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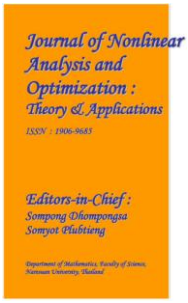


EVALUATION AND ANTI-INFLAMMATORY ACTIVITY EFFECT OF *ATROPINE* AGAINST SIMAZINE TOXICITY AND ENVIRONMENTAL FEASIBLE CONCENTRATIONS IN THE FRESHWATER FISH LABEO ROHITA (HAMILTON)

Dr. K. SUDHAKAR, Associate Professor, Department of Zoology, Kalaignar Karunanidhi Government Arts College, Tiruvannamalai-3, Tamil Nadu, India E-mail**:** ksudhakardr27@gmail.com
JAYACHANDRAN.K Department of Zoology,Annamalai University,PG Extension Centre, Villupuram-1, Tamilnadu, India
VIJAYAKUMAR.A Department of Zoology,Aravindar Arts and Science College,Thenpallipattu , Kalasapakkam -1

Abstract

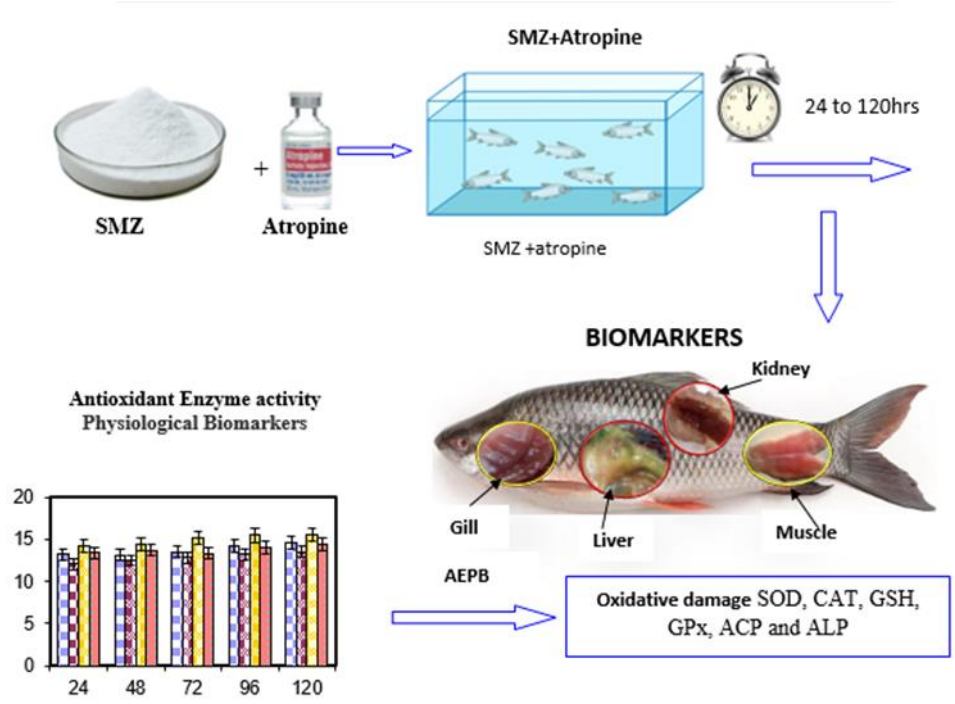
The present study was conducted to determine the toxicity and effects of herbicides simazine commercial formulation on the antioxidant enzyme system in the freshwater air breathing fish *Labeo rohita* .the effect of sub-concentrations of simazine in the *labeo rohita* fingerlings on enzyme activity in gill, liver, kidney and muscle has been reported. Antioxidant modification and the main antioxidant enzyme, are superoxide dismutase (SOD),catalase (CAT) have increased significantly following administration of simazine ,and atropine antidotes may restore these activities. In addition, the study findings show that atarzine cause oxidative stress formation improved and reduced glutathione (GSH) perpective. The effect of sub lethal concentrations of simazine in the *Labeo rohita* fingerlings on enzyme activity in gill, liver, kidney and muscle has been reported. The tests of sub lethal exposure were 24, 48, 72, 96 and 120 hours. The simazine dosage used was (0. 18 mg / l.) and atropine antidotes were 2.5 mg / l over 120 hours. Antioxidant modification and the m ain antioxidant enzymes are superoxide dismutase (SOD). Atropine exposure to simazine and antidotes resulted in substantial activation of SOD, CAT and GSH disease in the muscle and gill ,liver, kidney and muscles. The enzymatic activities of acid and alkaline phosphatases (ACP and ALP) change in a time-dependent manner, but adapt to the toxic environment over time. The ACP and ALP activities increase in gill and kidney, while a significant reduction in both the activities is observed in liver tissue. In addition to concentration and time dependent decrease in mortality rate, signs of stress in the form of behavioral changes were to observed. The antioxidants superoxide dismutase, catalase, and glutathione reductase, ACP and ALP activities reacted positively in a concentration dependent fashion, indicating the use of the antioxidants as possible toxicity biomarkers associated with freshwater fish exposure to contaminations.

Keywords:
Anti-inflammatory activity, atropine, simazine, environmentally feasible, *L. rohita*,

HIGHLIGHTS

- The effects of simazine (SMZ) and competitive antagonistic responsiveness of atropine were compared.
- Fish were exposed to SMZ and ATP for 24 and 120 hours.
- The activities of antioxidant enzyme activity physiological biomarkers were measured.
- SMZ promoted changes in enzyme activity in gill, liver, kidney and muscle a greater number of biomarkers compared to atropine (ATP).
- The herbicide protected the animal from the effects of atropine (ATP) indicating the use of the antioxidants as possible toxicity biomarkers.

GRAPHICAL ABSTRACT



1. Introduction

Public awareness of worldwide increase herbicides use and their adverse effects on ecosystems has been growing over the past decades. Herbicides may reach water bodies via agricultural runoff and leaching processes, as well as by direct applications to control noxious aquatic weeds. Once in the aquatic ecosystems, herbicides may reduce environmental quality and influence essential ecosystem functioning by reducing species diversity and community structures, modifying food chains, changing patterns of energy flow and nutrient cycling and changing the stability and resilience of ecosystems. Many environmental pollutants may induce the formation of reactive oxygen species (ROS) (Ahmad *et al*., 2000; Sevgiler *et al*., 2004). Due to their high reactivity, these species may cause damage to lipids, proteins, carbohydrates, or nucleic acids (Parvez and Raisuddin, 2005). Recently, there have been a great number of studies considering changes induced by environmental contamination in aquatic organisms (Lionetto *et al*., 2003; Sayeed *et al*., 2003). Herbicides and pesticides may produce a disruption of the ecological balance causing damage to non-target organisms, such as fish (Bretaudt *et al*., 2000). Deleterious effects of pesticides on human and animal health have been well documented in literature (Singh *et al.,* 2006, McCauley *et al*., 2006). Pesticide-induced oxidative stress has been also a focus of toxicological research for the last decade as a possible mechanism of toxicity (Ciccheti and Argentin, 2003; Akhgari et al., 2003; Abdollahi *et al.*, 2004). Antioxidant defenses such as catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) are involved to counteract the toxicity of reactive oxygen species (Orbea et al., 2002). Under normal conditions these antioxidants they protect the cells and tissues from oxidative damage.

The antioxidants in fish could be used as biomarkers of exposure to aquatic pollutants (Ahmad et al., 2000). Lipid peroxidation (LPO) is one of the molecular mechanisms involved in pesticide toxicity (Kehrer, 1993). Estimation of lipid peroxidation in particular has been found to have high predictive importance as revealed from a credible number of research papers describing its use as a biomarker (Lackner, 1998; Elia et al., 2002). Study of pesticide-induced effects on various antioxidants in fish and other aquatic organisms can provide the information about the ecotoxicological consequences of pesticide use. Recent studies indicate that the pesticide toxicity in fish may be related to an increased production of reactive oxygen species leading to oxidative damage. Reactive oxygen species (ROS) are products of electron transport chains, enzymes, and redox cycling and their production may be enhanced by xenobiotics (Winston *et al*., 1991). Oxidative stress occurs when reactive oxygen species overhelm the cellular defenses and damage proteins, membranes, and DNA

(Kelly *et al*., 1998). Oxidative stress is defined as a disruption of the pro-antioxidant balance, leading to potential damage.

CAT primarily occurs in peroxisomes and detoxifies H_2O_2 to O2 and water. GPx is the most important peroxidase for the detoxification of hydroperoxides.Although an important feature of these enzymes is their inducibility under conditions of oxidative stress and such inductions can serve as an important adaptation to these conditions, increased levels of oxidative stress cause lipid peroxidation and subsequently oxidative damage in organisms exposed to xenobiotics which stimulate the production of ROS (Livingstone *et al*., 2001). In addition, oxidative stress is believed to be one of their toxic side effects though endocrine disrupture chemicals primarily affect the endocrine functions. Accordingly, antioxidant enzymes are vital to neutralize endocrine disrupture chemicals-induced oxidative stress. Glutathione S-transferases (GSTs) which take part in detoxification and provide antioxidant defense to xenobiotically induced oxidative stress are the major contributors to xenobiotics detoxification pathways in fish (Yu *et al*., 2008).

Studies have also indicated that pesticide exposure is associated with chronic health problems or health symptoms such as respiratory problems, memory disorders, dermatologic conditions, cancer, depression, neurologic deficits, miscarriages, and birth defects Arcury *et al*.,2003, (Van Maele fabry, and Willems 2003). Various *in vitro* studies have shown the ability of simazine to induce genetic damage in human and animal cells (Surralles *et al*., 1995,). Further, it has been reported that pesticides such as alachlor, acephate, chlorpyriphos and simazine induce DNA damage (Moretti *et al*., 2000) and chromosomal aberrations among farm workers (Antonucci *et al*., 2000).

Widespread use of pesticides for agriculture or domestic purposes damage the non-target organisms, including fish of commercial importance. The investigation of the effects of pesticide on fish has diagnostic significance in evaluation of adverse effects of pesticides to human health (Begum and Vijayaraghavan., 1996) since fish have an important role in food chain. The fish endocrine system is fundamentally similar to that of mammals, and fish reproduction may be a relevant indicator on the mechanism of endocrine disruption by chemical compounds.Antioxidant enzymes play a key role in inactivation of reactive oxygen species (ROS) and thereby control oxidative stress as well as redox signaling. Both processes change across the life span of the organism and thus modulate its sensitivity and resistance against damage by free radicals (Vifia et al., 2003), (Wegwood and Black, 2004) and (Hu et al., 2007). A variety of hypotheses of aging have been proposed. Free radical theory explains cell and molecular damage by the attack of ROS. Aging changes can be attributed to development, genetic defects, the environmental unfavorable factors effects and diseases. The accumulation of the negative changes and the loss of the ability to repair or detoxify them increase the risk of death (Harman, 2003). The study of aging strategy, both endogenous and exogenous factors that it modulates in different species is very important for the understanding of the key mechanisms of senescence and its early prevention.

Pesticide-induced oxidative stress has been also a focus of toxicological research for the last decade as a possible mechanism of toxicity (Ciccheti and Argentin, 2003; Akhgari et al., 2003; Abdollahi et al., 2004). Antioxidant defenses such as catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) are involved to counteract the toxicity of reactive oxygen species (Orbea et al., 2002). Under normal conditions these antioxidants they protect the cells and tissues from oxidative damage. The antioxidants in fish could be used as biomarkers of exposure to aquatic pollutants (Ahmad et al., 2000). Study of pesticide-induced effects on various antioxidants in fish and other aquatic organisms can provide the information about the ecotoxicological consequences of pesticide use. In addition; oxidative stress is believed to be one of their toxic side effects though endocrine disrupture chemicals primarily affect the endocrine functions. Accordingly, antioxidant enzymes are vital to neutralize endocrine disrupture chemicals-induced oxidative stress. Glutathione S-transferases (GSTs) which take part in detoxification and provide antioxidant defense to xenobiotically induced oxidative stress are the major contributors to xenobiotics detoxification pathways in fish (Yu *et al.,* 2008). Pollutant-induced lipid peroxidation (LPO), as in the case of herbicides, has been observed in several fish species (Sevgiler *et al*., 2004). Variations in the activities of antioxidant enzymes have been proposed as indicators of pollutant mediated oxidative stress (Li *et al*., 2003).

The sensitivity of the cell to oxidants is attenuated by antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD). The antioxidant enzymes maintain a relatively low rate of the reactive and harmful .OH Oxidative stress occurs as a result of the effect of xenobiotics causing the disturbances in the antioxidant enzyme systems. Glutathione-S-transferase (GST) is a group of multifunctional enzyme involved in biotransformation and detoxification of xenobiotics (Smith and Litwack, 1980).

Hence, an attempt has been made to investigate the following enzymological parameters like SOD, CAT, GSH, $GP_{X,}$ ACP, ALP and LPO in the freshwater fish *Labeo rohita* exposed to sublethal concentration of simazine (group 2), simazine along with atropine (group 3) and atropine alone (group 4) for a period of 120 hrs.

2. MATERIALS AND METHODS

2.1. Collection and maintenance of the experimental animal.

The freshwater fish *Labeo rohita* were collected from the fish farm located in Puthur Nagapattinam district. The fishes were brought to the laboratory and transferred to the rectangular fiber glass tanks (100×175 cm) of 500 liters capacity containing chlorine free aerated well water fishes of the same size and weight were used irrespective of their sex for the experiments.

2.2. Procurement and rearing of experimental fishes

Labeo rohita commonly called 'rohu' is widely distributed in the freshwater of India. *Labeo rohita* was collected from the fish farm located in puthur. The collected fishes without least disturbance were transported in polythene bags filled half with water. About 100 fishes were put in each bag and water was well aerated, using pressurized air from a cylinder. This mode of transit proved successful since there was no mortality in all consignments throughout the course of this study. The fish brought to the laboratory were acclimatized in fibre aquarium for a fortnight before they were used for the experiment. The fish tanks (aquarium) were kept free from the fungal infection by washing with potassium permanganate solution. The fish were disinfected with 0.1% potassium permanganate solution and were maintained for three weeks in well aerated tap water. Prior to experimentation, they were acclimatized to experimental tanks for at least one week. The fishes measuring 10-12 cm in length and 9-14 gm in weight were selected irrespective of their sex for the experiments. The fish were fed daily on oil less groundnut cake. The unused food was removed after 2 hours and water was renewed daily.

The test fishes were critically screened for the sign of diseases, stress, physical damage and mortality. The injured severely diseased, abnormal and dead individuals were discarded. Feeding was discontinued two hours prior to the commencement of the experiments to reduce the additive effect of animal excreta in the test trough. (Arora *et al.*, 1972). The fishes were exposed to sublethal concentration for treated and control period of 120 hours. A control group was maintained with identical environment. The toxicant water and normal water was renewed every day. The fish were sacrificed both experimental and control groups on 120 hours.

2.3. Source of Simazine

The herbicide, simazine, used for the experiment was sourced from the present study commercial formulations of simazine (50% WP) with the trade name "Rasayanzine", manufactured by Krishi Rasayan Exports Ltd (India) was purchased from the market.

2.4. Source of Atropine

No 50/ B St Joseph College Complex, Chathiram Bus Stand, Trichy - 620002, Opposite Chathiram Bus Stand- Actiza Pharmaceutical Private Limited, India,245, 309 Royal Square, Utran,Surat, Gujarat, India Zip Code: 394105

2.5. Action of atropine

The most important therapeutic action of atropine is the inhibition of smooth muscle and glands innervated by postganglionic cholinergic nerves. It also has central nervous system activity, which may be stimulating or depressing depending upon the dose. Following the administration of usual clinical doses, atropine produces stimulation of the medulla and higher cerebral centers. This effect is

manifested by mild central vagal excitation and moderate respiratory stimulation. Atropine sulfate also acts peripherally as a competitive antagonist of the muscarinic actions of acetylcholine.

2.6. Tissue Sampling

The fish specimens were exposed to the three aforementioned test concentrations of simazine along with the control and the experiments continued for 15 days. At the end of every 1, 5, 7, 10 and 15 days five fishes were sacrificed by cervical decapitation. The liver was dissected out carefully, washed in ice-cold 1.15% KCl solution, blotted and weighed. They were then homogenized in homogenizing buffer (50mM Tris-HCl mixed with 1.15 KCL and pH adjusted to 7.4) using a motor-driven Teflon Potter–Elvejhem homogenizer. The resulting homogenate was centrifuged at 10,000 g for 20 min in a refrigerated centrifuge at $4C^0$.The clear supernatant collected were used for protein estimation and assaying the activity of enzymes.

2.7. Selection of herbicide

In the present investigation the herbicide simazine has been selected. Simazine is a broad leaf herbicide that has been used historically on agriculture and forestry. A herbicide is a chemical used to kill or otherwise manage certain species of plants considered to be pests. Plant pests, or weeds, compete with desired crop plants for light, water, nutrients, and space. This ecological interaction may decrease the productivity and yield of crop plants, thereby resulting in economic damage. Plants may also be judged to be weeds if they interfere with some desired aesthetic effect, as is the case of weeds in lawns.

2.8. Acute toxicity test

The LC_{50} values were calculated for fingerlings of *L. rohita* exposed to simazine at 24, 48, 72, 96 and 120 hrs and they were found to be 4.17, 2.56, 1.86, 1.54, 1.15, 0.95 and 0.82 mg/L respectively (Table 1). The toxicity curve (Fig. 1) showed the mode of action and the toxicity of simazine for different concentration at different periods. The peak action of the simazine was at 1.54 mg/L (24 hrs LC_{50}). The lethal (24 hrs LC_{50}) and sublethal (120 hrs LC_{50}) concentration of simazine for the fish *L. rohita* fingerlings were found to be 3.08 and 0.018 mg/L respectively.

2.9. Experimental design

Group I (untreated control) -	Kept on standard diet and clean water observed for 120 hours.
Group II (Simazine)	0.18mg/l of sub lethal concentration for 120 hours
Group III (Simazine and Atropine) -	0.18mg/l and 2.5mg/l of sub lethal concentration for 120 hours
Group IV (Atropine) -	2.5mg/l of sub lethal concentration for 120 hours

2.10. Enzymatic assay

2.10.1. Assay of superoxide dismutase (SOD) (EC 1.15.1.1)

Superoxide dismutase activity was determined by following the procedure of Kakkar *et al*. (1984). 0.5 mL tissue homogenate was diluted to 1 mL with water. Then 2.5 mL of ethanol and 1.5 mL chloroform (all reagents chilled) were added. This mixture was shaken for one minute at 4°C and then centrifuged. The enzyme activity in the supernatant was determined.

The assay mixture contained 1.2 mL of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.01 mL of 186 μM NADH, appropriately diluted enzyme preparation and water in a total volume of 3 mL. Reaction was initiated by the addition of NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1 mL glacial acetic acid. The reaction mixtures was stirred vigorously and shaken with 4 mL of *n*-butanol. The intensity of the chromogen in the butanol layer was measured at 560 nm against butanol blank. A system devoid of enzyme served as control. One unit of the enzyme activity was defined as the enzymes concentration required to inhibit the optical density at 560 nm of chromogen production by 50% in 1 min. Values were expressed as U/min/mg protein.

2.10.2. Assay of catalase (CAT) (EC 1.11.1.6)

The activity of catalase (CAT) was assayed by the method of Sinha (1972). 0.9 mL of phosphate buffer, 0.1 mL of tissue homogenate and 0.4 mL of hydrogen peroxide were added. After 60 seconds, 2 mL of dichromate acetic acid mixtures were added. The tubes were kept in a boiling water bath for 10 min and the colour developed was read at 620 nm. Standard in the range of 2-10 μM were taken and proceeded as test with blank containing reagent alone. The activity was expressed as μmole of H_2O_2 consumed (U)/min/mg protein.

2.10.3. Estimation of reduced glutathione (GSH)

Reduced glutathione was estimated by the method of Ellman (1959). A known weight of tissue was homogenized in phosphate buffer. From this 0.5 mL was pipetted out and precipitated with 2 mL of 5% TCA. 1 mL of the supernatant was taken after centrifugation/ 0.5 mL of plasma and added to it. 0.5 mL of Ellman's reagent and 3 mL of phosphate in a similar manner were added along with a blank containing 3.5 mL of buffer. The amount of glutathione was expressed as μg/mg protein.

2.10.4.Estimation of glutathione peroxidase (GPx)

Glutathione peroxidase (GSH-Px): GSH-Px activity was measured in the PMS by the method by Lawrence and Burk (1976). The reaction measured the rate of GSH oxidation by H_2O_2 catalyzed by the GSH-Px present in the PMS. The rate of GSSG formation was measured by following the decrease in absorbance at 340 nm as NADPH was converted to $NADP^+$ by glutathione reductase. The results were expressed as μg/min/mg protein.

2.10.5. Estimation of acid and alkaline phosphatase

Acid and alkaline phosphatase were assayed following the procedure adopted by Tenniswood *et al*. (1976). 100 mg of wet tissue from gill, liver, kidney and muscle were weighed and homogenized in a glass homogenizer using 10 mL distilled water. To each test tube 0.5 mL of substrate solution (*p*-nitrophenyl- phosphate) and 0.5 mL of 0.1 N citrate buffer were added. The test tubes with the above solution were kept in a water bath and maintained at 37°C for 5 min. Then, l mL of the tissue extract was added to the test tube. The test tube with the tissue extracts was than kept in water bath at 37°C for 30 min. After completion of 30 min the reaction was arrested in the extract by adding 3.8 mL of 0.1 N sodium hydroxide. The colour formed at the end was read at 415 nm grating spectrophotometer and the values expressed in μmole/min/mg protein.

2.10.6. Statistically analyses

The data obtained in the present work were expressed as means ± SE, percentage changes and were statistically analyzed using student t-test to compare means of treated data against their control ones and the result were considered significant at ($P<0.05$) and ($P<0.01$) level.

3.0. RESULT AND DISCUSSION

In the present investigation, sublethal concentration of simazine exposed fish (group-2) fingerlings of *Labeo rohita* shows an increase in lipid peroxidation and decrease in superoxide dismutase (SOD) and catalase (CAT) in gill,liver,kidney and muscle tissues have observed for 24,48,72,96 and 120 hours.

The significantly increased activities of SOD and CAT enzymes observed in the present study reflect the antioxidant response against oxidative stress caused by simazine exposure. This response could be due to the important role of SOD in the scavenging of superoxide free radicals, which helps to maintain a balance between oxidants and antioxidants. CAT and GPx act cooperatively as scavengers of H2O2 (both enzymes) and other hydroperoxides (GPx). Thus, the concomitant increase in CAT and GPx observed in the present study, may be due to exposure to higher dose of simazine, could be related with the increased H2O2 formation. Fish accumulate xenobiotic compounds, especially those with poor water solubility, from water or food. The uptake from water occurs because of the very intimate contact with the medium that carries the chemicals in solution or suspension and also because fish have to extract oxygen from the medium by passing enormous volumes of water over their gills.

Prieto et al. (2006) have reported that intraperitoneally applied microcystin (MC) significantly increased the SOD and CAT activities in various tissues of Tilapia. Jos et al. (2005) have also reported that subchronic dietary exposure to MC increased SOD and CAT activities in Tilapia. Furthermore, they observed that the increase of enzyme activity in liver was higher than that in gill. Li et al. (2003) also registered enhanced SOD and CAT activities in the hepatocytes of common carp (Cyprinus carpio L.), following exposure to MC-LR. Pinho et al. (2005) recodred that the increased SOD and CAT activities in the hepatopancreas tissue of the estuarine crab Chasmagnathus granulatus exposed to MCs were related to the production of ROS.

Values for SOD activity in different freshwater fish species Semotilus margarita, Catostomus commersoni, Sal_elinus namaycush, Oncorhynchus mykiss, Salelinus namaycush (Palace et al.,

1996) where similar to those found in Labeo rohita during this study. The SOD variations observed for fish from sites can also be linked to gonad maturity and food availability to a certain extent and can modulate the metabolic status (Viarengo et al., 1991). An increased CAT and SOD activity in dichlorvos and CuSO4 treated larvae further suggests that catalase functions to deal with H2O2 produced as a result of dismutation of O2 by SOD. Organophosphate compounds have been reported to inhibit mitochondrial ATP production through the uncoupling of oxidative phosphorylation (Videira et al., 2001) and this could lead to generation of reactive oxygen radicals (Ishii et al.,2004). ROS have been reported to be involved in the protein alteration (Briganti et al., 2003), and hence it is likely that these altered proteins could trigger induction in the exposed organism.

Dorval et al. (2003) and Dorval and Hontela (2003) provided evidence that endosulfan induce oxidative stress in adrenocortical cells of rainbow trout Oncorhynchus mykiss. Lipid peroxidation (LPO), antioxidant activities (CAT, GPx), and GSH levels in the liver and the adrenal tissue have been evaluated in white sucker Catostomus commersoni from the Yamaska, a river polluted by agricultural activities in Quebec (Canada) (Jocelyn et al., 2005).The increase in lipid peroxidation observed in perchloroethylene (PER) administered animals might be a consequence of higher levels of superoxide radicals which are produced in significant amounts in response to PER exposure or inhibition of free radicals scavenging metalloenzymes that play a key role in the defence against ROS by transforming superoxide anions into hydrogen peroxide (Yim et al., 1993). Antioxidant enzyme, such as superoxide dismutase (SOD) was changed in the liver of red and white muscle of fish with cadmium exposure (Almeider et al., 2002).

Comparative studies of fish species have found SOD and CAT content to be related to physical activity, with higher concentrations in the blood of more active, pelagic species (Filho et al., 1993; Filho, 1996 and Rudeva, 1997). In the present study the lack of catalase in gill may reflect a capacity to rid tissues of H2O2 through passive gill exchange, thereby eliminating the need for CAT such a mechanism, similar to the excretion of ammonia, is possible due to the high solubility and diffusion capacity of the H2O2 molecule (Filho et al., 1993 and Marcon and Filho, 1999).

The primary antioxidant protection against superoxide radicals and hydrogen peroxide is provided by the enzymes Superoxide dismutase (SOD) and Catalase (CAT) respectively (Chance et al., 1979 and Halliwell and Gutteridge, 1970). Consequently these antioxidant enzymes contribute to the maintenance of a relatively low level of the reactive and harmful species of hydroxyl radical (-OH), which is generated through the Haber Weiss reaction between O2- and H2O2 in the presence of simazine. The hydroxyl radical triggers the lipid peroxidation of membrane process that may be potentially dangerous to fish since they possess a high content of polyunsaturated fatty acids (Love, 1970). Reactive Oxygen Species (ROS) have shown to modify enzymes and induce signaling including activation of protein kinase phosphorylation (Schievan et al., 1993) and also act as second messenger for the expression of genes involved in the immune response (Satriano et al., 1993).

Parvez *et al.*, (2003) have reported the modulatory effect of copper on non enzymatic antioxidant in fresh water fish Channa punctatus. Campana et al. (2003) have studied the effect of lead on ALA-D activity on metallothionein levels and lipid peroxidation in blood, kidney and liver of the toad fish Halebatrachus didactylis. Shivashankara et al.(2002) have reported that chronic exposure of rats to fluoride causes both increasing and decreasing trends in antioxidant systems of the liver, leading to increased oxidative stress as indicated by elevated MDA level (Hai et al., 1997).

The combined effect of lead and zinc has causes changes in the liver SOD and Catalase detoxification system of carp (Dimitrova et al., 1994). In fish, modulation of antioxidant systems in liver by endosulfan and the modulatory effect of pre exposure to copper on the endosulfan-induced oxidative stress *in vivo* have been reported (Pandey *et al.,* 2001).

Buet *et al.* (2005) have observed that some heavy metals in the response of SOD and CAT has been significantly reduced by uranium exposure suggests a possible deterioration of the protective defense system of fish. They further state that potential impair of antioxidant defenses by uranium should make cells and the whole organism more susceptible to oxidative attacks and further deleterious effects. Under *et al.* (2001) have studied the effects of cypermethrin on antioxidant enzyme activities and lipid peroxidation in liver as evidenced by elevated levels of thiobarbituric acid reactive substance

(TBARS). In the present study, impairment of enzymatic antioxidant system may favour accumulation of free radicals that may be responsible for increased lipid peroxidation on simazine exposed fish.

Oxidative stress is a result of an increase in reactive oxygen species or an impairment of antioxidant enzymes. Several studies provide evidence that antioxidant may be used biomarkers of exposure to environmental pollutants [Di Giulio *et al.,* (1993) Hasspieter *et al.* (1994) and Regoli and Pricipate (1995)]. Moreover, Smilar report that various pesticides can induce oxidative stress in different tissues of mouse (Bagchi *et al.,* 1995; Hassoun and Stohs, 1996 and Bachowski *et al.,* 1998). Dautremepuits *et al*. (2002) have reported the inhibition of CAT and GST in carp after exposure to copper for 96h. SOD inhibition has been reported in carp after 48h of injection of copper by Varanka *et al*. (2001) and in gilthead seabream *Sparus aurata* by Pedrajas *et al.* (1995). Reactive Oxygen Species (ROS) produced in biological systems are detoxified by antioxidant defences. One of the important features of these antioxidant enzymes is their inducibility under conditions of oxidative stress and such induction can be an important adaptation to pollutant induced stress (Livingstone, 2001).

The SOD-CAT system provides the first defence against oxygen toxicity. The increase in these enzymes is probably the response towards increased ROS generation in pesticide toxicity (John *et al.,* 2001). High etoxazole concentrations cause an elevation in the SOD activity in all tissues of *Oreochromis niloticus* (Nevin Under *et al.,* 2004).

In the present study, simazine and atropine (group-3) exposure induced a noticeable concentration-dependent decrease in GSH content in the gill, liver, kidney and muscle. Thus, it is possible that an increase in the activities of these enzymes contributes to the elimination of ROS from the cell induced by simazine exposure.

Although increasing number of researchers have focused on the oxidative stress responses arising from xenobiotics, the reports on fish species were very limited and mainly examined the changes in activities of related antioxidant enzymes. For instance, Cazenave et al. (2006) reported that the activities of GR, GSH-PX, and CAT were enhanced in the liver of Corydoras paleatus after its exposure to 2 μg L−1 microcystin-RR. More recently, differential expression of the genes encoding these antioxidant proteins has been used to detect biological toxicity and or to monitor the impact of chemical pollutants (Sheader et al., 2006 and Woo et al., 2009).

Ebrahim and Sakthisekaran (1997) have observed that taurine and vitamin E directly scavenged the superoxide radicals and reduced the cellular damage caused by free radicals. Increased catalase activities in fish exposed to pro oxidants have been reported by Joachim Sturve *et al*. (2005). Scavenging of superoxide radicals by some spice principles have been documented (Krishnakantha and Lokesh, 1990; Kunchandy and Rao, 1990).

The liver is reported to be the primary organ for bioaccumulation, and thus, has been extensively studied in regards to the toxic effects of xenobiotics (Jin *et al*., 2008 and Liu *et al*., 2008). In general, oxidative stress effects were reflected more efficiently and effectively in the liver. For example, Elia *et al*. (2002) reported that 6 and 9 mg L^{-1} simazine exposures induced a significant change in the activities of GPx and SOD and induced a change in the content of MDA and GSH in the liver of bluegill sunfish after 96 h of exposure, while no response was found in the gill. Thus, the liver was viewed as a primary organ for simazine metabolism in zebrafish.

In the present study exposed to simazine (group-2) fish caused a significant reduction in lipid peroxidation and significant increase in antioxidant enzymes such as SOD and CAT in the gill, liver, kidney and muscle tissues of *Labeo rohita* when compared with control (group-1) and simazine along with atropine(group-3). In the present study, the increased SOD levels indicate an elevated antioxidant status. A parallel increase in SOD levels with an increase in the severity of poisoning shows that more the stress, more the free radicals are generated. This is additionally corroborated by the increased lipid peroxide levels. The free radical production is so high that it even overwhelms the elevated antioxidant (SOD) failing to check lipid peroxidation.

Therefore, elimination or detoxification of residual ROS and ROI on time is critical for host to protect itself from damage. The antioxidant enzymatic system is then recruited for protecting the host from the toxic effects by the activated oxygen species (Parkes *et al*., 1993). These enzymes include catalase (CAT), superoxide dismutase (SOD), glutathione reductase, glutathione peroxidase, and

glutaredoxin and thioredoxin reductase. Previous studies showed that a balance between the activities and the intracellular levels of these antioxidants was necessary for the survival of organisms and their health (sohal *et al.,* 1995). In animals, SOD detoxifies superoxide radicals by converting them to hydrogen peroxide and oxygen. Hydrogen peroxide is then detoxified by CAT and by glutathione peroxidase (GP_X). CAT is a tetrameric oxidoreductase that catalyzes the conversion of two molecules of hydrogen peroxide to two molecules of water and one molecule of oxygen. When injected intravenously with liposomes containing CAT and SOD, the rats exhibited increased survival of rates following the exposure to 100% oxygen (Turrens *et al*., 1984). Therefore, CAT is thought as a key enzyme of the antioxidant defense systems which can protect host cells by removing cytotoxic H_2O_2 (Bai *et al*., 2003).

The involvement of free radicals other than superoxide anions like hydroxyl free radical could not be ruled out, since the increased SOD levels were only partially effective in combating the oxidative damage. This calls for the investigation of the involvement of other antioxidant enzymes (Catalase) in conditions like simazine poisoning. A sharp increase in the lipid peroxide levels could have accelerated lipid peroxidation of the cell membrane leading to massive cell damage or death.

In the present investigation, sublethal concentration of simazine (group-2) exposed fish fingerlings of *Labeo rohita* shows an increase in ($GSH\text{-}P_x$) activity and increase in CAT and SOD activity in gill, liver, kidney and muscle tissues have observed for 24,48,72,96 and 120 hours. CAT enzyme is an important antioxidant component sharing the same function with glutathione peroxidase ($GSH\text{-}P_x$) for working primarily to degrade H_2O_2 to H_2O. Organic peroxides are the preferred substrate for $GSH\text{-}P_x$ in the presence of low H_2O_2 concentrations, but not at high level of H_2O_2, which are catalyzed by CAT (Yu *et al.,* 1994).

Previous study has found that CAT plays a relatively more important role in detoxification in invertebrates than vertebrates (Livingstone *et al.,* 1992). Generally, any significant increase in SOD activity is accompanied by a comparable enhancement in CAT and or GSH-Px activities (Warner *et al.,* 1998). Similar tendency was also found in our study. The high level of SOD activity was followed by the increased CAT activity accordingly in the hemocyte, serum, hepatopancreas and gill after LPS challenge. Taken together, our data revealed that the antioxidant enzyme activities like CAT and SOD varied in different tissues and cells against the LPS stress, suggesting that the enzyme activity along with their gene expression profile was tissue-specific as well as time-dependent under stress conditions.

On the other hand, the GP_x activity in the gills and muscles in MPc group showed a significantly lower activity than in group I. However, such a reduction was not observed in liver tissue. The decreased activity of GPx in gills and muscle observed in the present study could be related to the O_2 production (Bagnasco *et al*., 1991) or to the direct action of pesticides on the enzyme synthesis (Bainy *et al*., 1993).

Fatima *et al*. (2000) reported a low activity of GP_x in different fish tissues after exposure to paper mill effluent, indicating an inefficiency of these organs in neutralize the peroxide impacts. A similar decrease in GP_x activity in rat liver was reported after 90 days of treatment with lindane, an organochlorine pesticide (Bainy malathion reduced GP_x activity in mice erythrocytes (Yarsan *et al*., 1999). GP_x inhibition was reported after combined treatment with the pesticides 2, 4-D and azinphosmethyl in the brain of carp, *C. carpio* (Oruc *et al*., 2004), and in the liver of Nile tilapia, *Oreochromis niloticus* (Oruc and Uner, 2000). Enzyme activity can be decreased by negative feedback from excess of substrate or damage by oxidative modification (Tabatabaie and Floyd, 1994). A reduced GP_x activity could indicate that its antioxidant capacity was surpassed by the amount of hydroperoxide products of lipid peroxidation (Remacle *et al*., 1992). The GST activity is involved in xenobiotic detoxification and excretion of xenobiotics and their metabolites, including MP (Jokanovic, 2001). The increased GST activity was concomitant to the decreases in GSH content in all tissues analyzed. The GSH plays an important role in the detoxification of electrophiles and prevention of cellular oxidative stress (Hasspieler *et al*., 1994 and Sies, 1999). The considerable decline in the GSH tissue content during exposure to atropine may be due to an increased utilization of GSH, which can be converted into oxidized glutathione and an inefficient GSH regeneration.

The increase in GP_x activity observed, predominantly in liver and kidney, is similar to the results reported by Li *et al*. (2003) who studied the responses of the antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to microcystin-LR. The increase in GP_x activity probably reflects an adaptation to the oxidative conditions to which the fish had been exposed (Lenartova *et al*., 1997). However, GP_x activity decreased in gills after the longer exposure time, corroborating the LPO increase in this organ; this may be explained because gills are less efficient than kidney and liver at neutralizing the impact of peroxidative damage (Sayeed *et al*., 2003 and Ahmad *et al*., 2004).

In the present investigation *Labeo rohita* fish exposed to group 3 (simazine along with atropine) shows the antioxidant enzyme are recovered from the toxic effect of simazine .Wilfried Sanchez *et al*. (2005) have reported that copper accumulation was correlated to a recovery and return of antioxidant enzymes to the basal level suggesting that other detoxification mechanisms such as metallothioneins (MTs) have been involved to allow fish adaptation to copper. Chautan *et al*. (1990) have suggested that omega- 3 fatty acids may stimulate vitamin E incorporation into membranes to avoid lipid peroxidation resulting from higher membrane omega-3 fatty acids content. Further they have demonstrated that treatment with omega-3 fatty acids caused decreased lipid peroxidation in corpus striatum in rats. Sarsilmaz *et al*. (2003) have reported the increased CAT activity in erythrocytes, liver, kidney and heart in the animal with *C. deciduas* treatment indicating that the treatment may help to lower H_2O_2 concentration by its decomposition and subsequently oxidative stress.

The reduced levels of GSH in the simazine administered fish could be the result of either increased utilization of GSH for conjugation and/or participation of GSH as an antioxidant in terminating free radicals produced due to simazine induced toxicity. Administration of simazine (group-2) along with atropine (group-3) resulted in restoration of GSH levels to nearly control (group-1) values. Cells have wide array of enzymatic and non-enzymatic antioxidant defense systems. The enzymatic antioxidant system includes superoxide dismutase (SOD), catalase, glutathione peroxidase (GP_x), glutathione reductase (GR), glutathione-s-transferase (GST), glucose-6-phosphate dehydrogenase, etc. The increase in superoxide dismutase activity after simazine administration appears to be a adaptive response to increased generation of reactive oxygen species. It has been reported in the literature that exposure of animals to xenobiotics increases SOD activity in various tissues (Datta *et al*., 1992 and John et *al*., 2001). The increase in the activity of SOD in our study reflects compensatory mechanism to increased oxidative stress. Similar results have also been reported in other species. It is also reported that stimulation of GP_x catalase activities protect liver tissue from oxidation, thereby preventing accumulation of lipid peroxidation products and their subsequent secretion into circulation in hypercholesterolemic rabbits (Panasenko *et al*., 1992).

In the present investigation, sublethal concentration of simazine (group-2) exposed fish fingerlings of *Labeo rohita* shows an increase in ACP activity and decrease in ALP activity in gill, liver, kidney and muscle tissues have observed for 24,48,72,96 and 120 hours. An enhanced peroxidation of liposomal membranes due to $HgCl_2$ intoxication causes lysis of membrane and oozing out of the enzyme and hence results in an increased acid phosphatase activity (Mehra and Kanwar, 1986).

Alkaline phosphatase (ALP) activity was reported to be an indicator of the intensity of nutrient absorption in enterocytes of fish (Harpaz and Uni, 1999; Gawlicka *et al*., 2000). The herbivorous fishes are reported to have lesser ALP activities than carnivorous fishes (Harpaz and Uni, 1999). Changes in metabolite concentrations reflect biochemical adjustments of metabolism as diet composition changes.

Acid phosphatases act as marker enzymes for the detection of lysosomes in cell fractions and can be altered by the presence of xenobiotics (Cajaraville *et al*., 2000).Alkaline phosphatases are intrinsic plasma membrane enzymes found on the membranes of almost all animal cells. Both enzymatic activities have been studied in several organisms and the influence of heavy metals has been reported (Blasco *et al*., 1993). These enzymatic activities are involved in a variety of metabolic processes, such as molecule permeability, growth and cell differentiation and steroidogenesis (Ram and Sathyanesan, 1985), and their activities have been used to evaluate the effects of crude oil (Krajnovic- Ozretic and Ozretic, 1982) and methylparathion (Reddy and Rao, 1990) on prawns. The measurement of alkaline phosphatase activity is generally carried out in clinical and ecotoxicological

studies. In ecotoxicology, this enzyme may serve as an indicator of intoxication because of its sensitivity to metallic salts (Boge *et al*., ´ 1988).

In fish, gills are the first organ targeted by several xenobiotics because they have a very large interface area between the external and internal fish environment, and changes in gill epithelia have been considered to be good indicators of the effects of toxicants (Jiraungkoorskul *et al*., 2003). The literature shows that ACP and ALP activity decreases in the gills of fish exposed to various substances (Karuppasamy, 2000; Bhavan and Geraldine, 2004 and Das *et al*., 2004).The enzymatic activities of acid and alkaline phosphatases (ACP and ALP) changed in a time-dependent manner, but adapted to the toxic environment over time.

Significant reduction in ALP activity was observed in the present study,exposed to simazine along with atropine (group 3) fingerlings of *Labeo rohita* in gill, liver, kidney and muscle. The ACP and ALP activities increased in gill and kidney, while a significant reduction in both the activities was observed in liver tissue. The ACP activity reAChEd near control values in kidney but it was lower in liver and higher in plasma and gill tissue at the end of recovery period. However the ALP activity was near control values in the case of plasma and the kidney, whereas the liver showed marginally lower value and higher in gill, when compared to control.

Alkaline phosphatase has been shown to exhibit protein phosphatase activity by dephosphorylating proteins with phosphorylated tyrosine residues (Sarrouilhe *et al*., 1992).The elevation in alkaline phosphatase suggests an increase in the lysosomal mobilization and cell necrosis due to pesticide toxicity. Elevation of ACP activity in brain was reported earlier in stress-exposed *Channa punctatus* (Sastry and Sharma, 1980) and in *Labeo rohita* (Das, 1998).

Fig. 1. Changes in the level of lipid peroxidation (nmole/mg protein) activity in the freshwater fish *Labeo rohita* fingerlings exposed to 120 hrs sublethal concentration of simazine and antidote atropine

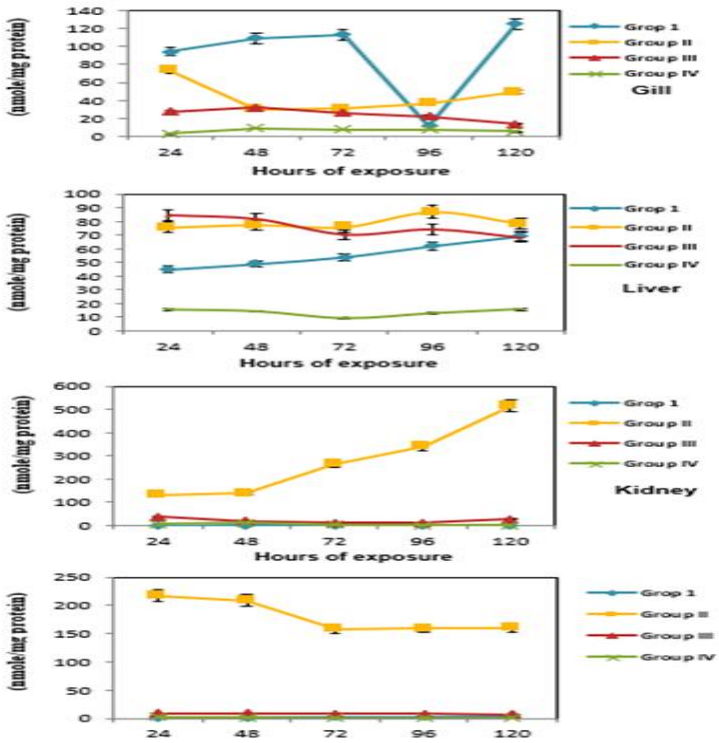


Fig. 2. **Changes in the level of superoxide dismutase (U/min/mg protein) activity in the freshwater fish *Labeo rohita* fingerlings exposed to 120 hrs sublethal concentration of simazine and antidote atropine**

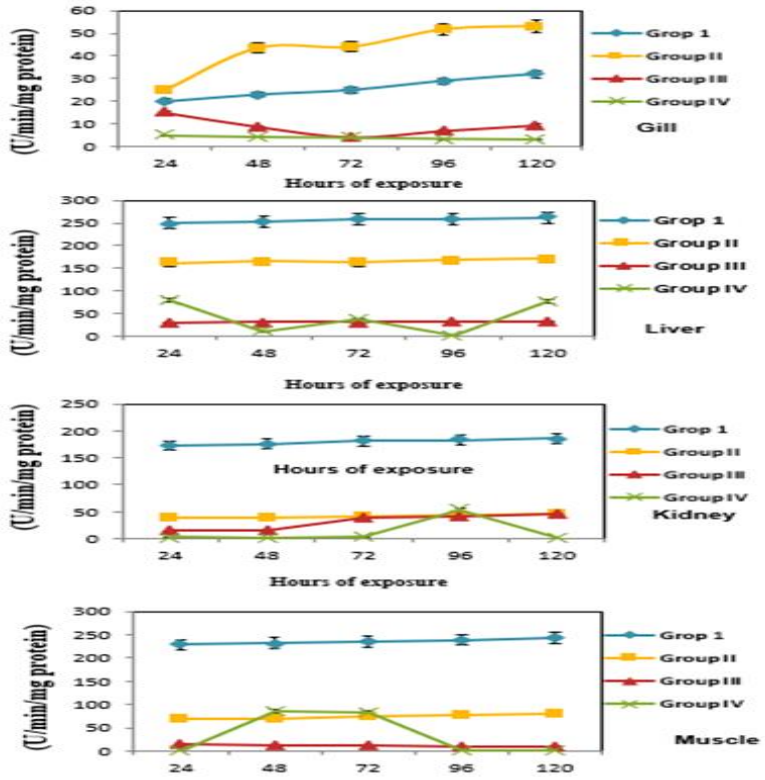


Fig. 3. **Changes in the level of catalase (µmole/min/mg protein) activity in the freshwater fish *Labeo rohita* fingerlings exposed to 120 hrs sublethal concentration of simazine and antidote atropine**

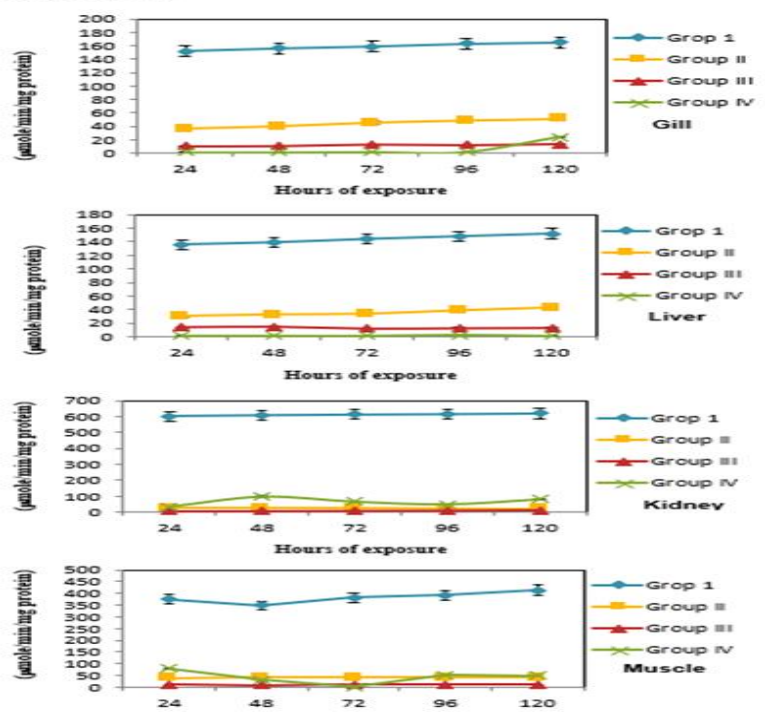


Fig. 4. **Changes in the level of reduced glutathione (µg/mg protein) activity in the freshwater fish *Labeo rohita* fingerlings exposed to 120 hrs sublethal concentration of simazine and antidote atropine**

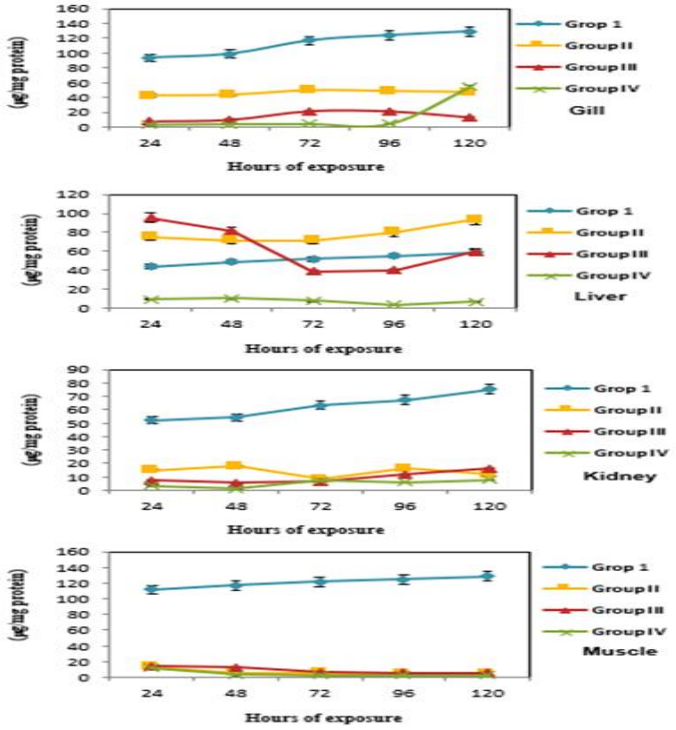


Fig. 5. **Changes in the level of glutathione peroxidase (µg/min/mg protein) activity in the freshwater fish *Labeo rohita* fingerlings exposed to 120 hrs sublethal concentration of simazine and antidote atropine**

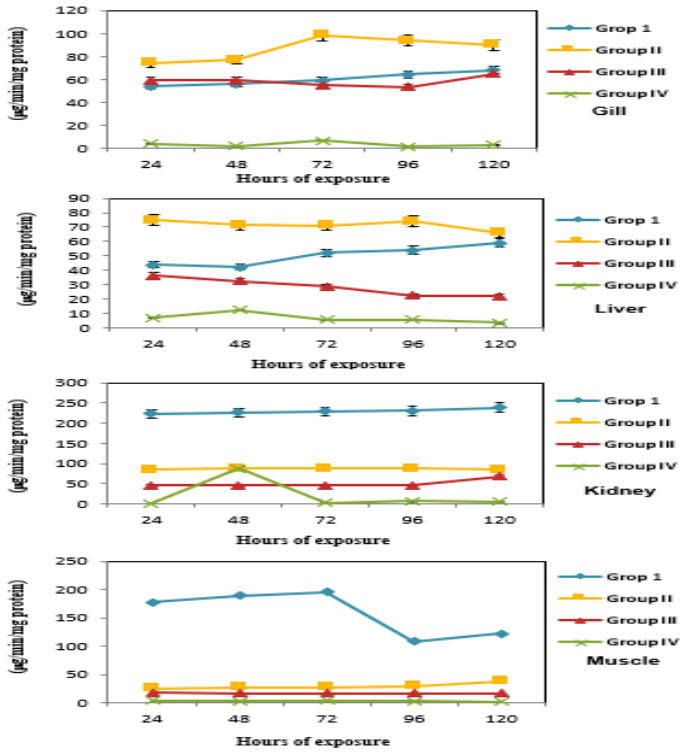


Fig.6. **Changes in the level of acid phosphatase (µmole/min/mg protein) activity in the freshwater fish *Labeo rohita* fingerlings on the effect of simazine and antidote atropine exposed to 120 hrs sublethal concentrations**

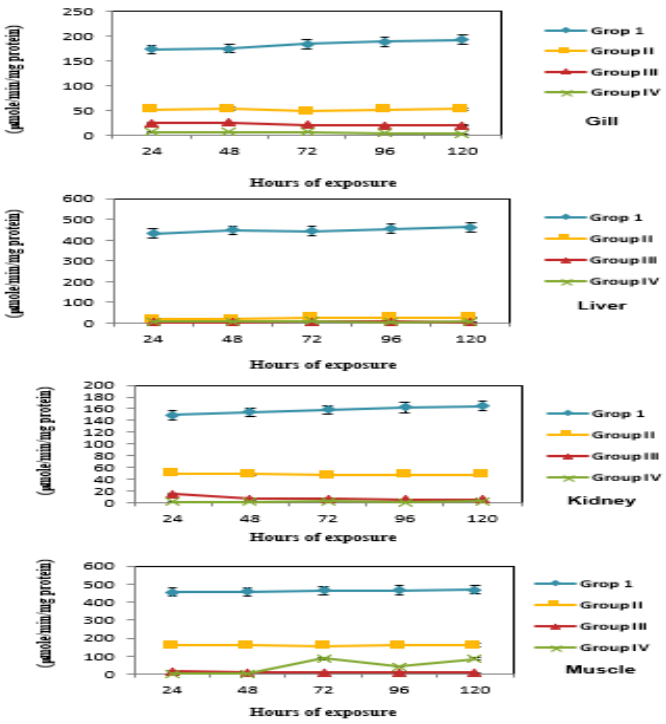
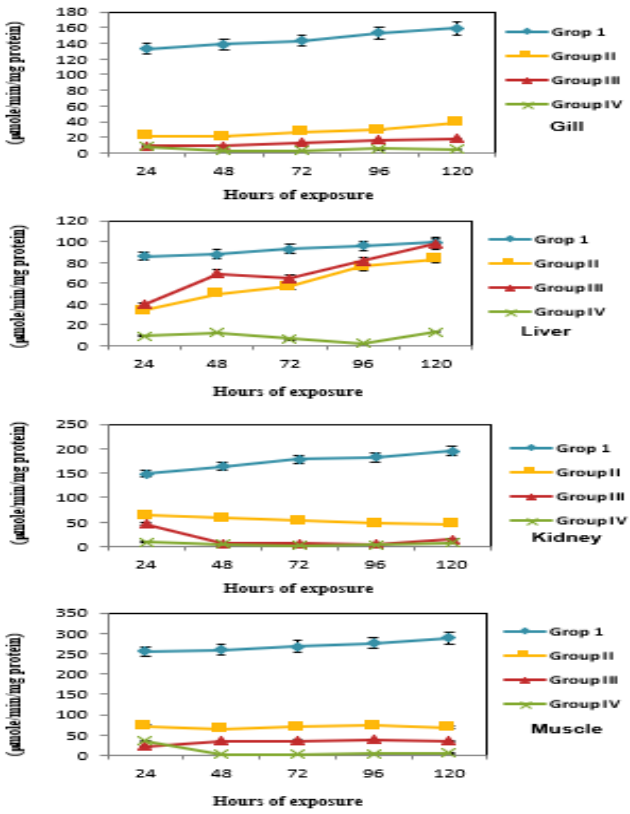


Fig. 7. **Changes in the level of alkaline phosphatase (µmole/min/mg protein) activity in the freshwater fish *Labeo rohita* fingerlings exposed to 120 hrs sublethal concentration of simazine and atropine**



Inhibition of ALP activity was also reported earlier in fishes exposed to different toxicants in different tissues (Rasatwar and Ilyas, 1984 and Gill *et al*., 1990) and serum (Newcomb, 1974). Such reduction of enzyme activity in tissues and blood can probably be attributed to the decrease in

intracellular pH of the substrate (tissues/blood) due to the nitrite buildup in blood and tissues of the exposed fish (Huang and Chen, 2002). Since ACP activity increases with increased acidic conditions, our findings of elevated ACP activities in serum and brain also might be attributed to pH reduction in these substrates. The reduction in ACP activity in gill in this study is in agreement with earlier reports (Gill *et al*., 1990, 1991) and can probably be attributed to the relatively higher pH at the gill surface due to contact with water (higher pH). Since ACP activity increases with increased acidic conditions, our findings of elevated ACP activities in serum and brain also might be attributed to pH reduction in these substrates. The fish exposed to simazine along with atropine (group 3) shows good oxidative tissues thus highlighting the protective action of antidote, atropine. The group 4 atropine alone exposed fish shows without any markable changes the potential antioxidant against simazine induced oxidative stress. The curative properties are related to enhancement of the regenerative process of gill, liver and kidney. The extent of regenerative process is greater in group 3. The antidote generally results in protective changes in the cellular organization.

CONCLUSION

It is concluded that, the findings of the present chelating investigations demonstrate a direct correlation between herbicide simazine exposure and antioxidant enzyme activity observed in selected tissues. Chemical analysis of simazine and fish tissues confirmed a causal relationship between simazine along with atropine exposure and the fish damages are found recovered. Changes occurring in the This present finding suggests that an antidote atropine of nerve agents targeting the various mechanisms for neuronal injury by simazine may prove successful. On the basis of overall observations, it may be concluded that exposure of simazine results in increased oxidative stress, alternate antioxidant station administration of atropine results in the maximum normalization of the toxic effect of simazine thus, highlighting the protective action of atropine.

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