

EXTRACTION OF TETRODOTOXIN FROM PUFFER FISH (*Tetrodon cutcutia*) AND ITS EFFECT ON COCKROACHES (*Periplaneta americana*)

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ABSTRACT

Tetrodon cutcutia is a fresh water fish available in the beels of the River Brahmaputra. It contains a non-proteinous toxin called tetrodotoxin. Collected from Dhemaji beel near the southern bank of Brahmaputra, the liver, muscle and skin of the fishes showed different lethal potency when administered on cockroaches. Crude extraction of tetrodotoxin from different tissues separately administered with food such as honey & sugar indicates rate of mortality differently. The contaminated food with the extraction of liver & skin causes mortality of cockroaches of same age group, 90% and 80% respectively at stipulated time. Extractions of muscles have little impact on cockroaches. The extractions are made in simple and crude method. The liver, muscle and skins are separated from live *Tetrodon* and homogenized and centrifuged at 20,000 RPM for 15 minutes. Mixing the tissues with 1.5 times of the weight of the tissue with double distilled water containing 0.5% acetic acid. The supernatant is heated upto 90°C stirring continuously to separate the protein contents present in the supernatant. Now the filtered solution is treated as tetrodotoxin. The composition of the toxin & purification is yet to be ascertained.

KEYWORDS : Tetrodotoxin, *Tetrodon cutcutia*, and Effect on Cockroaches (*Periplaneta americana*)

In biological research, plant products are commonly used to observe the effect on animals, but uses of animal product to animal is rare phenomenon. It is well known to all that certain chemical products which are produced by animals are beneficial to human being and certain products which cause tremendous harm to human, even death.

Tetrodotoxin (TTX) is also a toxic substance secreted mainly by *Tetrodon* & other certain group of animals. The name derives from tetrodontiformes an order of bony fishes, which includes globe fishes, porcupine fishes, *tetrodon*, ocean mola and tiger fishes etc. Besides these fishes tetrodotoxin is also found in (a) gastropod mollusc (b) egg of horse shoe crabs (c) Newts of genus *Taricha* (d) the skin of atelopid frogs (e) Skin and viscera of blue ringed octopus and some species of salamanders (Noguchi and Miyazawa, 2005).

To date little is known regarding the biosynthesis of TTX. No one has isolated enzyme responsible for the biosynthesis there are two ideas regarding the synthesis of tetrodotoxin.

It is mentioned above that tetrodotoxin is found in different groups of animals. According to Lee (2007), bacteria inside these animals make the toxin. These bacterial species, include strains of the family vibrionaceae, *Pseudomonas* species and *Photobacterium phosphoreum*. According to these ideas Puffer fish grown in culture do not produce tetrodotoxin. The blue ringed

octopus accumulates tetrodotoxin in a special salivary gland and infuses its prey with toxin by bite (Sheumack et al., 1984). Xanthid crabs contain tetrodotoxin (Noguchi et al., 1983). Tetrodotoxin in algae species *Jonia* is produced by a bacteria species *Alteromus*. The TTX producing bacteria and host organisms offers advantages to both partners, the bacteria gets a safe place to live, eat & reproduce and the host use the toxin for predation or defence. The blue ringed Octopuses employ TTX as a potent venom for securing prey (Johnson, 2002).

Tetrodotoxin is a very deadly toxic neurotoxin. Pure tetrodotoxin is a colourless, crystalline powder. It darkens above 220° C without decomposition. It blocks the sodium ion channels and thereby prevents nerve conduction (Narahashi et al., 1967; Kao, 1982). Tetrodotoxin administered animals exhibit paralytic behaviour, slow movement of its appendages and ultimate death. The list of evidences of Toxicity cases of TTX poisoning and death is long. The following event a) The deadly blow fish has been feared for thousands of years, b) The tomb of an Egyptian Pharaoh was engraved with blow fish image, c) The Bible warns against eating fish without fins and scales like the red sea puffers, d) Fugu (puffer fish) has been eaten in china and Japan. It is one of the most expensive foods in Japan (Davidson, 2006). A single fish cost \$ 200. Fugu chefs in Japan must pass a test and eat the fugu that he/she has prepared and only 25% of the applicant can pass a test; are the supporting facts. According to The Guardian Associated

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Press (2009) Weight for weight, Tetrodotoxin is ten times as deadly as the venom of banded krait. It is 10 to 100 times deadlier than cyanide. The estimated lethal dose for an adult in person is one to two milligram (Yuen & Tang, 2007). The question arises why tetrodotoxin produced by bacteria does not affect the organism which carries it. “ A single point mutation in the amino acid sequence of the Sodium-ion-channel makes it immune from being bound by TTX, TTX will not recognise the Channel in these organisms” (Lee & Ruben, 2008). The only known predator resistant to this toxin is the common garter snake (Lee, 2007).

Tetrodons are adorable looking with comical chubby. They are particular, curious and known as puffer fish because they have the ability to gulp air or water puffing up until they look like a round ball. The puffer fish are found in three different habituation i.e. marine, brackish water and fresh water. *Tetrodon cutcutia* is a fresh water fish found in the beels of river Brahmaputra. Besides Amazon puffer, fahaka puffer, golden puffer, Malabar puffer, pig nose puffer, spotted green puffer are also fresh water fish. Approximately 360 species puffer fishes are found most are marine less than 40% are brackish water, and only 29% species are truly fresh water.

Tetrodon cutcutia available in different beels of river Brahmaputra contain Tetrodotoxin. Generally these fishes are not in the list of edible fishes of Assamese society. But certain tribes of Mishng community use this fish along with other fishes as food. There are few cases of oseen bemar (Undetectable/ non-diagnosed disease) leads to death. Especially when they prepare 'namshing'(a method where fishes of same type dried and preserved) and eat . Interestingly those who suffered from oseen bemar had taken *Tetrodon cutcutia* (Gonga tup- Assamese; Dalli-pumpuh- Mishng) as namshing in large quantity. Domesticated duck ,hen, have died ,if fed Tetrodon. Crows don't take this fish .

There are some experiences of having toxicity in this fish by Dr. Mrs R. Barua using this toxin on house fly, which indicated positive results. In our experiment we have used the raw extracts of different parts of *Tetrodon cutcutia* on cockroaches to observe the variation in accumulation of toxins on different parts of Tetrodons.

MATERIALS AND METHODS

Collections of Tetrodon - *Tetrodon cutcutia* are collected from the beels of the river Brahmaputra available in the area. Tetrodons can be captured when flood water are recessed or when the fishes at the beels are captured extensively for commercial purposes. Tetrodons are collected from net along with other fishes in living condition and they are stocked in a pond in college campus. Large fishes are captured from the stock pond and kept in aquarium.

Large Tetrodons are dissected and liver, muscle and skin are separated. If ovary of the fish is found to be developed, it is also taken for processing. The dissected materials area weighted and 20grams of each material are taken in a beaker. 200ml of distilled water and 2ml of 0.5% of acetic acid is put into the beaker and homogenized at 30,000 RPM. The mixture is centrifuged at 20,000 RPM for 20 minutes. The supernatant is separated and it is warmed up to 95 degree Celsius on magnetic stirrer for at least half an hour. By this process the protein part in the solution are coagulated and separated by whattman No. 1 filter paper. The filtrate is considered as raw tetrodotoxin extract herein after called as extract. The pH of the extract is measured and maintained at in between 7-8.

Preparation of Experimental Specimen

Rearing of cockroaches- large numbers of mature male and female cockroaches are kept in large cages where abundant food and hiding places are provided to them. At one week interval the cages were checked and a sizeable number of oothecae were collected from the cages. The oothecae are kept in separate cages and they are allowed to hatch. Thus several batches of oothecae are formed and kept separately containing 60 to 70 oothecae. The net applied to the cages are thick so that hatched nymphs cannot go out of the cage. After hatching, each group of nymph was considered as similar age group and general food such as biscuits, cake, honey and water were supplied to them. The cockroaches take much time to attain maturity. After 12 moulting the cockroaches attain adulthood at 13th instar.

The rearing cockroaches are kept starved at least for one week. These cockroaches are transferred to separate cages where no other food are supplied except biscuit along

with honey mixed with extract of liver or skin or muscle so that the sample cockroaches have no option but to intake the contaminated food. In our experiment we could not use extraction from ovary. We have taken 30 cockroaches as one experimental unit in a cage and they were given extract mixed with honey or biscuits. After minute observation it is found that the starved cockroach eats/takes the mixed food.

The experiment were conducted for each material is extraction from liver, muscle and skin and were treated on different groups of cockroaches of the same age group. This is one type of treatment.

Another type of treatment was conducted by direct inserting of the crude extract by syringe. 0.2ml of extraction was injected through arthroial membrane of pleuron region of each cockroach and left in the cage. Extract of liver, muscle and skin were injected & left for observation.

The third type of experiment was conducted by spraying the crude extract of liver, skin and muscle on cockroaches.

There are certain problems regarding collection, carrying of the fishes. The *Tetrodons* die immediately if the water temperature rises. Further regarding treatment on cockroaches, it is found that as the cockroaches are omnivorous, it may not eat the food. Therefore it is necessary to keep the cockroaches starved before fed with the extract of mixing food. In case of in pushing of extraction it is taken care that the cockroaches do not get mechanical injury.

RESULTS

A. *Tetrodon* liver extract treatment

Crude liver extract employed directly by pushing injection. The cockroaches are injected with 0.2ml of crude extraction of liver of *Tetrodon*. The experiments were conducted in three batches of cockroaches of same age group. The average life span of treated cockroaches is found to be 48-96 hours. The treated cockroaches exhibit the following behaviour-

1. The movements of the cockroaches are slowed.
2. The antennal movements are stopped first.
3. After 18-20 hrs of treatment the cockroaches cling to the walls of cage and stop eating food.

4. Convulsion of different body parts is observed.
5. When disturbed the cockroaches move slowly.
The cockroaches ultimately become stiff and died.

Table 1: Observation on effect of Tetrodotoxin when injected

Sl. No.	Treatment Material	Amount used by injection (in ml)	No.of specimen treated	0-24 hr	24-48 hr	48-72 hr	72-96 hr	96-144 hr	144-168 hr	168-194 hr	194-218 hr	218-242 hr	242-266 hr	266-290 hr	290-314 hr	314-338 hr	338-362 hr	362-386 hr
1	Liver	0.2	30	0	3	27	0	0	0	0	0	0	0	0	0	0	0	0
2	Liver	0.2	30	2	7	16	5	0	0	0	0	0	0	0	0	0	0	0
3	Liver	0.2	30	0	5	19	4	2	0	0	0	0	0	0	0	0	0	0
4	Muscle	0.2	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	Muscle	0.2	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	Muscle	0.2	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	Skin	0.2	30	0	0	0	0	0	0	2	3	8	11	6	0	0	0	0
8	Skin	0.2	30	0	0	0	0	0	0	0	0	4	9	12	5	0	0	0
9	Skin	0.2	30	0	0	0	0	0	0	0	1	6	13	6	3	1	0	0

Table 2: Observation on the Effect of Tetrodotoxin When Mixed With Food

Sl. No.	Treatment Material	Amount used with honey	No.of specimen treated	0-24 hr	24-48 hr	48-72 hr	72-96 hr	96-144 hr	144-168 hr	168-194 hr	194-218 hr	218-242 hr	242-266 hr	266-290 hr	290-314 hr	314-338 hr	338-362 hr	362-386 hr	368-410 hr
1	Liver	5ml	30	0	0	0	0	1	4	3	11	8	1	0	0	0	0	0	0
2	Liver	5ml	30	0	0	0	0	0	3	2	8	11	0	6	0	0	0	0	0
3	Liver	5ml	30	0	0	0	0	2	0	2	6	4	7	5	4	0	0	0	0
4	Muscle	5ml	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	Muscle	5ml	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	Muscle	5ml	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	Skin	5ml	30	0	0	0	0	0	0	0	0	0	0	0	2	5	6	6	0
8	Skin	5ml	30	0	0	0	0	0	0	0	0	0	0	0	1	6	2	2	0
9	Skin	5ml	30	0	0	0	0	0	0	0	0	0	0	0	1	5	2	2	0

B. Crude Liver Extract Employed With Food Mixed With Honey and Biscuits

5ml of crude liver extract mixed with 1gm of honey and 1gm of sugar and the mixture is spread on biscuits and given as food to the cockroaches. It is observed that the cockroaches eat the biscuits regularly at least for three days, but after 4 day they become reluctant to eat the food.

The behavior of the cockroaches appeared as mentioned above but develops slowly. But most significantly the antennal movement reduction is appeared first.

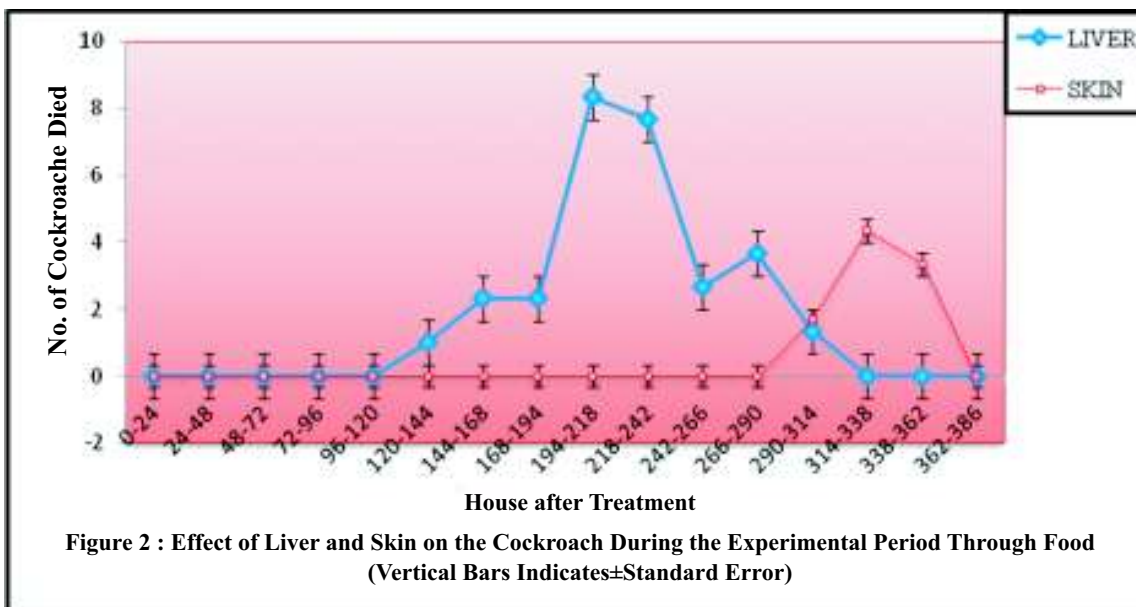
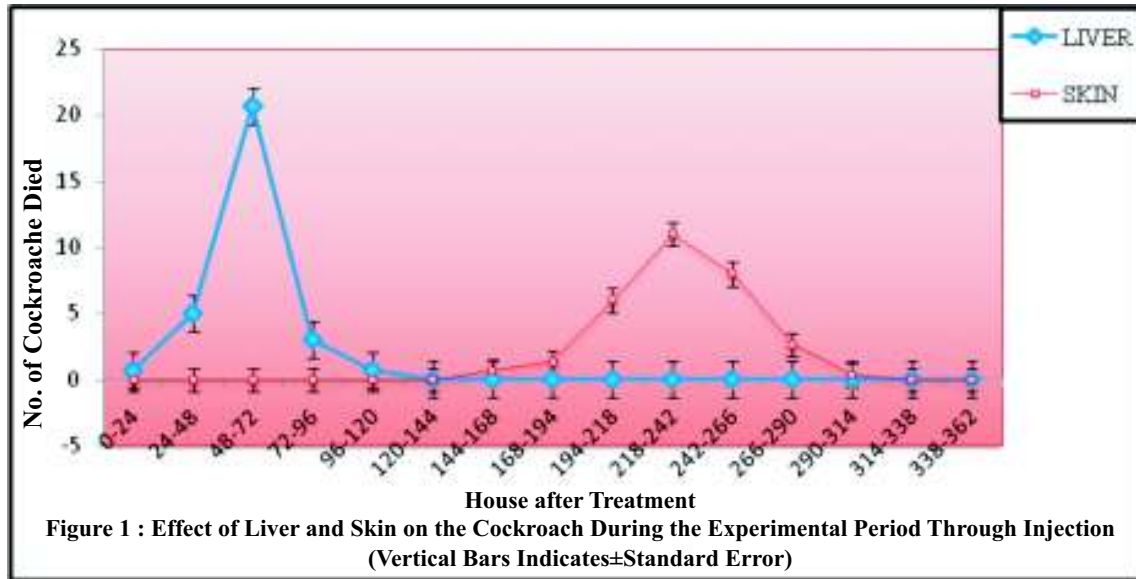
Same types of experiments have been conducted with the extract of muscle and skin. The data sheets are given in the table 2 respectively.

Most interestingly the cockroaches injected with muscle shows no adverse behavior like the cockroaches treated with liver. These cockroaches remain like as untreated one. The cockroaches those which are injected with extract of skin have shown no adverse behavior but after 5th day of injection it appears some behaviors mentioned above. From the above data sheet it is found that the cockroaches those were injected with muscle extract, and sprayed with extract of liver, muscle and skin have shown no toxic effect.

It indicates that the cockroaches died after effect of tetrodotoxin present in the extract. Figure 1 indicates that the mortality of cockroaches is highest in the period of 48-72 hours of treatment of liver extract. And in case of extract of skin, it is highest in between the period of 218 to 242 hours of treatment. Similar result is found from figure 2 in case of liver and skin when given as food to the cockroaches.

The death of the cockroaches starts from the day 6th of administration of contaminated food however, the mortality of these the treated cockroaches was found to be highest in the period of 194-218 hours when treated with liver extract. The effect of skin extract was found to be highest in the period of 314-338 hours after the treatment however death of cockroaches started only 12th day from the time of administration. (figure 2)

The administrations by injection of liver and skin extract have significant affect and they are negatively correlated.



The treatments with liver extract by injected and fed with food has found significant difference. The graphical representation further indicates that the TTX is more effective if it can be injected rather than fed with food or sprayed. Figure1 indicates that if the mortality is caused due to the toxicity of TTX, the concentration of TTX is highest in liver and then in skin and muscles.

CONCLUSION

In our experiment certain aspect are yet to be

completed to ascertain the effect of TTX in cockroaches however, we can assume that the TTX present in the crude extract does have certain effect on them. The treated cockroaches behaviour resembled that of TTX poisoning, such as, decreasing body movements, slow antennal movement and ultimate death of the specimen. The presence of TTX in the haemolymph of cockroaches when examined through TLC will ascertain the effect of TTX in cockroaches. The results of the experiments clearly indicate the strength of toxicity of liver extract is more than skin

extract. The muscle extract was found to have no impact on cockroaches. On the other hand the extracts had no impact on cockroaches when sprayed on them.

The experiment opens the door to investigate the matter in many folds.

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