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Assessment of toxicity in puffer fish (*Lagocephalus lunaris)* from South Indian coast

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Puffer fish intoxication is one of the most common fish intoxications along the coasts of Asia. *Lagocephalus lunaris* is the most commonly available species in the South Indian coast and it is eaten by the locals. This study was undertaken to assess the risk of poisoning due to consumption of *L. lunaris* collected along the Southeast Indian coast. Swiss albino mice were intraperitoneally injected daily with liver and muscle extracts of *L. lunaris* (1 ml/100 g of body weight), for 10 days. Control mice received injections of NaCl (0.9%). No mortality was recorded. The treatment led to: (1) decrease in body weight and increase in organ (liver and kidney) weights; (2) oxidative stress evidenced by an increase in lipid peroxidation and a decrease in antioxidant enzymes activities in tissues (blood cells, liver, and kidney); (3) a decrease in alanine aminotransferase and alkaline phosphatase activities in serum; and (4) development of mydriasis. The study suggests that *L. lunaris* collected from Southeast Indian coast is toxic, particularly, the muscle and therefore, it is not fit for consumption.

Key words: Fish toxins, food safety, antioxidant activity, toxicology, risk assessment.

INTRODUCTION

Puffer fish intoxication is the best known of all types of fish intoxications and has been recognized from ancient times. It is probably the most common fish intoxication along the coasts of Asia. (Ahasan et al., 2004; Hwang et al., 2002; Wu et al., 2005). The puffer fish are also variously known as toadfish, blowfish, globefish, swellfish and balloon fish, belonging to the order Tetraodontiformes (Halstead, 1967). They contain a potent neurotoxin known as tetrodotoxin (TTX) which has the ability to selectively block the ion transport of the sodium channel (Baselt, 2008). Puffer fish from the South Indian coast have not been studied yet. *Lagocephalus lunaris* popularly known as green toadfish is the most commonly available species in south Indian coast. This species is reported to be toxic elsewhere (Berry and Hassan, 1973; Monaliza and Samsur, 2011; Shiomi et al., 1985), but is eaten by the locals. This explicitly demands toxicity study. To determine the toxicity, standard mouse bioassay (Kawabata, 1978) was followed. In this case, the mice exhibited some symptoms but no mortality was recorded. This prompted us to carry out biochemical assays. Toxicity assessment of puffer fish *Lagocephalus lagocephalus* from Tunisian coast showed that the fish extracts could produce oxidative damages in liver, kidney and erythrocyte tissues of the rats (Sauodi et al., 2008a, 2008b). In this work, the toxicity of *L. lunaris* is investigated in Swiss albino mice. Mice were daily injected for 10 days with muscle and liver tissue extracts of *L. lunaris*. To assess the toxicity, the clinical symptoms, body weight (BW), organs (kidney and liver) weight and mortality were recorded. The activities of liver biomarker enzymes, alanine aminotransferase (ALT), and

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alkaline phosphatase (ALP) in serum were also recorded as they are important parameter in toxicity studies (Saoudi et al., 2008). Since a few marine toxins were found to induce oxidative stress (Dittmann and Wiegand, 2006; Li et al., 2003; Scinska et al., 2006), lipid peroxidation level and antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were also investigated in blood cells, liver and kidney of mice.

MATERIALS AND METHODS

Sample collection

Specimens of puffer fish *L. lunaris* (Bloch and Schneider, 1808) were collected from Chennai coast, Tamil Nadu in the Southeastern part of India, during the month of February, 2011 and transported to the laboratory in dry ice. Samples were frozen at -20°C until use. The specimens were identified by Professor S. S. Khora, Department of Medical Biotechnology, VIT University.

Fish extract preparation

Muscle and liver was carefully dissected from *L. lunaris*. 10 g of each organ/tissue was taken and minced properly. To each minced organ/tissue 2.5 volumes of 0.1% acetic acid was added and boiled in water bath for 10 min. Then these samples were cooled and centrifuged at 3000 rpm for 10 min and the supernatants were collected. This process was repeated thrice, to make up 5 volumes of the sample taken (Khora, 1991). The supernatants containing the toxins were finally stored at -30°C.

Animals

Adult male Swiss albino mice weighing about 25 to 35 g were obtained from VIT animal house (VIT University, Vellore, Tamil Nadu, India). They were housed at 22 ± 3°C with light/dark periods of 12 h and a minimum relative humidity of 40%. Mice were fed with a commercial balanced diet (V.R.K. Nutritional Solutions) and drinking water was offered *ad libitum*. This study was carried out in strict accordance with the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) Guidelines for laboratory animal facility. The protocol was approved by the Institutional Animal Ethical Committee, VIT University (Permit Number: VIT/SBST/IAEC/IIIrd/01; Registration number: 1333/c/10/CPCSEA; Date of registration: 30.03.2010). All surgery was performed under anesthesia by intraperitoneal injection of chloral hydrate, and all efforts were made to minimize suffering.

Experimental design

After acclimatizing to the laboratory conditions for one week, 54 mice were divided into three groups. They were daily intraperitoneally (i.p.) injected with (1) 1 ml of a saline solution (0.9% NaCl)/100 g of BW for the controls group (C), (2) 1 ml of muscle extract/100 g BW for the muscle treated group (M), and (3) 1 ml of liver extract/100 g BW for the liver treated group (L). After 2, 5 and 10 days of treatment, 6 mice of each group were sacrificed under anaesthesia by i.p. injection of chloral hydrate. The blood was collected without anticoagulant by heart puncture, centrifuged (4000 rpm/15 min, 4°C) and serum and blood cells were obtained and kept at -30°C. The liver and kidneys were removed, weighed, rinsed with ice cold saline and kept at -30°C. The frozen liver, kidney and blood cells samples were homogenized (1/2, w/v) in an ice cold buffer (TBS: 50 mM Tris, 150 mM NaCl, pH 7.4) and centrifuged at 5000 rpm for 30 min at 4°C, supernatants obtained were frozen at -30°C (Saoudi et al., 2008b).

Biochemical assays

In serum, activity of ALT and ALP was determined using commercial kits (Span Diagnostics, India). The enzyme activity was expressed is International Units (IU)/ml.

In blood cells, kidney and liver, superoxide dismutase (SOD) activity was determined according to the method of Marklund and Marklund (1974) which involves pyrogallol auto-oxidation at pH 8.0. It is expressed in units/mg protein. One unit of enzyme activity is defined as the amount of enzyme required to give 50% inhibition of pyrogallol auto-oxidation and the absorbance was read at 420 nm. Catalase (CAT) activity was determined by the method of Sinha (1972). In this method, dichromate in acetic acid was reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus produced was measured calorimetrically at 610 nm. The specific activity of catalase has been expressed as µmol of H_2O_2 consumed/min/mg protein. Glutathione peroxidase (GPx) activity was determined spectrophotometrically by using Ellmans reagent (DTNB) as a coloring reagent following the method described by Rotruck (1973) and the absorbance was read at 412 nm. The specific activity of GPx is expressed as µg of glutathione (GSH) utilized/min/mg protein.

Lipid peroxidation was determined by the procedure of Ohkawa et al. (1979) as thiobarbituric acid reactive substances (TBARS). It was expressed as nmol of malondialdehyde (MDA) formed/mg protein. The absorbance was read at 532 nm. The protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical analysis

Statistical analysis was performed using the Prism software package (GraphPad, InStat Version 3). Data were expressed as the mean and the standard deviation of the mean (SD). The analysis of variance (ANOVA) was used to compare the differences between the groups. Differences were considered significant at the 95% confidence level ($p < 0.05$).

RESULTS

Clinical manifestations

After the injection of muscle and liver tissue extract of *L. lunaris*, mice exhibited symptoms like hopping, scratching its body, stretching of hind limbs and lower back and concave curvature of spinal cord. The mice mostly remained motionless but no mortality was recorded. A significant decrease in the body weights of mice of M group was recorded. On the other hand a significant increase in the liver weights of mice of M and L groups were recorded especially after the 5th day of treatment

Table 1. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on body, liver and kidney weights of control and treated mice after 2, 5 and 10 days of treatment.

Parameter and treatment		Body weight (g)	Liver weight (g)	Kidney weight (g)
After 2 days	C	29.2 ± 2.68	1.22 ± 0.13	0.45 ± 0.07
	L	28 ± 1.58	1.47 ± 0.23	0.5 ± 0.04
	M	25.2 ± 1.79*	1.24 ± 0.9	0.4 ± 0.01$^{\#}$
After 5 days	C	28 ± 1.58	1.06 ± 0.12	0.38 ± 0.02
	L	25.8 ± 1.3	1.57 ± 0.06***	0.44 ± 0.05
	M	23 ± 2.82**	1.53 ± 0.2***	0.44 ± 0.01
After 10 days	C	28 ± 2.83	1.52 ± 0.18	0.42 ± 0.06
	L	25.25 ± 0.96	1.8 ± 0.09*	0.52 ± 0.06*
	M	21.33 ± 1.53**	1.87 ± 0.11*	0.57 ± 0.03*

Values are mean ± SD; n = 5; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001: significant from control. $^{\#}$p ≤ 0.05: M group vs. L group.

Table 2. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities in serum of control and treated mice after 2, 5 and 10 days of treatment.

Parameter and treatment		ALT (IU/L)	ALP (IU/L)
After 2 days	C	71.69 ± 1.18	50.39 ± 2.37
	L	71.41 ± 2.37	28.4 ± 1.75***
	M	64.65 ± 1.68***$^{\#\#\#}$	38.21 ± 1.65***$^{\#\#\#}$
After 5 days	C	87 ± 1.5	48.17 ± 1.5
	L	37.02 ± 1.6***	39.89 ± 1.04***
	M	33.33 ± 2.35***$^{\#}$	30.05 ± 1.22***$^{\#\#\#}$
After 10 days	C	86.64 ± 2.21	52.69 ± 2.24
	L	35.19 ± 1.4***	42.6 ± 1.72***
	M	18.12 ± 0.76***$^{\#\#\#}$	24.6 ± 1.3***$^{\#\#\#}$

Values are mean ± SD; n = 5; ***p ≤ 0.001: significant from control. $^{\#\#\#}$p ≤ 0.001: M group vs. L group.

and also a significant increase in kidney weights of mice belonging to M and L groups were recorded after 10th day (Table 1).

ALT and ALP activities in serum

After the 2nd day of treatment, no change in ALT activity in serum of mice belonging to L group was recorded while a significant decrease in ALT activity was recorded in mice belonging to M group. There was a significant decrease in the ALT activities after the 5 and 10th days of treatment in both M as well as L groups, the decrease being more evident in M group. Similarly, a significant decrease in the ALP activities in serum of mice belonging to M and L groups were recorded after the 2nd, 5th and 10th days of treatment (Table 2).

SOD activities in blood cells, liver and kidney

After the 2nd day of treatment, there was no significant difference in the SOD activities in blood cells, liver and kidney of treated mice when compared with control mice. But after the 5 and 10th days of treatment, there was an evidential decrease in the SOD activities in both the treated groups, M as well as L, the decrease being more evident in M group (Figures 1, 2, and 3).

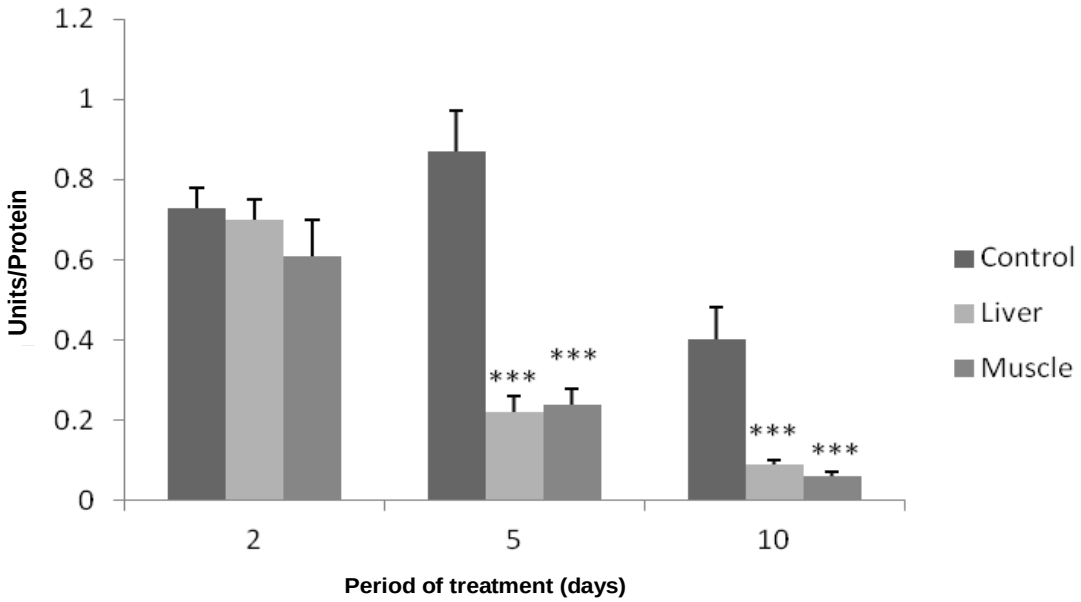

Figure 1. SOD activity in blood cells. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on superoxide dismutase (SOD) activity in blood cells of control and treated mice after 2, 5 and 10 days of treatment. *p ≤ 0.05; ***p ≤ 0.001: significant from control.

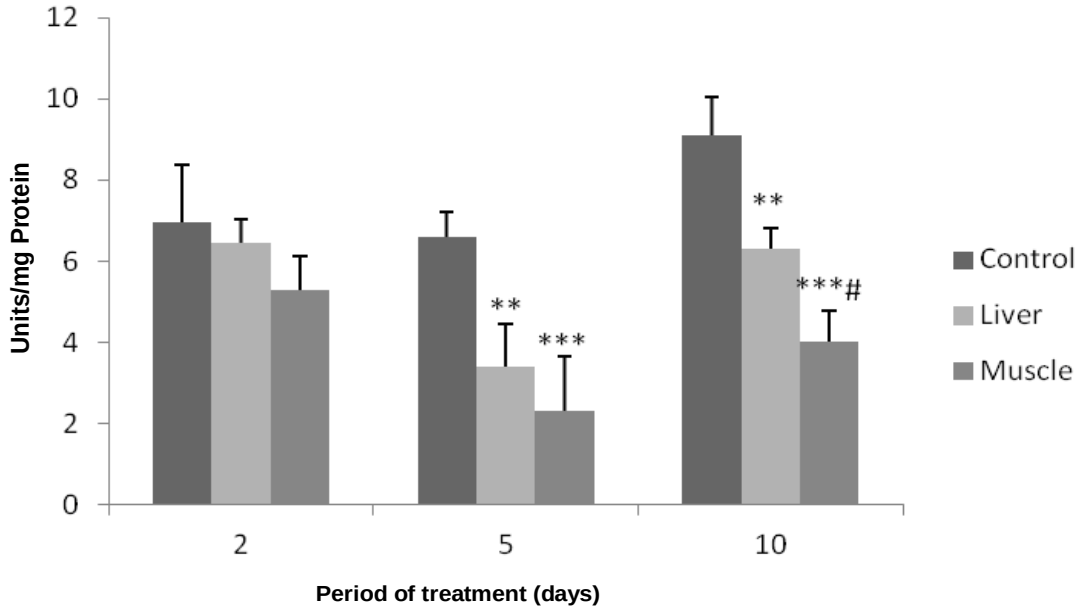

Figure 2. SOD activity in liver. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on superoxide dismutase (SOD) activity in liver of control and treated mice after 2, 5 and 10 days of treatment.**p ≤ 0.01; ***p ≤ 0.001: significant from control. $^{\#}$p ≤ 0.05: M group vs. L group.

CAT activities in blood cells, liver and kidney

Similarly, there was no significant difference in the CAT activities after the 2nd day of treatment, but a significant decrease in CAT activity was recorded after the 5 and 10th days of treatment in mice belonging to M and L groups, the decrease being more evident in M group (Figures 4, 5, and 6).

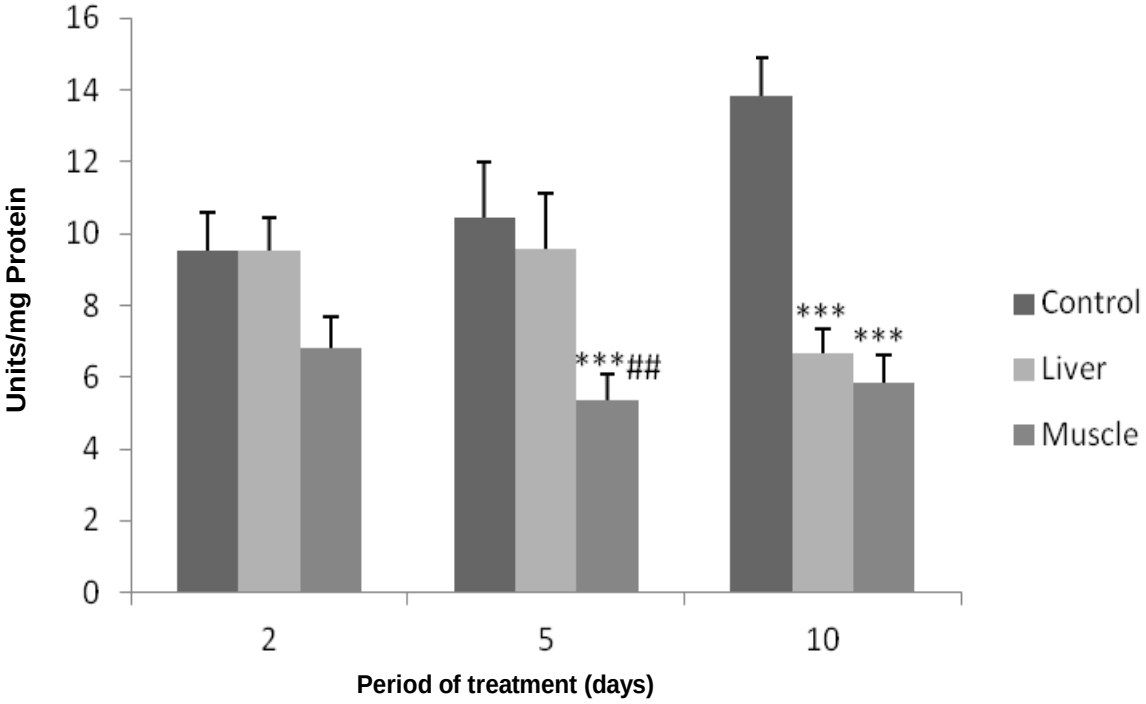

Figure 3. SOD activity in kidney. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on superoxide dismutase (SOD) activity in kidney of control and treated mice after 2, 5 and 10 days of treatment. **p ≤ 0.01; ***p ≤ 0.001: significant from control. $^{\#\#}$p ≤ 0.01: M group vs. L group.

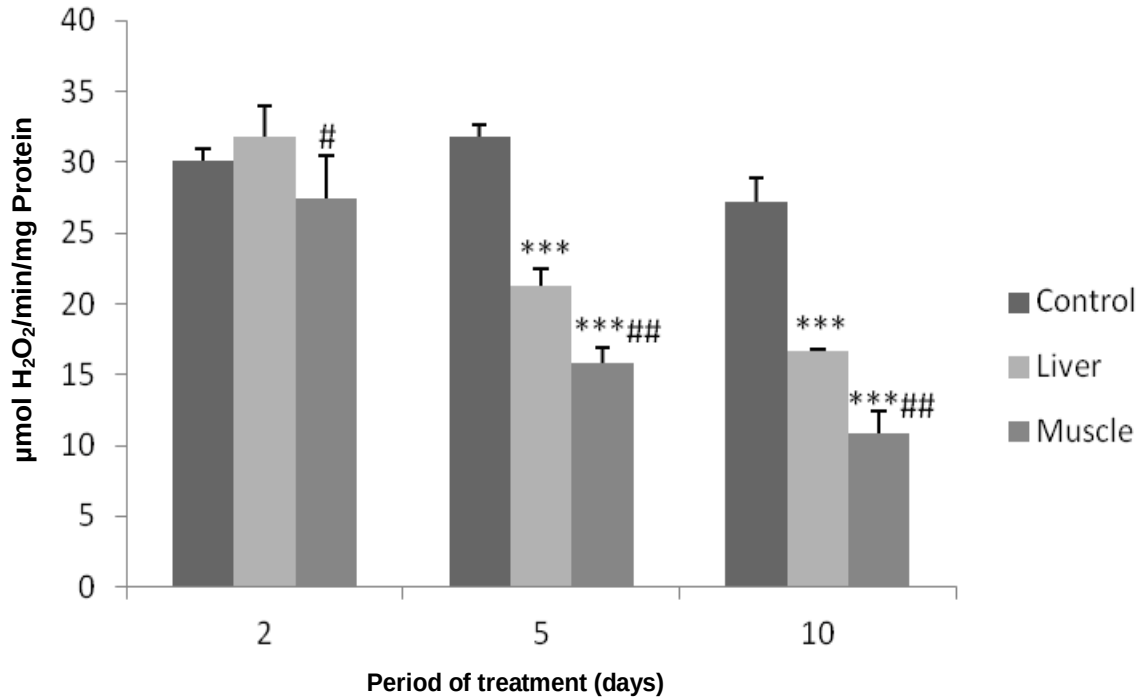

Figure 4. CAT activity in blood cells. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on catalase (CAT) activity in blood cells of control and treated mice after 2, 5 and 10 days of treatment. ***p ≤ 0.001: significant from control. $^{\#}$p ≤ 0.05; $^{\#\#}$p ≤ 0.01: M group vs. L group.

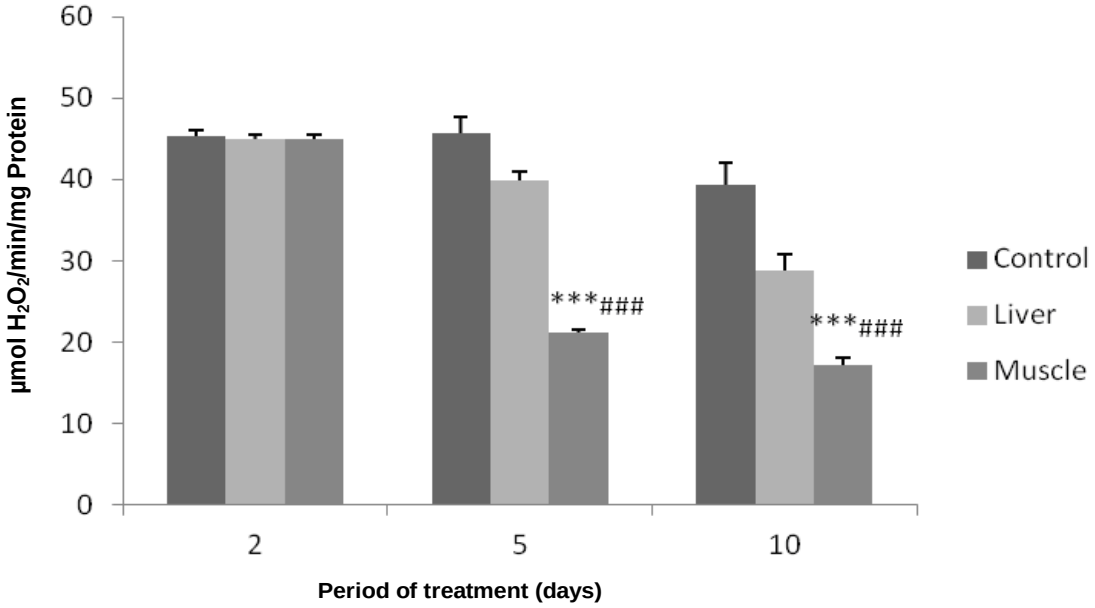

Figure 5. CAT activity in liver. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on catalase (CAT) activity in liver of control and treated mice after 2, 5 and 10 days of treatment. ***p ≤ 0.001: significant from control. $^{###}$p ≤ 0.001: M group vs. L group.

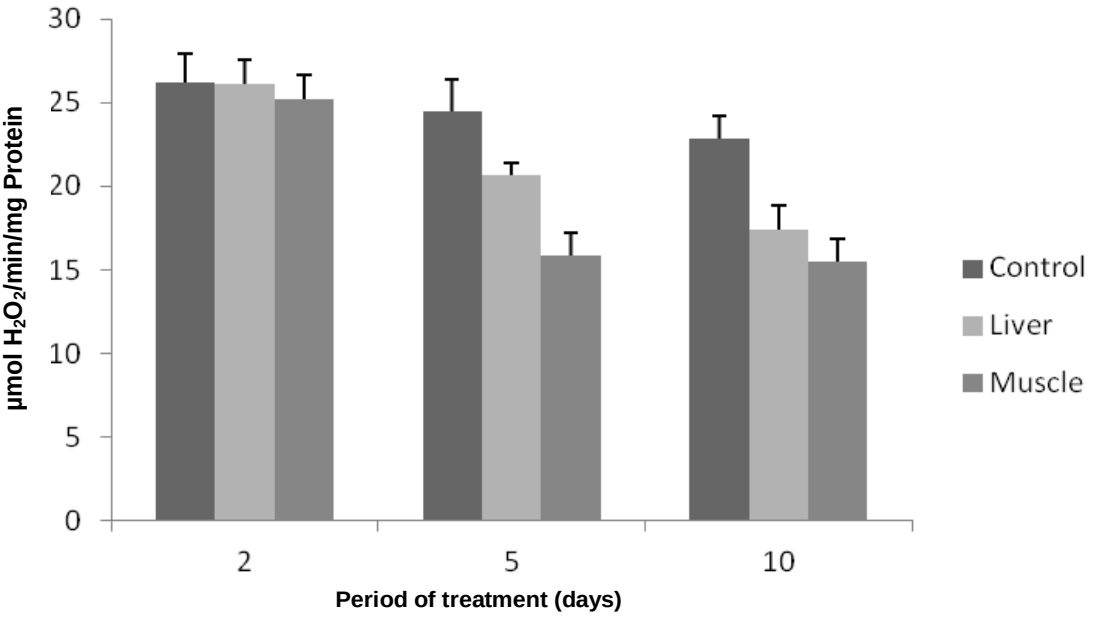

Figure 6. CAT activity in kidney. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on catalase (CAT) activity in kidney of control and treated mice after 2, 5 and 10 days of treatment. **p ≤ 0.01; ***p ≤ 0.001: significant from control. $^{##}$p ≤ 0.01: M group vs. L group.

GPx activities in blood cells, liver and kidney

Also, there was no significant difference recorded in the GPx activities after the 2nd day of treatment, but a significant decrease in GPx activity was recorded after the 5 and 10th days of treatment in mice belonging to M and L groups, again the decrease being more evident in M group (Figure 7, 8, and 9).

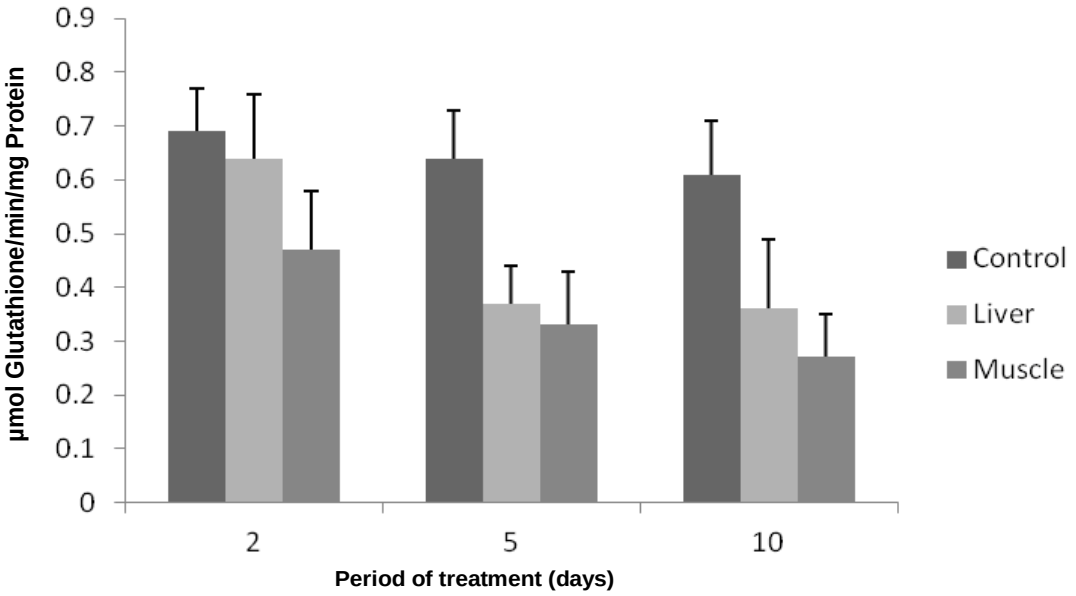

Figure 7. GPx activity in blood cells. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on glutathione peroxidase (GPx) activity in blood cells of control and treated mice after 2, 5 and 10 days of treatment. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001: significant from control.

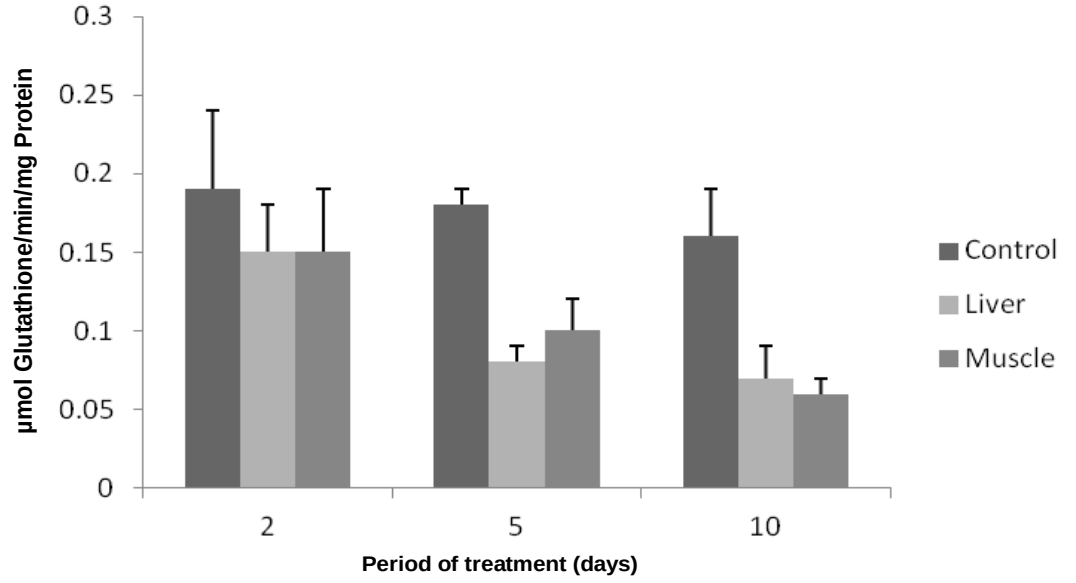

Figure 8. GPx activity in liver. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on glutathione peroxidase (GPx) activity in liver of control and treated mice after 2, 5 and 10 days of treatment. ***p ≤ 0.001: significant from control.

Lipid peroxidation levels in blood cells, liver and kidney

A significant increase in the TBARS levels were recorded in mice belonging to M as well as L groups and it was more evident in the blood cells than in liver and kidney of mice (Figures 10, 11, and 12).

DISCUSSION

This study showed that injection of *L. lunaris* liver and

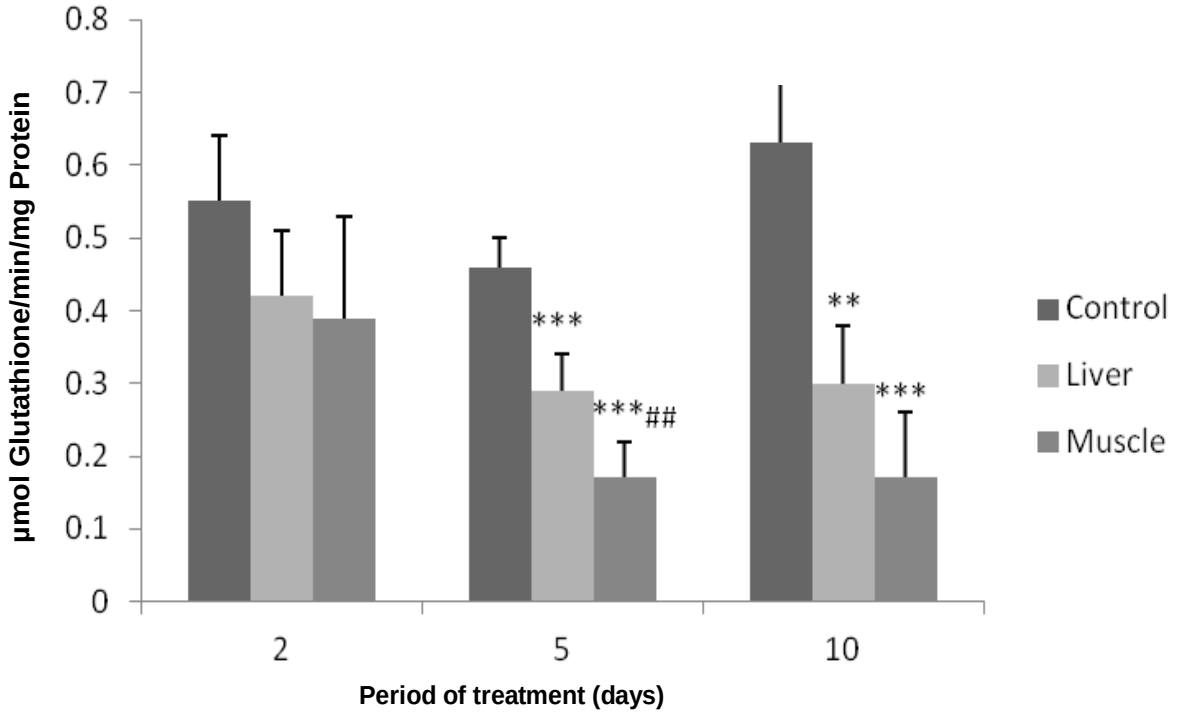

Figure 9. GPx activity in kidney. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on glutathione peroxidase (GPx) activity in kidney of control and treated mice after 2, 5 and 10 days of treatment. **p ≤ 0.01; ***p ≤ 0.001: significant from control. ##p ≤ 0.01: M group vs. L group.

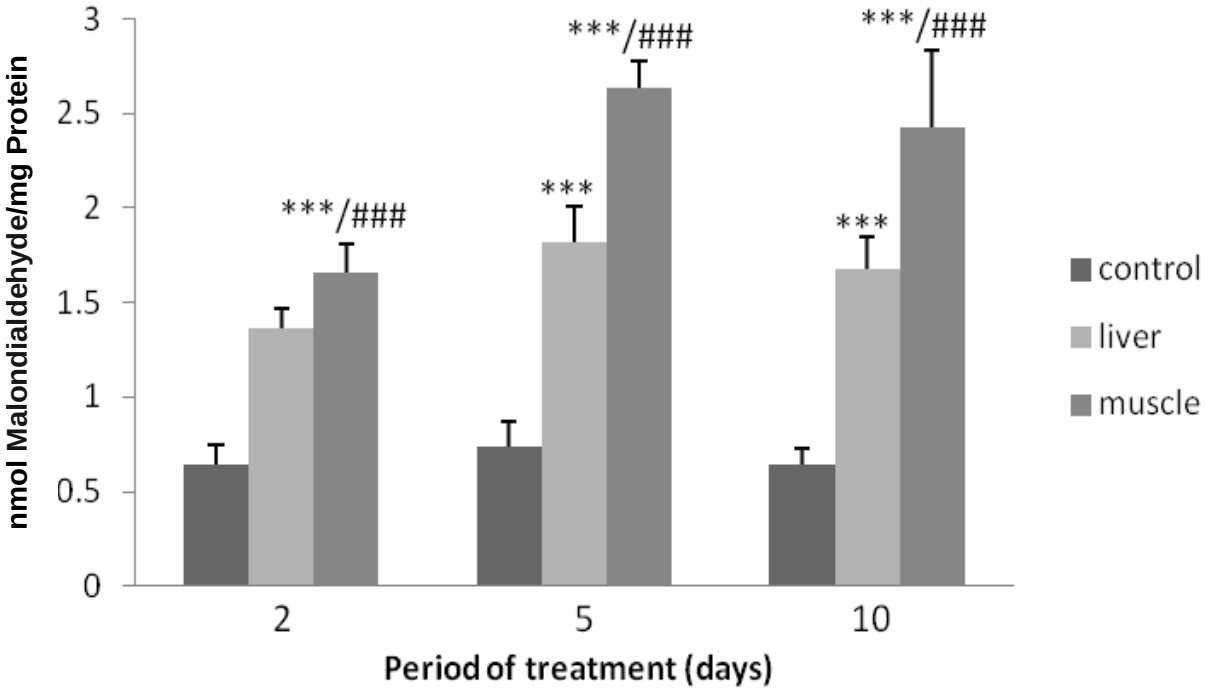

Figure 10. TBARS level in blood cells. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on lipid peroxidation (TBARS) level in blood cells of control and treated mice after 2, 5 and 10 days of treatment. ***p ≤ 0.001: significant from control. ###p ≤ 0.001: M group vs. L group.

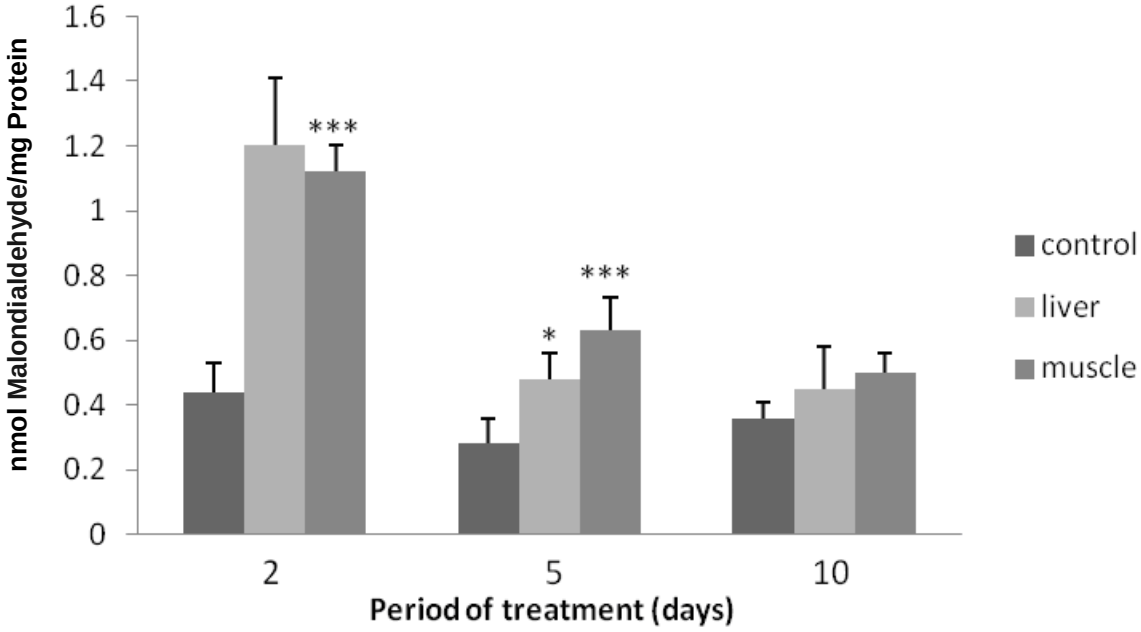

Figure 11. TBARS level in liver. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (L. lunaris) on lipid peroxidation (TBARS) level in liver of control and treated mice after 2, 5 and 10 days of treatment. *p ≤ 0.05;***p ≤ 0.001: significant from control.

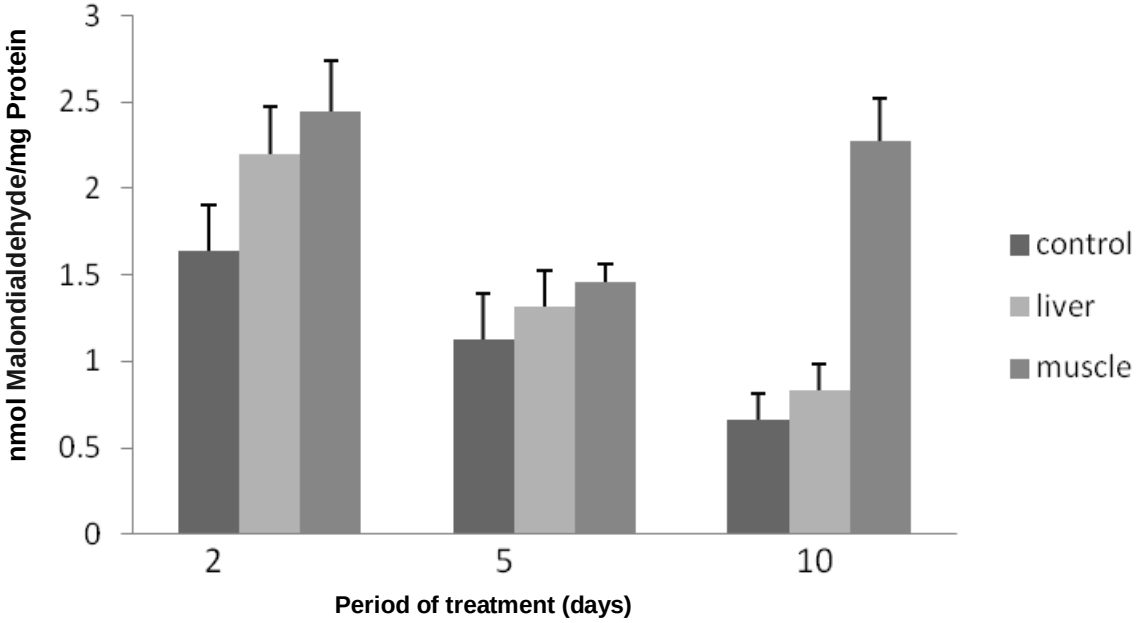

Figure 12. TBARS level in kidney. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on Lipid peroxidation (TBARS) level in kidney of control and treated mice after 2, 5 and 10 days of treatment. **p ≤ 0.01; ***p ≤ 0.001: significant from control. $^{###}$p ≤ 0.001: M group vs. L group.

muscle extracts into adult Swiss albino mice led to the decrease in body weight with significant increase in the organs (kidney and liver) weights which can be attributed to internal metabolic changes that may have occurred as same condition has been observed in other toxicity study (Soni et al., 2008).

The study showed a significant decrease in the ALP and ALT activities in serum of treated mice when compared with control. In liver enzyme, alkaline phosphatase is closely connected with lipid membrane in the canalicular zone, so that any interference with the bile flow, whether extra hepatic or intra hepatic leads to decrease in ALP activity (Vandenberghe, 1951). In this case, the muscle and tissue extracts of *L. lunaris* probably causes the cell membrane damage (lipid peroxidation) which leads to the imbalance between synthesis and degradation of enzyme protein, thus lowering the enzyme activity (Hardonk and Koudstaal, 1976). The findings of this study are in agreement with Saoudi et al. (2008b). A significant decrease in ALT activity was observed which could be due to the result of a down regulation of enzyme synthesis (Saoudi et al., 2008b). Interestingly, Solter et al. (1998, 2000) showed that ALT activity decreased with sub chronic exposure to hepatotoxin microcystin-LR. There was a significant decrease in the antioxidant enzyme (SOD, CAT, GPx) activities and therefore a high lipid peroxidation (TBARS) level which shows that the muscle and tissue extracts of *L. lunaris* resulted in oxidative stress. This study does not confirm the presence of a particular toxin since oxidative stress has been reported in various other toxicity studies (Ding et al., 1998; Li et al., 2003; Scinska et al., 2006).

From the 2nd day of treatment, a disorder in the eyes of most of the treated mice was observed. It started with a white spot at the centre of the pupil and as it progressed, the eye became reddish, the pupils were dilated, the membranes of the eyelids protruded and finally it was so much swollen that the mice could not open their eyes. The mice probably developed mydriasis due to the injection of toxin. Earlier mydriasis was reported in cats treated with ivory shell toxin (Hashimoto, 1976). Various toxins (botulinum toxin, prosurugatoxin, etc) have been known to induce mydriasis in rabbits and rats (Kosuge, 1985; Ishikawa, 2000). Perhaps, this is the first report of mydriasis due to puffer fish toxins in mice.

Generally, TTX is accumulated in the liver, gonads, intestine, muscle and skin of the puffer fish (Fuchi et al., 1991; Mahmud et al., 2000; Panichpisal et al., 2003). The liver and gonads are supposed to be more toxic when compared with the other parts. In another study, the skin was reported to be the most toxic part (Khora, 1991). Puffer fish accumulates TTX in their body through the food chain (Lee et al., 2003). Therefore, the toxicity of puffer fish also changes depending on the location they are collected from. It has also been reported that puffer fish raised in captivity are non toxic (Khora, 1994). Most of the earlier work on puffer fish showed the liver to be the most toxic part of puffer fish, and the muscle being the least toxic (Hashimoto, 1976; Matsui et al., 1981; Nagashima, 1999; Saoudi et al., 2008b). On the contrary, this study shows that the muscle can also be more toxic than liver.

Conclusively, this study reports for the first time, the development of mydriasis in mice due to puffer fish toxins. It also proves that the muscle of puffer fish can also be more toxic than the liver/gonad unlike earlier reports. Therefore, *L. lunaris* collected from Southeast Indian coast should not be consumed as it causes sub acute toxicity. Further studies are in progress to identify the implicated toxin.

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