



Short-term toxicity of chloroacetanilide herbicide on non-target organism: estimation of median-lethal concentration, hematological, biochemical, ion regulation, and antioxidants

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Abstract

Contamination of agricultural chemicals (including pesticides) raises concerns about the safety of the aquatic ecosystem. Pretilachlor is a chloroacetamide herbicide commonly used to control weeds in field crops. Hydrophobic activity can increase the risk of pretilachlor loading into the aquatic ecosystem. Like other pesticides, pretilachlor has the potential to be toxic to non-target organisms. In particular, the toxicity of pretilachlor is in its initial stages. So, to begin with the toxicity information, we studied the short-term toxic effects of pretilachlor on edible freshwater fish, *Labeo rohita*. To evaluate the toxicity of pretilachlor, we determined the median-lethal concentration of 96 h. Then, we studied sublethal (1/25th of 96 h LC₅₀) effects of pretilachlor on blood parameters (hematology, biochemical, and electrolytes) and tissue (gills, liver, and kidney) biomarkers (Na⁺/K⁺-ATPase: sodium–potassium adenosine triphosphatase, SOD: superoxide dismutase, CAT: catalase, GST: glutathione S-transferase, GPX: glutathione peroxidase, and LPO: lipoperoxidation) for 72 h. Compared with the control group, pretilachlor toxicity in the exposed group showed a significant change ($p < 0.05$) over the study periods. Hematological indices (excluding white blood cells and MCHC) decreased most. Sodium levels decreased, while chloride and potassium levels (excluding 24 h) increased. The basic energetic fish, glucose, and protein were declined. Pretilachlor inhibited gill Na⁺/K⁺-ATPase activity throughout the exposure periods. Pretilachlor induced oxidative stress, which we evidenced by the inhibition of SOD, CAT, GST, and GPX activities, and acceleration of LPO activity in the vital organs of fish. We conclude that pretilachlor has an effect on blood parameters and is a chemical toxic to several organs.

Keywords Chloroacetanilide · Pretilachlor · Carp · Biomarker · Toxicity · Oxidative stress

Introduction

Usage of insecticide, herbicide, and fungicide in developing countries was accelerated dramatically (5.5×10^4 tonnes/year); for example, China leads the world in pesticide usage (per hectare 1.5 times), and the average ratio of these pesticides is 28:43:26 (Lai 2017; Lan et al. 2019; Zaidon et al.

2019). Worldwide, almost \$30 billion worth of herbicides is used annually. To date, 1500 herbicides containing over 300 active ingredients have been registered in the global marketplace. In high food demand countries, annually, China uses nearly 1.6 million tons for agricultural purpose (Yang et al. 2019), and the use of chemical-based herbicides alone in India is increased up to 25% in the last 10 years (Sondhia 2019). Excessive use and inefficiencies in wastewater treatment processes have made herbicides a ubiquitous contaminant in the aquatic environment, as high as several μg levels (Nitschke and Schussler 1998; Ouyang et al. 2019). Most herbicides have a history of persisting in the aquatic environment. Since herbicides act as photosynthetic apparatus, the chances of affecting aquatic biota (phytoplankton) are higher (Yang et al. 2019; Zhao et al. 2019). Of the herbicides, the chloroacetanilide groups (N-alkoxyalkyl-N-chloroacetyl substituted derivatives of aniline) (pre-emergence class) are

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commonly used for many crops, including major crops such as rice and maize, worldwide, as a matter of course. During 2012 in the USA, nearly 90 million kg has been utilized. As they are more hydrophilic, the risk of accumulation and distribution in the aquatic medium is relatively higher (Mohanty and Jena 2019). The chloroacetanilide groups were also identified as genotoxic, endocrine disruptors, and carcinogens in animal studies (Jiang et al. 2016, 2018). Thus, few of chloroacetanilide group herbicides have been banned by the World Health Organization for use (Nykiel-Szymanska et al. 2019). The European Union guideline on the maximum allowable concentration for drinking water and surface water was 0.1 µg/L and 1.0 µg/L, respectively (Ji et al. 2017).

Pretilachlor (2-chloro-N-(2,6-diethylphenyl)-N-(2-propoxyethyl) acetamide) (M.W: 311.9, Pow: 4.08), a pre or post-emergence chloroacetanilide group, is a potent herbicide as both single and combined form with other xenobiotics in modern agricultural practice (Kumar et al. 2018; Mohanty and Jena 2019). The half-life of pretilachlor is 13.1 days and persistence is close to 200 days (Sondhia 2019). It is widely used in rice cultivation, but toxicity to non-target organisms is also reported (Soni and Verma 2020). It is a recommended weedicide for *Limnophila sessiliflora*, which is a major weed problem found in agricultural fields of Asian countries (China, India, Philippines, and Japan) (Wang et al. 2000), *Murdannia nudiflora* (L.) Brenan in Laguna, Philippines (Awan et al. 2016). For example, in Indian states (Punjab), the utilization of pretilachlor is estimated at 922.22 tons (Kaur and Kaur 2018), and annual load of pretilachlor, a common herbicide in the waterways of Lucban and Pagsanjan, Philippines, is approximately 600 kg (Fabro and Varca 2012). Maximum residue limits for pretilachlor in food stuff are 0.05 µg/g (Institute of Standards and Industrial Research of Iran, and Food Safety and Standards Authority of India), and 0.01 µg/g (European Union) (Razzaghi et al. 2018; Kaur and Kaur 2018). Unfortunately, the presence of pretilachlor is identified in the matrix of the human body (hair, cord blood, and meconium), at the level of µg/mL (Ostrea Jr. et al. 2008, 2009). Pretilachlor as a water contaminant ranges up to 1.6 µg/L (Shinano River, Japan) (Tanabe et al. 1996), 0.03 µg/L (detected 68.9% among herbicides in the Mekong Delta, Vietnam) (Toan et al. 2013), and 0.207 µg/L (Northern Greece) (Papadakis et al. 2015).

Freshwater fish are another food source for humans and have the potential to transfer a pesticide through the food web (Clasen et al. 2018). Fish toxicity assessment is widely used to find the potential effects of waterborne chemicals, because fish spend their entire life span in the aquatic ecosystem; additionally they are highly sensitive to slight

environmental alterations, easy to handle, and cost-efficient; most of the physiological and biochemical activities were similar to human. Fish bioassays (determination of median-lethal concentration: LC₅₀) have a direct and the valid toxic effect of any chemical substance. Furthermore, to calculate the hazard and risk assessment of chemicals, bioassays are the key component (Glaberman et al. 2019). Physiological and morphological changes can occur in carp with exposed to contaminants. Thus, reactions of hematological, biochemical, ionoregulatory, and antioxidant activities in fish are commonly used as biomarkers of stress to assess the effects of chemicals. Hematology shows the pathophysiology of fish in various conditions. Biochemicals, such as glucose and protein levels, indicate stress in fish. The ion-regulation parameters, concentration of inorganic ions (sodium: Na⁺, potassium: K⁺, and chloride: Cl⁻) in the blood and the gill membrane enzyme Na⁺/K⁺-ATPase (sodium–potassium adenosine triphosphatase) activity, reveal the internal and external stress in fish. Biochemistry and ion-regulation are linked to bioenergetics, so their responses are considered secondary indicators of stress in fish. The gills are the main organ in contact with waterborne contaminants, and the liver and kidneys are the main sites of metabolism and excretion of all chemicals. Thus, analyzing antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), and lipoperoxidation (LPO)) activities in the gills, liver, and kidney could be used to understand the health condition of the tissues, direct effect, and metabolism-mediated effects of the chemical (Poopal et al. 2017; Renuka et al. 2018). As a result, analysis of these endpoints could be useful in understanding the potential effects of pretilachlor on non-target organisms.

The World Health Organization has categorized pretilachlor under toxicity class as “U: unlikely to present acute hazard in normal use,” unlikely to present a hazard (WHO 2009). Studies have suggested that pretilachlor has the potential to be more toxic to fish than other organisms, including humans (Liu et al. 2011; Mo et al. 2015). Evidence suggests that exposure to pretilachlor changes normal endocrine and immune functions, apoptosis, antioxidant balance (Jiang et al. 2016), and behavior in fish (Soni and Verma 2018). The authors also mentioned that there is a lack of toxicity patterns for pretilachlor, so it is mandatory to understand the toxic effects on non-target organisms. Based on the above information, we have understood that the baseline toxicity data for the commonly used pretilachlor are missing. Hence, we aimed to study the short-term effects of pretilachlor on freshwater cultivable fish, *Labeo rohita*. To determine the potential effects of pretilachlor, LC₅₀ was determined and hematology, biochemistry, ion-regulation, and antioxidant activities were analyzed.

Materials and methods

Test specimen

The fish *Labeo rohita* (Mean weight: 5 ± 0.5 g and length: 6 ± 0.75 cm) was used as an experimental specimen. Fish fingerlings were purchased from Pungar fish farming, Bhavani Sagar Dam, Erode, TamilNadu, India. They were transported to the laboratory in a polyethylene bag with well-aerated water. Subsequently, they were acclimatized to laboratory conditions for 2 weeks; during the period, they were fed *ad libitum* twice a day with commercial foods. Water replenishment was carried out after the daily processes (removal of metabolic waste and uneaten food). Photoperiod of 12:12 h of light, the cycle of darkness has continued. Dechlorinated tap water was used with physio-chemical properties such as temperature (27.0 ± 0.5 °C), pH (7.1 ± 0.05), DO: dissolved oxygen (6.2 ± 0.05 mg/L), and total hardness (16.5 ± 0.5 mg/L) throughout the experiment. Acclimated fish were used for toxicity testing. The study on the maintenance and toxicity in animals was conducted according to the guidelines recommended by the OECD (Organization for Economic Co-operation and Development) and the Committee for the Monitoring and Surveillance of Animal Experiments (CPCSEA).

Chemicals and stock preparation

Pretilachlor (chloro-N-acetamide) was purchased from Sri Sakthi Agro Chemical, Gobichettipalayam, Erode, TamilNadu, India. Oxidized glutathione (GSSG) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1-chloro-2,4-dinitrobenzene (CDNB), and 5-dithiobis-2-nitrobenzoic acid (DTNB) were acquired from SD fine chemicals limited in Mumbai, India. Other chemicals used were locally sourced (India) and analytical in quality. A stock solution was prepared by dissolving an adequate amount of pretilachlor into the water. The stock of fresh pretilachlor was prepared for each assay.

Determination of median-lethal concentration for pretilachlor

Fish fingerlings from the stock were collected and starved for 24 h prior to bioassay. Preliminary toxicity tests were carried out by exposing fish (no. 4) to two sets of pretilachlor concentrations (1, 5, 10, and 20 mg/L). Based on the mortality result, fingerlings were grouped into two (control and treatment; 10 nos. each). Subsequently, for the treatment group, different concentrations of pretilachlor were mixed and mortality/survival rates were recorded every 24 h until the end of the exposure period (96 h). Similar patterns were followed for three replicates. In accordance with the result,

the second concentration will be determined. The dead fish were immediately removed from the water tank. The control group did not have any fatalities.

Short-term toxicity study and sampling

In the semi-static short-term study, $1/25^{\text{th}}$ of the 96 h LC_{50} was selected. Fish fingerlings were collected from the stock and starved for a day prior to the short-term toxicity test. Fingerlings were grouped into control and treatment, and three replicates. Specimens were introduced into each tank. Freshly prepared pretilachlor LC50 was added to the 96 h $1/25^{\text{th}}$ treatment group. The control group was kept free of pretilachlor. This study lasted 3 days. The diet was *ad libitum*, and the water was changed daily in order to prevent the accumulation of fecal matter and to eliminate the food not consumed. Next, the appropriate amount of toxicity and water was renewed for the treatment group. The appropriate amount of water was added to the control group. Fish fingerlings were healthy throughout the study periods. Fish were monitored on a daily basis.

Fingerlings (20 nos.) from the treatment group were collected at the end of each day. Blood was then drawn from the cardiac in a heparinized medical grade disposal syringe fitted with a 26-gauge needle. Blood was then collected in EDTA (ethylenediaminetetraacetic acid) tubes, and whole blood was used to analyze the blood indices. The remaining blood was centrifuged at 93.9 g chilled for 20 min and collected the supernatant for analysis of the target parameters. Then, the tissues (gills, liver, and kidney) were dissected from the fingerlings and utilized for target parameters. Similar steps were carried out in the control group.

Estimation of hematological parameters

Hemoglobin (Hb) levels in the blood of fish *L. rohita* were estimated by the method of Drabkin (1946), expressed in units' g/dL. The hematocrit rate (Hct) was estimated using the method described in Nelson and Morris (1989), expressed in %. Red blood cells (RBC) (millions/cu. mm) and white blood cells (WBC) (1000/cu. mm) in the fish were counted in the hemocytometer (Rusia and Sood 1992). RBC indices, such as mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC), were determined by adopting the following standard equations.

$$\text{MCV}(\text{cubicmicra}) = (\text{Hct}/\text{RBC}) \times 10$$

$$\text{MCH}(\text{picograms}) = (\text{Hb}/\text{RBC}) \times 10$$

$$\text{MCHC}(\text{g/dL}) = (\text{Hb}/\text{Hct}) \times 100$$

Estimation of plasma biochemical parameters

The amount of glucose in fish *L. rohita* was estimated by O-toluidine reagent method described in Cooper and Mc Daniel (1970). In summary, 0.1 mL of sample (supernatant) was mixed with 5 mL of O-toluidine reagent and labeled as “test”. Similarly, 0.1 mL of distilled water and standard was mixed individually with 5 mL of O-toluidine reagent and marked as “blank and standard,” respectively. The contents were then thoroughly mixed and stored in a hot water bath for 10 min. Following heating, the contents were kept under tap water for cooling. The optical density (OD) of the contents was measured using the UV spectrophotometer at 630 nm wavelength, against the blank. Glycemia was expressed as mg/100 mL.

Protein content was measured using phenol reagent (Lowry et al. 1951). In brief, the supernatant (0.10 mL) was mixed with distilled water (0.90) and used as a “test”. At the same time, 1 mL of distilled water was prepared as a blank. Then, solution A (50 mL of solution) (2 g of sodium carbonate dissolved in 100 mL of 0.1 N NaOH) and solution B (500 g of copper sulfate dissolved in 100 mL of 1% sodium potassium tartrate solution), 5.0 mL and 1 mL, respectively added to the mixture. The setup kept undisturbed under room temperature. After 10 min, 0.5 mL of Folin-phenol reagent was added to the mixture and then allowed the reaction to grow color. Simultaneously, a standard has been prepared (bovine serum albumin (1 mg) has been added to NaOH 1 N (10 mL) and supplemented to 100 mL by distilled water, kept in stock. To the stock (1.0 mL) 0.5 mL each of solution A and Folin-phenol reagent were added). The color intensity of the contents was measured at wavelength 720 nm using a UV spectrophotometer. Protein content was expressed at $\mu\text{g}/\text{mL}$.

Estimation of ion-regulation

Gill Na^+/K^+ -ATPase activity was determined by the method of Shiosaka et al. (1971). In short, 0.3 mL of Tris HCl buffer (pH 7.5), 0.1 mL of 0.02 M ATP, 0.1 mL of 100 mm of NaCl, and 0.1 mL of KCl (for Na^+/K^+ ATPase) were added to 0.1 mL of gill extract and used as tests. In the same manner, 0.1 mL of distilled water was added to the blank. The content was allowed for reacting in a warm state at 37 °C. After 15 min, the reaction was restrained by the addition of 2 mL of 5% TCA. The content was centrifuged at 500 rpm for 5 min at 4 °C. To the supernatant, 1 mL of ammonium molybdate and 0.4 mL of ANSA were added and stored at ambient temperature. After 10 min, the intensity of the color developed was measured (UV spectrophotometer) at 680 nm against the blank. Enzymatic activity was expressed at mg/h/g.

Plasma inorganic ion levels, Na^+ , K^+ , and Cl^- levels were estimated by following the methods of Maruna (1957), Sunderman and Sunderman (1958), and Schoenfeld and Lewellan (1964), respectively. Briefly, 1 mL of precipitating reagent was mixed with 0.01 mL of supernatant, used as a test. For the standard tube, 0.01 mL of precipitating reagent was added. Following this, the contents were mixed and stored at room temperature. After 5 min, content was centrifuged at 3000 rpm (2 min). The supernatant (0.02 mL) was then supplemented with the color reagent (1 mL) at room temperature. For the standard tube, 0.02 mL of the standard reagent was added. After 5 min, the color intensity of the contents was measured (against distilled water) at 530 nm using a UV spectrophotometer. The values for Na^+ were expressed in units, mmol/L.

To estimate K^+ levels, 0.05 mL supernatant was mixed with 1 mL boron reagent and marked as tests. In the same way, for the standard, 0.05 mL of potassium standard was added. Next, the contents were thoroughly mixed and stored at room temperature. After 10 min, the OD of the contents were estimated by using a UV spectrophotometer (at 620 nm), expressed as units, mmol/L.

Thiocyanate reagent (1000 μL) was mixed with 10 μL of supernatant (tests), and distilled water (blank), and chloride standard reagent (standard). Next, the contents were mixed at ambient temperature and undisturbed for 10 min. The color intensity was measured by using blank in UV spectrophotometer at 505 nm. The values expressed in mEq/L.

Estimation of antioxidant activity

SOD, CAT, GST, GPX, and LPO activities in various tissues (gills, liver, and kidney) were measured by methods described in Marklund and Marklund (1974), Aebi (1984), Habig et al. (1974), Rotruck et al. (1973), and Devasagayam and Tarachand (1987), respectively.

Briefly, to estimate SOD activity, 0.25 mL of supernatant was mixed with 0.25 mL of ethanol and 0.15 mL of chloroform at ice cold condition. The contents were centrifuged at 13,000 rpm. After 15 min, the supernatant was collected. To 0.5 mL of supernatant, 2 mL Tris buffer was added, and then by adding distilled water, the total volume was made up to 4.5 mL. Then, the content was mixed with pyrogallol (0.5 mL). Similarly, by adding Tris HCl (2 mL) and distilled water (2.0 mL of standard and 2.5 mL for blank), and pyrogallol (0.5 mL) only for standard, pyrogallol standard, and reagent blank were prepared. The contents were measured at 470 nm, and results expressed as units/mg protein.

To quantify CAT activity, briefly, 0.1 mL of supernatant was mixed with 1 mL phosphate buffer and 0.5 mL H_2O_2 . Then to the contents, 2 mL dichromate acetic acid solution was added and then incubated in a water bath. After

10 min, this was allowed to cool to develop color. Then, we measured the contents in UV spectrophotometer at 590, the readings expressed as units ($\mu\text{moles of H}_2\text{O}_2$ utilized/min/mg protein).

To observe GST activity, briefly, homogenate (0.1 mL) was mixed with phosphate buffer (1.0 mL) and distilled water (0.7 mL). Then incubated at 37 °C (5 min), then GSH (0.1 mL) was added and incubated. The content was measured at 340 nm using a UV spectrophotometer and the reading expressed as $\mu\text{moles of CDNB conjugated/min/mg protein}$.

GPx activity estimation, briefly, each 0.1 mL of buffer, sodium azide, reduced glutathione, and H_2O_2 , was added to 0.05 mL of supernatant. Then, 2 mL of distilled water was added and incubated for 3 min at 37 °C. Then, 0.5 mL of 10% TCA was mixed with the contents and centrifuged at 10,000 rpm for 10 min. Then, 0.15 mL of supernatant was mixed with 2 mL of disodium hydrogen phosphate (0.3 M) and 2 mL of DTNB (5,5'-dithiobis-[2-nitrobenzoate]). The OD of the content was measured against blank (phosphate solution and DTNB) using a UV spectrophotometer (412 nm). The readings were expressed as units ($\mu\text{g GSH formed/min/mg protein}$).

Lipid peroxidation activity estimation, briefly, supernatant (0.1 mL) was mixed with pH 8.2 Tris HCl buffer (0.5 mL), 10 mm KH_2PO_4 (0.15 mL), and distilled water (0.2 mL). Then, the contents were incubated (37 °C). After 20 min, the contents were added to 1 mL of TCA (10%) and 0.75 mL of TBA. The contents were centrifuged at 500 rpm for 10 min after incubating at boiling water bath for 10 min. The color intensity of the contents was measured in a UV spectrophotometer at 532 nm. The readings were expressed as moles of MDA/g protein.

Statistics analysis

All the calculations were examined in the MS-Office Excel worksheet. The worksheet values for hematological, biochemical, and ion regulation were analyzed using Student's "t" test (SPSS software – ver. 16). Significances were mentioned as asterisk "*" representing $p < 0.05$.

Results and discussion

Fish are highly vulnerable to pretilachlor-induced stress (Soni and Verma 2020). When fish exposed to different concentrations (preliminary tests) of pretilachlor, we observed serials of behavioral alterations, such as fast swimming, moving up and down, and swimming at the corners of the tank, at all concentrations. In addition, fish exposed to concentrations of 5 mg/L and higher were observed to have a loss of equilibrium, a large opercular opening, and a flipping

or floating of the belly upwards (dead sign). Only fast swimming was observed in the control group, which too no long lasting. Based on these behavioral signs, we exposed fish to concentrations less than 5 mg/L to determine the LC_{50} . Short-term ecotoxicological risks and the basic value of the acute toxicity of pesticides could be determined by analyzing the 96 h LC_{50} (Stara et al. 2019). The 96 h LC_{50} for pretilachlor was 4.7 mg/L, indicating that pretilachlor is highly toxic. The mortality rate of *L. rohita* was parallel to increasing concentrations of pretilachlor. In a recent study, the lethal concentration of pretilachlor was determined in freshwater fish *Clarias batrachus* (Linnaeus) at 5.84 mg/L (Soni and Verma 2018). The authors also reported that fish mortality increased with herbicide concentrations. Our findings indicate that pretilachlor is a serious concern for aquatic organisms. Observed behavioral abnormalities could include mediated behavioral responses to pretilachlor.

Hematological profiles of fish *L. rohita* under short-term study are shown in Fig. 1a-g. When compared with the control group, Hb content, HCT value, and RBC counts were decreased significantly ($p < 0.05$) in a pretilachlor treated group, whereas erythrocyte indices, such as MCV, and MCH values were found to be decreased significantly ($p < 0.05$) up to 48 h. The WBC counts (except day 1) and MCHC values were found to be increased significantly ($p < 0.05$). The values were ranged between 4.78–7.91 g/dL, 13.42–22.76 %, 1.26–2.98 millions/cu. mm, 10.23–23.89 1000/cu. mm, 52.85–122.1 cubic micra, 19.12–41.19 pg, and 34.88–37.93 g/dL for Hb, HCT, RBCs, WBCs, MCV, MCH, and MCHC parameters, respectively. The maximum percentage change decrease was 51.57%, 70.27%, and 67.01% for Hb, HCT, and RBCs, respectively. The maximum percentage change was 52.65% (increase) and 36.45% (decrease) for WBCs. The maximum percentage change was 39.95% and 38.52% (decrease), and 26.07% and 36.88% (increase), for MCV and MCH, respectively. A maximum percentage change increase was 10.29% for MCHC.

The decreased hemoglobin, hematocrit content, and RBCs observed might be due to the disruptive action (hemoglobi-nization or shrinkage of red blood cells) of the pesticides on the erythropoietic tissue. Leukocytes are involved in the control of immunological function and changes in WBC counts after exposure to pretilachlor may indicate non-specific immunity in fish. In the present investigation, the significant decrease in MCV and MCH might be due to compensate for impaired oxygen uptake due to gill damage caused by pretilachlor toxic. The high percentage of immature red blood cells in the circulation might be the reason for MCV and MCH decrease in the present investigation. Elevated MCHC levels reveal microcytic anemia in fish due to pretilachlor stress. Our results agreed with Prusty et al. (2011), Qureshi et al. (2016), Doherty et al. (2016), Ghayyur et al. (2021), Owolabi and Abdulkareem (2021), and Kumar et al. (2022). Chemical

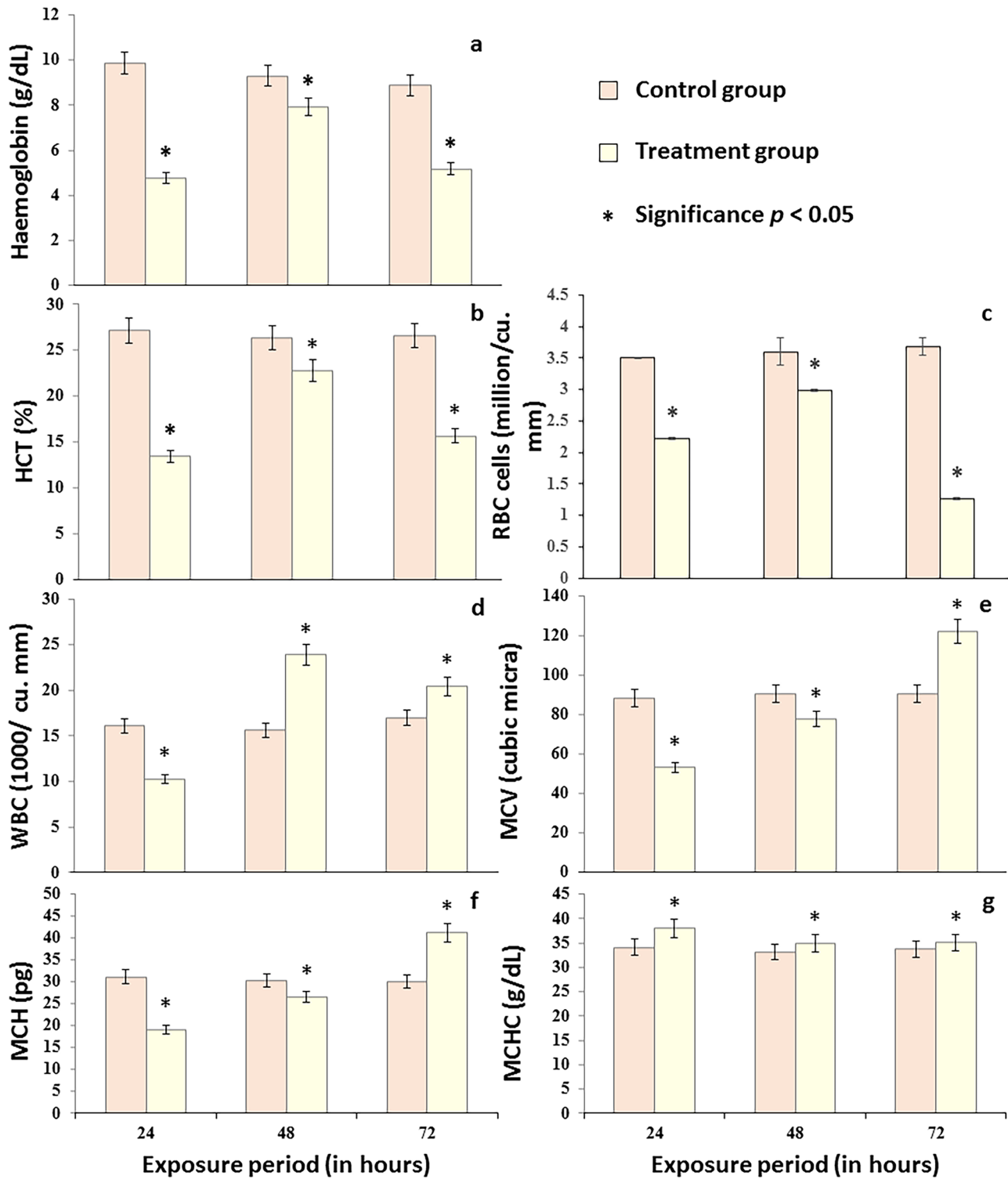


Fig. 1 Hematology of fish, *L. rohita* under short-term exposure periods

pesticides can affect blood cells by modifying the O_2 level, which could lead to severe conditions such as anemia and delayed physical activity. The RBC count and the Hb and HCT values of *C. batrachus* were declined under pesticide

treatment. This is an indication of the macrocytic anemic condition that occurred towards the defense action against the pesticide. In addition, a reduction in these blood indices may occur due to destruction caused by metabolic and

homeopathic activities under the stress of pesticides. Leukocytosis may be caused by the stimulation of lymphopoiesis or excessive release of lymphocytes from the tissues. Leukocytosis activates the immune system against stress from pesticides. The ability of fish to adapt to the toxic effects of pesticides may be evaluated by leukocytosis (Narra 2016).

Figure 2a and b reveal the plasma biochemical profiles of fish *L. rohita* under short-term study. When compared to the control group, plasma glucose (Fig. 2a) and protein (Fig. 2b) levels were significantly ($p < 0.05$) declined in a pretilachlor exposed group. Plasma glucose levels decreased steadily, while protein levels decreased gradually.

Similar types of results were reported by Owolabi and Abdulkareem (2021), Qureshi et al. (2016), and Tabassum et al. (2020). Glucose is a major power source for fish and other organisms. Glucose impairments are used as indicators of toxic stress. The toxicity of pesticides reduces fish glycemia. Metabolic depression caused by functional impairments in the vital organs of fish could result in the depletion of blood glucose. And stimulating the hypothalamus-pituitary-interrenal axis could increase the synthesis of corticosteroids and catecholamines. These pathways under pesticide stress could regulate the glucose homeostasis and promote catecholamine-induced glycogenolysis in the vital organs/tissues (skeletal muscles, liver, etc.) which could alter glucose uptake and affect glucose homeostasis in fish (Tabassum

et al. 2020). A decrease in protein level is an indication of the breakdown of protein to meet out the energy demand or due to interruption of regular synthesis of protein or due to denaturation of available protein or indirect effect of acceleration of immune response against pesticide stress (Narra 2016, Narra et al. 2017).

As shown in Fig. 3, plasma inorganic ion concentrations and gill tissue Na^+/K^+ -ATPase activity were altered significantly ($p < 0.05$) in a pretilachlor treated group than the control group. The plasma Na^+ level was decreased throughout the study period. The values were ranging from 124.2 to 182.5 mmol/L, and the highest percentage change was 34.92%. The plasma K^+ level was increased after 48 h of exposure periods, the values were ranged between 5.7 and 9.04 mmol/L, and the percentage change was 45.33% (increase) and 17.39% (decrease). Like Na^+ , the Cl^- level was increased, the values range from 106.6 to 120.8 mEq/L, and the highest percentage was 15.54%. Gill Na^+/K^+ -ATPase activity was inhibited throughout the study period, the values were ranging between 78.89 and 96.60 mg/h/g, and the higher percentage change was 24.14%.

In the present investigation, the decreased level of plasma Na^+ and K^+ during acute (72 h) treatment might have resulted from the lipophilic nature of the herbicide pretilachlor. The herbicide might accumulate on the gill surface and cause toxicity (either damage or alter the membrane permeability leading to lesser intake of electrolytes into the body or efflux of the same to the exterior). The observed increase in plasma K^+ and Cl^- level may be due to changes in the water ion equilibrium that occurred in inner cell and intercell under the effect of pretilachlor herbicide. The pretilachlor herbicide-induced inhibition of Na^+/K^+ -ATPase in fish gill after acute exposure probably disturbed the Na^+/K^+ pump, resulting in the limitation of Na^+/K^+ -ATPase synthesizing capability, or the amount of Na^+/K^+ -ATPase present could not cleave enough ATP under pretilachlor-induced stress.

Ion absorption of water is critical for freshwater fish to maintain the internal acid–base balance and ionic balance of blood and tissue. Pesticides have the potential to interact directly with enzymes and lipid molecules and disrupt Na^+/K^+ -ATPase activity and cell necrosis (Narra 2016). Freshwater fish exposed to pesticide showed alterations in electrolyte homeostasis, which could be due to osmoregulatory malfunction caused by damage to the gills. Furthermore, pesticides can inhibit the membrane-bound enzyme, like Na^+/K^+ -ATPase. This enzyme governs the electrolyte homeostatic properties of fish (Veedu et al. 2022). Pesticide toxicity may inhibit glycolytic pathway enzymes such as AK (adenylate kinase) and PK (pyruvate kinase), which may result in cellular functional impairment and loss of osmo-protection (Serafini et al. 2019). Alterations in ionic homeostasis may be attributable to pesticide-induced hormone imbalance (cortisol) (Katuli et al. 2014). The toxicity of pesticides may influence

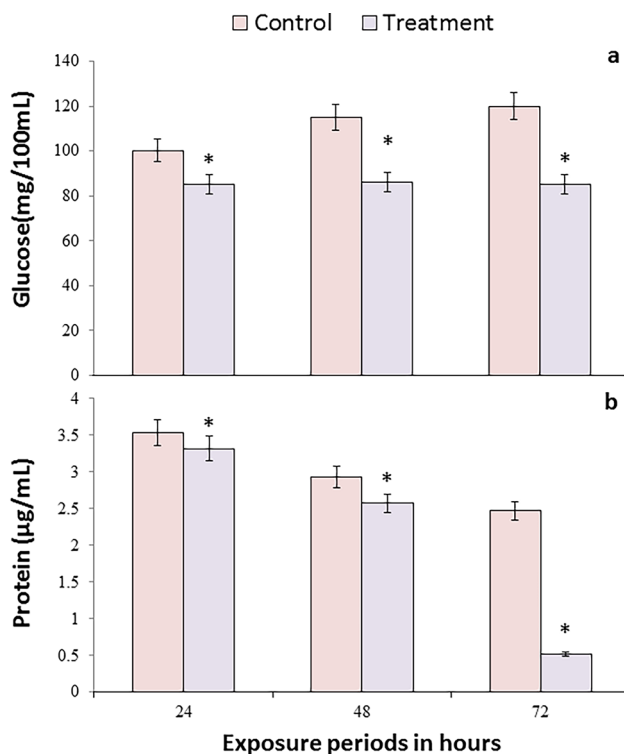


Fig. 2 Plasma biochemical levels of *L. rohita* under short-term exposure periods. Asterisk signifies statistical significance $p < 0.05$

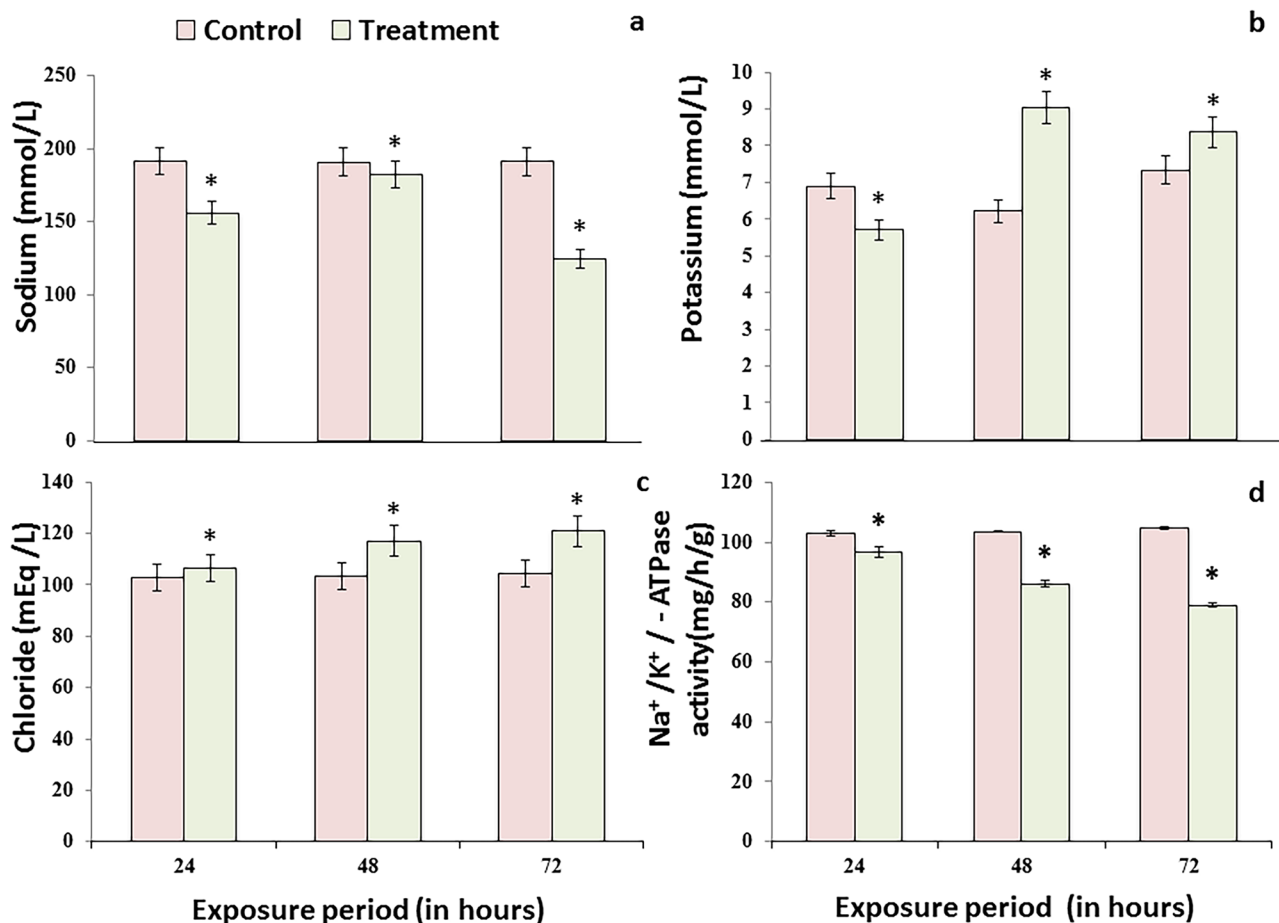


Fig. 3 Plasma electrolytes and gill Na^+/K^+ -ATPase activity of fish under short-term exposure periods. Asterisk signifies statistical significance $p < 0.05$

levels of synaptic transmission in tissues, which may result in inhibition of ATPases in fish (Begum 2011).

Gills, liver, and kidney tissue antioxidant activities of *L. rohita* under short-term study are illustrated in Figs. 4, 5, 6, and 7. Gill tissue (Fig. 4a-d) SOD (Fig. 4a), CAT (Fig. 4b), GST (Fig. 4c), and GPx (Fig. 4d) activities were decreased significantly ($p < 0.05$) in a pretilachlor treated group, when compared to the control group. The values of SOD, CAT, GST, and GPx activities were ranging from 21.1 to 25.5 units/mg protein, and 16.2 to 18.5 μmoles of H_2O_2 utilized/min/mg protein, 3.19 to 3.31 μmoles of CDNB conjugated/min/mg protein, and 5.5 to 5.99 μg GSH formed/min/mg protein, respectively. Higher percentage change was noted in SOD (37.97%) followed by GST (35%), GPx (14%), and CAT (10%). Similarly, liver antioxidant (Fig. 5a-d) activities also found to be declined significantly ($p < 0.05$) in a pretilachlor treated group, and the values were ranged between 21.1–25.5 units/mg protein, 16.2–18.5 μmoles of H_2O_2 utilized/min/mg protein, 3.19–3.71 μmoles of CDNB

conjugated/min/mg protein, and 5.12–6.99 μg GSH formed/min/mg protein, for SOD (Fig. 5a), CAT (Fig. 5b), GST (Fig. 5c), and GPx (Fig. 5d), respectively. A maximum percentage was noted at GST (46.38%), followed by GPx (41%), SOD (38.3%), and CAT (19.8%). As shown in Fig. 6a-d, kidney tissue antioxidants were also decreased in a pretilachlor treated group. The values were ranged from 21.1 to 25.5 units/mg protein, 13.2 to 16.5 μmoles of H_2O_2 utilized/min/mg protein, 2.29 to 4.19 μmoles of CDNB conjugated/min/mg protein, and 5.65 to 6.12 μg GSH formed/min/mg protein, for SOD (Fig. 6a), CAT (Fig. 6b), GST (Fig. 6c), and GPx (Fig. 6d), respectively. Percentage changes were found higher in GST (58.58%), followed by SOD (41.71%), CAT (27.86%), and GPx (25.75%). As shown in Fig. 7a-c, LPO activities in the gills (Fig. 7a), liver (Fig. 7b), and kidney (Fig. 7c) tissues were significantly ($p < 0.05$) elevated in a pretilachlor treated group, when compared to the control group. A decrease of SOD activity may be due to the enhanced production of pretilachlor-induced superoxide radical anions and decreased

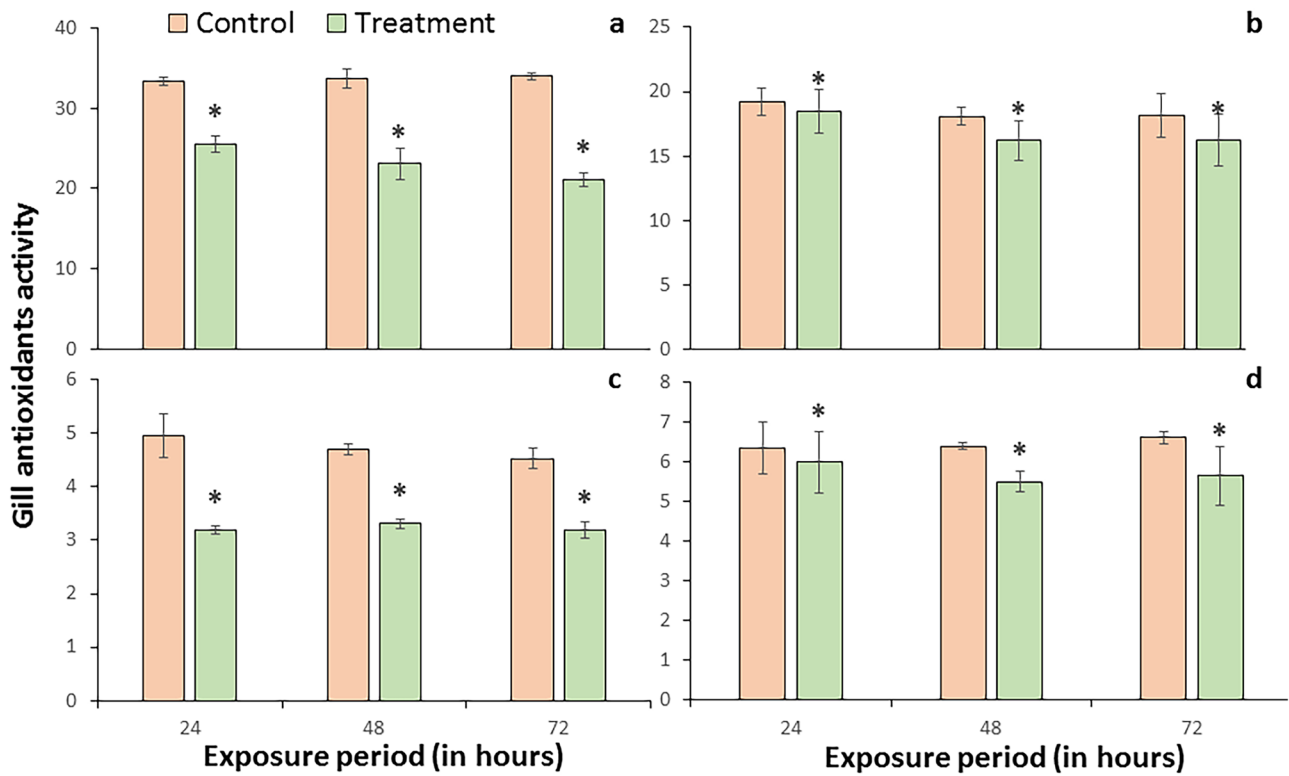


Fig. 4 Gill tissue antioxidant activity of *L. rohita* under short-term exposure periods. **a** SOD activity. **b** CAT activity. **c** GST activity. **d** GPx activity. Asterisk signifies statistical significance $p < 0.05$

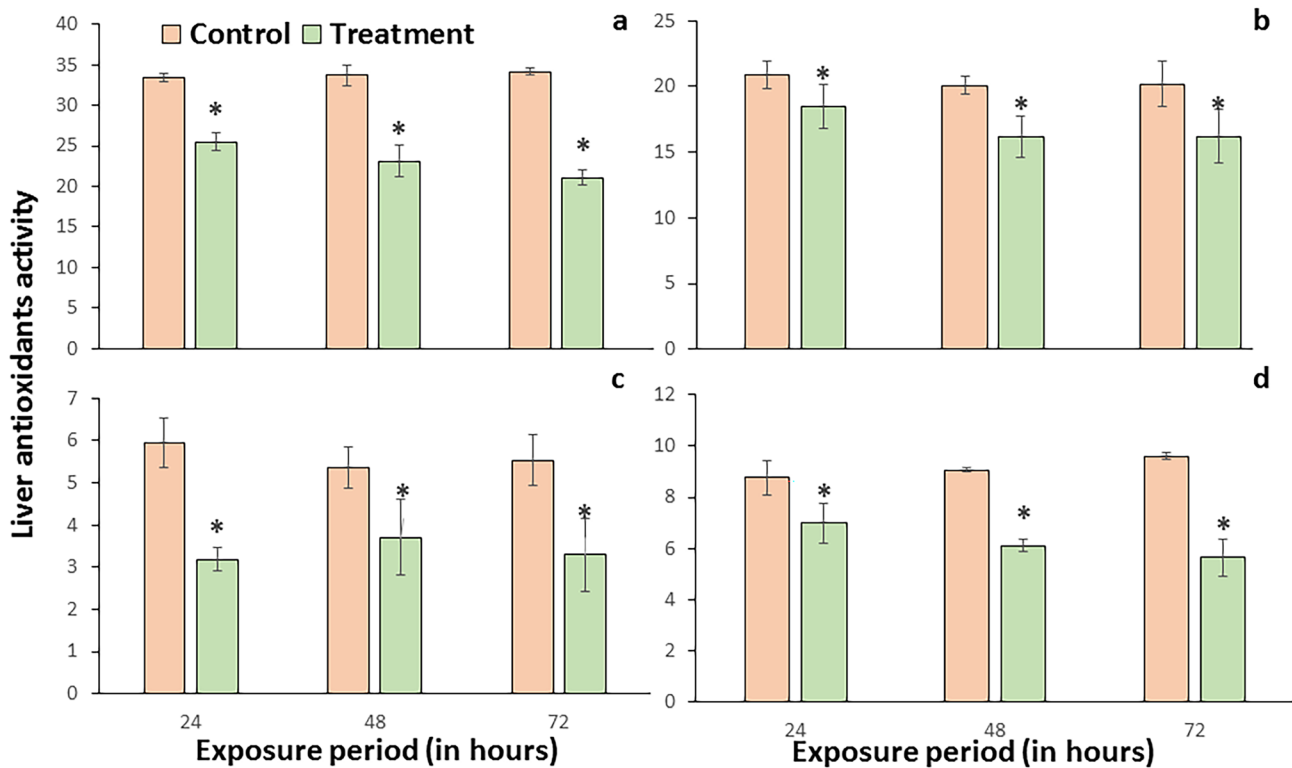


Fig. 5 Liver tissue antioxidant activity of *L. rohita* under short-term exposure periods. Asterisk signifies statistical significance $p < 0.05$

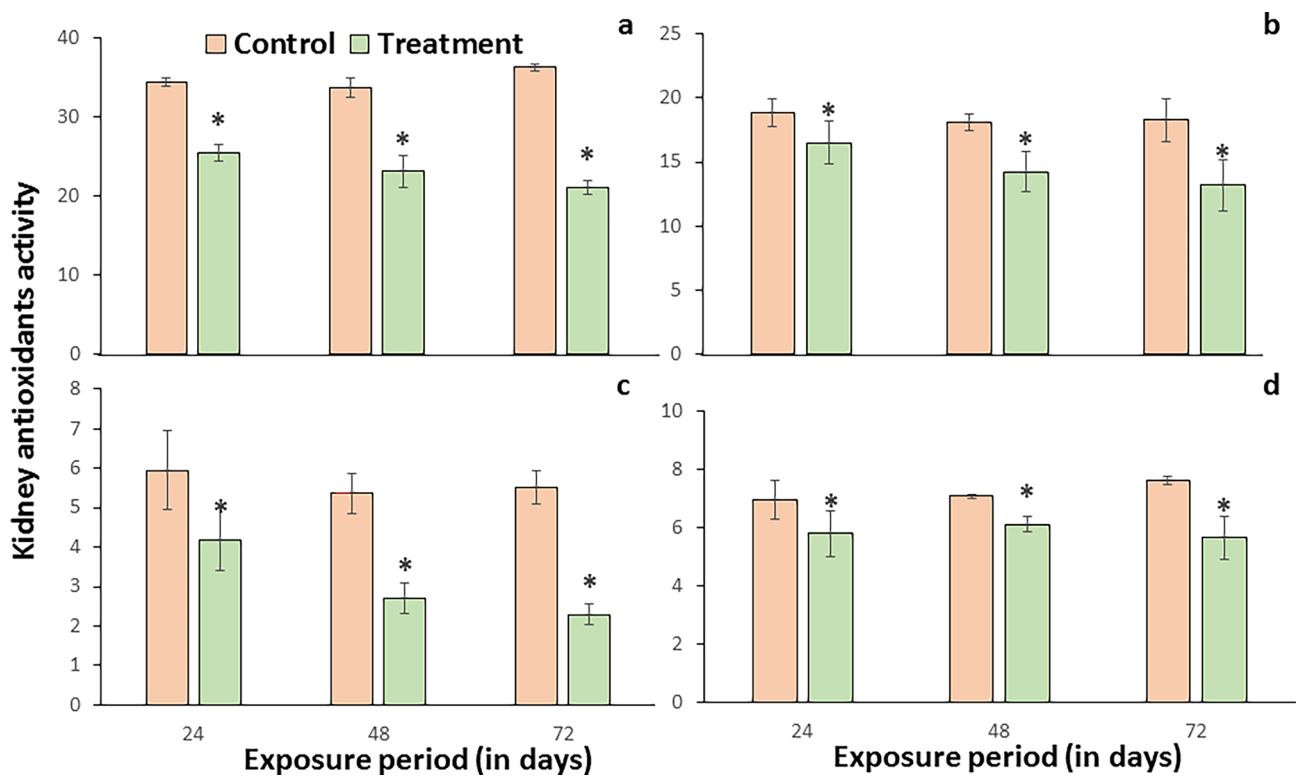


Fig. 6 Kidney tissue antioxidant activity of *L. rohita* under short-term exposure periods. Asterisk signifies statistical significance $p < 0.05$

antioxidant defense system mainly involves in the scavenging reactive oxygen species (ROS) to prevent the organ dysfunction. The decline of CAT activity might be due to the disturbance of normal oxidative process and direct binding of the soul group of enzyme molecule, which could increase hydrogen peroxides and superoxide radicals. The low GPx activity in various tissues of exposed fish demonstrated the inability of these organs to neutralize the impact of peroxides. The reduction in GST activity observed in fish tissues indicates that the detoxification mechanism of exposed fish over the long term is impaired. The rise in LPO may be due to reduced activity of enzymatic and non-enzymatic antioxidants, increasing recovery of free radicals.

The alterations in the antioxidants indicated oxidative stress in fish exposed to pesticide toxicity (Prusty et al. 2011; Doherty et al. 2016). Generally, oxidative stress is an unbalanced state between pro-oxidants and antioxidant ratios. Oxidative stress (LPO, DNA damage, protein oxidation, and cellular death) results from the production of reactive oxygen species (superoxide anion radical, H_2O_2 , and highly reactive OH). Pesticides are known to generate ROS in organisms. Fish has a well-developed defense system to balance those ROS. Antioxidants such as enzymatic and non-enzymatic antioxidants are very effective at offsetting available ROS (Jiang et al. 2016; Sachi et al. 2021). Excessive oxidizing agents could also inhibit antioxidant enzymes.

Hydrogen peroxide can inactivate SOD, superoxide anion can inactivate CAT, and likewise, oxidants can inactivate GST (Lushchak et al. 2009). Pesticide toxicity-mediated cell dysfunction could decline SOD activity; moreover, the liberation of superoxide free radicals (O_2 to H_2O) to maintain cell membrane integrity could also affect SOD homeostasis in vital organs of fish (Kumar et al. 2022). Decrease in CAT activity under pesticide insult resulted due to oxidative damage in vital organs (gills, liver, and kidney) or surplus of ROS. Inhibition of the multifunctional cytosolic enzyme and the GST detoxifying enzyme was declined in fish under pesticide toxicity, indicating a failure to detoxify the available free radicals. Similarly, GPx activity has also declined under pesticide treatment, indicating a lack of tripeptidic GSH (Tabassum et al. 2016). The decline in GPx activity in fish due to pesticide toxicity occurred due to the negative feedback mechanism of excess substrate or damage from oxidative adjustment. These alterations are an indication of inefficiency of antioxidant defense mechanism, which could be evidenced by the elevation of LPO levels (Narra 2016). Oxidative stress may result from respiratory disturbances in the mitochondria under the effect of a pesticide. Additionally, pesticide-mediated oxidants can react with lipids and cause interruption or damage to the structure and function of cell organelles and membranes, including energy homeostasis (Serafini et al. 2019).

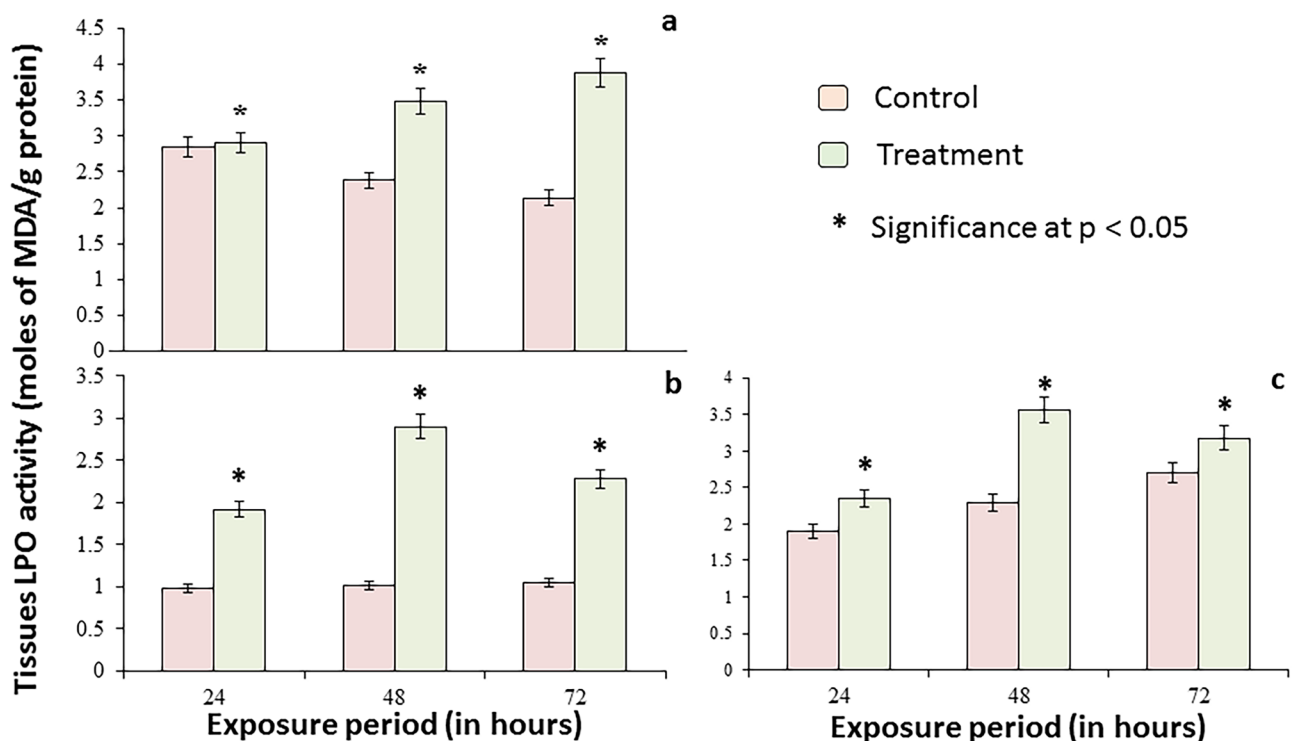


Fig. 7 Tissue antioxidant (LPO) activity of *L. rohita* under short-term exposure periods. **a** Gill tissue. **b** Liver tissue. **c** Kidney tissue. Asterisk signifies statistical significance $p < 0.05$

Conclusion

This study provides key baseline information on the toxicity of pretilachlor. The toxicity study indicated that pretilachlor is highly toxic to edible freshwater fish *L. rohita*. Like other pesticides, pretilachlor has a detrimental effect on the blood parameters (hematology, biochemistry, and electrolytes) of fish, even at a sublethal concentration ($1/25^{\text{th}}$ of 96 h LC_{50}). Pretilachlor is a multiorgan toxicant. Also, pretilachlor toxicity causes oxidative stress in fish. It is important to note that oxidative stress may have many effects on biological mechanisms, including neurological, reproductive, and development. Thus, the entry of pretilachlor raises concerns about the safety of the aquatic ecosystem. The excess use of pretilachlor should be avoided. The biomarkers investigated in this study could be used as early stress indicators for ecotoxicity of pesticides. Furthermore, studies on molecular toxicity and chronic effects of pretilachlor on non-target organisms are warranted.

Author contribution M.R.: supervision, review, funding acquisition. M.P.: methodology, data curation. R.T.: methodology, data curation. R.K.P.: methodology, writing—draft (original and revisions). Z.R.: Review. B.L.: writing—draft, review.

Data availability Not applicable.

Compliance with ethical standards

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Conflict of interest The authors declare no competing interests.

Ethical approval The experiment protocol was following the advices of university ethical committee—the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Informed consent For this type of study informed consent is not required.

Consent for publication For this type of study consent for publication is not required.

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