e-ISSN: 2455-667X

Annals of Natural Sciences
Vol. 1(1), December 2015: 32-38
Journal's URL: http://www.crsdindia.com/ans.html
Email: crsdindia@gmail.com

Annals of Natural Sciences

ORIGINAL ARTICLE

Extraction and Identification of toxin present in *Tetrodon cutcutia* (Ham) found in the River Brahmaputra and its tributaries, Assam, India

T.G. Devina[1], Tikendrajit Gogoi[2] and J.K. Nath[3]
Department of Zoology, D.K.D. College, Dergaon, Assam-785614, India
Email: [1] devinatg@yahoo.co.in, [2] tikengogoi@gmail.com, [3] mailtojugalnath@gmail.com

ABSTRACT

Tetrodotoxin is a highly poisonous, non-proteinous, neurotoxin. It is available in the ovary, liver and skin of Tetrodon found in the marine water. The name derives from Tetrodontiformes, an order of bony fishes. Besides these fishes, Tetrodotoxin is also found in (a) gastropod mollusc (b) egg of Horse shoe crabs(c) Newts of genus Taricha etc. We have studied the fresh water tetrodon i.e. Tetrodon cutcutia available in the River Brahmaputra, Assam, India. The extraction is made from liver, ovary and skin of T. cutcutia, isolated partially by TLC & then mouse bioassay is determined. Further analysis of the extract using NMR, IR , HPLC & ELISA techniques reveals that the compound predominant in these extracts contain Amine, Amide, = bonds, -C-C- stretch, allylic groups, etc. Another contrasting feature of the extracted derivatives of the compound is that it is toxic with certain brittle characteristics. The chief compound is Saxitoxin and its closely linked derivatives, which may vary from sample to sample. The compound may undergo some physical changes irreversibly upon application of some pressure but most of the basic chemical compositions are likely to remain intact with certain exceptions. The various forms of Saxitoxin are formed while developing from pyrimidine to Tetrodotoxin ring.

Key words: *Tetrodotoxin; Saxitoxin; Pyrimidine; Tetrodon cutcutia; HPLC; NMR*

Received: 26th Nov. 2015, Revised: 29th Nov. 2015, Accepted: 30th Nov. 2015

How to cite this article:
Devina T.G., Gogoi T. and Nath J.K. (2015): Extraction and Identification of toxin present in *Tetrodon cutcutia* (Ham) found in the River Brahmaputra and its tributaries, Assam, India. Annals of Natural Sciences, Vol. 1[1]: December, 2015: 32-38.

INTRODUCTION

Tetrodotoxin (TTX) is a potent neurotoxin which is found in the liver, ovary, testes & skin of Tetrodon and other certain group of animals. The name Tetrodotoxin is derived from Tetrodontiformes, an order of bony fishes. TTX is also found in gastropod mollusc, egg of horse shoe crabs, newts of genus Taricha, the skin of atelopid frog, skin & viscera of blue ringed octopus and some species of salamander (Noguchi & Miwazawa, 2005). According to Lee (2007), bacteria inside these animals make the toxin; which include strains of the family Vibrionaceae, Pseudomonas species and *Photobacterium phosphoreum*.

TTX is a very deadly toxic neurotoxin. It blocks the sodium channels and thereby prevents nerve conduction (Narahasi *et al.,* 1967; Kao, 1982). TTX administered animals exhibits paralytic behavior, slow movement of its appendages and ultimate death (Devina *et al.,* 2014). According to William H. Light "It is 10 to 100 times deadlier than cyanide". The estimated lethal dose for an adult person is one to two milligram (Yuen and Tang, 2007).

Tetrodons are found in marine, brackish & fresh water. *T. cutcutia* is a fresh water fish found in the river Brahmaputra, its tributaries & beels. Different puffer fishes found in various localities have varied in accumulation of Toxin in their body. (Mar Drugs, 2008).

ST= strongly toxic (>1000 MU/g tissue); MT= moderately toxic (100-1000 MU/g tissue); WT= weakly toxic (>10 MU/g tissue); t =<10 MU/g tissue; (-) =No data available.

MU is defined as the amount of toxin that kills a male mouse of 20 gm body weight in 30 minutes after intraperitoneal administration. No detail literature is available regarding toxin found in the body of *T. cutcutia.* The table 1 indicates the variety of *Tetrodon*s living in marine or brackish water found in different parts of the world. Researchers conducted on the *Tetrodon* in the rivers of Bangladesh are also fishes of brackish water contain TTX (Ahasan *et al.,* 2005).

The Tetrodon found in Brahmaputra valley are less poisonous than that of the species of this fish found in the ocean. We have found that cockroaches die when the extract is fed mixing the extract with honey applying on biscuits (Devina *et al.,* 2014).The aphis infested on flowers & legumes die 100%within 72 hours when sprayed on them. A 16 gm mouse dies within one hour after administration of 0.04 ml extract peritoneally.

In our experiment it is observed that the extract from fresh water *Tetrodon*, have the toxic effect but not as much as the marine fish. Therefore we have to analyze the composition of the compound present in *T. cutcutia.* "The presence of this poison has been known through its effect since antiquity, but its labial nature & it's extremely low concentrations in its natural milieu made the isolation of the toxic principle extraordinary difficult". This determination of the natural composition of Tetrodotoxin is a matter of very great difficulty. The toxin retains solvents very tenaciously; particularly moisture and other hydroxylic materials, and the presence within its molecule are large number of-OH and-NH groups. In these circumstances direct analysis for the elements gave only an approximate idea of its composition. The best conclusion available until a relatively recent in the structural investigations was that the composition of Tetrodotoxin lay within the limits defined by the expression $C_{10\text{-}12}H_{15\text{-}19}O_{8\text{-}10}N_3$ (Woodward, 1964).

In this experiment we have tried to analysis the chemical compound found in the extraction of *T. cutcutia* based on the above facts.

MATERIAL AND METHODS

*Tetrodon*s are collected from the beels of the river Brahmaputra. The large *Tetrodon*s are stored in the aquarium in the laboratory and the small Tetrodons are left in the pond for rearing. Livers, ovaries and skin are dissected out of the fishes and kept separately. Each of the organs is measured and added 1.5 times of the 0.5% acetic acid and homogenized at 30,000RPM.The homogenized materials are centrifuged at 20,000 RPM for 30 minutes. The supernatant is separated and pH of the solution is adjusted of 7.4 by adding Ammonia water. The process is conducted for each of the extract i.e. Liver, ovary and skin.

The solution is heated up to 95^{o}C in a magnetic stirrer to precipitate the sclera protein of the protein part and separated through ion-exchange resin. The filtrate is passed through a column of activated charcoal. Filtrate is kept stored. The activated charcoal in column is washed with 0.03 N of acetic acid. Both the filtrate are mixed and applied on mouse and insect pest to access the toxicity. Further the extraction is used for chemical analysis and identification of the compound present in the extract separately for liver and ovary. We have conducted chemical extraction of two samples; the first extraction is prepared from the fishes collected during September and October/2014 and the 2nd extraction is done from the fishes collected in the month of December/2014.

OBSERVATION AND DISCUSSION

Three mices are found to die after one hour of administration of 0.04 ml of the extract peritoneally. This indicates the toxin contain > 10 MU/g tissue.

A. Preliminary activity & identification:

a. From the crude observation and its basic initial experimentation, it looks like that the liquid extract may contain multiple compounds due to contrasting properties. Also the first requirement is to know whether the extract is a single compound or is it a mixture of compounds before applying instrumentation for identification. So the

imperative need is to find a suitable solvent for the extract and after testing many solvents in this aspect, it is found that acid water in equivalent proportions suits the best. The proportions of both the acid and water needs to be adjusted according to the requirement at the time of experiment which varies from sample to sample.

b. The next step is to find a suitable method to separate the compounds present in the extract. The separation is pertinent at this point as initial studies points to the presence of multiple compounds and we need to ascertain the credibility of the results before further proceeding in this aspect.

c. It may also happen that a particular compound is predominant in the liquid extract with many of its derivatives and their structures vary little in their typical bonding. The contrasting properties showed initially may be attributed to such derivatives of the chief compound.

d. If it would have been a single compound, then we can directly proceed for chemical analysis.

e. Two suitable methods for separation are –HPLC (High Performance Liquid Chromatography) & ELISA (Enzyme-linked Immunosorbent Assay).

f. In the next step, we have conducted NMR for chemical shift, ppm (parts per million determination in the purified of Liver (**Fig.1**) and ovary (**Fig.2**)

g. In the NMR (Ovary) attachment (Fig.2), shift @4.676 indicates that '**=**' (double bond) is confirmed in one of the compounds. Also shift @1.756 clearly indicates the presence of amine groups (may be either aliphatic or aromatic).

h. The other sample has also indicates similar characteristics with slight deviation (Fig.1), one strong peak @4.605/4.443 and one comparatively lower @1.713. There is also the possibility of the presence of a –ph OH / -CHOH group. (Fig.2)

i. After getting it separated we have used IR Spectroscopy also to determine the functional groups present in the compound in the extract. Stronger bonds and light atoms will vibrate at high stretching frequency (wave number).

B. Analysis Report Using Instrumentation after HPLC & ELISA:

The first confirmation is that the extract is not a single compound. It is a conglomeration of multiple compounds with $-NH_2$, -NH and double bonds.

NMR studies showed strong peaks @4.605 & @4.443 respectively. Peak @4.605 indicates –C=C-H (vinylic group) which may be from different derivative structures and the other peak @4.443 indicates -HC- R (e.g. R = - CH_3) group may be present.(Fig.1)

Another low intensity NMR peak Fig.1,@1.713 indicates the presence of C=C-CH_3 (allylic group) may be present. As the intensity of the peak is weak, this group may be present only in some of the derivatives of the main compound.

Fig. 1: NMR for chemical shift in the purified of Liver

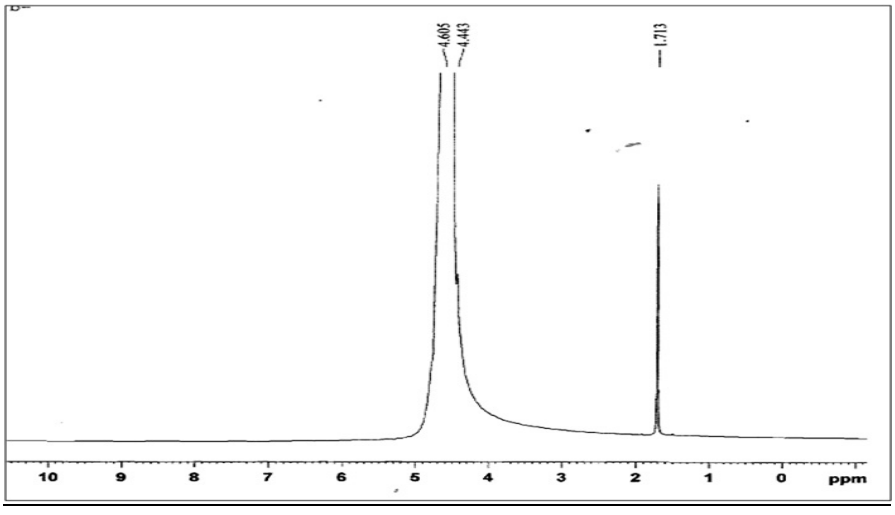


Fig. 2: NMR for chemical shift in the purified of Ovary

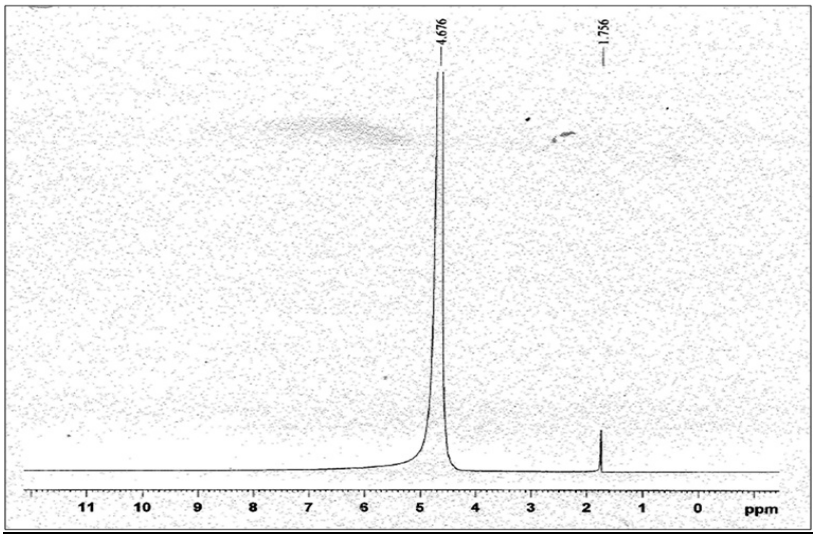
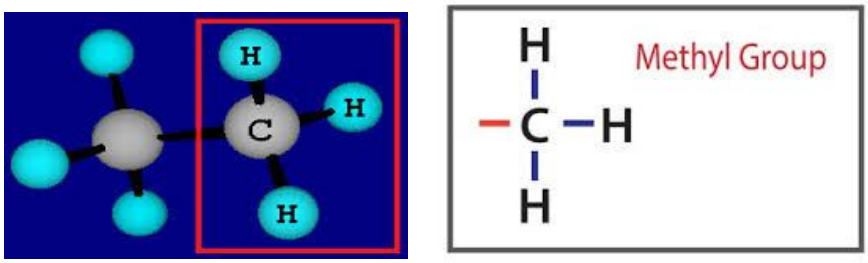


Fig. 3: (A). Methyl C-H bnding **(B).** -C-H bonding with other alkyl, allyl or phenolic group



Application of IR spectroscopy particular group frequency Cm^{-1} @3640-3610 shows the presence of an alcoholic /Phenolic group which is free hydroxyl. 3N-H IR frequency Cm^{-1} @ 3371 shows the presence of amines & amide. Since amines & amides are present, it has some basic characteristics. The compounds present are toxic with little brittle characteristics. It may undergo some physical changes when exposed to heating but the chemical composition remain almost the same expect some minor single bond to double bond conversion.

CONCLUSION

From the above result it confirms that the extraction made from liver, Ovary and skin of *Tetrodon cutcutia* available in the river Brahmaputra contain Toxin. The toxin found in *Tetrodon cutcutia is* not as much as toxic that are available in the puffer fish in marine water. The presences of toxin vary according to the seasons change. The basic structure of the toxin is pyrimidine which is transformed in to Saxitoxin and then to Tetrodotoxin within the body. During transformation different forms of the toxin are formed and ultimate compounds developed to Tetrodotoxin. The chief chemical compound present in the extract that we have analyzed from liver and Ovary is considered to be various forms of Saxitoxin which varies sample to sample. We have analyzed two structures extracted at different times one at September and other at December. Therefore it shows two structures of Saxitoxin. Various forms of Saxitoxin are formed while developing from Pyrimidine ring to Tetrodotoxin ring. The development of compound may be expressed as below.

Table 1: Mar Drugs

Family	Habitat	Species	Maximal toxicity					
			Ovary	Testis	Liver	Skin	Intestine	Muscle
Tetradontidae	Marine	Japanese pufferfish						
		Takifugu niphobles	S T	W T	S T	M T	S T	W T
		T.poectionotus	S T	M T	S T	M T	M T	W T
		T.paradalis	S T	W T	S T	M T	M T	t
		T.snyderi	S T	t	S T	M T	M T	W T
		T.porphyreus	S T	t	S T	M T	M T	T
		T.chinensis	S T	-	S T	-	-	-
		T.obscurus	S T	t	M T	M T	M T	T
		T.exascurus	S T	t	M T	M T	-	T
		T.pseudomnus	S T	t	W T	W T	W T	T
		T.chrysops	M T	t	M T	M T	W T	T
		T.vermicularis	M T	t	M T	M T	W T	T
		T.rubripes	M T	t	M T	T	W T	T
		T.xanthopterus	M T	t	M T	T	W T	T
		T.stictonotus	M T	t	M T	W T	t	T
		T. alboreticulatus	S T	-	W T	W T	M T	W T
		Pleuramacanthus sceleratus	S T	-	W T	W T	M T	W T
		Chelonodon patoca	M T	M T	M T	S T	-	M T
		Arothron firmamentum	M T	t	T	W T	t	T
		Canthigaster rivulata	t	-	W T	M T	W T	T
		Lagocephalus lunaris	t	t	T	M T	t	S T
		L.inermis	t	t	M T	T	t	T
		L.wheeleri	t	t	T	T	t	T
		L.gloveri	t	t	T	T	t	T
		Sphoeroides pachygaster	t	t	T	T	t	T
Marine	Marine	Chinese pufferfish						
		Takifugu flavidus	S T	M T	S T	M T	M T	W T
	Brackish	Thai pufferfish						
		Tetrodon nigroviridis	-	-	T	M T	W T	W T
		T.steindachneri	-	-	T	M T	t	T
Diodontidae	Marine	Japanese pufferfish						
		Diodon holocanthus	t	-	T	T	t	t
		Chilomycterus affinis	t	-	T	T	t	t
Ostraciidae	Marine	Japanese pufferfish						
		Ostracion immculatum	t	t	T	T	t	t
		Lactoria diaphana	t	t	T	T	t	t
		Aracana aculeuta	t	t	T	T	t	t

Fig. 4: Transformation from pyrimidine ring to Tetrodotoxin ring

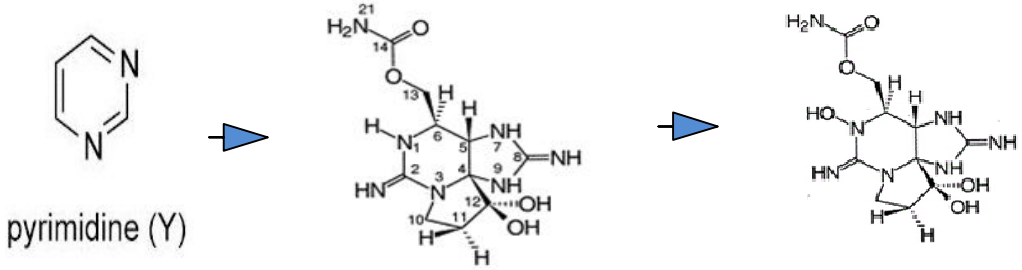


Fig. 5: The three dimensional structure of the toxin

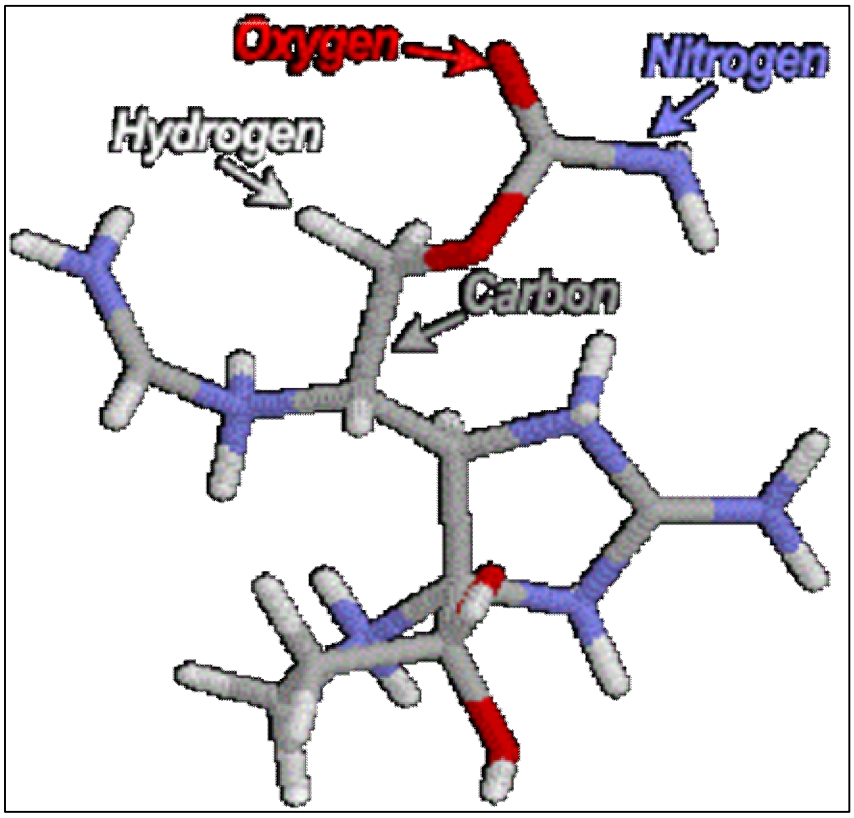
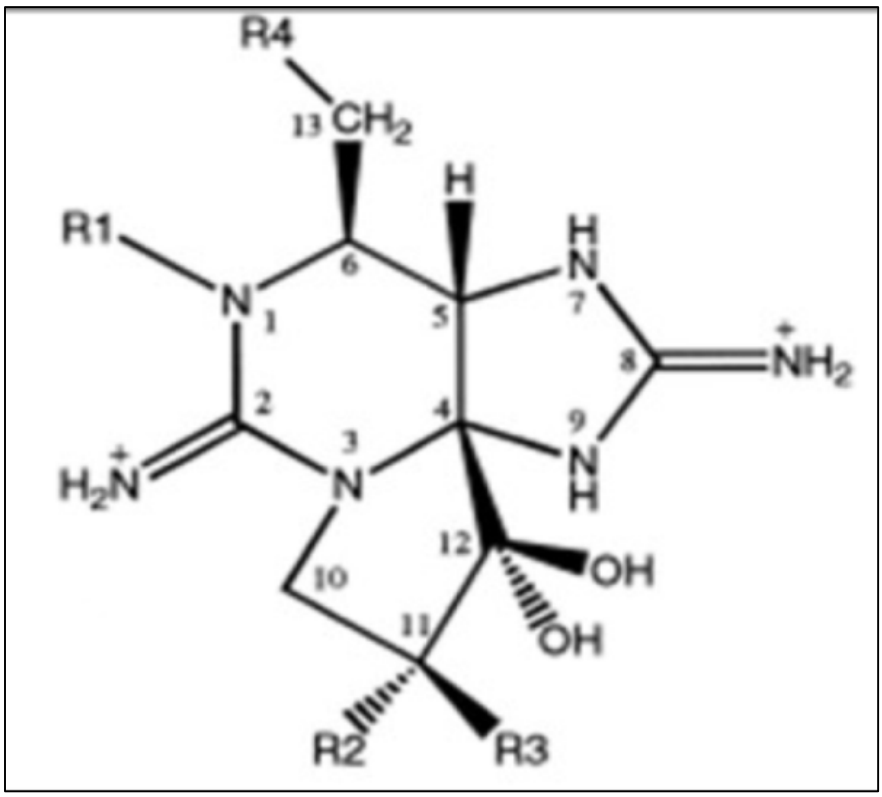


Fig.6 The near accurate structure of the compound



ACKNOWLEDGEMENT

We sincerely express our gratitude to researchers in IIT-Delhi and J.N.U. in their endeavour towards supporting us throughout the entire research work with their valuable inputs whenever required. We are also thankful to UGC, New Delhi for providing us financial support.

REFERENCES

1. Ahasan H.A.M.N., Mamun Al. A, Rasul C.H. and Roy K.P. (2005): Puffer fish poisoning (Tetrodotoxin) in Bangladesh; Clinical profile and role of anticholinesterase drugs. Tropical doctor; Academic journal. 35: 235.
2. Devina T.G, Gogoi T. and Nath J.K. (2014): Extraction of Tetrodotoxin from puffer fish (T*etrodon cutcutia*) and its effect on cockroaches (*Periplaneta americana*), Indian J.L.Sci., 3: 99-104.
3. Kao C.Y. (1982): Actions of nortetrodotoxin on frog muscle and squid axon. Toxicon. 20: 1043-1050.
4. Lee C. (2007): Adaptive Tetrodotoxin-Resistance in Garter snake sodium channel.
5. Narahashi T, Moore J.W. and Poston R.N. (1967): Tetrodotoxin derivatives: chemical structure and blockage of nerve membrane conductance. Science. 156: 976-979.
6. Noguchi T. and Miyazawa K. (2005): Distribution and origin of Tetrodotoxin. Toxin Reviews. 20:11-33.
7. Woodward R.B. (1964): The structure of Tetrodotoxin. Pure Appl. Chem. Harvard University, Cambridge, Massachusetts, USA. 9: 49-74.
8. Yuen T. and Tang A. (2007): Tetrodotoxin poisoning. Food safety focuses, 9th issue, April, 2007.