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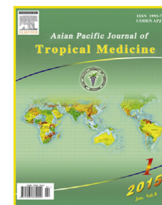

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Toxicity assessment and screening of tetrodotoxin in the oblong blowfish (*Takifugu oblongus*) from the Tamil Nadu Coast of Bay of Bengal, India

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ABSTRACT

Objective: To assess the toxicity of the pufferfish *Takifugu oblongus*, from Chennai coast, Tamil Nadu, India and to detect the presence of Tetrodotoxin (TTX).

Methods: The toxicity was evaluated by mouse bioassay using Swiss Albino mice which were expressed in mouse units (MU). Gross anatomical features were observed which is followed by histopathology of the dead mice tissues to establish the toxicity. Instrumental analysis for the presence of tetrodotoxin was also performed through GC–MS and HPLC.

Results: The toxicity of ovary was the maximum with 163 MU/g and lowest toxicity was observed in skin with 75.88 MU/g. Histopathological analyses of the dead mice showed various cellular degenerations and inflammations. The amount of Tetrodotoxin detected through GC–MS and HPLC was more reliable and sensitive than the customary mouse bioassay as instrumental analyses were able to detect even nanograms of the toxin.

Conclusions: The present study evidently proved that *Takifugu oblongus* is highly toxic and consumption of the same can pose serious threat for health and possible lethality to humans.

1. Introduction

Marine ecosystem remains the habitat of organisms from various phyla amidst which fishes are the most diversified group among the vertebrates. Several toxic marine organisms are reported till date and some fishes have exhibited toxicity at extremities. The coastline of the Bay of Bengal and Arabian Sea continues to be a rich fishing grounds in the South Asian region and India is one of the world's largest marine natural products exporting nations. The south east coast of India extends its coastal line from the state of Tamil Nadu to Odisha. Among the wide varieties of fishes found in these coastal waters, the pufferfishes form reasonable quantities. Pufferfishes belong to the family Tetraodontidae which encompasses 189 species in 28 genera [1]. Pufferfishes produce a potent neurotoxin named Tetrodotoxin (TTX) which is a sodium channel blocker in nerve cell membranes.

Various studies reported that consumption of these pufferfishes had caused food intoxications including deaths worldwide viz, Mexico, USA, Hong Kong, Japan, Korea, Taiwan, Malaysia, Bangladesh and even India is not spared [2–7]. Pufferfish poisoning kills approximately 20–100 people annually in Japan [8,9]. Ingestion of the cooked roe of *Chelenodon patoca* produced numerous deaths and that was the first report of pufferfish poisoning in India by Jones in 1956 [10]. The toxicity of pufferfishes vary based on varied species, sex, seasons of collection, geographical locations, and anatomical features as well [10–12]. In India, occurrence of pufferfish poisoning is sporadic, yet documenting toxicological profiles of the pufferfishes is still inadequate. In the past few years, some species of pufferfishes were assessed for toxicity from the Bengal Coast of India. Research on pufferfishes from southern parts of India is still lacking.

Takifugu oblongus (T. oblongus) (Bloch, 1786), commonly called the Lattice Blassop or Oblong Blowfish is one among the available species of pufferfish along the Indian coasts. Nevertheless, the toxicity of this species has been reported elsewhere, it is considered as delicacy in some of the countries [13,14]. *T. oblongus* was a disaster to Bangladesh in the years 1998 and 2002 causing several deaths due to its toxicity [13–16]. This study was undertaken to analyse the toxicity of the pufferfish *T. oblongus* collected from the coast of Chennai, Tamil Nadu by mouse bioassay. The occurrence of TTX in this species was also qualitatively and quantitatively analysed through chromatographic techniques such as gas chromatography–mass spectrometry (GC–MS) and HPLC.

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2. Materials and methods

2.1. Specimen collection

Specimens were collected from Kasimedu fishing harbour, Chennai, Tamil Nadu. The specimens were tightly packed, transferred in Icebox to the Medical Biotechnology Lab, VIT University and they were maintained in a deep freezer at −20 °C until use [2].

2.2. Experimental animals

Male Swiss albino mice weighing between (18–22) g were issued by and maintained in the Animal House, VIT University for experimentation [2]. The study had strictly followed the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The experimental procedures followed, were approved by the Institutional Animal Ethical Committee (IAEC), VIT University (registration No: 197/BC/06/CPCSEA). Chloroform was used as an anaesthetic agent for control mice before dissections and handling of mice was done ethically to reduce pain and suffering.

2.3. Preparation of toxic extracts

The specimens were dissected and the organs particularly Skin, Muscle, Liver and Ovary were excised from which the extract was prepared. Ten gram of every organ is weighed and separately homogenized in tissue homogenizer with 50 mL of 0.1% Acetic acid in water. The tissue homogenates were boiled for 10 min in water bath at 50 °C. They were cooled and centrifuged at 3 000 rpm for 10 min. The supernatants were collected and stored. The same procedure was followed thrice and the supernatants collected were combined, filtered and stored [17].

2.4. Mouse bioassay

Mouse bioassay is the gold standard for basic toxicity assessments. The mice were separated into 5 groups, with each group having 3 mice. First group is maintained as Control, 2nd group is injected with Liver extract, 3rd with Skin extract, 4th with Muscle extract and 5th with Ovary extract. The control group receives 1 mL of 0.1% acetic acid. One mL of the each extract (undiluted) was injected into groups of mice split accordingly. If the mice die before 4 min, the extracts were diluted with 5 mg/mL Phosphate buffered saline (PBS). The injected mice were observed for toxicity symptoms and death for few minutes [18]. The toxicity of the extracts in Mouse Units (MU/g) were calculated by the dose-death time relationship, where 1 MU is defined as the amount of pure toxin (approximately 0.22 μg) required to kill a 20 g male mouse of ddY strain within 30 min of injection [19]·

2.5. Histopathological examination

Brain, liver and kidneys of the mice which died followed by injection of the toxic extracts were excised to ascertain the toxicity of *T. oblongus*. They were fixed in 10% formalin for 24 h and processed further. Tissue sections were prepared for each organ, followed by Hematoxylin and Eosin (H&E) staining which is observed under light microscope and photographed [20].

2.6. Sample preparation for GC–MS

The crude tissue extracts, each of 500 μL was added with 200 μL of 3 N sodium hydroxide and 200 μL of internal standard (3 μg salicylic acid in 0.1% acetic acid), boiled for 30 min, cooled to room temperature. The mixture was defatted with 1 mL of *n*-butanol. The aqueous layer was added with 150 μL of 2 N hydrochloric acid, 4 mL of distilled water and 1 mL of *n*-butanol, vortexed for 5 min and centrifuged at 2500 rpm for 5 min to extract the toxin into the organic layer. The organic layer was dried with 0.2 g of anhydrous sodium sulphate. It was evaporated to dryness and 75 μL of derivatizing agent MSTFA (N-Methyl-N-TMS-triflouroacetamide) was added to the residue and heated at 60 °C for 30 min. To the cooled solution, 75 μL of *n*-dodecane was added, vortexed and the final sample was sent for GC–MS analysis [21]. Standard toxin was also processed the same way as the test samples, as the highly involatile tetrodotoxin cannot be directly detected through GC–MS.

2.7. GC–MS specifications

The samples were run on Shimadzu QP 2010 Plus system with Rtx-5MS column (30 m × 0.32 mm, 0.5 μm film thickness). The injector port and interface temperature were set to 250 °C and 280 °C, respectively. The column oven temperature was programmed from 100 °C to 280 °C (3 min hold) at a rate of 25 °C/min. One microlitre of the sample was run in split mode of the ratio 1.0. Helium gas used as carrier and the flow rate was set about 3.56 mL/min. Selective Ion Monitoring (SIM) mode was performed with the following selective qualitative ions of m/z 376, 392 and 407 and quantitative ion is m/z 392 [21]. The total run time was about 18.2 min that initiated from the 3rd min.

2.8. Sample preparation for HPLC

The presence of fats or proteins in the crude extracts may produce additional peaks during the analysis and Tetrodotoxin is neither a fatty nor a protein molecule. And so the crude tissue extracts were defatted with dichloromethane and deproteinized with acetonitrile. The acquired supernatant and was then applied on a Buchner funnel with activated charcoal under agitation and eluted. The toxin, if present gets adsorbed to the activated charcoal which is first washed with distilled water followed by washing with 0.1% acetic acid [22,23]. The toxin is eluted which is repeated thrice to elute the maximum amount of toxin.

2.9. High performance liquid chromatography (HPLC) specifications

The samples were detected for presence of TTX on Shimadzu SPD-20A Prominence with UV–Vis Detector and a reverse phase column (Hypersil™ BDS C18 column, 250 mm × 4 mm, 5 μm). 0.04% of phosphoric acid was used as mobile phase with a flow rate of 1 mL/min at 30 °C, and the UV wavelength set was 195 nm [22].

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3. Results

3.1. Mouse bioassay

As a result of mouse bioassay, the toxicity of the pufferfish *T. oblongus* was determined. Muscles of the pufferfish showed no toxicity symptoms in the injected mice. Ovary showed maximum toxicity with 163 MU/g, followed by the liver with 143.33 MU/g and skin with 75.88 MU/g. The approximate amount of TTX and the degree of toxicity were also expressed with the calculated mouse units (MU).

3.2. Gross physiological and anatomical observations

The injected mice showed intense hyper activity by running inside the cage in an excited manner, escape reaction, shivering

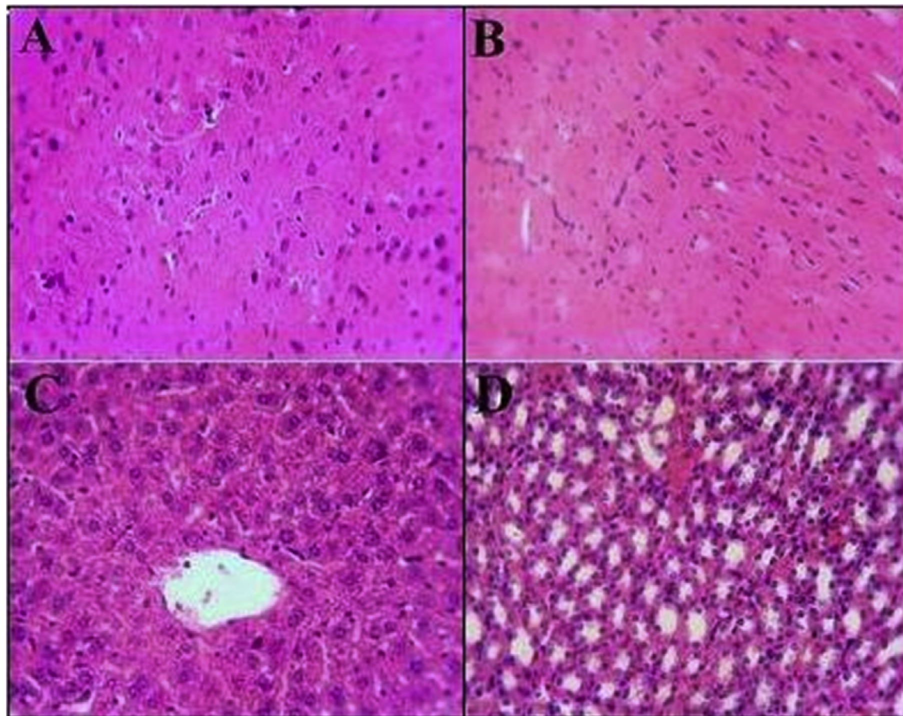

Figure 1. Histopathology of control mice showing no abnormalities (H&E ×400).
Note: (A) Brain (B) Heart (C) Liver (D) Kidney.

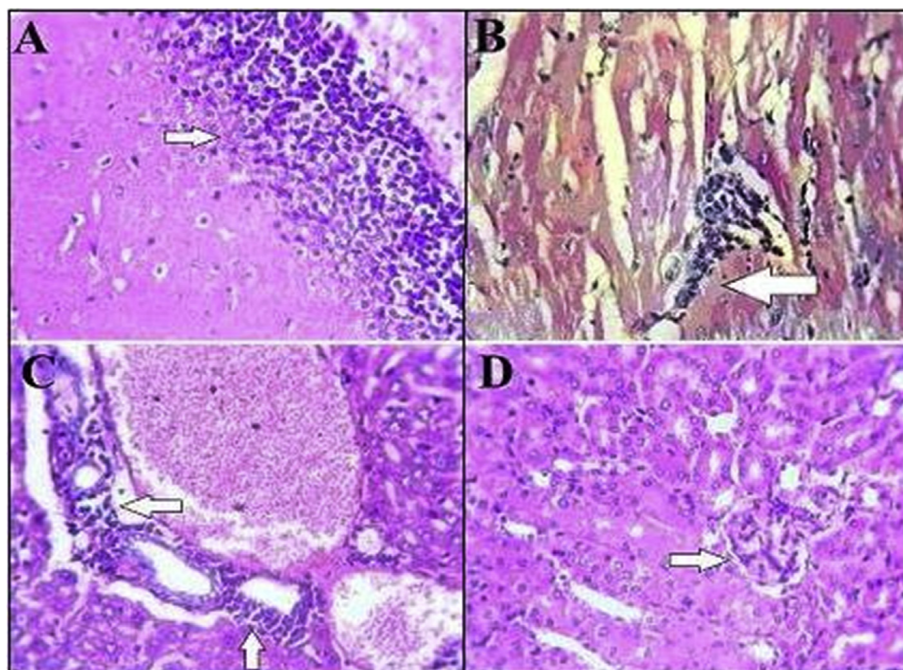

Figure 2. Histopathology of mice injected with skin extract (H&E ×400).
Notes: (A) Brain-Gliosis noticed (B) Heart-Cardiomyocytes seen in syncytial pattern (C) Liver Mild Periportal inflammation found (D) Kidney-Glomeruli seen with congestion.

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of the forelimbs, paralysis of the hind limbs, paleness of the tail, diarrhoea and were suffocating until its final gasping breath. Upon autopsy, blood clots were found in the anterior visceral parts. Dark discoloration was noticed in the heart and liver. These observations were further supported by the histopathological changes.

3.3. Histopathology

3.3.1. Control

The control mice injected with 0.1% acetic acid showed no notable changes in either of the organs viz, brain, heart, liver and kidneys in both pre and post-monsoon seasons (Figure 1A–D).

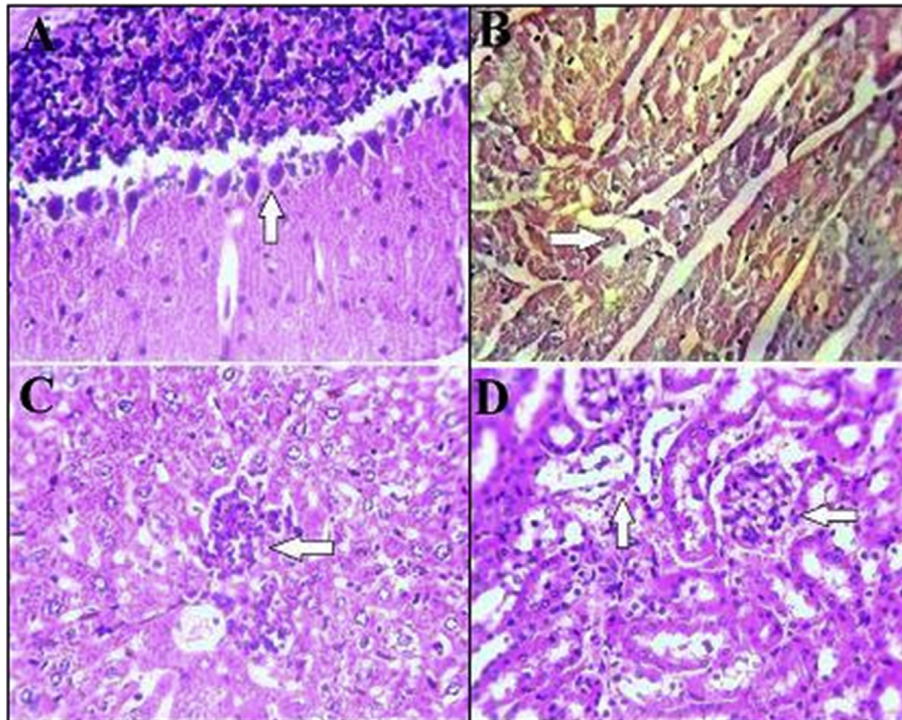

Figure 3. Histopathology of Mice injected with Liver extract (H&E ×400).
Notes: (A) Brain-Gliosis with haphazard astrocytes (B) Heart-Cardiomyocytes seen in syncytial pattern (C) Liver-Mild sinusoidal dilation and inflammations found (D) Kidney-Unremarkable vessels and tubules with necrosis and apoptosis.

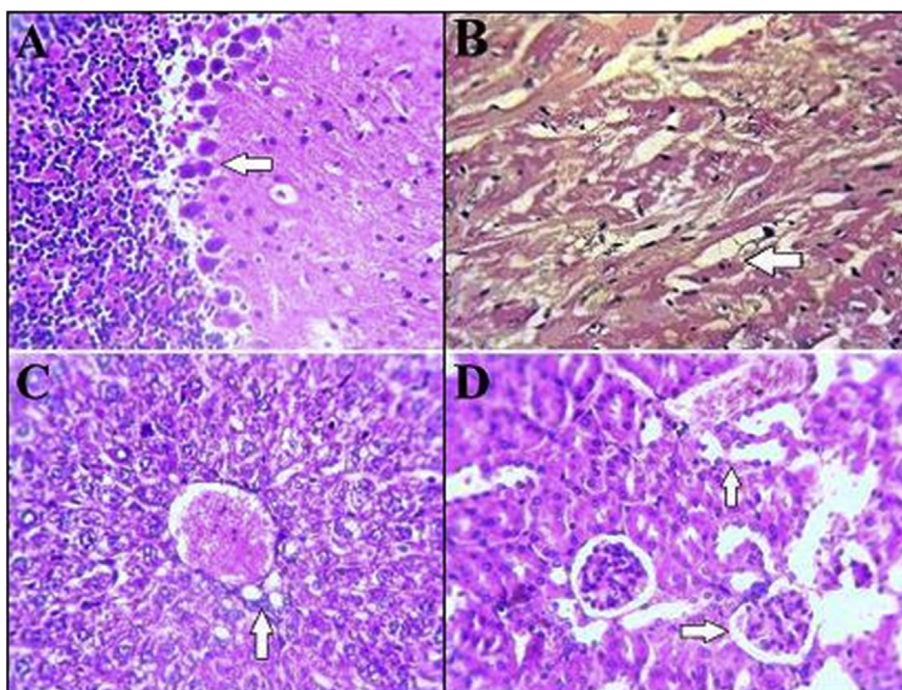

Figure 4. Histopathology of Mice injected with Ovary extract (H&E ×400).
Notes: (A) Brain-Gliosis with haphazard astrocytes (B) Heart-Cardiomyocytes arranged in syncytial pattern (C) Liver-Moderate periportal inflammation of lymphocytes (D) Kidney-Glomeruli with congestion seen.

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3.3.2. Effects of skin extract

Gliosis was seen and the molecular purkinje layer was observed with intact neurons in the brain of mice (Figure 2A). Cardiomyocytes were arranged in syncytial pattern in the heart tissues of mice and neither inflammations, nor infections were found (Figure 2B). Mild periportal inflammations were observed in the liver of mice (Figure 2C). Glomeruli with congestion were seen in mice kidneys (Figure 2D).

3.3.3. Effects of liver extract

Gliosis was observed in the brain of mice (Figure 3A). Heart tissues showed arrangement of cardiomyocytes in syncytial

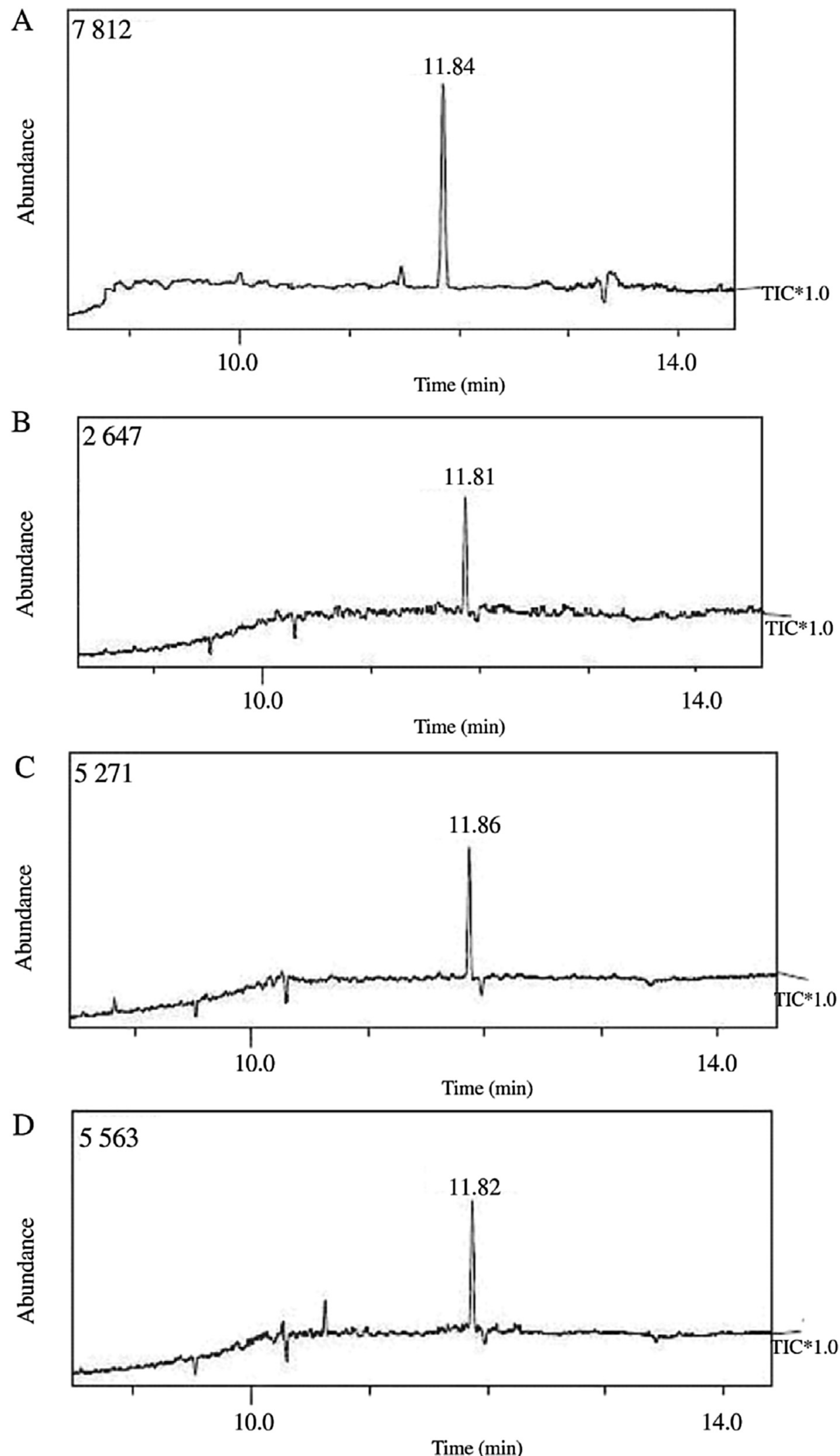

Figure 5. GC–MS Chromatograms in SIM Mode showing characteristic peaks for C9 base for the quantitative ion *m/z* 392.
Notes: (A) Standard TTX (B) Skin Sample (C) Liver Sample (D) Ovary sample.

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pattern and neither inflammations, nor infections were found (Figure 3B). Mild sinusoidal dilation and inflammations were found in the liver of mice (Figure 3C). Glomeruli with congestion, unremarkable vessels and tubules with necrosis and apoptosis were observed in the mice kidneys (Figure 3D).

3.3.4. Effects of ovary extract

Gliosis with haphazardly arranged astrocytes was noticed in the mice brain (Figure 4A). Cardiomyocytes were arranged in syncytial pattern in the heart tissues of mice and neither inflammations, nor infections were found (Figure 4B). Liver of mice showed mild to moderate periportal inflammation composed of lymphocytes, plasma cells and few eosinophils (Figure 4C). Glomeruli with congestion were seen in mice kidneys (Figure 4D).

3.4. GC–MS

Standard TTX (0.05 mg/mL) showed characteristic peaks for all the 3 qualitative ions viz, 376, 392 and 407 at the retention times 11.9, 11.84 and 11.87 revealing the presence of C9 base (2-amino-6-hydroxymethyl-8-hydroxyquinazoline), a TTX derivative (Figure 5A). Similar peaks were observed in the test samples. Skin sample showed peaks at 11.84, 11.81 and 11.83 (Figure 5B). Liver sample showed peaks at 11.81, 11.86 and 11.82 (Figure 5C). Ovary sample showed peaks at 11.88, 11.82 and 11.85 (Figure 5D). The relative intensity of the base peak at m/z 392 was 100%, hence it is alone considered for quantification of the toxin. The calibration curve was plotted with the standard and analyte concentration in the test samples was estimated. Ovary samples contained nearly 34.5 μg, liver samples with 32.5 μg and skin samples with 16.5 μg of TTX.

3.5. HPLC

Standard TTX (0.1 mg/mL) showed a characteristic peak at the retention time of 2.465 (Figure 6A). The 3 test samples showed characteristic peaks at similar retention times viz, 2.440 for skin sample, 2.417 for liver sample and 2.419 for ovary sample (Figure 6B–D). The calibration curve was plotted with the standard and analyte concentration in the test samples was estimated. Ovary sample contained about 51 μg, liver sample with 48 μg and skin sample with 18 μg of TTX.

4. Discussion

Food intoxications by consumption of pufferfishes is a serious threat in most coastal countries. The principle objective of the study was to assess toxicity of the pufferfish *T. oblongus* from the coast of Chennai, Tamil Nadu through standard methods. Mouse bioassay revealed that the liver, ovary and skin of the pufferfish are highly lethal causing death of injected mice in few minutes. The muscle extract was completely non toxic showing no symptoms of pufferfish poisoning. The toxicity of ovary was about 163 MU/g, liver was about 143.33 MU/g and skin showed least toxicity of 75.88 MU/g. This is in coincidence with the study of Ghosh *et al.* [2] which reported that ovaries were toxic than livers of the pufferfish *T. oblongus*, from the coastline of Bengal, in all the seasons [2]. Tani in 1945 reported that toxicity ≥10 MU is considered weakly toxic (+), ≥100 MU is moderate (++) and ≥1 000 MU is strongly toxic (+++), whereas, <10 MU is considered non-toxic (−) [19]. Kawabata in 1978 had reported that toxicity above 10 MU is hazardous for human consumption [24].

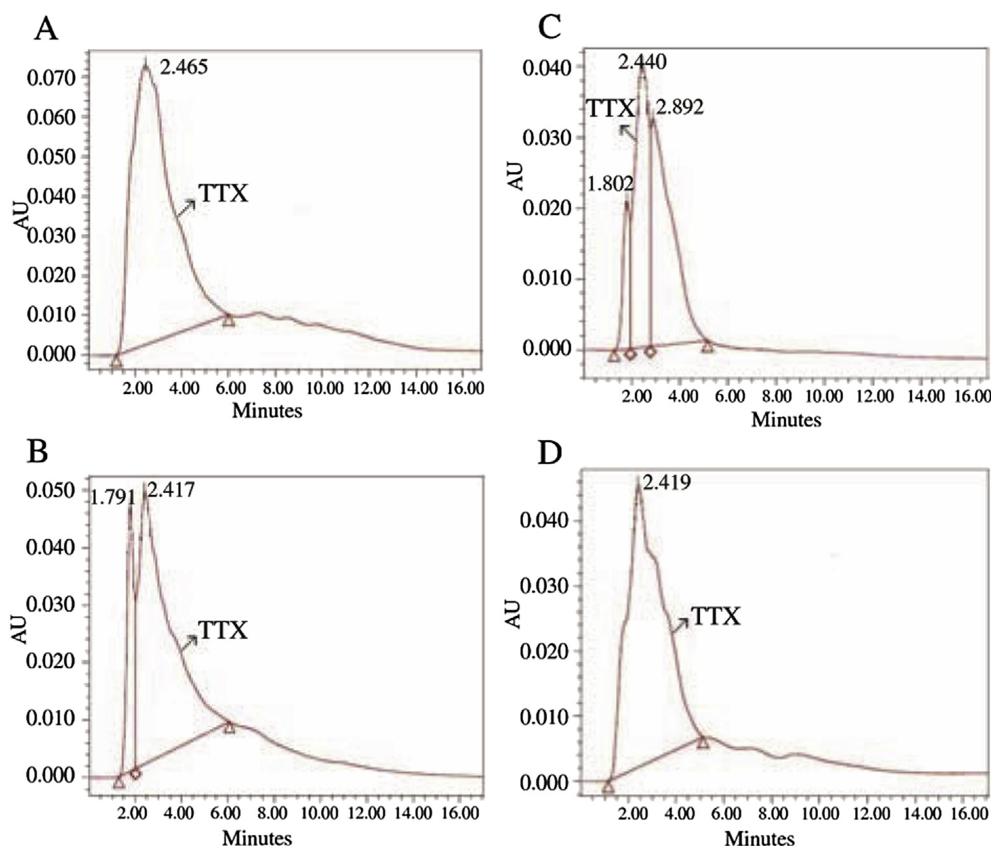

Figure 6. HPLC Chromatograms showing peaks for TTX at similar retention times.
Notes: (A) Standard TTX (B) Skin sample (C) Liver sample (D) Ovary sample.

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The presence of TTX in *T. oblongus* was detected and quantified through GC–MS and HPLC. Selective Ion Monitoring in GC–MS was more specific which revealed the presence of C9 base, by showing characteristic peaks for the *m/z* 376, 392 and 407, reducing unnecessary peaks corresponding to other *m/z* that interferes in general scanning mode. This is in accordance with the study of Man *et al.* in 2010 [21], which also showed characteristic peaks for all the 3 fragment ions but the Limit of Quantification (LOQ) was 0.1 ng of TTX and the Limit of Detection (LOD) was 0.05 ng. Wherein, the LOQ of the toxin in the present study is 0.05 mg and the LOD is 0.005 mg which is relatively higher than that of reported in the study of Man *et al*.

HPLC with UV detection confirmed and quantified TTX levels, similar to the study of Yan *et al*., in 2005 [18], which detected and quantified the isolated TTX from the bacteria *Serratia marcesens* isolated from Hong Kong pufferfishes. The LOQ of the toxin in the present study is 0.1 mg and LOD is 0.01 mg which is also higher than the LOQ of 0.01 mg achieved in the study of Yan *et al*. HPLC-UV detection is the better choice than HPLC-FLD, due to its simplicity, time-efficiency, lesser expenses and lesser damage to TTX structure [22,23]. Few miscellaneous peaks were also found along with TTX in both GC–MS and HPLC as the samples were just partially purified. But when compared to the conventional mouse bioassay, instrumental analyses give more precise and accurate detection levels of TTX and are more sensitive.

Chelenodon patoca is the most toxic species of pufferfish found in Indian waters as reported by Ghosh *et al*. [2]. On comparison with it, *T. oblongus* holds the next position in its toxicity levels. The data from mouse bioassay showed highest toxicity in ovary and least in skin which is in agreement with the amount of TTX detected through chromatographic techniques. Since the muscle of *T. oblongus* is non-toxic, it can be consumed when prepared under proper safety standards. Awareness regarding the potential threats of pufferfish consumption should be given to the public so that they refrain themselves from consuming them and could seek medical advice under emergencies.

Conclusively, the pufferfish *T. oblongus* which has been the cause of food poisoning outbreaks and deaths in many countries proved its toxicity of its liver and gonads at extremities. Hence, the consumption of this pufferfish species should be avoided to prevent the health hazards not only in the south eastern part of India but also in other nations. The present study has produced detailed data regarding the toxicity profile of the pufferfish *T. oblongus* from southern India.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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