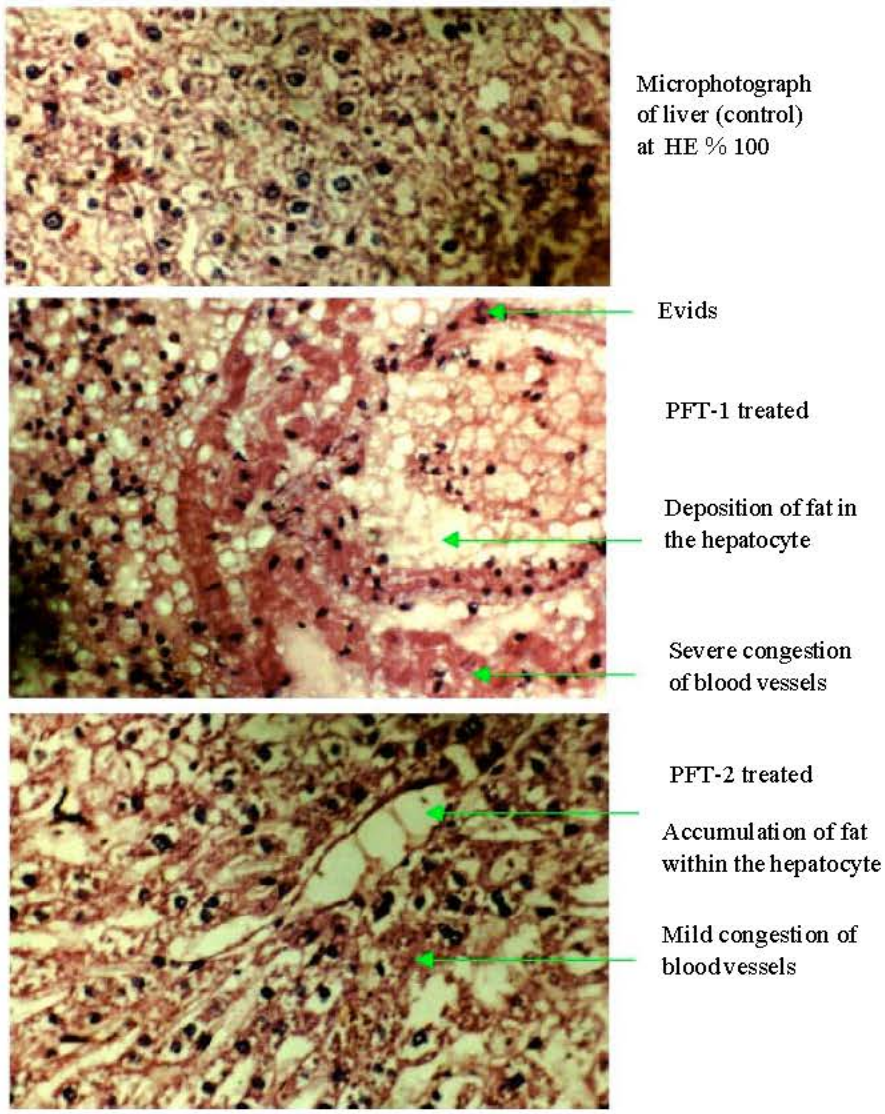

Fig. 2: Microphotograph showing histopathological changes in liver tissues of control and experimental rat after intraperitoneally injection of 2.25 µg rat day of the compounds

Histopathological observations: A marked detectable histopathological difference among the tissues of control (water only) and PFT-1 and PFT-2 treated rats (2.25 µg rat day for 14 consecutive days) were observed after the experimental period (Fig. 2). It was found that the tissues such as liver, kidney, lung and heart of the toxin treated rats were affected and the changes were summarized in Table 4. Although all the tissues of the experimental rats (toxin treated) were severely affected but the changes were more noticeable in liver as compared to that of others.

In this study, two toxins PFT-1 and PFT-2 were isolated from the same fish. Although PFT-1 is similar in structure to TTX but PFT-2 is a derivative of TTX, which contained a alcoholic side chain.

It can be concluded from the present data that the both PFT-1 and PFT-2 has high toxic effects in rats at dose and duration used in this study. Puffer fish possesses paralyzing/palytoxin (Tetrodotoxin, TTX and analogues) that are secreted upon stimulation (Nakamura and Yasumoto, 1985). 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) (Kodama *et al*., 1989; Schantz, 1986). Furthermore, some species of puffer fish have been reported to accumulate saxitoxin (STX) as the principal toxin. It was also reported that TTX is one of the most potent molecules that selectively blocks the voltage sensitive sodium channels of excitable tissues (Mosher, 1986).

Table 3: Effect of PFT-1 and PFT-2 on biochemical parameters of rat's blood after i.p. administration of 2.25 μg rat day for 14 consecutive days

		PFT-1 treated			PFT-2 treated				
Biochemical parameters	Group-C, n = 4 $M_1 \pm SD_1$	Group-A, n = 4 $M_1 \pm SD_1$	% of change	t_e (Calculated t-value)	Group-B, n = 4 $M_1 \pm SD_1$	% of change	t_e	t_c	Remark
SGPT (IU L^{-1})	8.750±0.82	10.00±0.50	14.28	2.51	9.70±0.25	10.85	2.47	2.447	S
SGOT (IU L^{-1})	10.000±0.70	11.70±0.50	17.00	3.16	11.50±0.50	15.00	3.14	2.447	S
SALP (IU L^{-1})	0.480±0.027	0.57±0.075	16.58	2.69	0.55±0.04	12.75	2.69	2.447	S
Serum bilirubin (m mol L^{-1})	0.317±0.048	0.38±0.025	19.87	2.44	0.33±0.075	6.45	2.44	2.447	S
Creatinine (mg %)	0.571±0.018	0.62±0.075	8.77	2.66	0.61±0.75	7.01	2.65	2.447	S
Urea (mmol L^{-1})	17.750±0.84	21.25±0.05	21.25	4.55	18.50±0.50	4.51	4.15	2.447	S

Group-C for control and 'S' indicates significance

Table 4: Changes observed in different tissues of rat's after treatment with the compounds, PFT-1 and PFT-2

		Types of effectiveness	
Concentration of toxin (μg mL^{-1})	Tissue	PFT-1 (Treated)	PFT-2 (Treated)
7.5	Liver	Severe congestion of blood vessels, deposition of fat in the hepatocyte. No inflammation and necrosis.	Mild congestion of blood vessels, accumulation of fat within the hepatocyte. No inflammation and necrosis.
	Heart	Mild congestion of blood vessels, inflammation and accumulation of fat within the cardio vascular cells.	Congestion of blood vessels and complexities in the cardiac system and the cells are collapsed.
	Kidney	Inflammation, vascular congestion and mild fatty change.	Inflammation and mild congestion of blood vessels.
	Lung	Mild congestion of blood vessels, inflammation and deposition of fat.	Mild congestion of blood vessels, inflammation and deposition of fat.
Control (300 μL of distilled water injected intraperitoneally)	All the tissues	There was no inflammation, necrosis, stromal oedema and congestion of blood vessels of the liver, lung, heart and kidney	

The histopathological study indicates that all the tissues as well as metabolic systems (based on changes in the biochemical parameters and histopathological changes) were affected after administration with the toxin, PFT-1 and PFT-2. The changes were observed to be more pronounced in liver as compared to the other tissues examined. Similar liver toxicity was previously found from Bangladeshi puffer fish (Zaman *et al*., 1997). But the toxicity showed in this study is not only PFT-1 and/or PFT-2, but might be other toxin(s). Therefore, the origin, mechanism of toxicity, or metabolic pathway of these components remains to be clarified.

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