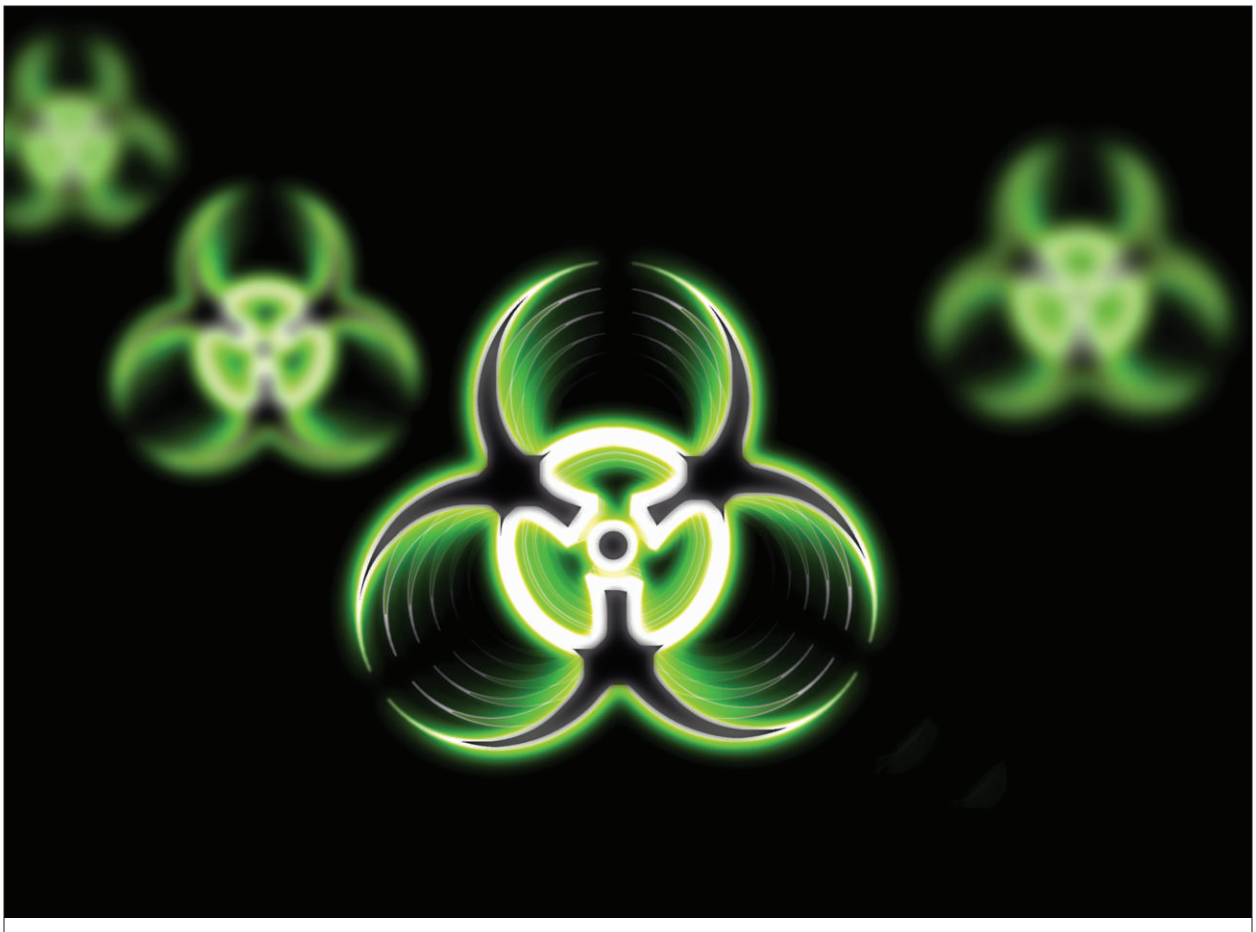

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Research Article
Genotoxicity of Tetrodotoxin Extracted from Different Organs of *Diodon hystrix* Puffer Fish from South East Indian Coast

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Abstract
In the present study, tetrodotoxin (TTX) extracted from eyes, skin, liver, intestine and gonads of *Diodon hystrix* were evaluated for toxicity study. The COX1 gene sequencing revealed 99% similarity to *D. hystrix*. This identification of *D. hystrix* from Chennai coastal region is the first molecular report providing new data about their geographical distribution. The GC-MS analysis revealed the presence of TTX toxin in all the crude extracts of the fish except eye. Human lymphocyte chromosome aberration assay was tested using crude extracts containing TTX (0.5 mg mL^{-1}) which caused spindle fibre aberration and comet assay showed lack of DNA damage. In support of genotoxicity, all the crude extracts containing TTX was tested on zebra fish, using 1 mL of 5 mg mL^{-1} concentration stock solution. The results suggest that extracts from skin, gonads and liver of *D. hystrix* caused 100% mortality within 72 h. Based on these observations it is suggested that *D. hystrix* from chennai coastal region as food is a potential risk to humans.

Key words: *Diodon hystrix*, genotoxicity, spindle fibre aberration, tetrodotoxin, zebra fish

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tretrodotoxin (TTX), one of the most potent sodium channel blocker and neurotoxin is found to harbour over 20 species of puffer fish (Noguchi *et al*., 2006). The TTX contamination in food, especially, puffer fish associated food poisoning has grown sporadically in Southwest Indian Ocean (Puech *et al*., 2014). In Asian countries cases like scombroid fish poisoning, paralytic shellfish poisoning and ciguatera fish poisoning were most of the sea food poisoning reported repeatedly (Arvanitoyannis *et al*., 2014). Ingesting the puffer fish causes true allergic reactions which were the first sign following symptoms like nausea, numbness, tingling sensation in the mouth and throat and muscle weakness. Respiratory failure and death were reported in severe and rare cases (Yong *et al*., 2013). Until now, no effective treatment or antidote for puffer fish poisoning is available and only cure was the habit of supportive care. Remarkably certain populations showed resistance to TTX. *Thamnophis sirtalis* known as garter snakes feed on tetrodotoxic newts (Geffeney *et al*., 2002) and *Mya arenaria* (Soft-shell clams) have shown resistance to saxitoxin (STX) which is similar to TTX (Bricelj *et al*., 2005). Mutation in the sodium channel gene would be the reason for resistance in clams. STX is also known for its neurotoxicity and is considered as highly potent paralytic shellfish poisoning toxin found commonly in mussels and crustaceans (Tian *et al*., 2014). Up to 30 structural analogues of TTX described and the mechanism of toxicity differs with structure (Yotsu-Yamashita *et al*., 1999). It is necessary to investigate the toxicity of TTX analogues as no studies have been reported till now on all these analogues. Only mass spectroscopy approaches are appropriate techniques to detect TTX and its analogues in samples (Bane *et al*., 2014). So far no standards are commercially available. High Performance Liquid Chromatography (HPLC) method has now been accepted by the European Union (EU) for screening method for paralytic shellfish poisoning toxins. But conversely, this technique is not capable of detecting TTX and hence effective method required for analysis of TTX (Campbell *et al*., 2013).

Until now there is no high affinity antibody has been reported for neutralization of TTX toxicity. Wang *et al*. (2014) developed an antibody scFv-T53 that showed high affinity specific to TTX antigen through phage display technology. The TTX is an incomplete antigen does not elicit immune response and hence to acquire a high affinity and specific serum titer antibody, it is necessary to conjugate the hapten with immunogenic molecule through monoclonal antibodies production by hybridoma technology against TTX. However reports are not in favour as monoclonal antibodies apt to lose their capability over period (Frame and Hu, 1990; Kessler *et al*., 1993). Studies are available on biosensors for rapid and robust detection of TTX (Yakes *et al*., 2014). Campbell *et al*. (2013) designed TX-7F monoclonal antibody to TTX which is extensively validated for the screening of TTX in gastropods and puffer fish. Similarly, Kreuzer *et al*. (2002) augmented a novel electrochemical immunosensors known as Screen Printed Electrode (SPE) for measurement of a diversity of seafood toxins including okadaic acid, brevetoxin, domoic acid and tetrodotoxin. The genotoxicity of puffer fish investigation though it has been extensively studied earlier to the best of our knowledge this is first report on toxicity of crude extract containing TTX from different organs such as eyes, skin, liver, intestine and gonads of *Diodon hystrix* collected first from Chennai coastal region of Tamil Nadu, India and providing new data about their geographical distribution.

MATERIALS AND METHODS

Black spotted porcupine puffer fish was collected from the coastal lines of Bay of Bengal in Chennai region, Tamil Nadu, during the month of June, 2014. Preliminary identification of the puffer fish was done by morphological observation based on fish database (www.fishbase.org). Fish samples were carefully transported to the laboratory under stored condition using dry ice. The collected puffer fishes were washed with double distilled water and the organs such as eyes, skin, liver, intestine and gonads of the puffer fish were carefully dissected. Genomic DNA was extracted from tissue samples using the method devised by Nishiguchi *et al*. (2002). Tissue samples were initially incubated in a tris-EDTA buffer (TE) for 24 h. Each tissue sample was digested in 500 µL of sodium chloride-tris-EDTA (STE) buffer containing SDS (0.2%) and ammonium acetate (250 µL of 10 M) at 55°C for 10 h. After digestion, small amount of tissue was grounded in a glass tube and incubated for 1 h. Samples were then centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a new tube and 2 mL of ice-cold ethanol (100%) was added and mixed gently. The tubes were placed at -18°C until DNA precipitates. Again tubes were centrifuged at 4°C at 14,000 rpm for 15 min, the resulting supernatant was separated and 2 mL of ice cold 70% ethanol was added. The tubes were once again allowed to spin at 4°C at 10,000 rpm for 15 min. Then ethanol was poured off and the tubes were dried completely. The pellets were re-suspended in 50 µL of TE buffer overnight at 4°C or for 30 min at 40°C. The precipitate was centrifuged for 20 min at 12,000 rpm in a micro centrifuge. Ethanol solution was discarded by decantation and pellet was washed with 1 mL of 70% ethanol. It was then rotated for 5 min at 12,000 rpm in a micro centrifuge. After discarding the ethanol solution it was let too dry in a vacuum

centrifuge (55°C). The pellet was again resuspended in 50 µL of TE buffer (pH 7.6) and the sample was incubated at 45°C to facilitate dissolution of the pellet. Cytochrome c oxidase (COX1) gene located on mitochondrial DNA has been used like for all animal barcoding studies. The LCO1490: 5 GGTCAACAAATCA TAAAGATATTGG-3 was used as a forward primer and $HCO_2$198: 5-TAAACTTCAGGGTGACCAAAAAATCA-3 was used as reverse primer (Folmer *et al*., 1994). The PCR thermal regime consisted of one cycle of 1 min at 94°C; five cycles of 1 min at 94°C, 1.5 min at 45°C and 1.5 min at 72°C, 35 cycles of 1 min at 94°C, 1.5 min at 50°C and 1 min at 72°C and a final cycle of 5 min at 72°C. The PCR products were analysed on 1% agarose gel for amplicons in 1X TBE buffer at 100 V. The amplified product was sequenced using ABI PRISM 3730 Genetic Analyser (Applied Biosystems). The sequences of COXI regions were compared against the sequences available from GenBank using the BLASTN program (Altschul *et al*., 1990) and progressively aligned using CLUSTAL W software (Thompson *et al*., 1994). The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) (Felsenstein, 1985). The MEGA4 package (Tamura *et al*., 2007) was used for all analyses.

Eyes, skin, liver, intestine and gonads organs were homogenized and suspended in 1% acetic acid in methanol (10 g tissues 100 mL^{-1} solvent). The homogenate were filtered through cheese cloth and the filtrate is further centrifuged at 12,000 rpm for 10 min at 4°C to remove tissue debris. The resultant supernatants were transferred to clean vials and were heated in water bath to evaporate methanol. Remaining liquid concentrates were lyophilized to obtain crude. To 1 mg of crude extracts, 5 mL of 1% acetic acid and 1 mL of Dragondorff's reagent was added and observed for precipitation.

Crude extracts were suspended in 2 mL of 2 M sodium hydroxide and kept in a boiling water bath for 45 min. After cooling to room temperature, samples were examined in UV/Vis spectroscopy between the ranges 190-800 nm. Whereas for GC-MS analysis, the crude extract was dissolved in 3 M sodium hydroxide (NaOH) and kept in boiling water bath for 30 min. The solution was then cooled to room temperature and pH was adjusted to 4.0 with 1 N hydrochloric acid (HCl). This was fractionated using 10-100% methanol. O-bisacetamide:trimethylchlorosilane:pyridine (2:1:1) was added to the resulting residue in order to generate trimethylsilyl (TMS) "C9-base" compounds. This solution was then analysed using GC-MS. In sterile culture tubes, 5 mL of Hikaryo XL RPMI ready-mix media and 0.5 mL of heparinized blood were added and the contents were mixed gently. These were incubated for 48 h in standing position and after 48 h of incubation, the cultures was treated with crude extracts of eyes, skin, liver, intestine and gonads organ (0.5 mg mL^{-1}) and again kept it in incubator for another 24 h. Then the cultures were thoroughly washed by centrifuging at 1500 rpm for 5 min. The supernatants were discarded and 5 mL of fresh RPMI 1640 medium was added to the pellets. About 60 µL of colchicine was added to all tubes and then incubated for 20 min at 37°C. Then the tubes were centrifuged at 1500 rpm for 10 min. The supernatant was removed and 6 mL of pre-warmed 0.075 M hypotonic solution was added. The content was mixed using a pasteur pipette and incubated at 37°C for 6 min. Then the tubes were centrifuged at 2000 rpm for 5 min. The supernatant was discarded and 6 mL of Carnoy's fixative was added and mixed vigorously and the tubes were kept in room temperature for 1-2 h. The tubes were centrifuged again at 1500 rpm and supernatant was removed. This step was repeated, until pellet becomes white. Clean slides were chilled in refrigerator and the cell mix was dropped over the slides and dried immediately on a hot plate. The slides were then placed in a coplin jar containing Giemsa staining for 4 min and washed in a coplin jar containing distilled water for 1 min. The slides were dried and then viewed under microscope for stained chromosome.

The leucocytes were cultured and treated with all the crude extracts of fish in the same way like genotoxicity assay but without colchicine. The cells were mixed with 1% low melting agarose and loaded on to the 1% normal agarose. The slides were immersed in lysing buffer for 1 h. After 1 h electrophoresis was performed using 300 mA for 30 min. At the end, the slides were stained with ethidium bromide, washed with distilled water and kept in 100% ethanol for 20 min. The dried slides were viewed under UV-transilluminator. Young male and female zebra fish of wild type were purchased from Vellore aquarium and kept in glass aquaria at temperature and air controlled environment. Fish were divided in to seven groups and fed twice daily with spirulina feed. Except the control group, remaining six groups were treated with crude extracts of puffer fish, respectively. About 1 mL of crude extract suspension (5 mg mL^{-1}) was added to the respective tank and mortality of the fishes was observed over a period of three days' time and the experiment was repeated thrice.

RESULTS

The COXI gene sequence of the studied puffer fish sample, demonstrated 100% similarity to *Diodon hystrix*. Confirming that the obtained puffer fish sample, for this study is *Diodon hystrix*. Phylogenetic tree of the COXI gene

sequence demonstrated a bootstrap value of 100 for the test sample and JQ 431688 *Diodon hystrix* as shown in Fig. 1.

Dragondorff's test showed strong precipitation for skin and liver extracts. Moderate precipitation for intestine and gonad extracts, while no precipitation. Figure 2 shows the precipitation in Dragondorff's test result.

The UV absorbance of the crude extracts demonstrated a characteristic shoulder peak over 276 nm in extracts of skin, liver, intestine and gonad. Saito *et al*. (1987) used same method in his investigation of evaluation of TTX and its derivatives in puffer fish. This primarily confirmed the presence of TTX in these organs. The GC-MS analysis demonstrated characteristic peaks at 8.33 and 8.66 Retention Time (RT) for skin, liver, intestine and gonad crude extracts. Neither of the peaks at the specified RT was observed in eye and kidney. The peaks at 8.33 and 8.66 RT represents the C9 base TMS derived from TTX under alkaline conditions.

Genotoxicity study on human leukocyte cells, demonstrated spindle fiber aberration for skin and liver extracts treated cells. Figure 3 shows the characteristic

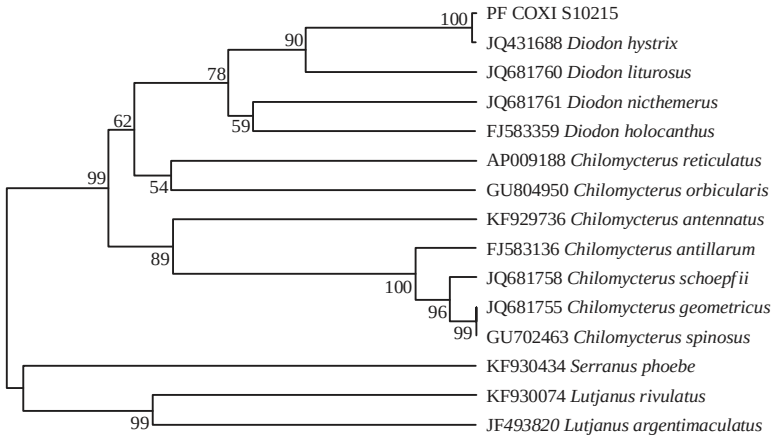

Fig. 1: Phylogenetic tree indicates the sample to be *Diodon hystrix*, with a boot strap value of 100 (GenBank No. KP876559)

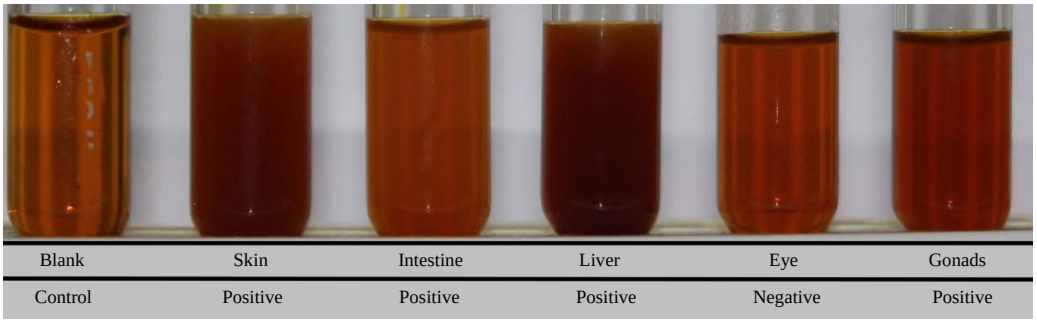

Fig. 2: Results of Dragondorff's test of different organs extracts of *Diodon hystrix*

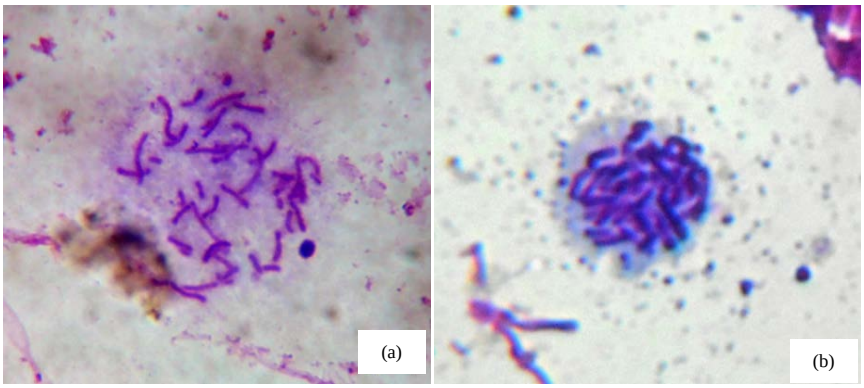

Fig. 3(a-b): Genotoxicity of TTX on human leukocyte culture (a) Control and (b) Spindle fibre aberration

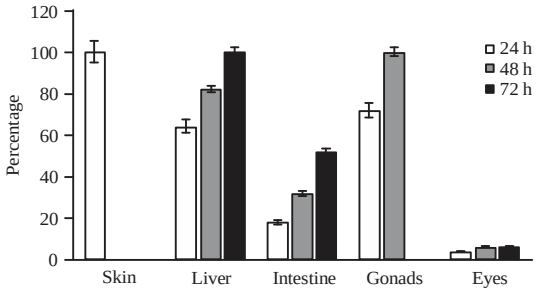

Fig. 4: Toxicity assay on zebra fish using different organs extracts of *Diodon hystrix*

clustering of chromosomes in extract treated cells. Concentration of TTX could have been very low in intestine and gonads.

The comet assay displayed no tail formation, confirming that there is no DNA damage caused by the extracts on the human leukocyte cells.

Zebra fish toxicity assay demonstrated highest toxicity for skin, liver and gonads. Skin extract showed 100% mortality in less than 24 h. Gonad extract showed 100% mortality in less than 48 h. Liver demonstrated 100% mortality in 72 h. Intestine and eye showed low mortality. Figure 4 shows the graphical representation of zebra fish toxicity.

DISCUSSION

Based on the morphological characteristics it was confirmed that the collected fish was black spotted porcupine fish. The genomic DNA obtained from the fish tissue was successfully amplified by PCR reaction to yield a product ~680 bp. The PCR product was sequenced and with the help of Clustal W, phylogenetic tree was generated (Fig. 1). Phylogenetic tree analysis revealed that mitochondrial DNA showed high similarity to *Diodon hystrix*, with a boot strap value of 100. The BLAST search displayed several COX1 region sequences of *Diodon hystrix* sequences with 99% similarity. Gene sequence was submitted in the NCBI GenBank database and KP876559 was the given accession number. Previous reports such as, Radhakrishnan and Nair (1981) and Hafeezullah (1988) has reported the presence of *Diodon hystrix* in the Arabian sea. Although, these reports did not provide a molecular level characterization, the pictorial representation of the studied *Didon hystrix* by Radhakrishnan and Nair (1981) was a perfect match to our sample. This phylogenic characterization is a first molecular level report, in addition to the previous reports of *Diodon hystrix* in Indian coast (Radhakrishnan and Nair, 1981; Hafeezullah, 1988).

Tetrodotoxin alkaloid toxin was identified by Dragondorff's test (Fig. 2). The test showed strong precipitation for skin and liver, moderate precipitation for intestine and gonads and no precipitation for eye crude extracts, respectively. Saito *et al*. (1987) used similar method in his investigation of evaluation of TTX. The results obtained in this study matched with the reports of Saito *et al*. (1987).

Human leucocyte cells demonstrated spindle fibre aberration in samples treated with skin and liver extracts (Fig. 3). The chromosomes were in a cluster while the control slides had well spread chromosomes. The clustering of chromosomes is a characteristic feature for spindle fibre aberration. Khora *et al*. (1997) demonstrated the genotoxicity of tetrodotoxin (TTX) extracted puffer fish *Arothron nigropunctatus* on the root meristem cells of *Allium cepa* where TTX inhibited mitosis at concentrations of ≥30 µM. This study showed that TTX caused spindle fibre aberrations and promotes Sister Chromatid Exchange (SCE) without any DNA breakages. Similarly, in the present study comet assay did not show any tailing in the slides suggesting that the crude extract does not cause any DNA damage to cells. Report by Khora *et al*. (1997) supports our study, that the spindle fibre aberrations observed in skin and liver crude extract treated cells, could be due to increased concentrations of TTX.

The effect of TTX in crude extracts on zebra fishes proved to be toxic. Skin, liver and gonad extracts were highly toxic showed 100% mortality within 72 h (Fig. 4). Crude extract from intestine was also toxic but was lower (52%) than skin, liver and gonads. Eye extracts showed 6% mortality among all the other, it demonstrated lowest toxicity. The toxicity analysis on zebra fish revealed, skin and gonad extracts of *D. hystrix* were extremely toxic. According to Mohid Nor *et al*. (2014) among the different tissues of puffer fish, liver had highest toxicity, followed by muscle and skin. Similarly, present study also reports highest toxicity in liver and skin, although, skin seemed to be greatly toxic than the liver. According to Alcock (2010) the ovary in an adult puffer fish has the highest concentration of toxin during reproducing season as the ovary accumulate more TTX transferred from the liver resulting in higher toxicity. Mohd Nor and Wan Norhana (2013) reported the presence of TTX and STX in dried puffer fish eggs. This report could also support this study that, the increased toxicity in gonads of *Diodon hystrix* could be due to presence of STX, although, it needs further studies to confirm the presence of STX in *Diodon hystrix*. So, based on reports by Mohd Nor and Wan Norhana (2013) it suggested that, the increased toxicity of gonads of *Diodon hystrix* could be due to presence of both TTX and STX.

Malpezzi *et al*. (1997) reported TTX on skin of *D. hystrix* which act as a repellent against predators. Nagashima *et al*. (2012) reported the toxicity of liver sample of *Lagocephalus inermis* and TTX was detected in the sample determined by LC-MS study. Wu *et al*. (2011) reported TTX poisoning in sea food. After ingestion of *Chelonodon patoca* puffer fish, the victim showed symptoms like perioral paresthesia, nausea, vomiting, ataxia, limb weakness, respiration failure and death within several hour. The urine, bile, cerebrospinal fluid, pleural effusion and pericardial effusion of the victim showed TTX. In recent years palytoxin (PlTX) compounds in fish, crustaceans, molluscs and echinoderms have been raising scientific concern. Aligizaki *et al*. (2011) reported PlTX in freshwater puffer fish.

CONCLUSION

Analytical study of the organ extracts proved the presence of TTX in skin, liver, intestine and gonads. Genotoxicity assay demonstrated that crude extracts of the *Diodon hystrix* organs causes spindle fibre aberrations. The genotoxic studies and zebra fish toxicity assay report was relevant in determining the harmfulness of consuming puffer fish. These scientific finding would raise the awareness among the public about the seafood disadvantages. This study gives the molecular confirmation of *Diodon hystrix* presence in Indian coast. The black spotted porcupine fish collected from Chennai, Tamil Nadu coast has been confirmed to contain TTX. Among the different organs of the fish, skin liver and gonads has been found to be highly toxic. The TTX has been confirmed by GC-MS analysis. Genotoxicity analysis using human leukocyte cell culture, demonstrated spindle fiber aberration. Comet assay using human leukocyte cells did not show any DNA damage. Zebra fish toxicity study was performed to analyze the toxicity of individual organ extract. This concludes that, *Diodon hystrix* present in Tamil Nadu coast is toxic and is not suitable for culinary purpose.

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