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Sub-Lethal Effects of Endosulfan+Chlorpyrifos Mixture on Biochemical Parameters and Blood Genotoxicity Markers of Two Cyprinidae Fish Species *Catla catla* and *Labeo rohita*

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Authors' Contribution
HN executed the research work. SA planned the research work. FL helped in lab work. FAand NH helped in writing the manuscript. TA and MAH performed statistical analysis. KA guided as member of supervisory committee.

Key words
Fish, Enzymes, Mixture toxicity, DNA damage, Chronic, Insecticides

ABSTRACT

The current study was performed to check the sub-lethal effects of endosulfan+chlorpyrifos mixture on biochemical parameters viz. peroxidase (POx), superoxide dismutase (SOD), catalase (CAT) and Phase-II glutathione (GST) in hepatic, neural, nephron, bronchial, cardiac and muscle tissue of *Catla catla* and *Labeo rohita*. The geno-toxic effects were studied in terms of DNA damage and nuclear abnormalities in the RBCs of both fish species. The fish was exposed to the mixture for 60 days and the fish sample was taken after the 15-day period. The negative control (NC) fish were kept in water having no insecticides. The results showed that during the first two samplings, an increase in activities of POx, SOD, and GST in all tissues of both fishes exposed to the insecticides mixture was noted as compared to the control. The trend of SOD level in organs of both fish species was noted as hepatic>neural>nephron>bronchial>cardiac>muscle tissue. The POx and GST levels in *L. rohita* and *C. catla* were observed as; hepatic>neural>bronchial>nephrotic>cardiac>muscle tissues. CAT activity was increased in the bronchial, hepatic, and nephrotic tissues of both fishes while it was reduced in cardiac, neural, and muscle tissues. The result of DNA damage showed that GDI and DN were higher during the first 15 days after that damage was lower. The *L. rohita* showed higher MN and NAs during the first 15 days of exposure after that damage was lower. However, *C. catla* showed an increase in the formation of MN and NAs throughout the exposure period.

INTRODUCTION

In the last few years, the application of agrochemicals like insecticides has been increased in the agricultural sector for improving the yield and quality of the crops (Doruchowski *et al*., 2017) with less work and time but this has negative consequences for the environment (Ullah, 2015). This practice has increased and becomes a necessary evil, especially in developing countries and those countries where agriculture is expected to be the backbone of the economy (Doruchowski *et al*., 2017). After application, these pesticides ultimately enter into the water bodies in significant quantities through contaminated water,

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Abbreviations
E, Endosulfan; C, Chlorpyrifos; GDI, Genetic Damage index; DN, Damaged nuclei; MN, Micronuclei; BIN, Binucleated nuclei; DEN, Deshaped nuclei; BN, Blebbed nuclei; DN, Dumble nuclei; NN, Notched nuclei

agricultural and urban run-off, bottom sediments, waste, atmospheric fall-out by rain and municipal water treatment, etc. (Kumari, 2020). Extensive use of these chemicals resulted in water pollution which poses a serious threat to freshwater biodiversity due to their ability to bioaccumulate and induce toxicity (Cui *et al*., 2015). Now, the assessment of toxicity associated with pesticides and their harmful effects on non-target aquatic animals has been a matter of worldwide concern (Matozzo *et al*., 2018).

The most commonly used insecticides in agriculture are organochlorine and organophosphate (Ullah *et al*., 2015, 2016). The metabolites of these insecticides finally enter the water bodies (Das and Mukherjee, 2003). Among the organophosphate, a widely used insecticide is chlorpyrifos with a long half-life and high stability (Wu *et al*., 2016). Exposure to pesticides to aquatic organisms especially fish led to toxic impacts, such as alteration in the acetylcholinesterase activity of *Cyprinus carpio* and behavior of *Labeo rohita* as well as impairment in *Gobio cypris rarus* embryos and larvae development (Wang *et al*., 2015; Mustafa *et al*., 2014; Zhu *et al*., 2014). Endosulfan belongs to organochlorine insecticide also broadly used throughout the world and can affect aquatic life through its bio-magnification and disturb homeostasis and metabolic activities and also induce damage to DNA (Indirabai *et al*., 2010; Adhikari *et al*., 2006; Ullah, 2015). It also induced the formation of reactive oxygen species (ROS) (Ghaffar *et al*., 2015) causing oxidative stress in aquatic organisms, especially fish, by modulation of antioxidant systems in fish (Shao *et al*., 2012). The organisms kept balance in the formation and elimination of ROS with the help of an antioxidant defense system including superoxide dismutase (SOD), peroxidase (POx), catalase (CAT), and glutathione-S-transferase (GST) (Kilili *et al*., 2004; Valavanidis *et al*., 2006). Against oxidative stress, the first line of defense is SOD which transfers the free oxygen radicals into hydrogen peroxide and molecular oxygen, that is further converted into H_2O and O_2 by POx and CAT enzymes (Zheng *et al*., 2016; Cheng *et al*., 2018). GST is a Phase-II enzyme present almost in all species and plays a role in the detoxification of toxicants (Hayes *et al*., 2005).

The unnecessary production of ROS may also cause DNA damage like oxidation and breakage of DNA strands (Oruc *et al*., 2014). The toxicological and safety effects evaluation of pesticides is necessary due to their deleterious effects such as cancer, chromosomal aberrations, gonadotoxicity, infertility, and fetal malformations (Ahmad *et al*., 2012). The alkaline single-cell gel electrophoresis assay also famous as the comet assay identify the DNA damage in term of alkali-labile sites, strand breaks, and delayed-repair sites (Ng and Romano, 2013). The other most promising test is micronucleus (MN) which is associated with nuclear abnormalities (NAs) and has also been used in the field of ecotoxicology to detect abnormalities at the chromosomal level (Bolognesi and Hayashi, 2011). Both these simple tests are widely applied due to their high sensitivity and statistical power to assess the genotoxic impacts.

Another major problem in environmental risk evaluation is that aquatic bodies contain different insecticides in a mixture form rather than a single chemical (Schreiner *et al*., 2016). Fish are good specimen which is not only used to assess the quality of aquatic system but their physiological systems are also used as valuable biomarkers to detect pollution. In this context, the present study provides more information about the effects of organophosphate and organochlorine pesticides on the enzymes and DNA functioning in non-target organisms the fish.

MATERIALS AND METHODS

Experimental specimens and sub-lethal trail

The two fish species, *Catla catla* and *Labeo rohita* (90-day old) from the Cyprinidae family were got from the fish seed Hatchery, Faisalabad. Both fishes were, live transferred to the Toxicology laboratory at the Fisheries Research Farm of UAF. Fishes were acclimatized to the laboratory environment by keeping them in the rectangular cemented tanks for 14 weeks. The experiment was conducted with 20 specimens of both species with equal weight and size, separately, kept in a 100-L aquarium facilitated with an oxygen pump. The control fish were kept in water having no insecticide.

Fishes were kept in $1/3^{rd}$ of LC_{50} of endosulfan+chlorpyrifos mixture, separately, for 60 days. The LC_{50} (96 h) concentration of the mixture for *L. rohita* and *C. catla* was calculated as 1.95±0.02 and 1.35±0.01 μgL^{-1}, respectively (Naz *et al*., 2019a, b). The sampling of fish (n=5) were done after 15, 30, 45 and 60 days interval labeled as D1, D2, D3, and D4, respectively. In the sub-lethal trial, no mortality was observed. During the trial, water pH (7), temperature (28°C), and total hardness (220 mgL^{-1}) were also kept constant. Fish of positive control (PC) were injected with a dose of cyclophosphamide at 20 μgg^{-1} of body weight to study the blood genotoxic markers.

Preparation of insecticides solutions

The clean water having no insecticide was used for control. The stock-I solution was made by mixing 1g of technical grade endosulfan (97% purity) and chlorpyrifos (98% purity), separately, in 95% analytical grade methanol (100ml). The stock-II solution, E+C mixture of insecticide of required ratio (1:1) were made in deionized water.

Tissue enzymes markers

After the sub-lethal trial, activities of enzymes viz. superoxide dismutase SOD, CAT and POx, and phase-II GST were assessed in cardiac, bronchial, muscle, nephritic, neural and hepatic tissues of both fishes. The homogenates of tissues were ready according to the procedure given by Zia *et al*. (2007). Giannopolitis and Ries (1977) protocol was used to analyze the SOD activity. Chance and Mehaly (1977) procedure was adopted to quantify the CAT and POx activities. Mannervik (1985) method was followed to calculate the GST activity.

Blood genotoxic markers

Comet/ SCGE assay

The blood was collected from the caudal vein of the fish and treated according to Singh *et al*. (1988). According to Jose *et al*. (2011) the damaged DNA was evaluated. The length of the tail was used to classify five types of damaged DNA known as comets. The following formula was applied to quantify the DNA damage:

$$\text{Damaged cell (\%)} = \text{Types II} + \text{III} + \text{IV}$$

$$\text{GDI} = \frac{(\text{Type I}) + 2(\text{Type II}) + 3(\text{Type III}) + 4(\text{Type IV})}{\text{Type 0} + \text{Type I} + \text{Type II} + \text{Type III} + \text{Type IV}}$$

Micronucleus test

The slides for micronuclei were prepared according to Barsiene *et al*. (2004) method. Fenech *et al*. (2003) procedure was followed to score the micronuclei and other nuclear anomalies in the blood of fishes. To compute MN frequency, the following formula was used:

$$\text{MN\%} = \frac{\text{Number of cells containing micronucles}}{\text{Total number of cells counted}} \times 100$$

Data analyses

The obtained data was statistically analyzed through the 8.1 version of statistics software. The ANOVA (a linear model) under CRD was applied to data to see the differences among tissues for enzyme activities followed by the student Newman-Keul test for mean comparison. The data obtained from the comet assay and MN test was analyzed through a non-parametric Mann-Whitney U-test. The significance level was set as $p>0.05$.

RESULTS AND DISCUSSION

Tissue enzymes markers

The results of this study showed that E+C-exposed fish species showed significant change in antioxidant enzymes viz. SOD, POD, GST and CAT as compared to control group. The change in antioxidant enzymeactivities may be a response againt oxidative stress due to free radicals. During the first two samplings (D1 and D2) an increase in activity of SOD, POx, and GST in all tissues of both fishes exposed to the E+C mixture was noted as compared to the control. However, a decline was noted in D3 and D4 sampling. The trend of SOD level in both fish species was noted as: hepatic> neural>nephron>bronchial>cardiac>muscle tissue. The POx and GST level in *L. rohita* and *C. catla* was observed as: hepatic>neural>bronchial>nephrotic>cardiac>muscle tissues. In the present study, CAT activity was initially (D1 and D2 sampling) increased in the bronchial, hepatic, and nephrotic tissues of both fishes. While it was decreased in neural, cardiac, and muscle tissues of E+C mixture exposed fishes throughout the experiment (Fig. 1). Similar findings were observed by Naz *et al*. (2022) for *Cirrhinus mrigala* when exposed to three different binary mixtures of insecticides viz. endosulfan, chlorpyrifos and bifenthrin. According to Webb *et al*. (2005) changes in enzyme activity, as well as a reduction, reveal that contaminants have shown reaction inside the body of fish. Several parameters, including species of fish toxicant dose, and period of exposure influence the duration and amplitude of these reactions (Piazza *et al*., 2015). Several authors had reported specie and dose-specific responses of enzymes, due to persistent organic pollutants (either increase or decrease in enzyme activity) (Lu *et al*., 2013; Koenig *et al*., 2012). Antioxidant enzymes work in the defense mechanism of the fish body. These act to defend fish against oxidative stress while a decrease in their activity disrupt the redox status of the cell. Reactive oxygen species (ROS) are induced by POP exposure to cells. Enzymes get back to normal activity when ROS got removed from the fish bodies (Stara *et al*., 2012; Ural, 2013). Various organs are under the effect by pesticide (Limon-Pacheco and Gonsebatt, 2009) like the liver, heart, stomach, intestine, spleen, kidney, gallbladder, muscle, swim-bladder, brain, operculum, gills, vertebra and gonads; however, all these are not commonly used but they also could serve as valuable evidence in terms of ecotoxicology (Jovicic *et al*., 2014).

The metabolites of endosulfan are more persistent and toxic as compared to the original form (Awasthi *et al*., 2000). According to Salvo *et al*. (2012), activity of antioxidant enzymes (SOD, CAT, GST, and GPx) in the liver of *Cyprinus carpio* were considerably altered by sub-lethal endosulfan exposure. The activities of SOD and GST in cardiac tissues were dramatically increased after exposure to endosulfan (Jalili *et al*., 2007).

Organophosphate pesticides (OP) use two pathways to induce ROS. The first choice is oxidation-reduction cycle, which is catalyzed by cytochrome P 450S. The chemical link –P= O, which was changed

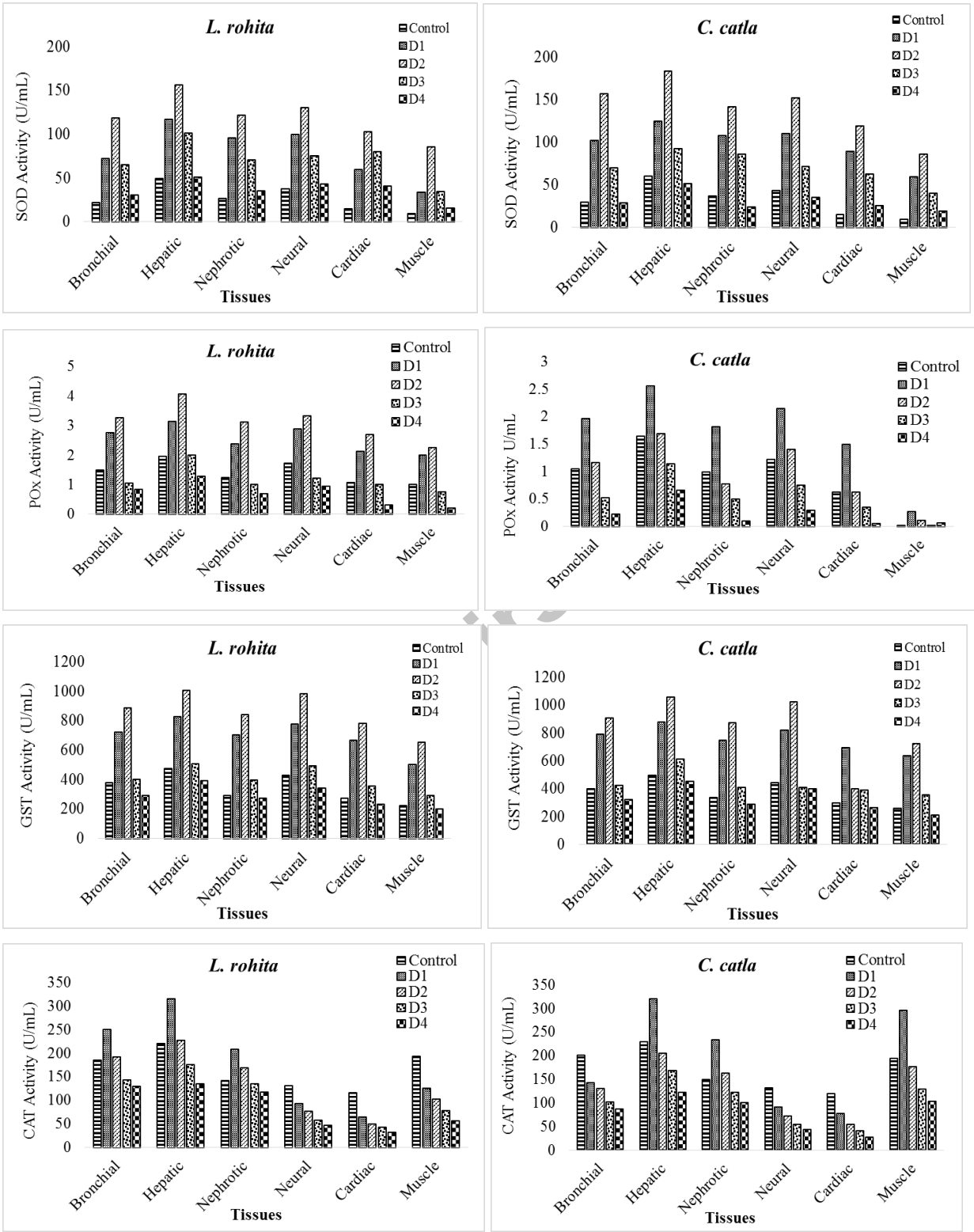

Fig. 1. Activity of antioxidant enzymes in different tissues of *C. catla* and *L. rohita.*
Samples of tissues were taken after 15 days (D1), 30 days (D2), 45 days (D3) and 60 days (D4).

from the –P=S or was previously present in organophosphate insecticides, may easily gain an electron and transfer into the oxygen molecule to form superoxide anion, which can subsequently be produced other ROS such as hydroxyl ion (Kovacic, 2003). Secondly, these ROS are limited by antioxidant enzyme otherwise, these causes an excessive accumulation of ROS. Plasma membrane and organelle enzyme activities as well as nerve conductance are interrupted by OP (Karaoz *et al*., 2002). Similarly, many researchers reported the fluctuation in GST, POx, SOD, and CAT activities in different fish species exposed to insecticides (Wang *et al*., 2009; Abdullah *et al*., 2018; Ozok, 2020; Deb and Das, 2021; Naz *et al*., 2021). The chlorpyrifos+endosulfan mixture induced fluctuations in GST, SOD, CAT, and POx activities in various organs (liver, brain, gills, heart, kidney, and muscle) of fish, *Labeo rohita* (Naz *et al*., 2019a) and *Catla catla* (Naz *et al*., 2021). According to Usman *et al*. (2020) decline in CAT activity in various tissues depends on the type of toxicants and exposure duration. Siddique *et al*. (2020, 2021) observed an initial rise in GST activity of *L. rohita* up to 28 days after that it decreased up to 56 days.

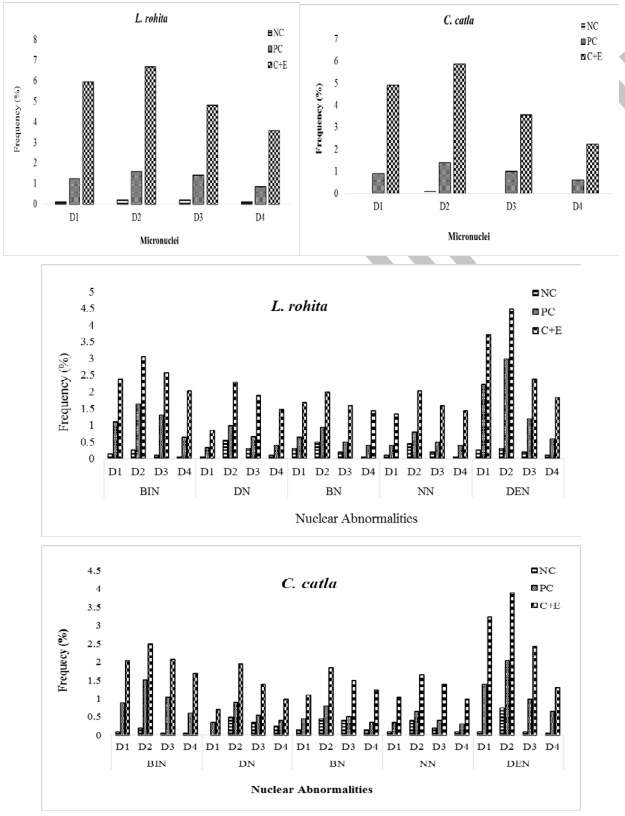

Fig. 2. Micronuclei and nuclear anomalies in RBC of *C. catla* and *L. rohita.*

Blood genotoxicity markers

Results showed that *L. rohita* had higher MN and NAs in RBCs during the first sampling (D1) of exposure to the E+C mixture after that damage was lower throughout the experiment. However, *C. catla* showed an increase in the formation of MN and NAs in RBCs throughout the exposure period. The result of geno-toxicity showed that GDI and DN were higher during the first sampling (D1) after that damage was lower (Figs. 2, 3). The fish, *Cirrhina mrigala* showed exposure depended on changes in DNA damage, MN, and NAs during chronic exposure to three different mixtures of insecticides (Naz *et al*., 2022). In aquatic environments, the SCGE/CA is a frequently applied method for detecting geno-toxicity (Frenzilli *et al*., 2009). This assay offers the benefit of identifying individual cells with damaged DNA (Buschini *et al*., 2004; Lee and Steinert, 2003). According to Lee and Steinert (2003), interactions between DNA molecules and contaminants can appear in a variety of ways, including DNA damage caused by ROS, DNA repair inhibition, compound action directly on DNA, and metabolites' interaction with DNA. ROS has been found to cause cellular and DNA damage at levels above normal (Cadet *et al*., 2003).

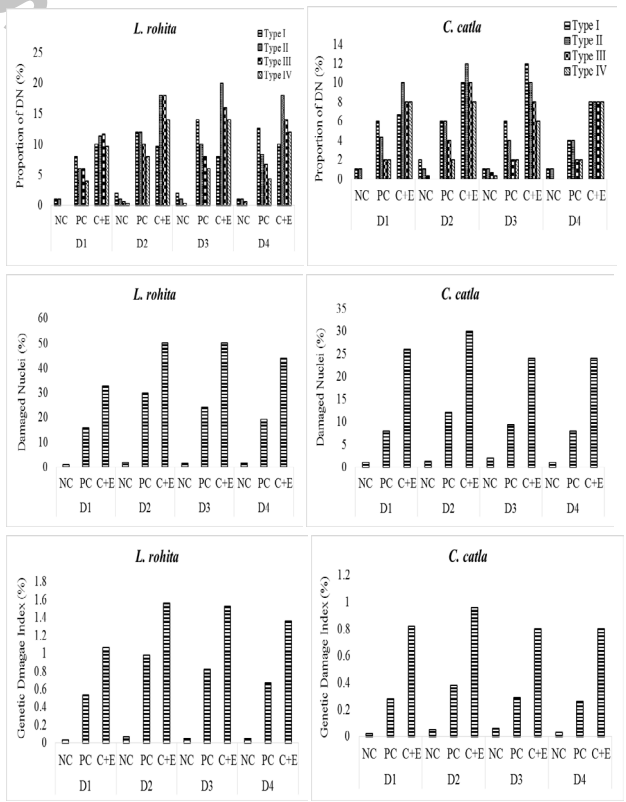

Fig. 3. Percentage of comet types, damaged nuclei and genetic damage index in RBC of *C. catla* and *L. rohita.*

With the oxidative potential of hydroxyl radical and indiscriminate reactivity with cellular constituents like lipids in cell membranes, DNA, and enzyme proteins, the hydroxyl radical is the most significant free radical of biological and toxicological importance, with a lifetime of a few nanoseconds (Jackson and Loeb, 2001). Another method of DNA damage caused by pesticide exposure is the presence of heavy metals like chromium, iron, cadmium, nickel, copper, zinc, lead , and manganese in these pesticides (Hayat *et al*., 2007). Through Fenton-like processes, these metal cations affect the polyanionic DNA (Ercal *et al*., 2001). Fenech and Ferguson (2001) reported the ability of live organisms to build and control particular enzyme systems for restoring DNA damage. Two electrophilic groups, alkyl and phosphoryl groups that are produced by the metabolism of organophosphate are ideal targets for nucleophilic attack. Through the phosphorylation process, this could interact with DNA (Ali *et al*., 2009). Several researchers successfully applied to quantify the insecticides induced DNA damage in terms of damaged nuclei and GDI in various fish species viz., *Catla catla* (Naz *et al*., 2019b), *Labeo rohita* (Nataraj *et al*., 2020), *Cyprinus carpio* (Hemalatha *et al*., 2020), silver carp (Ullah *et al*., 2019) and *Cyprinus carpio* (Ambreen and Javed, 2019).

The toxicity of aneugenic and clastogenic aquatic contaminants is evaluated by the use of MN frequency in fish erythrocytes (Udroiu, 2006; Ferraro *et al*., 2004). Tubulin polymerization failure may be linked to nuclear changes such as BL and LB (Vardavas *et al*., 2016). Furthermore, NAs are produced as a result of complications in the development of mitotic fuse due to the chemical's aneugenic activity (de-Campos Ventura *et al*., 2008). As in previous research, the signification formation of NAs frequencies in fishes was noted after exposure to QP-containing pesticides (Sadiqul *et al*., 2016), carbosulfan, glyphosate, atrazine (Nwani *et al*., 2014) and formalin (Mert *et al*., 2015). Similarly, MN and NAs formation in erythrocytes of insecticides exposed fish species by using micronucleus test were also recorded by many authors (Mitkovska and Chassovnikarvo, 2020; Naz *et al*., 2021; Davico *et al*., 2020).

CONCLUSION

The findings of the current study suggest that the fish enzyme activities, nuclear anomalies, and comet assay are valuable potent diagnostic tools for monitoring insecticide toxicity in the aquatic environments. However, this study suggests that extensive use of insecticides should be minimized or applied under strict environmental regulations. Farmers should adopt another strategy like biological control to kill pests instead of insecticides.

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IRB approval and ethical statement

The present study was approved by the Institutional Biosafety and Bioethics Committee of University of Agriculture, Faisalabad and was conducted following institutional guidelines for ethical conduct.

Statement of conflict of interest

The authors have declared no conlict of interest.

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