Cell. Mol. Biol. 58 (1): 153-159
Published on December 22, 2012
DOI 10.1170/T935

Cellular & Molecular Biology
http://www.cellmolbiol.com

ASSESSMENT OF STRESS IN EFFECT TO PYRETHROID INSECTICIDES, λ-CYHALOTHRIN AND CYPERMETHRIN, IN A FRESHWATER FISH, *Channa punctatus* (BLOCH)

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Abstract

The present study was planned to see the changes in the levels of different biochemical stress markers such as the level of lipid peroxidation and the specific activities of lactate dehydrogenase (LDH), acid and alkaline phosphatases in different organs such as brain, liver, kidney, gills and muscle of a freshwater muddy fish, *Channa punctatus* in effect to pyrethroid insecticides, cypermethrin and λ-cyhalothrin treated for 96 h. The results showed significant increase in the levels of lipid peroxidation as well as the activities of LDH, acid and alkaline phosphatases in a dose dependent manner. The remarkable increase in the levels of these stress biomarkers indicates strong stress inducing potential of these insecticides in fishes. The importance of the current study lies in indicating the potential risk of muddy freshwater fishes due to strong soil binding property of pyrethroids along with their slow metabolism in fishes as compared to that of mammals.

Key words: Pyrethroids, cypermethrin, λ-cyhalothrin, *Channa punctatus,* stress biomarkers.

Article information

Received on May 14, 2012
Accepted on June 14, 2012

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INTRODUCTION

Though pesticides have contributed considerably to human welfare, their adverse impacts on non-target organisms are significant (15, 21). An estimated 85-90% of pesticides never even reach their target organisms (34). The contamination of surface water by pesticides used in agriculture has become a global problem (36). Insecticides comprise one of major portion of the different pesticides used in various indoor and outdoor pesticides. Among insecticides usage, pyrethroids cover a greater chunk. These pyrethroids are metabolized and eliminated significantly more slowly by fish than by mammals or birds (7). The half-lives for elimination of several pyrethroids by fish are all greater than 48 h, while elimination half-lives for birds and mammals range from 6 to 12 h (7). These factors along with strong soil binding property of pyrethroids cumulatively increase the risk of freshwater muddy fishes inhabiting bottom region of water bodies receiving agricultural run-offs. The lipophilic nature of pyrethroids exacerbate their toxicity by facilitating rapid access to the various tissues which in turn increase their interaction with central nervous system, for which these pesticides have high affinity (2).

These pyrethroids are known to exert their neurotoxicity primarily through interference with sodium channel function in the central nervous system. Type II pyrethroids (e.g. cypermethrin and λ-cyhalothrin) can also affect chloride and calcium channels that are important for proper nerve function (8). The insecticides, cypermethrin and λ-cyhalothrin, selected for the current study belong to type II group of pyrethroids. Cypermethrin (both the cis- and trans-isomers) is metabolized via the cleavage of the ester bond to phenoxybenzoic acid and cyclopropane carbolic acid. Ester cleavage is much slower in certain fish species than in other animal species, the main metabolic pathway being hydroxylation of the phenoxybenzoic and the cyclopropyl moieties (18). λ-cyhalothrin is also cleaved at the ester bond as the first metabolic step followed by hydroxylation at various sides of both breakdown products (22).

Both pyrethroids have been found to cause significant morphological and behavioral stress in freshwater fishes by Kumar et al. (25, 26). Few earlier reports also gave indication of strong stress inducing potential of pyrethroids. Sarkar et al. (2005) (35) found cypermethrin to be potential toxicant as it resulted in significant changes such as hyperplasia, disintegration of hepatic mass and focal coagulative necrosis in rohu (*Labeo rohita*). Korkmaz et al. (2009) (24) reported severe histo-pathological lesions and marked decline in protein level and glycogen level in different organs of Nile tilapia (*Oreochromis niloticus*) in response to the treatment of cypermethrin for 10 days. The freshwater fish, *Clarias batrachus*, has been reported to exhibit significant decrease in the activity of Na^+-K^+ ATPase and the level of glycogen content along with simultaneous elevation in the level of glycogen phosphorylase in response to cypermethrin intoxication (5) indicating its strong capacity of inducing strong stress in fish. Ogueji and Auta (2007) (29) found λ-cyhalothrin to exert profound effects on serum glucose, protein, cholesterol, triglycerides, alkaline phosphatase, glutamic pyruvic acid transaminase and glutamic oxaloacetic acid transaminase in African catfish, *Clarias gariepinus*. The present study aims to consolidate earlier reports of stress inducing potential of pyrethroids by studying the effects of both pyrethroids (cypermethrin and λ-cyhalothrin) on different biochemical stress markers in freshwater muddy fish, *Channa punctatus.*

MATERIALS AND METHODS

Sub-acute bioassays

The freshwater fish, *C. punctatus* were treated at sub-acute concentrations of 0.8, 1.2 and 1.6 µg/l of λ-cyhalothrin and 40, 60 and 80 µg/l of cypermethrin which were equi-

valent to 10, 15 and 20% of 96 h LC_{50} values obtained in acute bioassays with *C. punctatus* (25). Each static renewal bioassay was carried out in five replicates in order to increase the accuracy of the results. Four fishes were kept in each aquarium (dimension: 30 cm x 30 cm x 30 cm) so as to minimize the stress. The water quality was tested before exposure following the guidelines of American Public Health Association (1985) (1) which showed the mean values as follows: temperature 26 ± 1.2°C, pH 6.9 ± 0.9, dissolved oxygen (DO) 6.9 ± 0.4 mg/l and total hardness 113.3 ± 2 mg/l. The water was replenished along with insecticides at every 24 hr intervals with proper provision of aeration through the air bubblers to maintain the quality of water as well as to prevent the degradation of both of the insecticides. During the experiment, the fishes were kept with proper arrangement of their feeding at least twice (morning and evening) a day and due care was taken to maintain their normal biological clock i.e. 12 h of light and 12 h of dark. The feeding was stopped 24 h prior to their sacrifice. The stock and test solution of λ-cyhalothrin was prepared by dissolving the insecticides in acetone. The fishes kept in insecticide free medium with added amount of 3.2 µl acetone/l of aquatic medium served as the control. Two formula grade pyrethroid insecticides namely Colt®25 (25% EC cypermethrin procured from PI Industries Ltd., Division: Pesticides India, 237, G.I.D.C., Ankleshwar-394116, India as a gift) and REEVA-5 (5% EC λ-cyhalothrin received from Rallis India Ltd. Factory: IDA, phase II, Patancheru-502319, India as a gift) were used as toxicant in all sub - acute toxicity studies.

Biochemical assays

The fishes were sacrificed by decapitation and dissected after 96 h of exposure to each of the insecticides, and different organs were surgically removed, thoroughly washed in cold normal saline (0.69% NaCl, 4-6° C), blotted dry, weighed and proceeded further to the preparation of different cell free extracts to be used for carrying out biochemical assays or stored at -20°C till further use. The cell free extracts were prepared by homogenization through Potter-Elvehjam homogenizer fitted with Teflon coated pestle. The homogenates were, further, centrifuged in a refrigerated centrifuge (Sigma, model: 3K30) at different speeds and duration as per requirement of different methods. The supernatant was used for biochemical estimations. The optical density of different samples were taken using double beam UV-vis spectrophotometer (ELICO, model: SL-160) with quartz cuvettes (3.0 ml, 1.0 cm light path) against the suitable blank.

Assay of lipid peroxidation (LPO)

Preparation of cell-free extract

The tissues were excised, rinsed in isotonic ice-cold NaCl (0.69%) solution, blotted dry and weighed. A 10% (w/v) homogenate was prepared in phosphate buffer (100 mM, pH 7.4) containing 150 mM KCl. The homogenate was centrifuged at 9,000 *g* for 30 min. The pellet was discarded and the cell-free supernatant was used for estimation of lipid peroxidation.

Assay of LPO

Lipid peroxidation (LPO) was determined in cytosolic fraction of rat brain and hepatic tissue homogenates by colorimetric estimation of malondialdehyde (MDA)/ thiobarbituric acid reactive substances (TBARS) formed using the method of Niehaus & Samuelsson (1968) (28). The homogenate (0.1 ml) was mixed with 0.9 ml of 0.1 M phosphate buffer (pH - 7.4). The resulting solution was further mixed with 2.5 ml of a reagent composed of 0.375% thiobarbituric acid (TBA), 15% trichloroacetic acid (TCA) and 0.025 N hydrochloric acid (HCl) followed by its incubation at 100°C for 15 min. The solution was cooled and centrifuged at 5,000 *g* for 10 min. The intensity of the developed colour of supernatant was monitored at 540 nm. The results were expressed as nM MDA released/min/mg protein using the extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Assay of lactate dehydrogenase (LDH) activity

Preparation of cell free extract

The 10% (w/v) homogenates were prepared in 0.25 M sucrose solution and kept for 30 min with intermittent stirring and centrifuged at 10,000 *g* for 30 min in a refrigerated centrifuge. The supernatants were collected and used as enzyme source after their incubation in an ice bath for 10 min.

Assay of LDH activity

Lactate dehydrogenase (LDH, EC 1.1.1.28) activity in the cell free extracts was measured by a NADH linked optical assay following the method of Horecker & Kornberg (1948) (16). The reaction mixture (3.0 ml) in quartz cuvettes of 1.0 cm light path contained 50 µl of 2.4 mM NADH, 0.1 ml of 50 mM sodium pyruvate, 1.0 ml of 0.2 M Tris HCl buffer (pH 7.4), 1.0 ml of 0.1M KCl, 50 µl of cell free extracts and 0.8 ml of distilled water. The reaction was started by adding the enzyme source and decrease in optical density at 340 nm was read at 30 seconds interval by monitoring the rate of oxidation of NADH to NAD^+ (or reduction of pyruvate to lactate). LDH activity was expressed as nmole NADH oxidized/min/mg protein. The molar extinction coefficient of NADH at 340 nm (6.22×10^3 M^{-1} cm^{-1}) was used for calculating the enzyme activity.

Assay of acid and alkaline phosphatase activities

Preparation of cell free extract

The 10% (w/v) homogenates were prepared in 50 mM sodium acetate buffer for assaying acid phosphatase and in 25 mM bicarbonate buffer for assaying alkaline phosphatase. The corresponding homogenates were centrifuged at 15,000 *g* for 15 min in a refrigerated centrifuge and the supernatants were collected and used as enzyme source.

Assay of activities of phosphatases

The activities of acid (EC 3.1.3.2) and alkaline phosphatases (EC 3.1.3.1) were measured colorimetrically in cytosolic fractions of different organs by the method as described by Plummer (1971) (32). For assay of acid phosphatase activity, the reaction mixture (3.0 ml) contained 1.3 ml of 100 mM sodium acetate buffer (pH 4.5), 0.5 ml of 2.5 mM p-nitrophenyl phosphate, 100 µl of cell free extract and 1.1 ml of distilled water. The mixture was incubated for 20 min at 37°C. The reaction was stopped by addition of NaOH (2.0 ml, 1N) which developed yellow colour. The intensity of yellow colour was measured colorimetrically at 405 nm. The p-nitrophenol was used as standard. For assay of alkaline phosphatase activity, the reaction mixture (2.4 ml) contained 1.5 ml of 2.0 mM p-

nitrophenyl phosphate prepared in bicarbonate buffer (50 mM, pH 9.5), 20 µl of cell free extract and 0.48 ml of distilled water. The solution was incubated for 30 min at 37°C with intermittent shaking. The reaction was terminated by addition of NaOH (0.4 ml, 0.1N) which produced yellow colour. The intensity of the colour was measured colorimetrically at 410 nm. The p-nitrophenol was used as standard. The activity of acid phosphatase was calculated as following:

ΔOD × 0.1/0.242 × incubation time (min) × protein (mg)

µM p-nitrophenol formed/min/mg protein

where 0.1 µM p-nitrophenol corresponds to OD equal to 0.242 at λ_{max} 405nm

The activity of alkaline phosphatase was calculated as following:

ΔOD × 2/0.257 × incubation time (min) × protein (mg)

µM p-nitrophenol formed/min/mg protein

where 2.0 µM p-nitrophenol corresponds to OD equal to 0.257 at λ_{max} 410nm

Statistical Analyses

The values were presented as means ± standard error of mean (SEM) of observed data of five replicates. Graph pad prism version 3.0 (GraphPad Prism Software Inc., San Diego, CA, USA) was used to analyze the data. One-way analysis of variance (ANOVA) with Dunnett post-test was performed to compare the changes at each of the insecticide concentrations from that of control. In the graphical representation of data, each bar represents the values as means ± Standard Error of Means (SEM) of five replicates of experiments. * and ** represent the values significant at $p < 0.05$ and $p < 0.01$, respectively.

RESULTS

The data depicted in Table 1 showed significant increment in the degree of lipid peroxidation (LPO) in liver and kidney of *C. punctatus* in effect to pyrethroids at 15 and 20% concentrations of their 96 h LC_{50} values. Cypermethrin treatment resulted in more increase in kidney than liver. λ-cyhalothrin exposure was also found to cause more lipid peroxidation in kidney as compared to liver. The result showing comparative efficacy of cypermethrin and λ-cyhalothrin with respect to increase in the level of lipid peroxidation revealed λ-cyhalothrin to be more effective as compared to that of cypermethrin (Table1). In brain, cypermethrin was more efficient in increasing the levels of LPO (Table 1).

The activity of LDH was observed to be enhanced re-

Table 1. Effect of cypermethrin and λ-cyhalothrin on the degree of lipid peroxidation (nM MDA released/min/mg protein) in different tissues of *Channa punctatus* exposed for 96 h.

Degree of lipid peroxidation (nM MDA released/min/mg protein)							
Tissues	**Control**	**Cypermethrin (µg/l)**			**λ-cyhalothrin (µg/l)**		
		40	**60**	**80**	**1.2**	**1.8**	**2.4**
Brain	0.36±0.07	0.48±0.05^{ns} (+33.33)	0.56±0.08^{ns} (+55.56)	0.64±0.08* (+77.78)	0.44±0.07^{ns} (+22.22)	0.51±0.05^{ns} (+41.67)	0.57±0.07^{ns} (+58.33)
Gills	0.5±0.05	0.58±0.07^{ns} (+16)	0.64±0.05^{ns} (+28)	0.69±0.05^{ns} (+38)	0.56±0.07^{ns} (+12)	0.61±0.05^{ns} (+22)	0.65±0.07^{ns} (+30)
Liver	0.67±0.05	0.86±0.06^{ns} (+28.36)	0.95±0.08* (+41.79)	1.09±0.06** (62.69)	0.92±0.06^{ns} (+37.31)	1.03±0.08* (+53.73)	1.19±0.11** (+77.61)
Kidney	1.43±0.15	1.87±0.17^{ns} (+30.77)	2.14±0.11** (+49.65)	2.41±0.11** (+68.53)	1.99±0.28^{ns} (+39.16)	2.25±0.22* (+57.34)	2.57±0.24** (+79.72)
Muscle	0.51±0.07	0.63±0.07^{ns} (+23.53)	0.68±0.08^{ns} (+33.33)	0.76±0.17^{ns} (+49.02)	0.65±0.1^{ns} (+27.45)	0.71±0.1^{ns} (+39.22)	0.78±0.09^{ns} (+52.94)

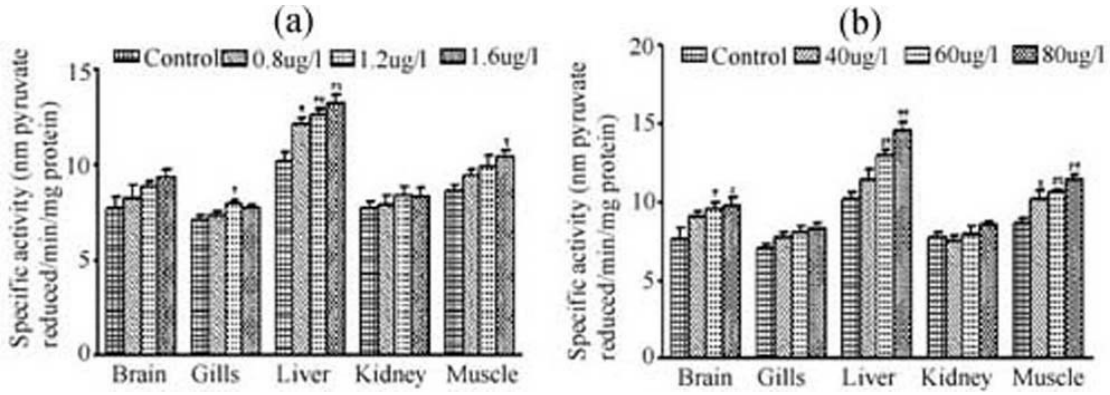

Figure 1. (a). Effect of λ-cyhalothrin on the specific activity of lactate dehydrogenase (nM pyruvate reduced/min/mg protein) in different tissues of *C. punctatus* exposed for 96 h. The activity of enzyme was assayed according to the procedure as described in Materials and Methods. The concentration of λ-cyhalothrin was 0 (, Control), 0.8 µg/l, 1.2 µg/l and 1.6 µg/l. One-way analysis of variance (ANOVA) with Dunnett post-test was performed to compare the changes at each of the insecticide concentrations from that of control. Each bar represents the values as means ± SEM of five replicates of experiments. * and ** represent the values significant at $p < 0.05$ and $p < 0.01$, respectively. **(b).** Effect of cypermethrin on the specific activity of lactate dehydrogenase (nM pyruvate reduced/min/mg protein) in different tissues of *C. punctatus* exposed for 96 h. The activity of enzyme was assayed according to the procedure as described in Materials and Methods. The concentration of cypermethrin was 0 (,Control), 40 µg/l, 60 µg/l and 80 µg/l. One-way analysis of variance (ANOVA) with Dunnett post-test was performed to compare the changes at each of the insecticide concentrations from that of control. Each bar represents the values as means ± SEM of five replicates of experiments. * and ** represent the values significant at $p < 0.05$ and $p < 0.01$, respectively.

markably in liver, muscle and brain of fish under stress induced by both pyrethroids. Cypermethrin as well as λ-cyhalothrin treatment caused highest elevation in the level of LDH in liver followed by muscle and brain. In pyrethroid specific comparison, enhancement in the activity of LDH was more significant under cypermethrin exposure as compared to that of λ-cyhalothrin (Figures 1a and 1b).

The separate treatment of both pyrethroids resulted in significant elevation in the activity of acid phosphatase in brain, liver and kidney of fish (Figures 2a and 2b). The increase was maximum in kidney followed by liver and brain. The study of the specific activity of alkaline phosphatase showed its maximum increase in liver and kidney in effect to either of pyrethroids (Figures 3a and 3b). In organ specific comparison, liver showed more increase in the activity as compared to that of kidney under cypermethrin treatment whereas λ-cyhalothrin exposure resulted in comparatively more increase in the activity of acid and alkaline phosphatase liver than that of kidney. The pyrethroid specific comparison showed λ-cyhalothrin treatment to be comparatively more prominent with respect to the changes in the activities of acid and alkaline phosphatases.

DISCUSSION

The level of lipid peroxidation is an important biomarker of oxidative stress. The results showed significant increment in the degree of lipid peroxidation in different organs of fish in response to the individual treatments of both pyrethroids in a dose dependent manner. This provides a direct evidence of pyrethroid induced oxidative stress in fish. The dose dependent elevation in the level of lipid peroxidation in effect to pyrethroids are in conformity with earlier studies with other oxidative stress biomarkers like cellular stress proteins hsp 60, 70 and 90 in Japanese Medaka (*Oryzias latipes*) (17), lipid peroxidation in mice (41), the production of reactive oxygen species (ROS) in rat (13, 23, 31), osmotic fragility of erythrocytes in rat (9) and thiobarbituric acid reactive substances (TBARS) levels in rabbit erythrocytes (11). The kidney and liver were observed to be the main target organs of lipid peroxidation in effect to the treatment of cypermethrin as well as λ-cyhalothrin. The results showed that the organs involved in detoxification were highly affected by pyrethroids which could be due to overproduction of free radicals via activation of cytochrome P450 isozyme (38). The various

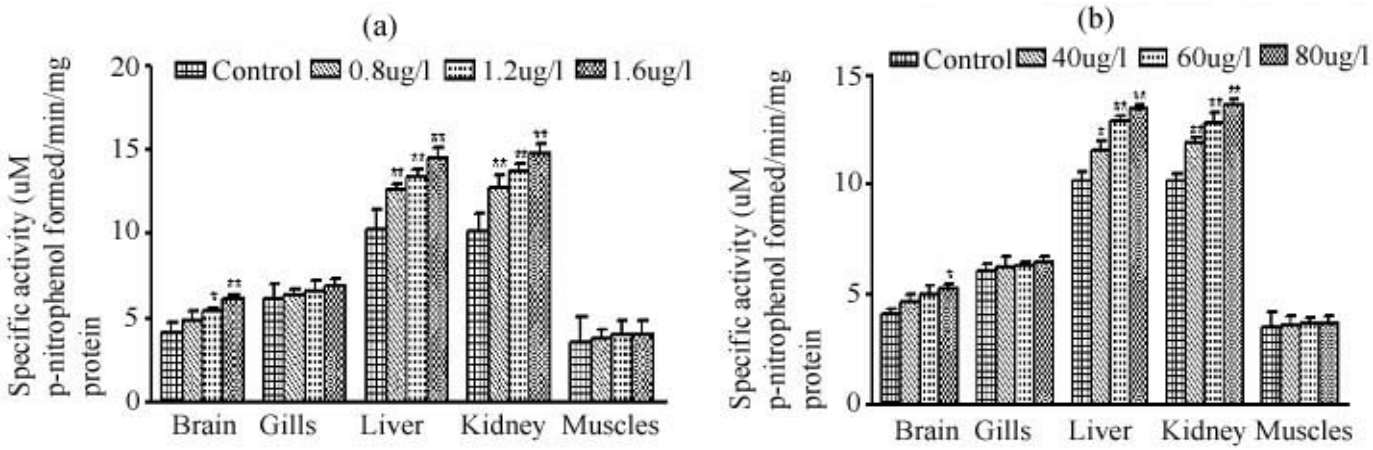

Figure 2. (a). Effect of λ-cyhalothrin on the specific activity of acid phosphatase (μM p-nitrophenol formed/min/mg protein) in different tissues of *C. punctatus* exposed for 96 h. The activity of enzyme was assayed according to the procedure as described in Materials and Methods. The concentration of λ-cyhalothrin was 0 (, Control), 0.8 μg/l, 1.2 μg/l and 1.6 μg/l. One-way analysis of variance (ANOVA) with Dunnett post-test was performed to compare the changes at each of the insecticide concentrations from that of control. Each bar represents the values as means ± SEM of five replicates of experiments. * and ** represent the values significant at $p < 0.05$ and $p < 0.01$, respectively. **(b).** Effect of cypermethrin on the specific activity of acid phosphatase (μM p-nitrophenol formed/min/mg protein) in different tissues of *C. punctatus* exposed for 96 h. The activity of enzyme was assayed according to the procedure as described in Materials and Methods. The concentration of cypermethrin was 0 (,Control), 40 μg/l, 60 μg/l and 80 μg/l. One-way analysis of variance (ANOVA) with Dunnett post-test was performed to compare the changes at each of the insecticide concentrations from that of control. Each bar represents the values as means ± SEM of five replicates of experiments. * and ** represent the values significant at $p < 0.05$ and $p < 0.01$, respectively.

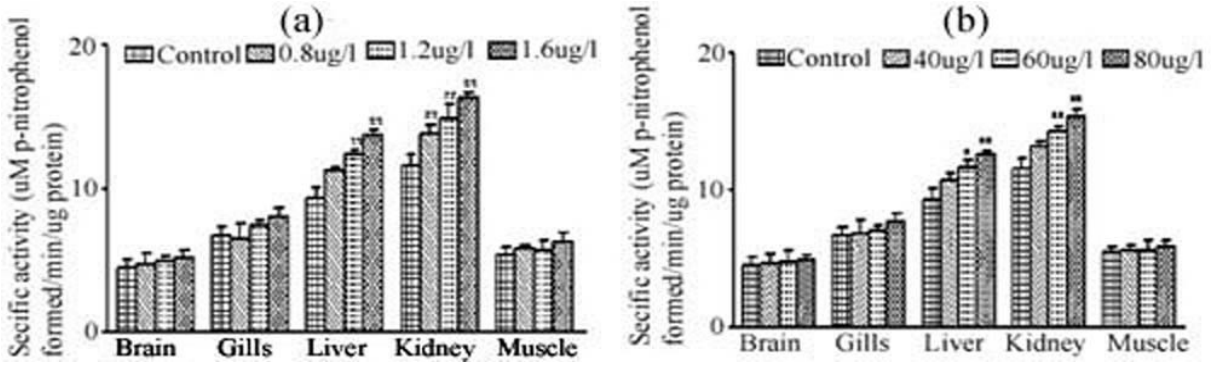

Figure 3. (a). Effect of λ-cyhalothrin on the specific activity of alkaline phosphatase (μM p-nitrophenol formed/min/mg protein) in different tissues of *C. punctatus* exposed for 96 h. The activity of enzyme was assayed according to the procedure as described in Materials and Methods. The concentration of λ-cyhalothrin was 0 (, Control), 0.8 μg/l, 1.2 μg/l and 1.6 μg/l. One-way analysis of variance (ANOVA) with Dunnett post-test was performed to compare the changes at each of the insecticide concentrations from that of control. Each bar represents the values as means ± SEM of five replicates of experiments. * and ** represent the values significant at $p < 0.05$ and $p < 0.01$, respectively. **(3b).** Effect of cypermethrin on the specific activity of alkaline phosphatase (μM p-nitrophenol formed/min/mg protein) in different tissues of *C. punctatus* exposed for 96 h. The activity of enzyme was assayed according to the procedure as described in Materials and Methods. The concentration of cypermethrin was 0 (,Control), 40 μg/l, 60 μg/l and 80 μg/l. One-way analysis of variance (ANOVA) with Dunnett post-test was performed to compare the changes at each of the insecticide concentrations from that of control. Each bar represents the values as means ± SEM of five replicates of experiments. * and ** represent the values significant at $p < 0.05$ and $p < 0.01$, respectively.

metabolic activities associated with these organs, therefore, could be altered very much under such stress conditions. The comparative potential of λ-cyhalothrin and cypermethrin to induce oxidative stress was tissues specific as well as insecticide specific which is in accordance with various earlier studies with different pyrethroids (38, 3, 14).

The results demonstrated in figures 1a and 1b revealed remarkable enhancement in the activity of LDH in liver, muscle and brain in effect to the stress induced by both pyrethroids. Cypermethrin as well as λ-cyhalothrin treatment caused highest elevation in the level of LDH in liver followed by muscle and brain of *C. punctatus*. The elevation in the activity of LDH indicated stress-based tissue impairment (39) in different organs of fish as well as metabolic changes i.e. the glycogen catabolism and glucose shift towards the formation of lactate in stressed fish, primarily in the muscle tissue (37). This increase might be due to the development of anaerobic conditions in fishes (33) which, therefore, could have resorted to an alternative mechanism of energy production for compensating the stress induced by pyrethroids (30). The differential enhancements in the activity of LDH in both fishes in effect to the stress induce by cypermethrin and λ-cyhalothrin reaffirmed the various earlier investigations of pyrethroid induced alterations in energy metabolism (27, 4).

The activities of acid and alkaline phosphatases are good stress indicators of a toxicant in fish (40). Any alteration in phosphatase activity is a direct manifestation of damage or dysfunction in experimental organs of fish (29). Acid phosphatase is a lysosomal enzyme that hydrolyses the ester linkage of phosphate esters and helps in autolysis of cell after its death. Alkaline phosphatase, a brush border enzyme, splits various phosphate esters at an alkaline pH and mediates membrane transport. The present study showed significant but differential enhancements in the activity of acid and alkaline phosphatases in different organs of both fishes in response to the individual treatment of pyrethroids. The strong toxic action of toxicant probably ruptures the cellular and lysosomal membrane that contains the hydrolytic enzymes, resulting in their increase. Both the enzymes are believed to be associated with metabolism of glycogen, phosphoproteins, phospholipids, nucleotides, carbohydrate, proteins and transport of metabolites. Thus, alterations in their activities may result in distorted transport, glycogen level, cell growth and proliferation (6).

The biochemical changes observed in liver profile might also be correlated to hepatocyte damage as reported earlier for *Labeo rohita* exposed to cypermethrin (19). The present findings of pyrethroid specific variability in the activity of phosphatases were in conformity with earlier reports of the differential induction of phosphatase activity in response to different pyrethroids. Johal et al. (2002) (20) reported an increase in acid phosphatase and reduction in alkaline phosphatase activity in muscle, liver and kidney tissues of *Heteropneustes fossilis* on exposure to fenvalerate. The acid phosphatase level remained unchanged while the alkaline phosphatase level in the brain of *Labeo rohita* was depleted when exposed to sub-lethal levels of the pyrethroid, cypermethrin (10). Velisek et al. (2006) (40) found an increase in activity of ALP in rainbow trout after exposure to cypermethrin (3.14 μg/l). λ-cyhalothrin was also reported to cause time dependent increment in the activity of alkaline phosphatase in *C. gariepinus* (29). Long term exposure (28 d) of Nile Tilapia (*Oreochromis niloticus* L.) to sub-acute dose (1.46 μg/l) of another pyrethroid, deltamethrin, also caused an increase in serum alkaline phosphatase (12).

In conclusion, both pyrethroids were able to cause significant increase in the level of lipid peroxidation along with the specific activities of different stress biomarker enzymes even in short period of 96 h at their ppm (parts per million) level concentrations which signifies strong stress inducing potential of these pyrethroids in *C. punctatus*. The importance of present study lies in drawing attention towards the risk of poisoning effects of these pyrethroids especially to those freshwater fishes which usually live in muddy region of water because of the strong soil binding property of pyrethroids along with their slow metabolism in fishes as compared to that of mammals.

ACKNOWLEDGEMENTS

The authors express their gratitude to the Head, Department of Zoology, for providing Central Instrumentation Facility developed with the financial assistance from University Grant Commission, India under its Special Assistance Programme (UGC-SAP) and Department of Science and Technology, Ministry of Science, India under its fund for improvement of Science and Technology Infrastructure in Universities (DST-FIST) for carrying out this work. Financial assistance to one of the authors (AK) from the Council of Scientific and Industrial Research (CSIR), India, in the form of NET-SRF (F.No.9/1 (274)/2005-EMR-I) is gratefully acknowledged.

Other articles in this theme issue include references (42-69).

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