Journal of Advanced Zoology

ISSN: 0253-7214

*Volume **45** Issue **3** Year **2024** Page 406-**417***

Effects of Clothianidin on Biochemical Parameters of Adult Zebrafish

Shreya Patil[1], Roshan C. D'souza[1]*

[1*]*Department of Zoology, Sophia College for Women (Autonomous), Mumbai, India*

***Corresponding Author:** Roshan C. D'souza*
E-mail: roshan.dsouza@sophiacollege.edu.in

ABSTRACT

The ubiquitous use of insecticides leads to detrimental effects on non-target organisms due to accidental exposure. Neonicotinoid insecticides are popularly used worldwide for their high affinity for arthropod nicotinic receptors which effectively kill insect pests. Low affinity towards vertebrate nicotinic receptors, make them safer as compared to traditional insecticides. Recent studies demonstrated that neonicotinoid exposure can cause some toxicity in vertebrates including humans. Zebrafish is one of the popular model organisms to study ecotoxicity. This is the first study on adult zebrafish to report the effect of novel neonicotinoid, Clothianidin on mortality, liver antioxidant stress profile, liver function profile and brain acetylcholinesterase (AChE). Observations were made over two treatment periods, 96 hours, and 21 days, in five groups exposed to varying concentrations of Clothianidin viz. 30mg/L, 50mg/L, 70mg/L, 90mg/L, 110mg/L and a control. Although no mortality was observed, Clothianidin exposure led to increased activity of superoxide dismutase (SOD) enzyme, lipid peroxidation and decreased catalase (CAT), glutathione-S-transferase (GST). Treated groups also showed increased concentrations of liver enzyme alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and acid phosphatase (ACP). These results indicate that Clothianidin causes disturbances in the hepatic function. A prominent decreasing trend observed in brain AChE shows that Clothianidin inhibits AChE in adult zebrafish. Further investigations on DNA damage and gene expression studies could be conducted to understand the exact mechanism of action of Clothianidin at molecular level.

Keywords** – **Neonicotinoids, Zebrafish, Clothianidin, Hepatotoxicity, Antioxidant stress enzymes

Introduction

Agriculture holds enormous significance for development of any country, standing as one of its important sectors. Use of pesticides increases the agricultural yield exponentially by protecting the plants from pest attack and diseases. In India, around 40% of the total cultivated area is treated with pesticides[1]. More than half of the pesticides produced globally are utilized in Asia[2]. A study conducted in agricultural workers in Puducherry, South India showed that more than 40% of workers were not using protective equipment during pesticide spraying, waste management guidelines were not followed while disposing the pesticide containers, and

leftover pesticides are generally buried in the soil which causes soil and water pollution[3]. Pesticides also have an important role in public health as part of controlling vectors and carriers of disease-causing agents. The use of household pesticides as well as unsafe handling practices are highly prevalent in Indian rural and urban communities[4,5]. Pesticide exposure, either occupationally or environmentally, can cause a range of human health problems like immune suppression, hormone disruption, diminished intelligence, reproductive abnormalities and cancer[6].

Neonicotinoids such as Clothianidin, imidacloprid, and thiamethoxam are broad-spectrum insecticides that exhibit activity against sucking insects such as aphids, whiteflies, leafhoppers and several species of flies and moths. They are generally used as seed dressings for a variety of oilseed crops, cereals, beets, and potatoes etc. Neonicotinoids are nicotinic agonists that interact with the nicotinic acetylcholine receptors (nAChER) in a different way than nicotine. Clothianidin is a novel neonicotinoid insecticide that possesses a thiazolyl ring. It is effective against Hemiptera, Thysanoptera, Diptera, Coleoptera and Lepidoptera pests. It can be applied to crops by a broad variety of treatment methods[7]. Neonicotinoids have low affinity for vertebrate nicotinic receptors as compared to that of the insects. Hence, they generally show low acute toxicity to mammals, birds, and fish as compared to traditional insecticides[8], but some recent studies also display that neonicotinoid pesticides indeed cause some toxicity in amphibians, fish and mammals including humans[9-16]. In recent years, neonicotinoids and their metabolites have been detected in various environmental and biological samples[17]. Limited data is available on the fate of Clothianidin under realistic agricultural and other fields of use[18]. The Groundwater Ubiquity Scores suggest that Clothianidin has moderate to high potential to leach to groundwater[19]. It has been observed that the thiamethoxam and its metabolite-Clothianidin bioaccumulates on prolonged exposure[20]. Studies have demonstrated the adverse effects of a chronic exposure to sublethal doses of Clothianidin on foraging and dance communication in honey bees[21]. However, the effect of chronic exposure to Clothianidin on vertebrates has not been studied elaborately. Monitoring and analyzing the effects of xenobiotics such as pesticides in the aquatic ecosystems is crucial in protecting human health and the environment. The zebrafish is one of the widely used NIH approved model organisms. It is a preferred model in research fields because of its small size, low maintenance cost, short breeding cycle, high fecundity, and transparent embryos. Recent studies have reported that use of zebrafish can aid in assessing the toxicity environmental contaminants[22].

In current literature, very few studies are available that focus on evaluation of toxicity of Clothianidin on Zebrafish model organism[23-27]. The present study aims to understand the effect of sublethal dose of neonicotinoid - Clothianidin on Zebrafish on 96 hours (Acute) and 21 days (Chronic) exposure. In this study we assessed the effect of Clothianidin, on liver antioxidant stress profile, liver function profile and brain AChE in zebrafish.

Materials and Methods

Model animal

Zebrafish, (*Danio* sp.*)* were maintained under the standard laboratory conditions at the CCSEA registered zebrafish breeding and maintenance facility of Sophia College for Women, Mumbai, Registration Number 1936/PO/Re/S/17/CPCSEA. Individuals of both sexes with average body length of about 2.5 to 3.5cm were selected randomly for the study.

Study agent

Clothianidin of 96.5% technical grade was procured from R. V. Agri Corporation, Navsari, Gujarat, India.

Other reagents

AR Grade chemicals used for antioxidant stress enzyme assays were procured from SISCO Research Laboratories Pvt. Ltd. Enzyme assay kits from ARKRAY Healthcare Pvt. Ltd. were used for AST, ALT, and ALP analysis, while ACP analysis was conducted using kits from Coral Clinical Systems (Tulip Diagnostics Pvt. Ltd.). Lipid peroxidation analysis was carried out using kits from HiMedia (Product Code - CCK023).

Experimental design

Adult zebrafish were acclimatized for two weeks in laboratory conditions, at temperature 28±0.5°C, light-dark cycle of 14:10 hours. After acclimatization, healthy individuals of equal numbers of both sexes were selected and divided in the six groups of 12 animals each. The males and females were maintained in separate glass aquariums holding 5L of water with 6 adult zebrafish in each. One group was maintained as a control and five groups were exposed with varying concentrations of study agent, namely, 30mg/L, 50mg/L, 70mg/L, 90mg/L, 110mg/L for 96 hours to study the effect of acute exposure and for 21 days to study the effect of chronic

exposure of Clothianidin on adult zebrafish. Fish were fed with ground powder of readymade fish food pellets, twice a day during acclimatization as well as entire research period. Throughout the study period, the water levels in the tanks were maintained meticulously by the semi-static renewal method by replacing the approximately 1/3 of the tank water with fresh water. The appropriate volume of stock standard solution was added into the tank to get the specific working concentration of study agent in the tank as per OECD guidelines[28]. The water in the aquarium was monitored daily for various physicochemical parameters like pH, temperature, ammonia, nitrate, and nitrites and maintained constant. At the end of the exposure periods, animals were sacrificed ethically by immersing in ice cold water. Liver and brain tissues were harvested, cleaned in normal saline, blotted, and processed immediately for enzyme assays. Maintenance of animals, treatment, euthanizing, and further handling of tissues were conducted as per the guidelines and OECD Test No. 203[28,29].

Antioxidant stress profile of liver

Liver and brain homogenate was prepared separately by pooling the tissue obtained from all the individuals of respective treatment groups at the end of exposure periods. Specific activity assays for CAT[30], Peroxidase[31], GST[32], SOD[33] and Lipid peroxidation[34] were carried out using liver homogenate. Specific activity assay for enzyme AChE[35] was performed using brain tissue. Catalytic activities of all analyzed enzymes were normalized to protein concentration, determined by Folin Lowry method[36]. Amount of lipid peroxidation was expressed as nmol per gram wet weight of tissue. Additionally, assays were performed for enzymes ALT[37], AST[37], ALP[38] and ACP[39] from liver tissue homogenate. Spectrophotometric analysis was performed using Double Beam UV-VIS Spectrophotometer LMSPUV -1200.

Statistical Analysis

All statistical analyses of data were performed using GraphPad Prism 9. Results were expressed as Mean $\pm$ SD. One-way analysis of variance (ANOVA) was used for determining the statistical significance between treatment groups at different concentrations and two-way ANOVA was used to find significance between treatment groups at different time duration, both followed by Bonferroni test for post-hoc analysis. Differences at $P < 0.05$ were considered significant.

Results and Discussion

Neonicotinoids are commonly used pesticides in agriculture as well as in residential areas due to their effective mode of action and comparatively lower toxicity to vertebrate organisms. However, some recent studies have thrown light on the possible side effects of neonicotinoids such as thiamethoxam, sulfoxaflor, imidacloprid, nitenpyram on non-target vertebrate species including humans. The present study focuses on the effects of acute and chronic treatment of Clothianidin on the mortality, liver oxidative stress profile, liver function profile and brain AChE of adult zebrafish.

Mortality

No significant mortality was observed in any of the experimental groups as compared to that of the control group. Less than 2% mortality was observed overall during the treatment period. This shows that the margin of safety of Clothianidin could be broad and it may not cause lethality in adult zebrafish at the concentrations up to 110mg/L for 21 days. LC50 for other fish species such as Bluegill sunfish (*Lepomis macrochirus*), Rainbow trout *(Oncorhynchus mykiss)* and Sheepshead minnow *(Cyprinodon variegatus)* has been reported to be 117 ppm for 96 hr, 105 ppm for 96 hr and >93.6 ppm for 96 hr respectively[40-42]. As practically no mortality was observed in the present study, LC50 of Clothianidin for zebrafish is >110 ppm for 21 days.

Oxidative Stress Enzyme Profile

SOD

SOD is an important antioxidant enzyme that protects the cells by preventing the free radical damage. Several environmental pollutants are known to either induce or inhibit the SOD response. In the present study, the acute as well as chronic exposure to Clothianidin caused significant increase in the specific activity of SOD at two highest concentrations, 90 mg/L and 110 mg/L (Fig 1. A, B). However, the effect of chronic treatment was observed to be insignificant than that of acute (Fig 1. C). A study showing effect of another neonicotinoid, thiamethoxam, of which Clothianidin is a metabolite, on zebrafish liver has also reported the dramatic increase in SOD levels on acute exposure to pesticide but not on chronic exposure[43]. Similar results were observed in a study assessing the toxicity of one of the neonicotinoids, imidacloprid on zebrafish where a noticeable increase in SOD was observed in the early exposure but towards the end of the exposure period SOD was inhibited[44]. In present study, significant increase in the SOD level on acute exposure is observed to be consistent in chronic

treatment. This shows that the toxic effect of Clothianidin induces the formation of superoxide ions which in turn causes increase in the SOD activity in order to prevent the subsequent cell damage.

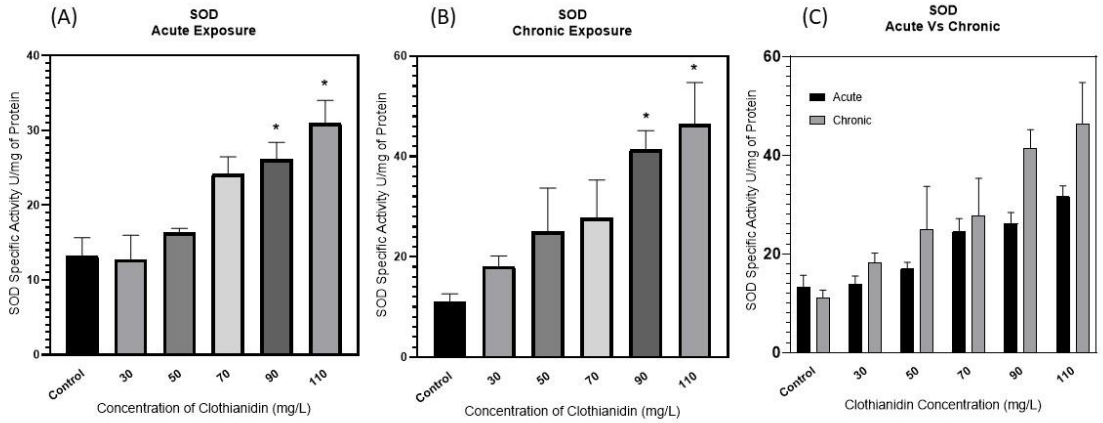

Fig 1. Mean values of specific activity of SOD enzymes following clothianidin exposure for (A) 96 hours (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentration at P<0.05 are marked as *.

CAT

CAT is one of the marker enzymes of antioxidant system which reduces the elevated concentration of reactive oxygen species in liver cells. In the present study, the specific activity of CAT showed a significant decrease at the highest treatment concentration on acute exposure to Clothianidin. However, it did not cause significant change in any other treatment groups as compared to control (Fig 2. A, B). Decreased CAT activity could be a result of DNA damage caused due to increased concentration of ROS on exposure to Clothianidin. No significant change was observed between acute and chronic treatment period (Fig 2. C). This may suggest that the cells are able to recover from the damage caused by elevating ROS concentration by Clothianidin. This can be a result of collective response of all the antioxidant enzyme system. Contrarily, a study assessing the effect of imidacloprid, increased the CAT activity during early exposure in adult zebrafish followed by decrease to the control level[44].

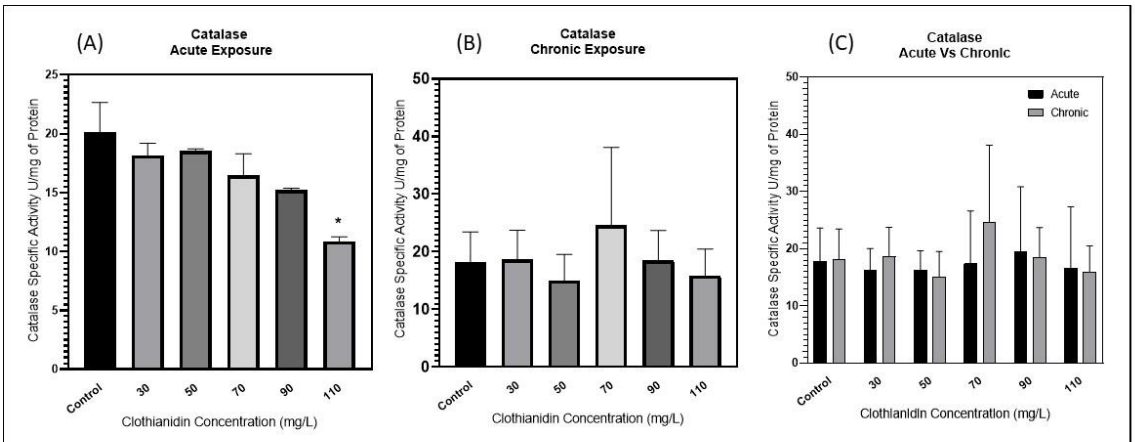

Fig 2. Mean values of specific activity of CAT enzymes following clothianidin exposure for (A) 96 hours (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentration at P<0.05 are marked as *.

GST

Primarily, GSTs play a role in detoxification of xenobiotics by catalyzing the interaction with GSH on the xenobiotic substrates. This prevents the interaction of xenobiotics with the crucial cellular proteins and nucleic acids thereby preventing the DNA damage. In the present study, chronic exposure to Clothianidin caused significant decrease in the specific activity of GST at all concentrations, however the values were not

significantly different as compared to the control group on acute exposure to the Clothianidin (Fig 3. A, B). The time dependent effect of Clothianidin treatment was observed to be significantly different at three highest concentration, 70mg/L, 90 mg/L and 110 mg/L (Fig 3. C). It shows that Clothianidin significantly decreases the specific activity of GST on chronic exposure, but does not induce any significant change on acute exposure. The exact mechanism of effect of Clothianidin on GST is not clear. Similar inhibiting effect was observed on GST and SOD in zebrafish on exposure to imidacloprid for 28 days[44]. In a study conducted on a neonicotinoid Sulfoxaflor, diminishing activity of GST was observed in the zebrafish gills[45]. Contrarily, GST activity increased in Zebrafish on 28-day treatment with thiamethoxam[43].

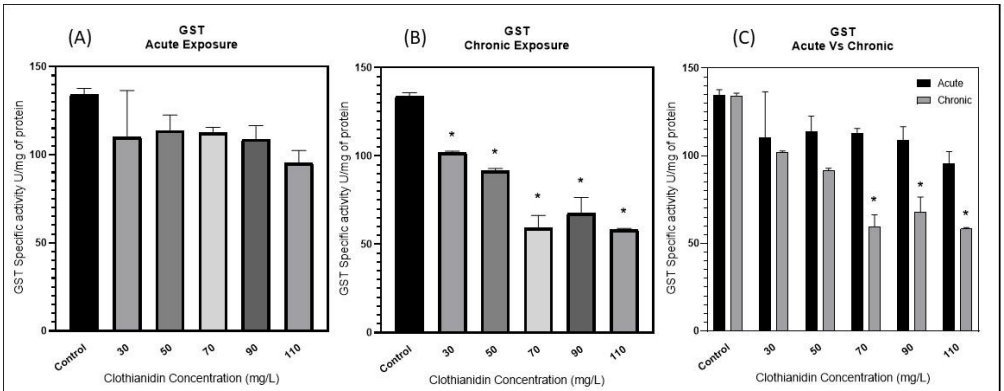

Fig 3. Mean values of specific activity of GST enzymes following clothianidin exposure for (A) 96 hours (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentration at P<0.05 are marked as *.

Peroxidase

Peroxidases is an important group of anti-oxidant enzymes involved in detoxifying hydrogen peroxide and other free radicals to protect the organ from oxidative stress. The specific activity of enzyme peroxidase did not cause any significant change in both the treatment groups, except a significant decrease at 70mg/L treatment concentration for Acute exposure period (Fig 4. A, B). The comparison between acute and chronic treatment did not show significant difference (Fig 4. C). One of the other neonicotinoids, sulfoxaflor is known to increase the level of Glutathione peroxidase in zebrafish gills[45].

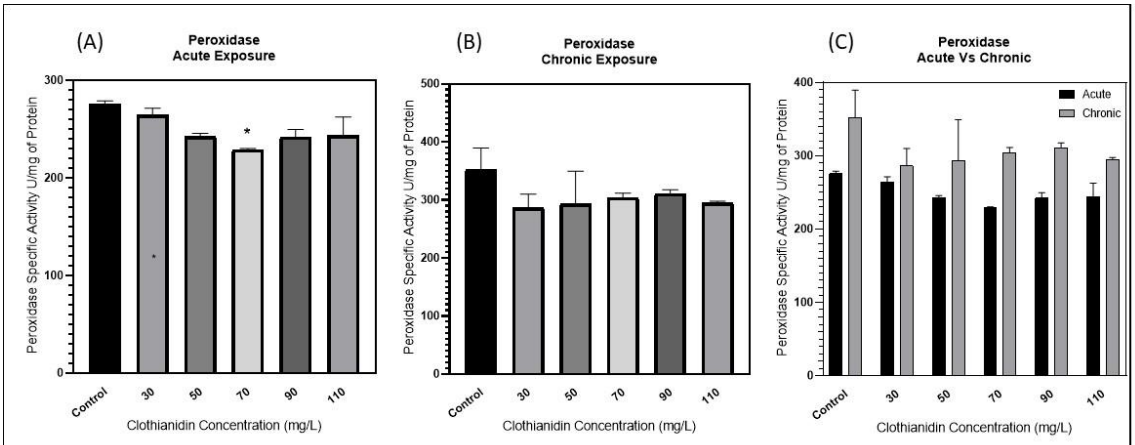

Fig 4. Mean values of specific activity of Peroxidase enzymes following clothianidin exposure for (A) 96 hours (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentration at P<0.05 are marked as *.

Lipid Peroxidation

Lipid peroxidation is a free radical oxidation of polyunsaturated fatty acids to form reactive aldehydes such as malondialdehyde (MDA). These reactive aldehydes can induce adaptive stress response to repair the oxidative damage caused, which would result into increased activity of antioxidant enzymes. If the oxidative damage is beyond repair capacity of antioxidant system, cells induce apoptosis. Both the cases lead to molecular cell

damage which may change the normal physiological conditions. Measurement of lipid peroxidation level in terms of MDA produced in the tissue facilitates the understanding of level of cell damage[46]. An overall increase in lipid peroxidation was observed in acute as well as chronic treatment groups. The effect was observed to be significant at all concentration treatment groups above 50mg/L of acute and above 70mg/L of chronic treatment groups (Fig 5. A, B). The difference between the acute and chronic treatment groups found significant only at 70mg/L (Fig 5. C). The results clearly indicate that Clothianidin induces oxidative stress in liver tissues of adult zebrafish during both the exposure periods. Similar effect was observed in zebrafish on exposure to other neonicotinoids. On treatment with thiamethoxam, lipid peroxidation was slightly elevated on days 21 and 28[43]. Imidacloprid significantly increases the lipid peroxidation by elevating the malondialdehyde (MDA) content on the 21st day treatment[44]. Sulfoxaflor also caused oxidative damage in the gill of zebrafish by increasing lipid peroxidation[45]. In other fish species, rainbow trout, the treatment of Clothianidin resulted in a significant increase in MDA in juvenile fish indicating oxidative damage[47].

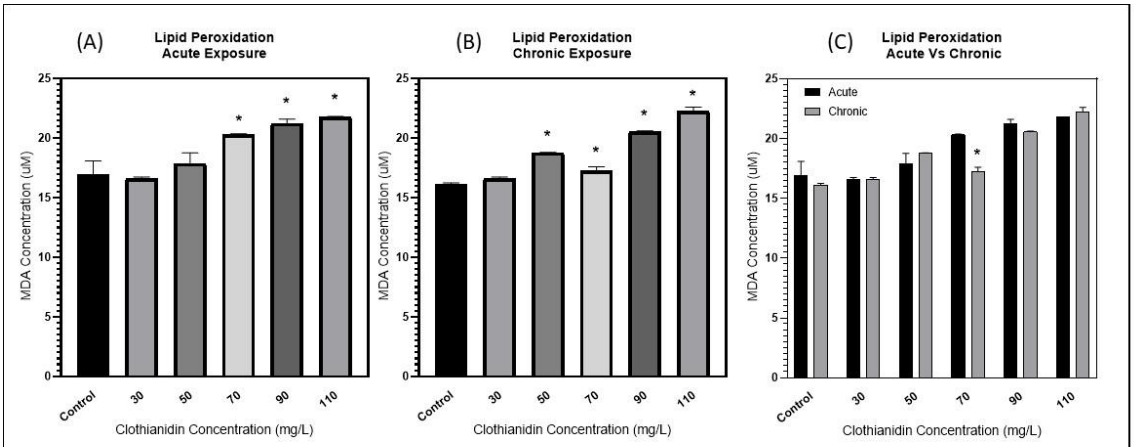

Fig 5. Mean values of MDA concentration uM (lipid peroxidation) following clothianidin exposure for (A) 96 hours (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish , (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentration at $P<0.05$ are marked as *.

AChE

Neonicotinoids are designed to hinder the breakdown of acetylcholine in insects, by binding to the active site of AChE, a key enzyme in the nervous system. This inhibition disrupts normal neurotransmission due to accumulation of acetylcholine, leading to muscle spasms, paralysis, and death. In current study, a decreasing trend was observed in the specific activity of AChE in both acute and chronic treatment groups as compared to control group. The change was observed to be significant at two highest concentrations of 90mg/L and 110 mg/L of the acute treatment groups (Fig 6. A, B). There was no significant difference between the acute and chronic treatment groups at any of the concentrations (Fig 6. C). This shows that Clothianidin is a potent inhibitor of AChE in brain tissue of adult zebrafish. Neonicotinoids are known to inhibit the specific activity of AChE by blocking the active gated synaptic channels in invertebrates[48]. Clothianidin also inhibited the activity of acetylcholinesterase (AChE) in other fish species like rainbow trout[49] and mosquitofish *Gambusia affinis*[50]. In *Labeo rohita*, 42 days treatment to Clothianidin, imidacloprid, and their mixture markedly reduced AChE enzyme activities in the brain[51]. Four neonicotinoids including Clothianidin can block the activity of purified eel AChE in vitro in a concentration dependent manner[52]. However, contrary to the effect of Clothianidin seen in the current study on adult zebrafish, thiamethoxam elevated AChE activity in zebrafish larvae at 28 dpf[53].

Recent studies conducted using other vertebrate model organisms also showed that Clothianidin causes alterations in the antioxidant enzyme system. Significant increase in specific activity of SOD, CAT, GST, lipid peroxidation levels were observed in *Labeo rohita* on exposure to Clothianidin and imidacloprid[51]. A study performed on rainbow trout showed that environmentally relevant concentrations of Clothianidin cause toxic effect on nervous system by inhibition of AChE. It may also mediate the inhibition of membrane bound enzyme which is evident from the sustained lipid peroxidation levels in different tissues[54]. Also, increased protein carbonyl and MDA levels triggered antioxidant response in rainbow trout on exposure to Clothianidin and inhibited the acetylcholinesterase (AChE) activity and lowered tissue protein levels[49].

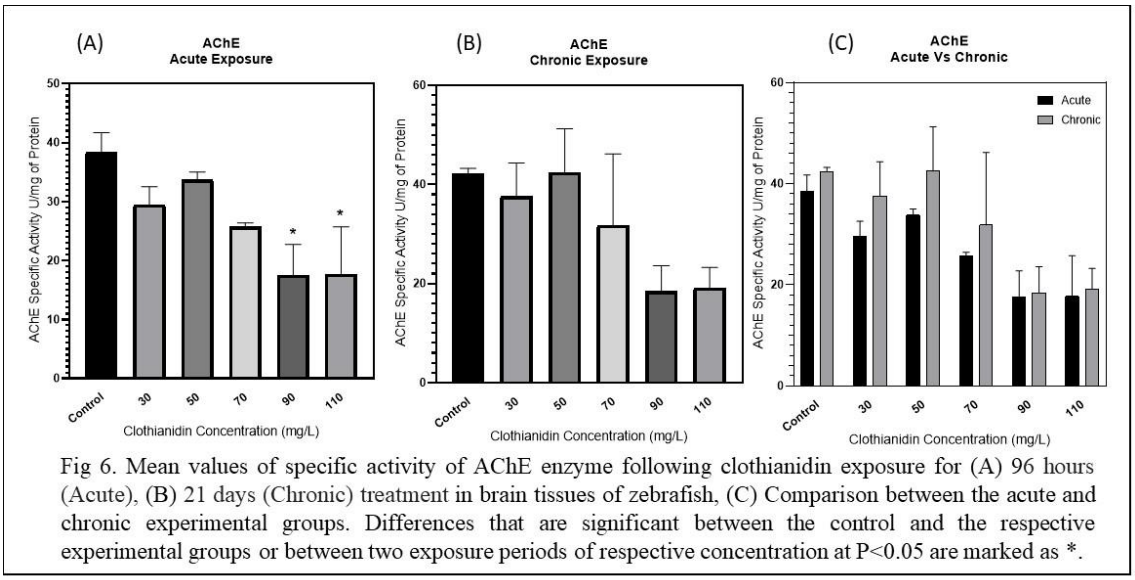

Fig 6. Mean values of specific activity of AChE enzyme following clothianidin exposure for (A) 96 hours (Acute), (B) 21 days (Chronic) treatment in brain tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentration at P<0.05 are marked as *.

Liver Function Enzyme Assays

AST and ALT

AST and ALT are commonly measured as biomarkers for detecting liver injury. In present study, concentration of AST observed to be increased in dose dependent manner in acute and chronic treatment groups however the increase was significant only at two highest concentrations, namely, 90mg/L and 110mg/L of chronic treatment groups (Fig 7. A, B). The comparison between acute and chronic treatment groups also showed significant difference at two highest concentrations (Fig 7. C). Concentration of ALT showed significant increase in the highest concentration of treatment group 110mg/L on acute exposure (Fig 8. A). However, the increase in concentration of ALT in chronic treatment groups was found to be insignificant (Fig 8. B).

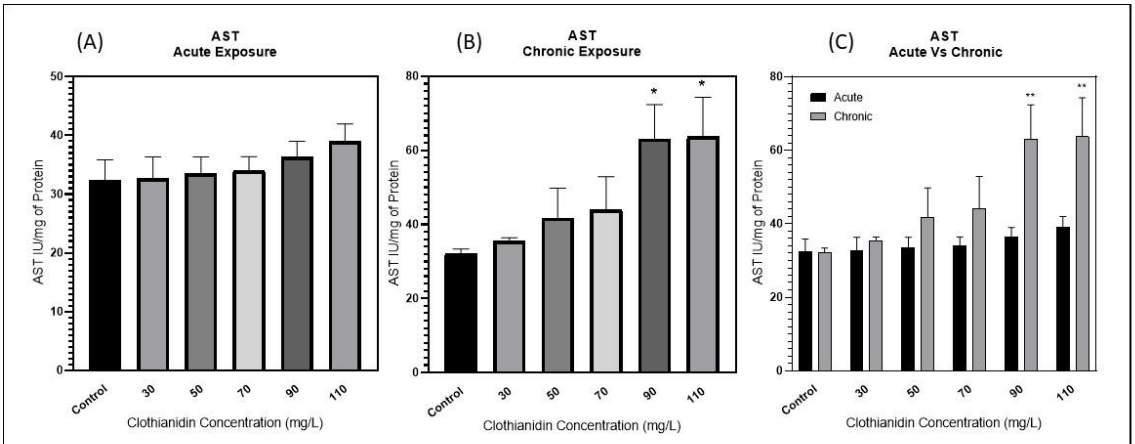

Fig 7. Mean values of concentration of AST enzyme following clothianidin exposure for (A) 96 hours (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentration at P<0.05 are marked as *.

The difference between acute and chronic treatment groups was observed to be insignificant at all concentrations (Fig 8. C). In rainbow trout, on exposure to Clothianidin, both ALT and AST activities showed statistically insignificant fluctuations in blood serum[47]. The elevated level of either ALT or AST suggests increased synthesis of amino acids or increased transamination process from fatty acids or glucose in response to Clothianidin toxicity.

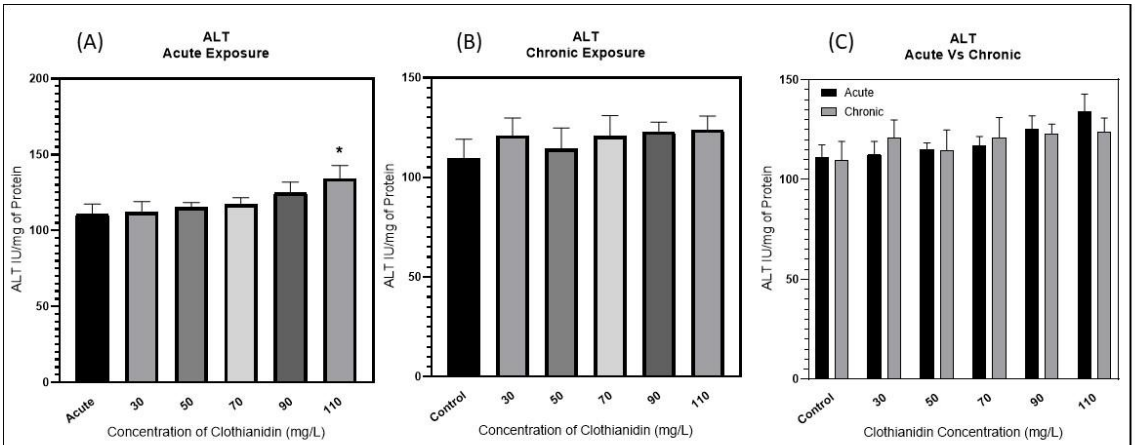

Fig 8. Mean values of concentration of ALT enzyme following clothianidin exposure for (A) 96 hours (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentration at P<0.05 are marked as *.

ACP and ALP

In assessment of toxicological effects of xenobiotics, ACP and ALP are important liver enzymes that are studied to detect the physiological alteration caused due to the toxin exposure. ACP, a lysosomal enzyme and ALP, a plasma membrane-bound enzyme, is often measured to assess the integrity of the lysosomes and plasma membrane, respectively[55]. The concentration of ACP and ALP showed increase in the higher concentration treatment groups, which was observed to be significant at 110mg/L in acute treatment groups and at two highest concentrations 90mg/L and 110mg/L in chronic treatment groups (Fig 9. A, B), (Fig 10 A, B).

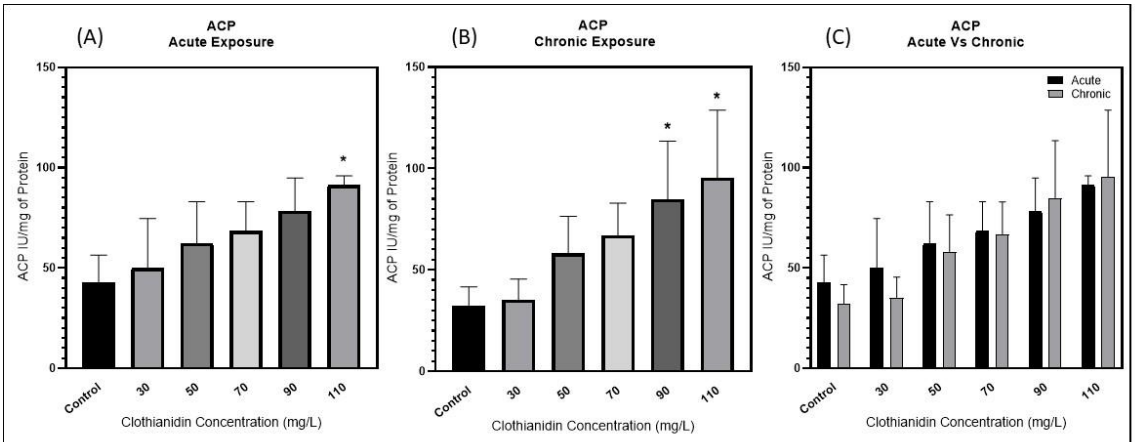

Fig 9. Mean values of concentration of ACP enzyme following clothianidin exposure for (A) 96 hours (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups. Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentration at P<0.05 are marked as *.

The Variation between the acute and chronic treatment groups was found to be insignificant (Fig 9. C), (Fig 10 C). Clothianidin treatment did not cause any significant changes in ALP levels of other fish species like in rainbow trout[47].

It has been reported that Clothianidin has higher tendency to accumulate in liver tissues and intestine as hepatobiliary system plays an important role in the metabolism and elimination of Clothianidin[23]. Hence analysis of oxidative stress enzyme system activity and other biomarker enzyme levels is important in assessing the toxicity of Clothianidin in liver tissue.

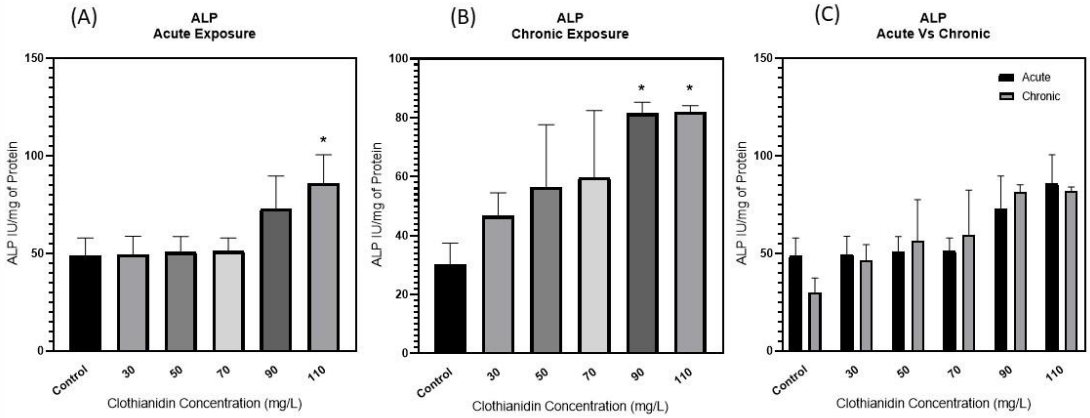

Fig 10. Mean values of concentration of ALP enzyme following clothianidin exposure for (A) 96 hours (Acute), (B) 21 days (Chronic) treatment in liver tissues of zebrafish, (C) Comparison between the acute and chronic experimental groups, Differences that are significant between the control and the respective experimental groups or between two exposure periods of respective concentration at P<0.05 are marked as *.

Conclusion

The present study reports the effect of Clothianidin on mortality and some biochemical parameters such as liver antioxidant stress profile SOD, CAT, GST, Peroxidase, Lipid peroxidation; liver function biomarker enzymes AST, ALT, ACP, ALP and brain AChE in adult zebrafish. Clothianidin being one of the neonicotinoids did not cause mortality in any of the study groups. This shows that the LC50 of Clothianidin for adult zebrafish is higher than the exposure concentrations of Clothianidin. However, Clothianidin exposure showed some changes in the biochemical parameters at acute as well as chronic treatment groups. At higher concentration, Clothianidin caused significant increase in SOD whereas significant decrease in CAT. Elevated lipid peroxidation values were observed in all except the lowest concentration in both exposure periods. However, overall changes observed in activities of GST and Peroxidases were insignificant. Increasing concentrations of liver biomarker enzymes AST, ALT, ACP and ALP were observed in all treatment groups. These results indicate that Clothianidin causes disturbances in the oxidative stress profile as well as liver function enzymes and may cause liver toxicity at its higher concentration and on prolonged exposure. A prominent decreasing trend observed in AChE activity in brain tissue shows that Clothianidin can inhibit AChE in adult zebrafish by blocking the nAChER. Additional research, such as analyzing DNA damage and gene expressions, are needed to fully understand the mechanism of Clothianidin action on adult zebrafish.

Acknowledgement

The authors would like to acknowledge the infrastructural support given by the Suman Tulsiani Research Centre and the CCSEA registered zebrafish maintenance facility of Sophia College for Women (Autonomous), Mumbai.

References

1. Koli, P. & Bhardwaj, N. R. (2018). Status and use of pesticides in forage crops in India. Journal of pesticide Science, 43(4), 225-232.
2. Nayak, P. & Solanki, H. (2021). Pesticides and Indian agriculture—A review. *Int J Res Granthaalayah*, *9*(5), 250-263.
3. Mohanty, M. K., Behera, B. K., Jena, S. K., Srikanth, S., Mogane, C., Samal, S. & Behera, A. A. (2013). Knowledge attitude and practice of pesticide use among agricultural workers in Puducherry, South India. *Journal of forensic and legal medicine*, *20*(8), 1028-1031.
4. Chitra, G. A., Kaur, P., Bhatnagar, T., Manickam, P. & Murhekar, M. V. (2013). High prevalence of household pesticides and their unsafe use in rural South India. *International journal of occupational medicine and environmental health*, *26*, 275-282.
5. Mittal, S., Kaur, G. & Vishwakarma, G. S. (2014). Effects of environmental pesticides on the health of rural communities in the Malwa Region of Punjab, India: a review. *Human and Ecological Risk Assessment: An International Journal*, *20*(2), 366-387.
6. Aktar, M. W., Sengupta, D. & Chowdhury, A. (2009). Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary toxicology*, *2*(1), 1.

7. Uneme, H., Konobe, M., Akayama, A., Yokota, T. & Mizuta, K. (2006). Discovery and development of a novel insecticide 'clothianidin'. *Sumitomo kagaku*, *2*, 1-14.
8. Tomizawa, M. & Casida J. E. Neonicotinoid insecticide toxicology: mechanisms of Selective Action. Annual Review of Pharmacology and Toxicology. 2005; 45:247- 268.
9. Holtswarth, J. N., Rowland, F. E., Puglis, H. J., Hladik, M. L. & Webb, E. B. (2019). Effects of the neonicotinoid insecticide clothianidin on southern leopard frog (Rana sphenocephala) tadpole behavior. *Bulletin of Environmental Contamination and Toxicology*, *103*, 717-722.
10. Robinson, S. A., Chlebak, R. J., Young, S. D., Dalton, R. L., Gavel, M. J., Prosser, R. S., ... & de Solla, S. R. (2021). Clothianidin alters leukocyte profiles and elevates measures of oxidative stress in tadpoles of the amphibian, Rana pipiens. *Environmental pollution*, *284*, 117149.
11. Li, L., Liang, H., Zhao, T., Liu, Y., Yan, S. & Zhu, W. (2022). Differential effects of thiamethoxam and clothianidin exposure on their tissue distribution and chronic toxicity in mice. *Chemico-Biological Interactions*, *366*, 110149.
12. Yanai, S., Hirano, T., Omotehara, T., Takada, T., Yoneda, N., Kubota, N., ... & Hoshi, N. (2017). Prenatal and early postnatal NOAEL-dose clothianidin exposure leads to a reduction of germ cells in juvenile male mice. *Journal of Veterinary Medical Science*, *79*(7), 1196-1203.
13. Marlatt, V. L., Leung, T. Y. G., Calbick, S., Metcalfe, C. & Kennedy, C. (2019). Sub-lethal effects of a neonicotinoid, clothianidin, on wild early life stage sockeye salmon (Oncorhynchus nerka). *Aquatic Toxicology*, *217*, 105335.
14. Hirano, T., Yanai, S., Omotehara, T., Hashimoto, R., Umemura, Y., Kubota, N., ... & Hoshi, N. (2015). The combined effect of clothianidin and environmental stress on the behavioral and reproductive function in male mice. *Journal of Veterinary Medical Science*, *77*(10), 1207-1215.
15. Li, P., Ann, J. & Akk, G. (2011). Activation and modulation of human α4β2 nicotinic acetylcholine receptors by the neonicotinoids clothianidin and imidacloprid. *Journal of neuroscience research*, *89*(8), 1295-1301.
16. Di Prisco, G., Iannaccone, M., Ianniello, F., Ferrara, R., Caprio, E., Pennacchio, F. & Capparelli, R. (2017). The neonicotinoid insecticide Clothianidin adversely affects immune signaling in a human cell line. *Scientific reports*, *7*(1), 13446.
17. Andria M. Cimino, Abee L. Boyles, Kristina A. Thayer & Melissa J. Perry (2017). Effects of Neonicotinoid Pesticide Exposure on Human Health: A Systematic Review. Environmental Health Perspectives 125:2
18. Xu, T., Dyer, D. G., McConnell, L. L., Bondarenko, S., Allen, R. & Heinemann, O. (2016). Clothianidin in agricultural soils and uptake into corn pollen and canola nectar after multiyear seed treatment applications. *Environmental toxicology and chemistry*, *35*(2), 311-321.
19. Li, Y., Su, P., Li, Y., Wen, K., Bi, G. & Cox, M. (2018). Adsorption-desorption and degradation of insecticides clothianidin and thiamethoxam in agricultural soils. *Chemosphere*, *207*, 708-714.
20. Wang, Y., Zhang, Y., Zeng, T., Li, W., Yang, L. & Guo, B. (2019). Accumulation and toxicity of thiamethoxam and its metabolite clothianidin to the gonads of Eremias argus. *Science of the Total Environment*, *667*, 586-593.
21. Tison, L., Duer, A., Púčiková, V., Greggers, U. & Menzel, R. (2020). Detrimental effects of clothianidin on foraging and dance communication in honey bees. *PLoS One*, *15*(10), e0241134.
22. Dai, Y. J., Jia, Y. F., Chen, N., Bian, W. P., Li, Q. K., Ma, Y. B., ... & Pei, D. S. (2014). Zebrafish as a model system to study toxicology. *Environmental toxicology and chemistry*, *33*(1), 11-17.
23. Yang, Y., Su, L., Huang, Y., Zhang, X., Li, C., Wang, J., ... & Zhao, Y. H. (2022). Bio-uptake, tissue distribution and metabolism of a neonicotinoid insecticide clothianidin in zebrafish. *Environmental Pollution*, *292*, 118317.
24. Hawkey, A. B., Unal, D., Holloway, Z. R. & Levin, E. D. (2023). Developmental exposure of zebrafish to neonicotinoid pesticides: Long-term effects on neurobehavioral function. *NeuroToxicology*, *96*, 240-253.
25. Wu, S., Li, X., Liu, X., Yang, G., An, X., Wang, Q. & Wang, Y. (2018). Joint toxic effects of triazophos and imidacloprid on zebrafish (Danio rerio). Environmental pollution
26. McManus, M. M., Crago, J., Seenivasan Subbiah, C. & Schmidt, J. E. (2018). Neurobehavioral and gene expression effects of neonicotinoids on embryo-larval zebrafish (danio rerio). *Neonicotinoid in the Aquatic Environment*, *103*.
27. Von Hellfeld, R., Ovcharova, V., Bevan, S., Lazaridi, M. A., Bauch, C., Walker, P., ... & Braunbeck, T. (2022). Zebrafish embryo neonicotinoid developmental neurotoxicity in the FET test and behavioral assays. *ALTEX-Alternatives to Animal Experiments*.
28. OECD (2019), Test No. 203: Fish, Acute Toxicity Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

29. Westerfield, M. (2000). The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). 4th ed., Univ. of Oregon Press, Eugene
30. Takahara, S., Hamilton, H. B., Neel, J. V., Kobara, T. Y., Ogura, Y. & Nishimura, E. T. (1960). Hypocatalasemia: a new genetic carrier state. The Journal of Clinical Investigation, 39(4), 610-619
31. Kochba, J., Lavee, S. & Spiegel-Roy, P. (1977). Differences in peroxidase activity and isoenzymes in embryogenic ane non-embryogenic 'Shamouti'orange ovular callus lines. Plant and Cell Physiology, 18(2), 463-467.
32. Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974). Glutathione S-transferases the first enzymatic step in mercapturic acid formation. Journal of biological Chemistry, 249(22), 7130-7139.
33. Misra, H. P. & Fridovich, I. (1977). Superoxide dismutase: a photochemical augmentation assay. Archives of Biochemistry and Biophysics, 181(1), 308-312.
34. Yagi, K. (1998). Simple assay for the level of total lipid peroxides in serum or plasma. Free radical and antioxidant protocols, 101-106.
35. Ellman, G. L., Courtney, K. D., Andres Jr, V. & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical pharmacology*, *7*(2), 88-95.
36. Lowry, O., Rosebrough, N., Farr, A. L. & Randall, R. (1951). Protein measurement with the Folin phenol reagent. *Journal of biological chemistry*, *193*(1), 265-275.
37. Reitman, S. & Frankel, S. (1957). A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *American journal of clinical pathology*, *28*(1), 56-63.
38. Kind, P. R. N. & King, E. (1954). Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. *Journal of clinical Pathology*, *7*(4), 322.
39. King, E. J. & Armstrong, A. R. (1934). A convenient method for determining serum and bile phosphatase activity. *Canadian Medical Association Journal*, *31*(4), 376.
40. *USEPA/Office of Pesticide Programs; Pesticide Fact Sheet- Clothianidin (May 2003). Available from, as of November 7, 2018: https://iaspub.epa.gov/apex/pesticides/f?p=chemicalsearch:1*
41. *USEPA/Office of Pesticide Programs; Pesticide Fact Sheet- Clothianidin (May 2003). Available from, as of November 7, 2018: https://iaspub.epa.gov/apex/pesticides/f?p=chemicalsearch:1*
42. *USEPA/Office of Pesticide Programs; Pesticide Fact Sheet- Clothianidin (May 2003). Available from, as of November 7, 2018: https://iaspub.epa.gov/apex/pesticides/f?p=chemicalsearch:1*
43. Yan, S. H., Wang, J. H., Zhu, L. S., Chen, A. M. & Wang, J. (2016). Thiamethoxam induces oxidative stress and antioxidant response in zebrafish (*Danio rerio* livers. *Environmental toxicology*, *31*(12), 2006-2015.
44. Ge, W., Yan, S., Wang, J., Zhu, L., Chen, A. & Wang, J. (2015). Oxidative stress and DNA damage induced by imidacloprid in zebrafish (Danio rerio). *Journal of agricultural and food chemistry*, *63*(6), 1856-1862.
45. Piner Benli, P. & Çelik, M. (2021). Glutathione and its dependent enzymes' modulatory responses to neonicotinoid insecticide sulfoxaflor induced oxidative damage in zebrafish in vivo. *Science Progress*, *104*(2), 00368504211028361.
46. Ayala, A., Muñoz, M. F. & Argüelles, S. (2014). Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxidative medicine and cellular longevity, 2014.
47. Dogan, D., Deveci, H. A. & Nur, G. (2021). Manifestations of oxidative stress and liver injury in clothianidin exposed Oncorhynchus mykiss. *Toxicology Research*, *10*(3), 501 510.
48. Somogyvári, D., Farkas, A., Mörtl, M. & Győri, J. (2022). Behavioral and biochemical alterations induced by acute clothianidin and imidacloprid exposure in the killer shrimp, Dikerogammarus villosus. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 261, 109421.
49. Fakhereddin, T. & Doğan, D. (2021). Pro-oxidant potency of clothianidin in rainbow trout. *Arhiv za higijenu rada i toksikologiju*, *72*(2), 172-172.
50. Cheghib, Y., Chouahda, S. & Soltani, N. (2020). Side-effects of a neonicotinoid insecticide (actara®) on a non-target larvivorous fish Gambusia affinis: growth and biomarker responses. The Egyptian Journal of Aquatic Research, 46(2), 167-172.
51. Gajula, S. K., Kanthala, S. K., Narra, M. R. & Vanamala, V. D. (2023). Individual and interactive biochemical profile damages in Labeo rohita (Cyprinidae) by imidacloprid and clothianidin. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *271*, 109689.
52. Győri, J., Farkas, A., Stolyar, O., Székács, A., Mörtl, M. & Vehovszky, Á. (2017). Inhibitory effects of four neonicotinoid active ingredients on acetylcholine esterase activity. Acta Biologica Hungarica, 68(4), 345-357.

53. Zhang, J. G., Ma, D. D., Xiong, Q., Qiu, S. Q., Huang, G. Y., Shi, W. J. & Ying, G. G. (2021). Imidacloprid and thiamethoxam affect synaptic transmission in zebrafish. Ecotoxicology and Environmental Safety, 227, 112917.
54. Yang, Y., Su, L., Huang, Y., Zhang, X., Li, C., Wang, J., ... & Zhao, Y. H. (2022). Bio-uptake, tissue distribution and metabolism of a neonicotinoid insecticide clothianidin in zebrafish. *Environmental Pollution*, *292*, 118317.
55. Jiang, H., Yang, H., Kong, X., Wang, S., Liu, D. & Shi, S. (2012). Response of acid and alkaline phosphatase activities to copper exposure and recovery in freshwater fish Carassius auratus gibelio var. Life Science Journal, 9(3), 233-245.