



Original Research Article

Taxonomy and Toxicological Evaluation of Tetraodontiformes from Kasimedu, Chennai, India

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ABSTRACT

The present study discusses the Taxonomy and Toxicological Evaluation of Tetraodontiformes from coastal waters of Kasimedu, Chennai, India. The buffer fish has a pelagic, or open-ocean, life stage. Spawning occurs after males slowly push females to the water surface. The eggs are spherical and buoyant, floating in the water. Hatching occurs roughly after four days. Live specimens of *Lagocephalus scleratus* were procured from Kasimedu landing center, Chennai. Crude mucus toxin extraction was performed from buffer fish and toxicity test were done. The level of monocytes, neutrophils, Haemoglobin content, were not found in decreasing trend with increment of crude toxin in treated rat groups. But there is no change in eosinophiles, basophiles. The level of blood glucose, urea, creatinine, PCV were found decreased in treated rat groups when compared with the control. The MCV had positive correlation with crude toxin administration.

Keywords

Tetraodontiformes,
Medicinal use,
Kasimedu,
Survey,
Treatment.

Introduction

Tetraodontiformes is an order of primarily marine and estuarine fish. The order includes many familiar species which are variously called puffers, balloonfish, blowfish, bubblefish, globefish, swellfish, toadfish, and toadies. They are morphologically similar to the closely related porcupine fish, which have large conspicuous spines (unlike the small, almost sandpaper-like spines of Tetraodontidae). The scientific name, Tetraodontidae, refers to the four large teeth, fused into an upper and lower plate, which are used for crushing

the shells of crustaceans and mollusks, and red worms, their natural prey. Puffer Fish are the second most poisonous vertebrate in the world, the first being a Golden Poison Frog. The skin and certain internal organs of many Tetraodontidae are highly toxic to humans, but nevertheless the meat of some species is considered a delicacy in both Japan (as *fugu*) and Korea (as *bok-uh*). If one is caught while fishing, it is recommended that thick gloves are worn to avoid poisoning and getting bitten when removing the hook.

Members of the Tetraodontidae family are found near shore in temperate and tropical seas worldwide and completely absent from cold waters. Pufferfish are mostly found in coastal regions, though some are oceanic (e.g., *Lagocephalus lagocephalus*) or live in the deep sea (e.g., *Sphoeroides pachygaster*). The Tetraodontidae contains at least 121 species of puffers in 19 genera. They are typically small to medium in size, although a few species can reach lengths of 100 centimetres (39 in). A large number of puffers are found in brackish and fresh waters: at least 39 marine species enter brackish or freshwater to feed or breed (e.g., *Arothron hispidus*), and a further 28 species are completely freshwater fish in distribution and never enter the sea (e.g., *Colomesus asellus*). It is surprising that some select species are even known to live in entirely polluted water.

The puffers's unique and distinctive natural defenses are great due to their particular form of locomotion. Puffers use a combination of pectoral, dorsal, anal, and caudal fins for propulsion that make them highly maneuverable but very slow, and therefore comparatively easy targets for predators. As a defense mechanism, puffers have the ability to inflate rapidly, filling their extremely elastic stomachs with water (or air when outside the water) until they are almost spherical in shape. Thus, a hungry predator stalking the puffers may suddenly find itself facing what seems to be a much larger fish and pause, giving the puffers an opportunity to retreat to safety. When lifted out of water there is a risk that puffers inflate with air. This may result in problems deflating again afterwards. When this happens with aquarium specimens the recommended course of action for fishkeepers is to hold the puffer underwater by the tail, head upwards, and shake the fish gently until the air escapes out of the mouth.

Puffers are able to move their eyes independently, and many species can change the color or intensity of their patterns in response to environmental changes. In these respects they are somewhat similar to the terrestrial chameleon. Although most puffers are drab, many have bright colors and distinctive markings and make no attempt to hide from predators. Due to some unknown selection pressure, intronic and extragenic sequences have been drastically reduced within this family. As a result, they have the smallest-known genomes yet found amongst the vertebrate animals, while containing a genetic repertoire very similar to other fish and thus comparable to vertebrates generally. Since these genomes are relatively compact it is relatively fast and inexpensive to compile their complete sequences, as has been done for two species (*Takifugurubripes* and *Tetraodonnigro viridis*).

The buffer fish has a pelagic, or open-ocean, life stage. Spawning occurs after males slowly push females to the water surface. The eggs are spherical and buoyant, floating in the water. Hatching occurs roughly after four days. The larvae are predominately yellow with scattered red spots. They are well developed with a functional mouth, eyes, and a swim bladder. Larvae less than ten days old are covered with a thin shell. After the first ten days, the shell is lost and the spines begin to develop. The larvae undergo a metamorphosis approximately three weeks after hatching. During this time, all the fins and fin rays are present and the teeth are formed. The red and yellow colors of the larvae do not persist into the juvenile phase and are replaced by the olives and browns; characteristic of adults. Dark spots also appear on the juvenile's underside. Pelagic juveniles are often associated with floating sargassum, and these spots may serve as camouflage from predators such as

dolphin that swim below the seaweeds. Juveniles retain spotting until they move inshore and become adults. The juvenile puffer fish does not undergo another metamorphosis to become an adult. All changes now are external and include elongation of the spines and normal body growth

The puffer's toxin is called tetrodotoxin. Some puffers also produce a powerful neurotoxin in their internal organs, making them an unpleasant, possibly lethal, meal for any predatory fish that eats one. This neurotoxin is found primarily in the ovaries and liver, although smaller amounts exist in the intestines and skin, as well as trace amounts in muscle tissue and in its blood. This is likely an example of aposematism. Puffer's toxin evolved as a response to aquatic predators such as larger fish, rather than for use against humans. Note also, not all puffers are poisonous; *Takifuguoblungus*, for example, is one of the few puffers that is not poisonous. However, it should be noted that puffer's neurotoxin is not necessarily as toxic to other animals as it is to humans, and puffers are eaten routinely by some species of fish, such as lizardfish and tiger sharks. It is also found within other animals such as the blue-ringed octopus, cone snail, and in certain varieties of newt. Tetrodotoxin is produced within the puffers by bacteria, which are acquired through food. This means that puffers raised in captivity do not contain tetrodotoxin, and therefore are not poisonous until they come into contact with the bacteria. The puffer itself has immunity to the poison due to a mutation in the protein sequence of the Sodium ion channel on the cell membranes. Tetrodotoxin is an exceptionally lethal poison. Tetrodotoxin is said to be nearly 100 times deadlier than potassium cyanide. In animal studies with mice, 8 µgtetrodotoxin per kilogram of body weight killed 50% of the mice (see also

LD₅₀). It is estimated that a single puffer has enough poison to kill 30 adult humans.

Puffer poisoning usually results from consumption of incorrectly prepared puffer soup, *chiri* or occasionally from raw puffer meat, *sashimi fugu*. While *chiri* is much more likely to cause death, *sashimi fugu* often causes intoxication, light-headedness, and numbness of the lips, and is often eaten for this reason. Puffer's (tetrodotoxin) poisoning will cause deadening of the tongue and lips, dizziness, and vomiting. These are followed by numbness and prickling over the body, rapid heart rate, decreased blood pressure, and muscle paralysis. Death results from suffocation as diaphragm muscles are paralyzed. Patients who live longer than 24 hours are expected to survive, although the poison can cause comas lasting several days. Many people report being fully conscious during the entirety of the coma, and can often remember everything that was said while they were supposedly unconscious. In Voodoo, puffer's poison must be ingested by the victim for the black magic of creating "zombies," most likely because of the pseudocomatose effect. Pufferfish, called *pakpao*, are also consumed in Thailand, usually by mistake, at times these fish are eaten because they are cheaper to buy, and there is little awareness or monitoring of the situation. Patients are regularly hospitalized or die as there are no specific preparations to remove the toxin before eating. Treatment consists of supportive care and intestinal decontamination with gastric lavage and activated charcoal. Case reports suggest that anticholinesterases such as edrophonium may be effective. Saxitoxin, the cause of PSP (paralytic shellfish poisoning, red tide), can also be found in puffers. Cases of neurologic symptoms, including numbness and tingling of the lips and mouth, have been reported to arise rapidly after the

consumption of puffers caught in the area of Titusville, Florida. These symptoms are generally resolved within hours to days, although one affected individual required intubation for 72 hours. As a result of such cases, Florida banned the harvesting of puffers from certain bodies of water. A drug called Tectin that is derived from tetrodotoxin is being developed as a potent pain reliever. Administered in very small quantities it can bring relief to those suffering from intense chronic pain, such as that experienced by some cancer patients. Other uses, such as helping opiate addicts through withdrawal, are also being studied.

Materials and Methods

Study Area

The Tetraodontiforms were collected from Kasimedu landing center, Chennai. There are about 500 mechanized boats operating from the Madras fishing harbor. Except for 30 to 40 gill netters, all are trawlers. Besides mechanized boats, traditional craft and nets are operated from the harbour by traditional fishermen. The peak auctioning hours, when most of the boats land, are between 6.30 a.m. and 8.30 a.m. and again between 2.30 p.m. and 5.30 p.m. Except for a few vessels belonging to CMFRI, Fishery Survey of India, and TNFDC, all boats are privately owned. At the auctioning place, there are 50 or more sheds for processing exportable species, for packing, and for icing the fish. Nearly 100 more sheds are scattered around the harbour. People engaged in these activities live near the harbour, the main exception being some women fish traders. Before the fishing harbour was built, a traditional fishing village, Kasimedu, was the centre of activities in the area. Kasimedu's village council still exerts authority over the auctioning place. Members collect Rs.0.25 per basket of fish

bought here. All the catch, except the large fish and exportable species is auctioned. At times large fish may also be sold this way. The major marine species of commercial importance are big prawn, lobsters, quality fish like seer and pomfret, small fish like sardines, mackerels, cuttlefish, mussels, and trash fish (all the smaller varieties). Market outlets: Retail and wholesale city markets, suburban markets, other town and city markets in Tamil Nadu, other Indian town and city markets and foreign markets.

Taxonomical study

Specimens belong to the order tetraodontiforms were collected in Kasimedu Landing center, Chennai for the period of six months. All the species were identified up to species level with standard references (Fish base, 2009).

Toxicity studies

Specimen collection

Live specimens of *Lagocephalus scleratus* were procured from Kasimedu landing center, Chennai and transported to the laboratory where they were stored frozen at -20° C until use. The average size of the fish was 21 cm and 53 gm. Taxonomic identification of fish was done following Talwar and Jhingran (1991).

Extraction of crude mucus toxin

Mucus from 15 fishes were scraped off using a scalpel and was homogenised for 10 minutes in 25 ml phosphate buffer saline (pH 7.5). The mixture thus obtained was centrifuged twice at 15,000 rpm for 15 minutes. The residue was discarded and the supernatant was collected. This was then lyophilised in a freeze-dry system

(LABCONCO) to obtain crude toxin and stored in refrigerator at 4°C.

Experimental animal

Adult male albino rats of wistar strain 4 – 6 weeks old, weighing 180 – 230g were obtained from the laboratory animal center of Vapery Medical College. The animals were housed in large spacious cages, maintained in controlled environment of temperature, humidity and light/dark cycles. They were fed with standard pelleted diet obtained from Hindustan Lever Limited, Bangalore and water *ad libitum*. The experiment was carried out at Dept of Marine Studies and Coastal Resources Management, Tamil Nadu, India.

Acute toxicity studies

Mice were divided into 3 groups of 10 mice each. The crude toxic under study was injected intraperitoneally in different doses to different groups. The animals were then observed for signs of toxicity and mortality for every 2 hours and at the end of 9 days, number of animals dead was counted in each group.

Percentage death was then calculated and was transferred to probits using probit table. Probits were then plotted in Y-axis against the log dose of the drug in x-axis. The LD₅₀ was determined by the dose that corresponds to probit 5. Mouse unit (M.U.) was calculated by modifying the method described by the A. O. A. C. (1995). In this case, 1 M.U. was defined as the amount of toxin required to kill a male strain of mice weighing 20gms in 100 seconds.

Mice bioassay

The sample for injection was prepared by diluting the lyophilised samples in phosphate buffer saline (pH 7.5) @ 1

mg/ml. This prepared mucus was injected intraperitoneally in doses varying from 0.1 to 0.35 ml.

Fifteen albino rats were taken and divided in to four groups. Apart control, Group I injected the crude toxin of 0.1 mg per mice per day. Group II injected the crude toxin of 0.25 mg per mice per day. The group III injected the crude toxin of 0.35 mg per mice per day. This dose was given to the animals for 9 days. The injected mice were kept under observation in cages and their activities were noted. The time of injection and the time of death were recorded. A control was maintained with each set of experiment by injecting an equal volume of 0.15 M phosphate buffer saline (pH 7.5). Triplicate sets were maintained for each dose administered. All the animals were sacrificed at 9th day, different viscera's were separated, grossly inspected and then subjected to histological studies. Physical observations were also made in elite organs such as liver, heart; lung and kidney. Blood was drawn from the heart directly and the blood cell parameters *viz.*, RBC, WBC and differential counts such as polymorphs, lymphocytes, eosinbophiles, monocytes, basophiles, neutrophils and platelets were analyzed by using standard procedures. Besides that the content of hemoglobin, packed cell volume, blood glucose were analyzed based on the standard procedures. Moreover the urea, creatinine, Mean corpuscular volume (MCV) contents were also analyzed by using standard methods.

Histopathological study

Kidney and liver were excised from the mice which died upon envenomation and was fixed in 10% formalin. It was then rinsed in three changes of 70% (v/v) alcohol to remove the excess fixative. The tissues were dehydrated in ascending grades (50%,

70%, 90%, 100%) of alcohol for 1 hour each. The samples were then cleared in xylene for two hours and embedded in paraffin wax thrice, each time kept for 45 minutes. Once the samples were blocked, it was allowed to solidify and the surplus amount was trimmed off. Sections of 10µm thickness were made using a hand rotary microtome. The “ribbon” thus obtained was kept with its shiny side downwards onto the surface of warm water in the water bath where the temperature was maintained 2-3° C lesser than the melting point of paraffin wax.

The best section among them was picked up on a microscopic slide. The excess water was removed using a blotting paper. Dewaxing was done by drying the slides in hot plate for 2-3 hours and by clearing them in xylene. Samples were then hydrated in descending grades of alcohol. The staining was done by using Delafield's haematoxyline for 7 minutes, dipped once in acid alcohol and then for 3 minutes in Scotts tap water. This were then passed through descending grades of alcohol (3 minutes each), followed by Eosin stain (3 min) and absolute alcohol (1 dip) and finally cleared in xylene. These were mounted on DPX. Prepared sections were examined and photographed on a phase contrast microscope.

Result and Discussion

A total of twelve species belong to the order tetraodontiforms were collected from the study area (Kasimedu landing center). The list of species, common name and distribution frequency are given in table 1.

The species *Lagocephalus inermis*, *Arothronim maculatus*, *Torquigener hypselogoneion*, and *Lagocephaluslunaris* were noticed higher distribution frequency

(100%) which means the availability of these species in all seasons. The species, *Diodonholocanthus* and *Arothronstellatus* showed restricted distribution (4%) and found only in chosen season. The collected species and its detailed taxonomical information given in plates (Plates 1-12).

A total amount of 190 mg lyophilised mucus toxins were extracted using phosphate buffer saline (pH 7.5) from 10 numbers of *Lagocephalus* (4.226 kg –total wt of the fishes). A quantity of 1mg lyophilised toxin were obtained from 10ml of the thehomogenised samples of skin mucus extract. Primor and Zlotkin (1975) reported that in the skin of *Paradachirusmarmoratus*, the same constituent was responsible for both the lethal and hemolytic activities. The minimum lethal dose (MLD) of the lyophilised mucus toxin was found to be 1.75ml at a concentration of 1mg/ml at which the death occurred in six hours. Sub-lethal doses (0.85, 0.80, 0.75) elicited symptoms similar to that of the lethal dose, but the mouse were found to recover in a few minutes or hours. The general symptoms shown were the dragging of hind limbs, scratching of the snout area, movement along the corners of the cage, excessive urination and defaecation, apathy, palpitation, respiratory distress, convulsions and loss of balance. In the case of the lethal doses, apart from the general symptoms described above, violent jumping was also noticed. The toxin contains two lethal factors, as the symptoms shown by the mice on envenomation resembles those reported by Shiomiet *al.*, (1986) in the case of *Plotosuslineatus*. Similar symptoms were also shown with the goby toxin, *Gobiuscriniger* in mice (Noguchi and Hashimoto, 1971). There is no hyperactivity shown by the mice in the present study with the findings of Bhimachar (1994); Dattaet

al., (1982) with the toxin from *H. fossils* on catfishes and frogs respectively.

Microscopic examination of the prepared sections of liver and kidney of the envenomated mice revealed moderate reduction in cells regeneration. Tubular necrosis and prominence of pyknotic nuclei (condensed nuclei) in certain areas were observed in the kidney. The liver revealed intracellular vacuolization, nuclear condensation (pyknosis) and degeneration of hepatocytes. Necrosis was also pronounced at certain areas. The results are shown in Plates 13-14.

Histopathological observation points out that the main target organs were the kidney and the liver. Moderately damage to the kidney cells confirms that the kidney was affected by the toxin, next being the liver in decreasing order. The damage to the hepatocytes may be due to the storage of the toxin in the liver for detoxification. It is possible that the toxin, which is manifested by the symptoms like palpitation and respiratory distress, only indirectly affects the heart. The induced respiratory failure may not be due to the direct effect on heart, but may be due to the combined effect of hypertension, cardiogenic shock and CNS manifestation as shown in the case of *Plotosus canius* (Auddy, 1994).

The symptoms like apathy, paralysis, loss of balance and convulsions indicates that the toxin might have affected the CNS. Deo (1995) and Absuma (1997) also reported these symptoms in *H. fossils*. Tetanic convulsions in frogs due to *H. fossils* venom was reported by Bhimachar (1944). The convulsions incurred may be due to the presence of prostaglandin like substances in the toxin; Datta (1982) has explained the relation between prostaglandin like substances and convulsions. Presence of yet

another chemical called acetylcholine along with a toxic component that affects the neuromuscular transmission, without affecting the heart function is also suspected in the epidermal secretion because of the similarity in symptoms to that of the lionfish spine toxin (Cohen and Olek, 1989).

The blood parameters such as RBC and WBC were analyzed in both control and treated group of mice and found that, the WBC and RBC were showing increasing trend in all mice groups (Fig.3 and 4). It understood that the count of RBC and WBC would be increased by the increased level of toxin. The drastic changes noticed between the group 1 and 2 with group 3 is due to one to two fold increased level of crude toxin administration to the group 3.

Likewise, the lymphocytes, neutrophils, eosinophils, basophils and monocytes were analysed in all mice groups (Fig 4). Lymphocytes count was increased towards control to group 3 as with increased dosage administration from group 1 to group 3. It is clear that increasing in doses of crude toxin from *Lagocephalus scleratus* influencing lymphocytes production in blood. But monocytes and neutrophils were noticed to decrease than the control. However, the count of monocytes found to increase between the experimental groups, but neutrophils count was reduced up to 32% from control to group 3.

Basophils and eosinophils were observed 0.1% even in group 3 alone. It concluded that the immunity level is moderately induced by the crude toxin of *Lagocephalus scleratus*. The level of hemoglobin was analysed and showed the decreasing trend towards the increasing dosage of crude toxin from the skin of *Lagocephalus scleratus* (Fig.5). So, the amount of crude toxin in the skin of *Lagocephalus scleratus* caused

deficiency in oxygen content of the mice which was observed physically in group 3.

The platelets were observed in all groups of mice including the control mice (fig.6). The drastic increases of platelets were noticed from control to experimental samples as with the increased level of dosages. The first dosage of crude toxin caused 1: 6 ratio of platelets proliferation in the blood mice and third increased dosage caused 1: 10 ratio in 9 days. At the end of the experiment, the count of platelets increased from 1,00,000 cells to 10,00,000 cell with in 216 hours. Absence of variation in groups of 2 leads to immature in the point of growth (104.5 gms).

Packed Cell volume, Mean Corpuscular Volume (MCV) and creatinine were examined in the blood of mice after the experiment. MCV showed positive correlation, creatinine and PCV had negative correlation (Table 2). The level of creatinine decreased towards the dosage increment. It means that it might have reduced metabolic rate in the body and ultimate reduction of creatinine to be produced.

The blood sugar showed slight elevation in group I but drastic decreases in group II. It was noted that the group II was injected with high amount of crude toxin (0.25 mg). It is two and half fold more than group I. So, the crude toxin caused drastic elevation of sugar level in blood with minor dose and rigorous decline in larger dosage (Table. 2).

The urea in the blood of treated mice showed that the group I had strong elevation and slight decline in group II (Table.2). This

statement shows clearly that minimum level of crude toxin from *Lagocephalus scleratus* caused to induce the rate to the metabolism more in mice and the level increased to the optimum level, it leads to low metabolic rate and detrimental to the mice. So, the crude toxin needed for the normal metabolic activity of the mice might be 0.15 mg.

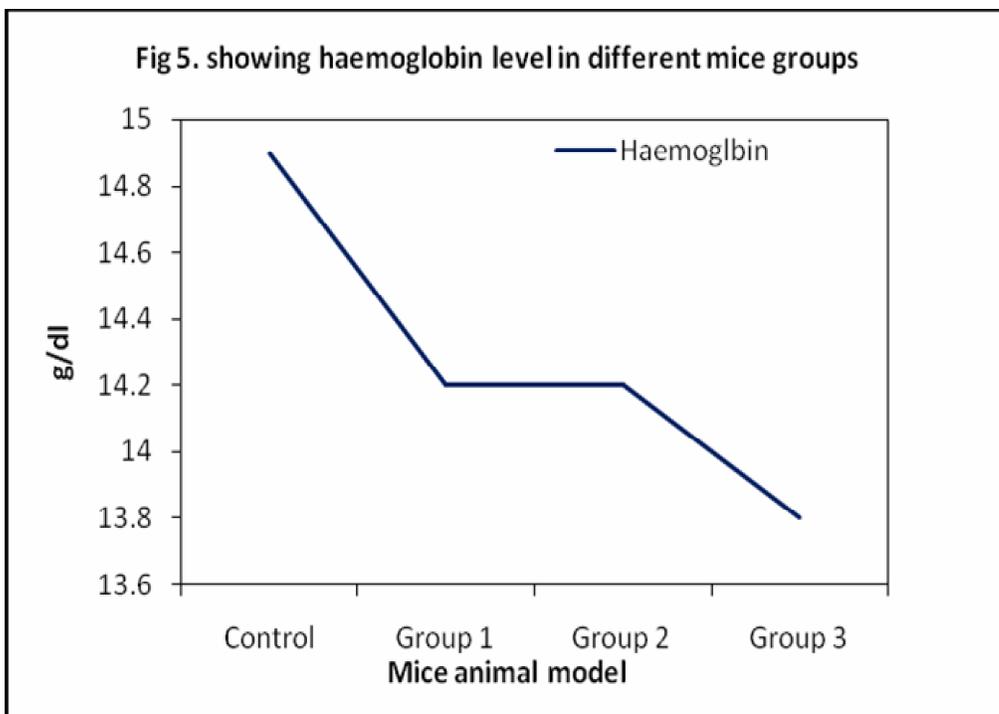
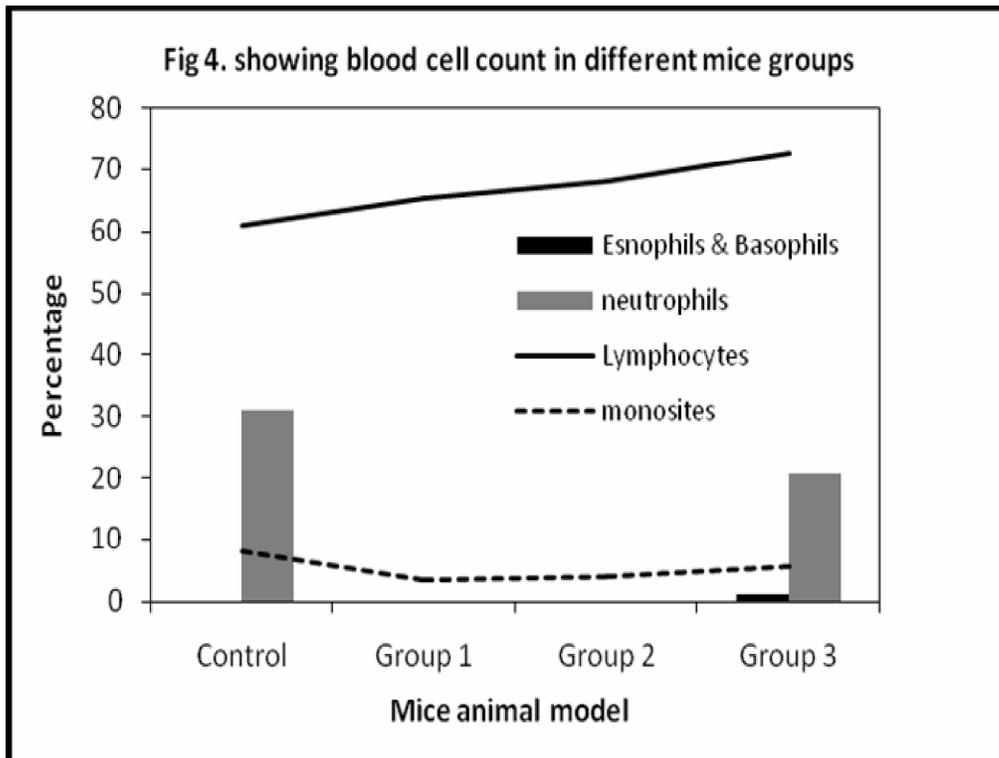
There are totally 12 species belongs to tetraodontiforms were collected in the study area within six months study period. Among the 12 species, four species were 100%, three species were 24%, two species were 4%, and one species was 48%, 40%, and 12% respectively showed distribution frequency. For toxicological studies, *Lagocephalus scleratus* was chosen as it was found to be a predominant fish for consumption. The crude toxins treated mice models showed sign of side effects in elite organs such as kidney and liver. The liver and kidney were moderately affected showed necrosis in kidney and palpitation and respiratory disorder due to accumulation of toxin in liver. The level of blood cell parameters such as WBC, RBC, platelets, lymphocytes, were found increased in mice compared with the control towards the increased level of administration. The level of monocytes, neutrophils, Haemoglobin content, were not found in decreasing trend with increment of crude toxin in treated rat groups. But there is no change in eosinophiles, basophiles. The level of blood glucose, urea, creatinine, PCV were found decreased in treated rat groups when compared with the control. The MCV had positive correlation with crude toxin administration.

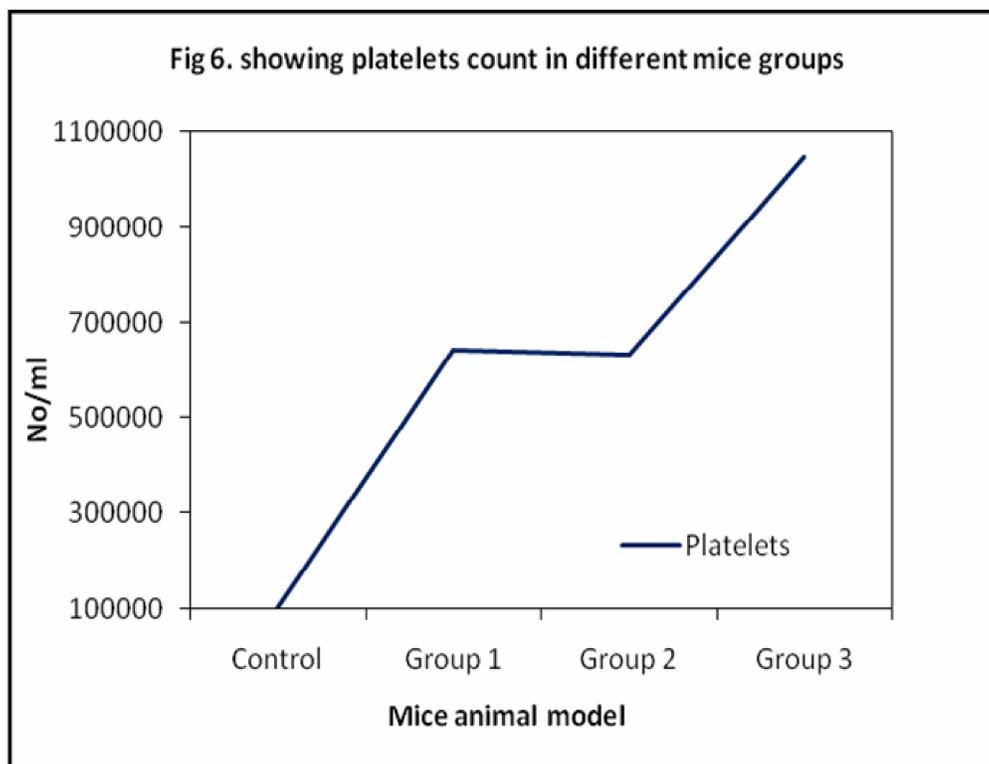
Table.1 showing the list of species, common name and its distribution frequency

S.No	Name of the species (family)	Common Name	Distribution Frequency (%)
01.	<i>Lagocephalus scleratus</i> (Tetraodontidae)	Silver stripe blaasop puffer fish	48
02.	<i>Lagocephalus inermis</i> (Tetraodontidae)	Smooth blaasop puffer fish	100
03.	<i>Lagocephalus lunaris</i> (Tetraodontidae)	Green Rough-Backed puffer fish	100
04.	<i>Chelonodon patoca</i> (Tetraodontidae)	Milk spotted puffer fish	40
05.	<i>Torquigener hypselogeneion</i> (Tetraodontidae)	Orange-spotted toad fish	100
06.	<i>Arothron stellatus</i> (Tetraodontidae)	Starry toad fish	4
07.	<i>Arothron immaculatus</i> (Tetraodontidae)	Immaculate puffer fish	100
08.	<i>Arothron reticularis</i> (Tetraodontidae)	Reticulated puffer fish	12
09.	<i>Diodon holocanthus</i> (Diodontidae)	Long-spine porcupine fish	4
10	<i>Paramonacanthus choirocephalus</i> (Monacanthidae)	Pig faced leather jacket fish	24
11	<i>Tetrosomus gibbosus</i> (Ostraciidae)	Humpback turret fish	24
12	<i>Triacanthus biaculeatus</i> (Triacanthidae)	Short-Nosed Tripod-fish	24

Table.2 Level of PCV, blood glucose, urea, creatinine and MCV in different groups of mice Groups

Mice Groups	Blood Glucose (mg/dl)	PCV (%)	Urea (mg/dl)	Creatinine (mg/dl)	MCV
Control	116.1	49	23.38	1	48.3
Group 1	140.2	45.7	28.39	0.9	49.7
Group 2	92	47.1	22.32	0.9	49.1
Group 3	-	42.6	-	-	51.2





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