Beta-Cyfluthrin-Induced Hepatic Alterations in Zebrafish: Enzymatic Profiles and Oxidative Stress Responses

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Abstract

The widespread use of pesticides poses a serious risk to both human and environmental health. Many pesticides find their way into aquatic environments as runoff. Broad-spectrum insecticides like synthetic pyrethroids can reach waterways and affect non-target species. Beta-Cyfluthrin is a widely used synthetic pyrethroid which affects both the central and peripheral nervous systems. The current study investigates the impact of beta-Cyfluthrin on the liver of zebrafish (*Danio* sp.). Adult zebrafish were exposed to concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 µg/L of beta-Cyfluthrin for a period of 4 and 21 days respectively (n=20). Biochemical biomarkers for oxidative stress and liver function were assessed. It was observed that exposure to beta-Cyfluthrin led to variations in biochemical markers in both acute and chronic exposure groups. Liver function tests revealed elevated levels of alanine transaminase (ALT) and aspartate transaminase (AST), alongside decreased levels of acid phosphatase (ACP) and alkaline phosphatase (ALP). Lipid peroxidation exhibited a concentration and time-dependent increase, while superoxide dismutase (SOD) and peroxidase (PER) levels declined in both acute and chronic exposure groups. Catalase (CAT) initially increased in acute groups but decreased in chronic ones. Glutathione S-transferase (GST) levels exhibited an increase at lower concentrations but a decrease at higher concentrations of beta-Cyfluthrin. This research suggests that exposure to beta-Cyfluthrin leads to hepatic damage in adult zebrafish. This indicates a potential risk to non-target aquatic species, such as fish, in ecosystems where beta-Cyfluthrin is used as an insecticide.

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Keywords: Zebrafish, beta-Cyfluthrin, pyrethroids, hepatic toxicity, oxidative stress

1. Introduction

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As an agricultural nation, more than 60% of Indians depend on agriculture and its related industries for their livelihood. India produces all types of crops like food grains, horticultural and commercial crops [1]. This in turn leads to the high incidence of pests and diseases and the consequent usage of a multitude of agrochemicals for the protection of crop plants. India is the fourth leading manufacturer of agrochemicals in the world [2]. A significant proportion of the pesticides used reach aquatic ecosystems as agricultural runoff and affect non-target aquatic organisms adversely.

Synthetic pyrethroids (SP) are some of the most widely used pesticides [3]. SPs have low mammalian and avian toxicity but are highly toxic to fishes and aquatic invertebrates [4, 5, 6].

Beta-Cyfluthrin is a carboxylic ester and is characterised as a type II SP. Type II pyrethroids possess α-cyano group and are characterized by increased biological activity compared to type I pyrethroids. They affect the nervous system and inhibit the transmission of neurotransmitters like GABA to the receptors by closing calcium channels, leading to dysfunction of both the peripheral and central nervous systems [7, 8].

In India, beta-Cyfluthrin is widely used for both domestic and agricultural purposes. It is marketed under several brand names, including Solfac® and Responsar® (beta-Cyfluthrin) for house flies, cockroaches, and mosquitoes in homes, and Solomon® (beta-Cyfluthrin + Imidacloprid) for pests like girdle beetles, aphids, and fruit borers on brinjal and soybean crops [9]. Very little ecotoxicological data is available for beta-cyfluthrin. When irradiated with light, the half-life of beta-Cyfluthrin is 16 hours in aqueous solutions [10]. Beta-Cyfluthrin is expected to adsorb to suspended solids and sediments in water, based on the Koc range. The estimated Bioconcentration Factor (BCF) of beta-Cyfluthrin is 170, which suggests that the potential for bioaccumulation in aquatic organisms is high [11, 12]. Hence, it is very important to study the effects of beta-cyfluthrin on aquatic organisms.

In this current investigation, we examined how exposure to low levels of beta-Cyfluthrin in the environment affects zebrafish (*Danio* sp.). Zebrafish was selected as the model animal due to its small size, rapid development, ease of maintenance, large number of eggs laid in a single spawning, and its physiologic similarity to other vertebrates including humans [13, 14]. Since the liver is the main detoxification centre for xenobiotics, exposure to toxic substances like synthetic pyrethroids can impact liver function, affecting enzyme activity and tissue structure. Liver enzymes are sensitive indicators of liver damage and dysfunction caused by chemicals. Thus, we analyzed specific activities of enzymes such as AST, ALT, ALP and acid phosphatase ACP in liver tissue. Other synthetic pyrethroids have been shown to induce oxidative stress responses, leading to necrosis. Therefore, we also conducted assays on oxidative stress marker enzymes - SOD, CAT, PER, GST and lipid peroxidation in liver tissue.

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2. Materials and Methods

2.1 Chemicals

Beta-Cyfluthrin[cyano-(4-fluoro-3-phenoxyphenyl)methyl]-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate, 96.4%] was obtained from Sigma Aldrich [15]. A stock solution of 1 mg/L concentration was prepared using acetone as a solvent medium and stored in the dark at 4ºC. A solvent control (SC) was prepared with a concentration of 4 µl/L of acetone. Based on pilot trials conducted on the lethal toxicity of beta-Cyfluthrin, test solutions of 0.25, 0.5, 1.0, 2.0 and 4.0 µg/L were prepared by diluting the stock solution.

The kits for enzyme assays were procured from ARKRAY Healthcare Pvt. Ltd. for AST, ALT, and ALP analysis, while ACP analysis was done using kits from Coral Clinical Systems (Tulip Diagnostics Pvt. Ltd.). AR Grade chemicals for antioxidant stress enzyme assays were obtained from SISCO Research Laboratories Pvt Ltd. Lipid peroxidation analysis was carried out using HiMedia (Product Code - CCK023) kits. The physicochemical parameters of the aquarium water in the aquarium was monitored daily for pH, temperature, ammonia, nitrate, and nitrites using commercially available API Freshwater Master kit. All the other chemicals were of analytical grade and were obtained from S D Fine-Chem.

2.2 Experimental design

Adult zebrafish (*Danio* sp.), around four months old were obtained from Zebrafish Breeding and Maintenance facility, Sophia College for Women. They were housed as per the standard laboratory protocols at the Zebrafish Breeding and Maintenance facility registered under the Committee for Control and Supervision of Experiments on Animals (CCSEA) at Sophia College for Women, Mumbai, Registration Number 1936/PO/Re/S/17/CPCSEA.

The fishes were then transferred to static tanks and acclimatized for a period of two weeks at 28±1ºC and exposed to a light:dark cycle of 14:10 hours at a stocking density of 20 fishes in 10 litres of charcoal-filtered, UV purified tap water. They were fed with readymade fish food granules *ad libitum*, twice a day throughout the study period. The tanks were cleaned daily and any waste matter was siphoned out. After acclimatization, healthy individuals of both genders, with an average body length ranging from 2.5 to 3.5 cm were randomly chosen for inclusion in the study. To study the effect of acute and chronic exposure of beta-Cyfluthrin on adult zebrafish, different groups were exposed to varying concentrations for 4 days and 21 days respectively. Within each treatment period, seven groups of 20 adults each were created, one group as Control (C), one as SC and 5 test concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 µg/L of beta-Cyfluthrin.

To maintain the relevant concentration of beta-Cyfluthrin in the tank, 30% of the aquarium water was replaced by semi-static renewal method and the appropriate volume of stock solution was added into the tank to get the specific working solution as per OECD guidelines [16]. At the end of the treatment periods, the animals were sacrificed ethically by immersing in ice-cold water. The liver was immediately dissected out, rinsed in saline, blotted, and processed for enzyme assays under ice cold conditions. All the protocols regarding maintenance of animals, exposure to test chemical, euthanization, and handling of tissues were conducted as per the guidelines of OECD Test No. 203 and 230 [16, 17].

The liver homogenate was prepared by pooling the tissue obtained from all the individuals of the respective treatment groups. The antioxidant stress profile was evaluated by determining the specific activity of CAT [18], PER [19], SOD [20], GST [21] in terms of protein concentration of the liver homogenate. Lipid peroxidation was expressed as malondialdehyde (MDA) concentration in terms of protein concentration of the liver homogenate [22]. The protein concentration was determined by Folin Lowry method [23]. The liver enzyme assays for ALT, AST, ACP and ALP [24, 25, 26] were determined in terms of protein concentration. Double Beam UV-VIS Spectrophotometer LMSPUV -1200 was used for spectrophotometric analysis. All the assays were replicated twice for a treatment group.

2.3 Statistical Analysis

The data was presented as Mean ± standard deviation (SD). Statistical differences between control and treatment groups were evaluated by one-way ANOVA for significance between treatment groups at different concentrations and two-way ANOVA was used to find significance between treatment groups at different time durations, followed by Bonferroni and Holm pairwise comparison of means for post-hoc analysis. The differences were considered statistically significant when $p<0.05$ and extremely significant when $p<0.01$.

3. Results and Discussion

Among commonly used insecticides in agricultural as well as domestic settings, SPs are the highly effective insecticides against their target groups. Their toxicity to birds and mammals is very low, however, they are highly toxic to fishes and other aquatic invertebrates. This can result in the inadvertent impact of these insecticides on non-target species in aquatic ecosystems due to agricultural runoff. The current study investigated the effect of sub-lethal concentrations of acute and chronic exposure to beta-Cyfluthrin, a widely used synthetic insecticide on adult zebrafish (*Danio* sp.) liver antioxidant stress profile and liver function profile.

3.1 Mortality

No significant mortality was observed in any of the control or experimental groups. In the chronic group, fishes exposed to concentrations of 1.0, 2.0 and 4.0 µg/L showed less than 10% mortality at the end of the study period. There was no mortality in the acute group. This shows that the LC50 of beta-Cyfluthrin on zebrafish is greater than 4.0 µg/L.

3.2 Liver Function Profile

AST and ALT

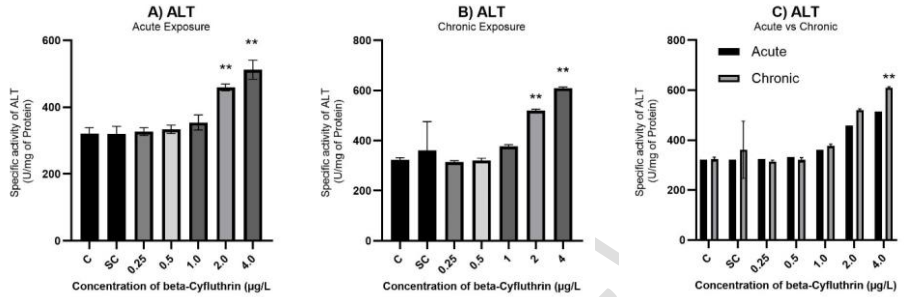

Fig.1. Mean values of specific activity of ALT enzyme following exposure for (A) 4 days (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentrations at P< 0.01 are marked as **.

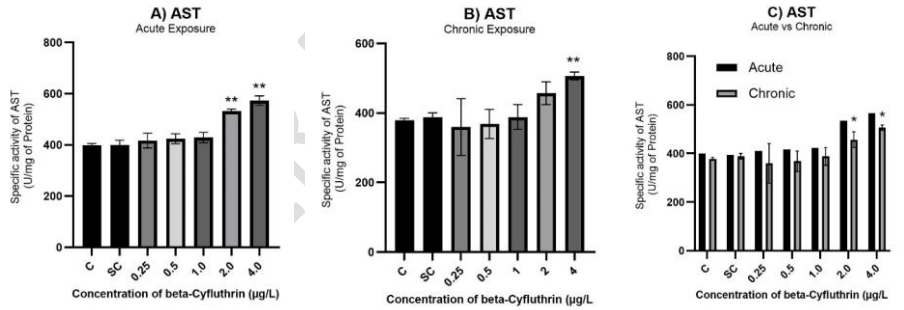

Fig.2. Mean values of specific activity of AST enzyme following exposure for (A) 4 days (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentrations at P< 0.05 are marked as * and P< 0.01 as **.

In the present study, specific activity of ALT and AST increased in a dose and time dependent manner for both acute and chronic treatment groups when compared to the Control and Solvent control groups (Fig. 1A, 1B, 2A, 2B). The increase was significant at concentrations of 2.0 and 4.0 µg/L for both the treatment periods for ALT and AST.

Comparison of acute and control groups showed significant increase in ALT activity in the chronic group at concentration of 4.0 µg/L, while AST activity decreased significantly in the chronic group at 2.0 and 4.0 µg/L (Fig. 1C). African sharptooth catfish *(Clarias gariepinus)* exposed to synthetic pyrethroids, cypermethrin and deltamethrin showed concentration-specific increases in ALT, AST and ALP [27]. Zebrafish exposed to sublethal concentrations of fenvalerate showed increased activity of ALT and AST in the liver [28]. Increased activity of ALT and AST could be due to an increase in the synthesis of amino acids or increase in the transamination process from fatty acids in response to xenobiotics [28]. AST and ALT activity levels are frequently used as biochemical indicators of xenobiotic injury to the liver. Increased activity of transaminases in the liver could indicate deterioration of cellular functions and loss of tissue integrity due to necrosis, edema, cholestasis and cellular inflammation [29, 30, 31]. The decrease in AST (Fig 2C) in the chronic group could indicate the breakdown of liver cytoarchitechture after prolonged stress.

ALP and ACP

In present study, specific activity of ACP and ALP decreased in a dose and time dependent manner for both acute and chronic treatment groups when compared to the Control and Solvent control groups (Fig. 3A, 3B, 4A, 4B). The decrease in ACP activity was significant at concentrations of 4.0 µg/L for the acute group while there was no significant change for ALP. The decrease in ACP was significant at concentrations of 2.0 and 4.0 µg/L for the chronic group while there significant decrease at 1.0, 2.0 and 4.0 µg/L for ALP. Comparison of acute and control groups showed significant decrease in ACP activity levels in the chronic group at concentrations of 2.0 and 4.0 µg/L, while ALP activity decreased significantly in the chronic group at 1.0, 2.0 and 4.0 µg/L (Fig. 3C and 4C). ACP and ALP activity in the liver and gills of zebrafish exposed to alphamethrin showed significant decrease [32]. *Labeo rohita* fingerlings exposed to sub-lethal concentrations of Cypermethrin showed a similar decrease in ALP [33]. In *Channa punctatus*, ACP activity decreased after exposure to fenvalerate [28]. However, contrary to our findings, in *Heteropneustes fossilis*, chronic exposure to fenvalerate resulted in an increase of ALP activity in the liver [28]. Hepatic cellular damage can lead to significant alteration in phosphatase activity. Decreased enzyme synthesis could be a result of organ dysfunction [34]. The decrease in ALP can be an indicator of hepatic parenchymal damage and necrosis. Any damage in the hepatic cells may result in an alteration in phosphatase activity [35].

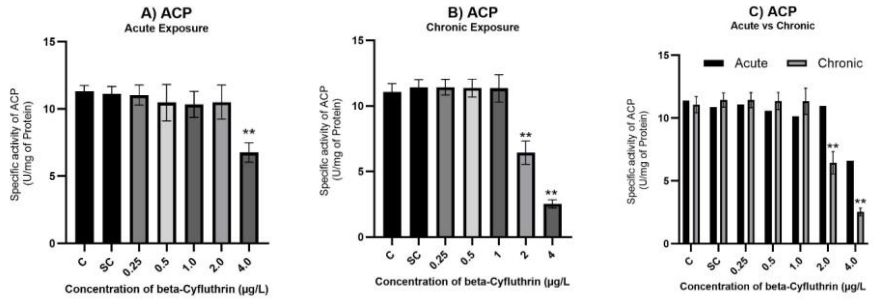

Fig.3. Mean values of specific activity of ACP enzyme following exposure for (A) 4 days (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentrations at P< 0.01 are marked as **.

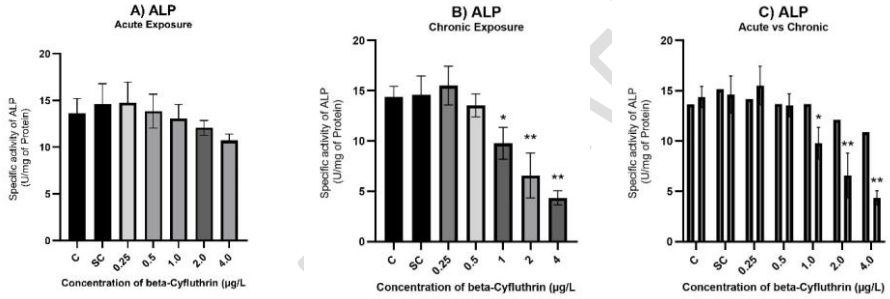

Fig.4. Mean values of specific activity of ALP enzyme following exposure for (A) 4 days (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentrations at P< 0.05 are marked as * and P< 0.01 as **.

3.3 Oxidative Stress Response Profile

Oxidative stress results from an imbalance between the levels of reactive oxygen species (ROS) and antioxidants. Exposure to xenobiotic stressors like pesticides leads to oxidative stress resulting in the production of ROS. Fishes, like other organisms, utilize antioxidant enzymes like SOD and CAT to combat ROS. These enzymes work to transform superoxide anions into hydrogen peroxide, subsequently breaking them down into water and oxygen, aiming to mitigate the detrimental effects of excessive ROS [36].

Reactive aldehydes like malonialdehyde (MDA) formed during lipid peroxidation of polyunsaturated fatty acids can activate the adaptive stress response during xenobiotic insult. This will lead to increased activity of the antioxidant system. Failure of the antioxidant system would lead to cellular apoptosis. An increase in lipid peroxidation is a good biomarker for the presence of xenobiotic stress in an organism as it indicates increased production of ROS [37, 38].

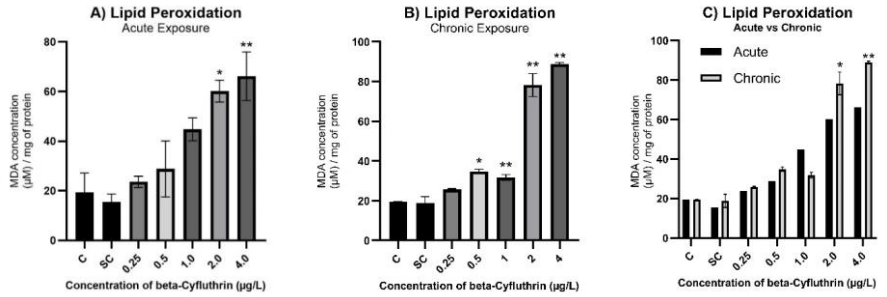

Fig.5. Mean values of MDA concentration (lipid peroxidation) following exposure for (A) 4 days (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the Control (C), Solvent Control (SC) and the respective experimental groups or between two exposure periods of respective concentrations at P< 0.05 are marked as * and P< 0.01 as **.

In the current study, there was a significant increase in lipid peroxidation in the liver of fishes exposed to 2.0 and 4.0 µg/L of beta-Cyfluthrin in both the acute and chronic groups (Fig. 5A and 5C). Comparison between the respective concentrations of acute and chronic treatments showed significant increase in the chronic group at concentrations of 2.0 and 4.0 µg/L (Fig. 5C). This is in concurrence with other studies in which an increase in LPO was observed in the liver and kidney of *Oreochromis nilticus* and *Cyprinus carpio (L)* exposed to cypermethrin and deltamethrin [39, 40, 41]. Freshwater mussel *Unio elongatulus euchres* exposed to deltamethrin showed similar increase in lipid peroxidation [42]. Common carp (*Cyprinus carpio* L.) exposed to deltamethrin also showed an increase in lipid peroxidation [43]. The liver is the main detoxification organ and serves as the location for numerous oxidative processes. The highest production of free radicals occurs there. Exposure of the liver to beta-Cyfluthrin could have led to an increase in ROS, which could be associated to the metabolism of beta-Cyfluthrin to the peroxidation of membrane lipids of the liver [44, 45, 46].

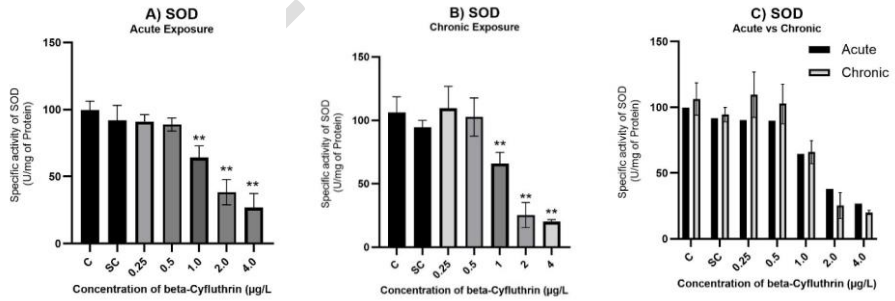

Fig.6. Mean values of specific activity of SOD enzyme following exposure for (A) 4 days (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentrations at P< 0.01 are marked as **.

SOD is a metalloenzyme which plays a crucial role as an antioxidant. It is the primary defence against superoxide radicals, the major initial form of ROS produced by mitochondria following a xenobiotic insult. It catalyzes the dismutation of superoxide anion free radical into molecular oxygen and hydrogen peroxide [47]. In this study, SOD activity decreased significantly in both acute and chronic groups at concentrations of 1.0, 2.0 and 4.0 µg/L of beta-Cyfluthrin (Fig. 6A and 6B). Comparison between acute and chronic groups showed an initial increase in the chronic group at lower concentrations, followed by a decrease at concentrations 2.0 and 4.0 µg/L. However, the decrease was not significant (Fig. 6C). In a similar study, fenvalerate, a type II synthetic pyrethroid exposure resulted in decreased SOD activity in zebrafish liver after 28 days [28]. Nile tilapia (*Oreochromis niloticus*) exposed to chlorpyrifos, an organophosphate insecticide and common carp (*Cyprinus carpio*) exposed to hexachlorobenzene also showed a decrease in SOD activity [48, 49]. However, contrary to our findings, exposure of zebrafish to Cypermethrin lead to a significant increase in SOD activity. *Labeo rohita* exposed to beta-Cypermetnrin also increased SOD activity in the liver [50, 51]. An increase in the antioxidant activity of SOD indicates attempts by the oxidative stress response system to eliminate ROS. However, our studies show slight elevation at lower concentrations followed by a reduction in SOD activity. This trend can be interpreted as initial attempts by the antioxidant system to get rid of ROS at lower concentrations. Reduction of SOD activity at higher concentrations and prolonged treatment periods could indicate an overwhelmed antioxidant capacity potentially leading to cell damage [48, 49].

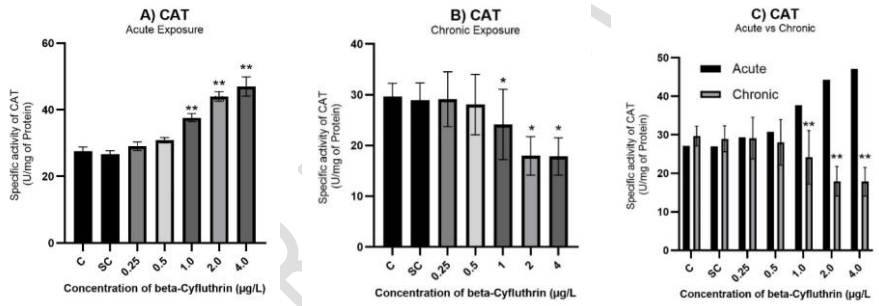

Fig.7. Mean values of specific activity of CAT enzyme following exposure for (A) 4 days (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentrations at $P< 0.05$ are marked as * and $P< 0.01$ as **.

Catalase (CAT) is a ubiquitous antioxidant enzyme, which degrades the hydrogen peroxide produced by SOD activity into water and oxygen [51, 52]. Hence it is responsible for the removal of hydrogen peroxide under toxic conditions. Exposure to beta-Cyfluthrin led to an increase in CAT activity with increasing concentrations in the acute treatment group (Fig. 7A), while the chronic group showed a decreasing trend (Fig. 7B). Comparison between acute and chronic trend shows a significant reduction in the chronic group when compared to acute group ate concentrations of 1.0, 2.0 and 4.0 µg/L of beta-Cyfluthrin (Fig. 7C). Zebrafish exposed to dimethoate, an organophosphate showed a gradual decline in the activity of CAT in the liver at 7, 14 and 21 days [53]. Zebrafish exposed to Alphemethrin showed a time and dose dependent reduction in CAT activity [54]. Tripathi and Singh also reported a reduction of CAT activity in the brain, liver and skeletal muscles of *Channa*

punctatus exposed to alphamethrin [55]. However, larval stages of zebrafish exposed to Cypermethrin showed an increase in CAT activity [56]. The increase in CAT activity with increasing concentrations in the acute group could stem from the antioxidant system trying to degrade hydrogen peroxide into water and oxygen. However, chronic exposure could have led to decreased CAT activity due to excess production of hydrogen peroxide. This could be due to the increased flux of superoxide radicals which has been shown to inhibit CAT [57].

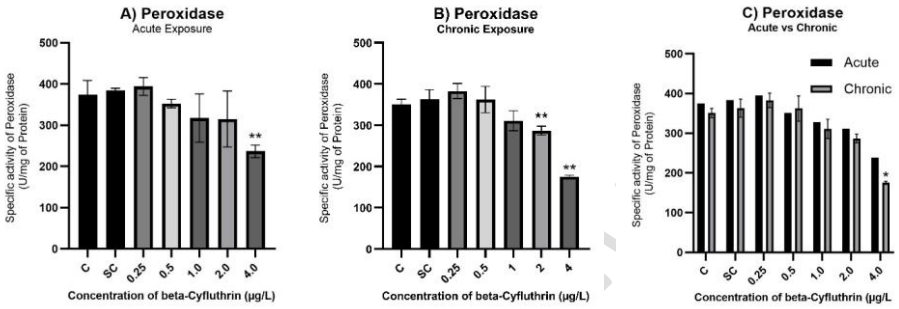

Fig.8. Mean values of specific activity of peroxidase enzyme following exposure for (A) 4 days (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentrations at $P< 0.05$ are marked as * and $P< 0.01$ as **.

Peroxidases are a family of enzymes classified as oxidoreductases. Its function is to break down hydrogen peroxide. Thus it plays an important role in the antioxidant stress response system. In the current study, peroxidase activity showed a significant decrease at higher concentrations in both acute and chronic groups (Fig. 8A and 8B). The decrease in the chronic group was more compared to the acute group (Fig. 8C). Earlier studies show that the livers of mice exposed to deltamethrin showed a significant decrease in peroxidase [58]. Ullah reported an increase in peroxidase activity in the liver of Grass carp, *Ctenopharyngodon idella* [59]. Exposure of rats to pesticides chlorpyrifos (CPF), methyl parathion (MPT) and malathion (MLT) resulted in significant decrease in Glutathione peroxidase activity [60].

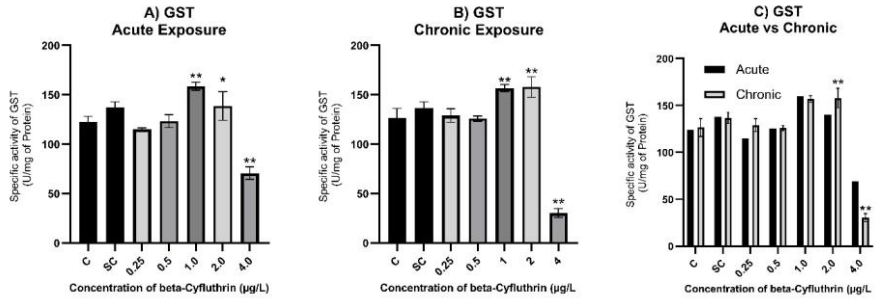

Fig.9. Mean values of specific activity of GST enzyme following exposure for (A) 4 days (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentrations at P< 0.05 are marked as * and P< 0.01 as **.

GST is responsible for detoxification and neutralizes toxic compounds through the SH group of reduced GSH. [61, 62]. In the current study, the specific activity of GST increased in zebrafish exposed to concentrations of 1.0 and 2.0 µg/L of beta-Cyfluthrin, followed by a significant reduction at 4.0 µg/L in both the acute and chronic groups (Fig. 9A and 9B). Comparison acute and chronic groups showed increase in the chronic group at 2.0 µg/L, followed by decrease at 4.0 µg/L of beta-Cyfluthrin (Fig. 9C). Prior research conducted on rats has reported increase in GST activity when exposed to permethrin [63] and cypermethrin. Freshwater *Channa punctatus* exposed to deltamethrin showed a similar increase [39]. Kong et al. 2021 found that exposure to deltamethrin, a type II pyrethroid over 28 days caused a decrease in GST levels in snakehead fish, *Channa argus* [64]. Long-term exposure of deltamethrin on Common carp, *Cyprinus carpio* showed a similar reduction in GST activity [65]. Ensibi et al 2013 studied the effects of deltamethrin on liver biomarkers in Common Carp, *Cyprinus carpio* L. found that while there was no significant change in the GST levels during short-term exposure, chronic exposure resulted in a reduction of GST levels [43]. Exposure to Lambda-cyhalothrin, a type II pyrethroid in *Cyprinus carpio* L. for 45 days showed a significant increase in liver GST in the initial stage followed by a subsequent reduction [66]. The above findings are in agreement with the present study. Exposure to beta-Cyfluthrin could have led to increased levels of ROS, which would have triggered the stress-response mechanism [67]. This would lead to an increased generation of glutathione (GSH) [68]. The subsequent decrease in GST activity at higher concentrations and increasing exposure periods could be a result of the reduced efficiency of the detoxification mechanism of the liver.

4. Conclusion

In the current study, there was no significant mortality indicating that the LC50 of beta-Cyfluthrin is greater than 4.0 µg/L. It was observed that exposure to beta-Cyfluthrin resulted in significant variations in biochemical markers, both in acute and chronic groups. Liver function tests indicated an increase in ALT and AST levels, while ACP and ALP decreased. This change was more significant in chronic groups, indicating that longer exposure could lead to liver damage. Lipid peroxidation increased in a concentration and time dependent manner, indicating increasing levels of ROS. SOD and PER levels decreased in both acute and chronic groups, while CAT increased initially in acute groups, but decreased in chronic

groups. GST showed an increase in lower concentrations and a decrease in higher concentrations.

In summary, this study highlights the adverse effect of beta-Cyfluthrin on biochemical markers of the liver which can help create more effective regulatory measures to protect ecosystems and reduce their inadvertent effects on non-target species.

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