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Malathion induced oxidative stress leads to histopathological and biochemical toxicity in the liver of rohu (*Labeo rohita*, Hamilton) at acute concentration

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ABSTRACT

Organophosphorus pesticides form a diverse group of chemicals, having a wide range of physicochemical properties with crucial toxicological actions and endpoints. These are extensively used to control pests of different food (fruits, vegetables, tea, etc.) and non-food (tobacco, cotton, etc.) crops. Malathion is an important widely used organophosphorus pesticide but its hepatotoxic effects on fish are not well studied. Therefore, the current study was designed to investigate the hepatotoxic effects of Malathion on rohu (*Labeo rohita*) fish in a semi-static system using different parameters. The LC_{50} of Malathion was found to be 5 μg/L for rohu for 96 h through Probit analysis and was used for further toxicity testing. To find the hepatotoxic effects of Malathion, changes in different biochemical indices including protein contents, Lipid Peroxidation (LPO), activities of four protein metabolic enzymes [Aspartate Aminotransferase (AAT), Lactate Dehydrogenase (LDH), Alanine Aminotransferase (AlAT), and Glutamate Dehydrogenase (GDH)], seven antioxidant enzymes [Catalase (CAT), Superoxide Dismutase (SOD), Peroxidase (POD), Glutathione (GSH), Glutathione Reductase (GR), Glutathione-s-transferase (GST), and Glutathione Peroxidase (GSH-Px)], DNA damage [in term of comet tail length, tail moment, DNA percentage in tail, and olive tail moment], reactive oxygen species (ROS), and Histopathological alterations were assayed. Malathion exposure led to a time-reliant significant ($P < 0.05$) decrease in protein contents and a significant ($P < 0.05$) increase in ROS, LPO, enzymatic activities, and DNA damage. The histopathological examination of the liver showed different changes including hepatic necrosis, fatty infiltration, hemorrhage vacuolation, glycogen vacuolation, congestion, and cellular swelling. The current study clearly revealed Malathion as a potent hepatotoxic pesticide; therefore the injudicious, indiscriminate and extensive use of Malathion should be prohibited or at least reduced and strictly monitored.

1. Introduction

Pesticides are widely used around the world to boost agricultural yield with low labor and in less time. Different types (insecticides, herbicides, fungicides, bactericides etc.) and classes (organophosphorus, organochlorine, pyrethroids etc.) of pesticides are employed in agricultural fields, homes, and industries for very long, based on the target species and their efficacy against them (Ullah et al., 2018). Among all the types, insecticides are widely employed, while across classes, the most widely employed ones are organophosphorus based pesticides. Organophosphorus pesticides (OPs) are used for the control of pests, both vertebrates and invertebrates, and to some extent in the control of weed plants (Patil and David, 2009). There are more than 200 OPs but these are literally formulated to thousands of various products (Hill, 2003).

OPs are of intense environmental concern since very long due to their poor biodegradability and hydrolyzation. The concern is elevated by the efficient absorbance, and rapid redistribution and disposal of the organophosphorus compounds to various organs of the exposed organisms, interfering membranes' dependent processes such as nerve conductance, plasma membrane, and enzymatic activities (Karaoz et al., 2002). OPs induce oxidative damage due to higher ROS (reactive

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oxygen species) production that can lead to damage to proteins, lipids, and DNA, loss of enzymatic activities and their structural integrity, and ultimately activate inflammatory processes (Ozyurt et al., 2004).

Pesticides lead to water bodies from different sources including runoffs from agricultural fields, industrial effluents, and domestic wastes. These pesticides directly affect the aquatic flora and fauna by leading to different toxicological effects/endpoints and indirectly affect human health upon the use and consumption of the water or the edibles from these water bodies such as fish that bio-accumulated these pesticides (Cerejeira et al., 2003). Aquatic organisms are widely employed for evaluating the toxic effects of different chemicals because they are exposed to chemicals either directly (run-offs) or indirectly (trophic chains). Among the non-target organisms, fish is extensively employed for chemicals toxicity testing and risk assessment studies on account of its wide consumption and worth, both economically and nutritionally (Ullah, 2015). The cellular and biochemical responses of fish are widely assessed for investigating the potential risks associated with the aquatic pollutants (Lakra and Nagpure, 2009). Fish is prioritized in ecotoxicological studies due to their higher sensitivity to the environmental contaminants, even at a very low concentration (Ullah and Zorriehzahra, 2015). Different organs of fish are used for evaluating the biochemical and physiological toxic effects/endpoints of chemicals in risk assessment studies including gills, brain, blood peripheral erythrocytes, and liver (Ullah et al., 2016a; Ullah et al., 2016b). Among these organs, the liver holds the prime position for being the direct target of the pollutants, detoxification center, and has an enormous potential of clearly indicating the pathological and physiological alterations, even at minute scale and in response to the tiny concentration of the pollutants. The biochemical parameters are assessed as health indicators for detecting the functional and structural status of fish under stress (Pimpão et al., 2007; Suvetha et al., 2010).

OPs are in use since very long, such as Malathion is in use since the 1950s, therefore these pesticides are present in almost every water body and ecosystems. These are uptaken by aquatic organisms from the ambient water, sediments, food, and particulate matters suspended in the water column (Patil and David, 2009). Therefore, the modern toxicology and current knowledge are mostly based on toxicological studies involving aquatic organisms. These organisms are employed as model systems for assessing the basic processes contributing to cellular damage, tissue injury, free radicals protection, and physiological alterations including aging, different diseases/disorders, and genotoxic effects (Volodymyr, 2011).

Malathion is a broad spectrum nonsystemic organophosphate acaricide/insecticide. It is one of the earliest and widely used insecticides for agricultural and non-agricultural purposes. Malathion is allowed to be used directly to water bodies for controlling larval mosquitoes. Over 8 lac kg Malathion is used on cotton crop in the US alone (NAAS, 2008). Malathion was introduced in the 1950s. It can result in some serious troubles in the exposed organisms, more specifically in the aquatic ones. Malathion is classified as highly toxic to very highly toxic to some aquatic vertebrates including fish. It is better known for causing severe disturbances in metabolism, nerve poisoning (blocking of active sites of acetylcholinesterase enzyme leading to tremors, convulsion, and ultimately death), protein metabolism, neural physiology, histology, biochemical aspects, and genotoxicity in non-targeted species including freshwater mussels and fish (Patil and David, 2009; U.S. EPA, 2012; Ullah et al., 2016c). The concentration of Malathion reported from realistic environment varies in different ranges such as 0.008–0.012 μg/L (rivers joining the Chesapeake Bay), up to 0.16 μg/L (urban streams), and up to 15 μg/L (Colorado wetland) (Fordham et al., 2001; Webb and Crain, 2006). Some studies reported severely threatening and very higher concentrations of Malathion (8.12 μg/L to 105.2 μg/L) in water samples from various sites (Karmakar et al., 2016).

Owing to the hostile consequences of Malathion in different model organisms, a plethora of well-documented literature regarding the toxic effects of Malathion is available online. Exposure to Malathion led to various toxic effects in different fish species such as, behavioral alteration and respiratory dysfunction in *Labeo rohita* (Patil and David, 2008), altered acetylcholinesterase activities in *Ctenopharyngodon idella* (El Ella, 2008), histochemical and histological alterations in ovary, brain, and liver of *Ophiocephalus punctatus* (Pugazhvendan et al., 2009), histopathological alterations in kidney, ovary, and liver of *Heteropneustes fossilis* (Deka and Mahanta, 2012), changes in the immune response, hematological profile, and oxidants/antioxidants status in *Cyprinus carpio* (Mişe Yonar et al., 2014), altered behavior and hematological parameters in *Capoeta damascina* (Shahbazi et al., 2015), hematological changes and morphological alterations in the gills of *Cyprinus carpio* (Sharmin et al., 2016), and DNA damage in the gills of *Labeo rohita* (Ullah et al., 2016a).

Similarly, exposure to Malathion led to different toxic effects in other model organisms including frog (Fordham et al., 2001), earthworms (Espinoza-Navarro and Bustos-Obregón, 2005), Japenese quails (Nain et al., 2011), rats (Lasram et al., 2008, 2014; Moore et al., 2011; Ojha and Srivastava, 2014; Geng et al., 2015), and mice (Penna-Videau et al., 2012; Slimen et al., 2014; Mamun et al., 2015). Malathion induced hepatotoxicity (Kalender et al., 2010), oxidative stress (Alp et al., 2011), and metabolic disorders (Lasram et al., 2009) in rats are well studied and documented, however, comprehensive studies regarding the aforementioned effects of Malathion in fish is scanty.

The toxicity mechanism is a complex consequence of biochemical events, initiated between a biological target and a chemical based on their interaction, which culminates in explicit toxicity expression and different toxicological effects/endpoints. In most of the cases, the initial interaction of the cellular target with the chemical is vital but is not sufficient for eliciting the sings and characteristics of the toxicity, warranting exposure concerns to that chemical. In case of the OPs, the primary interaction between the macromolecular targets and the pesticides leads to a number of events, that results in various possible toxic effects, sequels, and cholinergic signs (Pope, 1999). That's why, various parameters were included in the current study to get a clear spectrum of the consequences of Malathion mediated toxicity in the liver of rohu, such as different biochemical indices including protein contents, lipid peroxidation, protein metabolic enzymes, antioxidant enzymes, DNA, ROS, and gross anatomical, morphological, and histopathological alterations were investigated.

Rohu, *Labeo rohita* (Hamilton, 1822), is a freshwater fish species, widely distributed in Asian countries, more specifically in India, Pakistan, Sri Lanka, Bangladesh, Myanmar, and Nepal. Rohu is a bottom feeding vegetarian, but young fry consumes zooplankton. Rohu is found in tropical and temperate regions, breed in running water around June-July, having 52 diploi chromosomes, fecundity varies from 226,000 to 2,794,000 (depend on the length and weight of ovary), having ossified endoskeleton, and is employed as a model fish/type specimen of bony fish on account of its wide consumption. Detailed description about rohu can be read/retrieved at Biology discussion forum (Tanika, 2018).

The liver was selected for the study, as it is one of the main organs, performing a wide variety of body functions such as maintaining the internal environment, the flow of nutrients, controlling the metabolism of fats, proteins, and carbohydrates, detoxification etc. Moreover, the liver was selected as the target organ for evaluating Malathion induced toxic effects on account of being a center for lipid and glucose homeostasis, and enzymes production (Lasram et al., 2015). Currently, there is no reimbursing way to the absence of liver's functions; however, research regarding the hepatotoxic effects of commercial grade Malathion at an acute concentration on major carps is still scanty. Therefore, the current study was aimed to investigate the acute hepatotoxic effects of Malathion in the widely consumed major carp, rohu (*Labeo rohita*). The current study will broaden the ideal toxicity markers used to characterize the development of oxidative stress that leads to various toxic effects in the liver of the exposed organisms because of involving/exploring different parameters/indices.

2. Materials and methods

2.1. Fish Handling and Acclimatization

Healthy and uniform sized (8.8 ± 1.21 cm length and 6.9 ± 1.32 g weight) fingerlings (90 days of age) were purchased from a local hatchery, transported to the laboratory through closed-system live hauling method, and were distributed in fiberglass tanks after careful conditioning through water mixing from polyethylene bags to avoid any damage to the fish. The fish were acclimatized for fifteen days before starting the experiment and were fed 35% basal protein diet (small dried pellets) at the rate of 5% body weight, two times a day (Table S1). The aquaria were fit with aerating stones. During the experiment, water was exchanged on daily basis, with sustaining a 12:12 h of photoperiod. The feed remains and wastes were siphoned off, on a daily basis, to avoid stress to the fish.

The water quality parameters including DO, pH, ammonia, hardness, and temperature were assessed using DO meter, pH meter, and water quality checker meter (Horiba U10) on a daily basis. An attempt was made to keep the physico-chemical parameters within the optimum range by removing the dead fish and wastes instantly to avoid any kind of deterioration of the water quality. The recorded physico-chemical parameters of the water were in varying ranges such as temperature ranged between 24.4 ℃ and 25.9 ℃, dissolved oxygen 6.2–7.5 mg/L, pH 6.7–7.5, ammonia < 0.25 ppm, total hardness 162–179 mg/L, and conductivity 240–290 μS/cm.

2.2. Experimental design

The current experiment was conducted in triplicate and semi-static closed system. Even sized healthy and active fish were distributed into 6 aquaria (1.5 g/L stocking density), three aquaria serving as a control group and three as a treated group. After 24, 48, 72, and 96 h, 9 fish for biochemical (liver from 3 fish were pooled as a single sample) and 6 fish for histopathological examination were captured from each aquarium before changing the water and restoring the Malathion concentration. The captured fish were anesthetized using MS222 (60 mg/L). The liver was dissected out on the ice box and stored at − 20 ℃ for further biochemical analysis and observations, including the change in protein content/level, lipid peroxidation, the activities of different enzymes including Aspartate Aminotransferase (AAT), Glutamate Dehydrogenase (GDH), Alanine Aminotransferase (AlAT), Lactate Dehydrogenase (LDH), Catalase (CAT), Superoxide Dismutase (SOD), Peroxidase (POD), Glutathione (GSH), Glutathione Reductase (GR), Glutathione-s-transferase (GST), Glutathione Peroxidase (GSH-Px), Deoxyribonucleic Acid (DNA), and ROS. For Histopathological studies, the liver was weighed and transferred to NBF (neutral buffered formalin).

2.3. Test chemical

Commercial grade Malathion (50% EC) was procured from the local market (Table S2) for the study because it is the most widely employed grade in field practices (Pandey et al., 2005). The stock solution of Malathion (commercial formulation) was procured in Acetone, of which the required dilution amount was used in the study. The fish in the control received acetone (0.005%) of the same volume used for Malathion exposure in treatment group. No toxic effects of acetone were observed.

2.4. Lethal concentration (LC_{50}) determination

The lethal concentration (LC_{50}) of Malathion against rohu was estimated through Probit analysis using the semi-static method. A total of ten fish per aquarium (20 L water) were exposed to different concentrations of Malathion. The fish exposed to different Malathion concentrations were observed keenly, mortality was noted, and dead fish were removed instantly to avoid water contamination. The LC_{50} for Malathion for 96 h was calculated using Probit method (CI of 95%) by following Finney (1971), which is recommended as a standard procedure for pesticides toxicity evaluation by OECD (1996).

Table 1
Determination of lethal concentration (LC_{50}) of Malathion against *L. rohita* for 96 h.

S. No	Conc. (μg/L)	Log Conc.	Fish exposed	Mortality (%)	Probit's mortality value (95% CI)
1	2.0	0.477	10	0	0.00
2	3.0	0.544	10	10	3.72
3	4.0	0.602	10	30	4.48
4	5.0	0.653	10	50	5.00
5	6.0	0.699	10	70	5.52
6	7.0	0.740	10	90	6.28
7	8.0	0.778	10	100	8.09

Increase in Malathion concentration led to increased mortality of rohu (Table 1). A concentration of 5 μg/L was observed to be LC_{50} of Malathion to rohu by causing 50% mortality, tested through Probit Analysis (Ullah et al., 2016c). The percent mortality was plotted against log concentration and the Probit mortality was plotted against log concentration, both of which showed log concentration 0.65 (5 μg/L) to be LC_{50} (Fig. S1).

2.5. Biochemical analysis

Due to the small size of the liver, samples from three fish were pooled together and a total of nine specimens forming three triplicates from the same aquarium were employed for the biochemical studies. Standard protocols, as followed by Ullah et al. (2016a), were followed.

Level of the reactive oxygen species (ROS) was assessed by following the procedures of Contreras et al. (2005) through DCF-DA (2′,7′-dichlorofluorescein diacetate). The pooled samples of the liver (0.1–1 g wet wt.) were incubated in 10 μM DCF-DA (100 ml) in a water bath (37 ℃) for 30 min in methanol. The DCF fluorescence was observed using spectrofluorometer at 488 nm and 525 nm excitation and emission wavelengths, respectively. The standard curve (0–500 nM DCF) was used to obtain fluorescence values.

The total protein contents were analyzed by following Lowry et al. (1951). A total of 90 mg liver was taken out, homogenized (phosphate buffer), and were centrifuged for 20 min at 10,000 rpm at 4 ℃. The stock solution (1 mg/ml) of the standard BSA (bovine serum albumin) was prepared by dissolving BSA (15 mg) in distilled water (15 ml). A solution (No. 1) was prepared by mixing 2% Na_2CO_3 (50 ml) and 0.1 N NaOH (50 ml) solution. Another solution (No. 2) was prepared by mixing 1.56% CuSO4 (10 ml) solution with 2.37% Sodium Potassium Tartrate (10 ml) solution. Folin-Ciocalteau reagent was prepared by mixing 2 ml commercial reagent with water (equal amount). An alkaline solution was procured after mixing Solution No. 2 (2 ml) with Solution No. 1 (100 ml). After that, the sample was thawed, and 0.1 ml sample was mixed with 1 ml alkaline solution, incubated (10 min), then 1:1 Folin-Ciocalteau phenol reagent was added into each tube, vortexed for proper mixing, and after 30 min incubation, the optical density was observed (595 nm) through spectrophotometer. The concentration of protein was calculated using BSA standard curve.

Lipid Peroxidation (LPO, μmol/ min/ mg protein) level was assayed by following Wright et al. (1981). For preparing reaction mixture (1 ml), 0.58 ml phosphate buffer (pH 7.4, 0.1 M), 0.2 ml ascorbic acid (100 mM), and 0.02 ml ferric chloride (100 mM) were mixed with 0.2 ml supernatant. The prepared solution was incubated at 37 ℃ in a water bath for an hour, and 10% trichloroacetic acid (1 ml) was added to stop the reaction. All the tubes were then boiled for 20 min in a water bath after addition of thiobarbituric acid (1 ml), then cooled via ice

bath, and were then centrifuged for 10 min at 2500 × *g*. The change in absorbance of the solution was recorded (535 nm) after one min using a spectrophotometer.

Catalase (CAT, μmol/ min/ mg protein), and Peroxidase (POD, μmol/ min/ mg protein) were assayed by following Chance and Maehly (1955). For evaluating the CAT activity (briefly), 50 mM phosphate buffer (2.5 ml, pH 5), and 5.9 mM H_2O_2 (0.4 ml) were mixed with 0.1 ml enzyme extract. For POD activity evaluation (briefly), 50 mM phosphate buffer (2.5 ml, pH 5), 40 mM H_2O_2 (0.3 ml), and 20 mM guaiacol (0.1 ml) were mixed with 0.1 ml enzyme extract for making reaction solution. The change in the absorbance of the reaction solution was recorded after one min using spectrophotometer (CAT at 240 nm, and POD at 470 nm)

Superoxide Dismutase (SOD, μmol/ min/ mg protein) was assayed by following Kakkar et al. (1984) with some modification. The reaction mixture was prepared by mixing 0.1 ml phenazine methosulphate (186 μM), 1.2 ml sodium pyrophosphate buffer (0.052 mM, pH 7), and supernatant (0.3 m). NADH (0.2 ml, 780 μM) was added to initiate enzyme reaction and was stopped by addition of glacial acetic acid (1 ml) after one min. Chromogen amount formed was measured via color intensity recording using spectrophotometer (560 nm).

Glutathione Reductase (GR, μmol NADPH oxidized/min/mg protein) was assayed by following Carlberg and Mannervik (1975). Reaction solution was prepared by mixing 0.1 M phosphate buffer (1.65 ml, pH 7.6), 0.5 mM EDTA (0.1 ml), 0.1 mM NADPH (0.1 ml), 1 mM oxidized glutathione (0.05 ml), and supernatant (0.1 ml). The absorbance change was recorded after one min using spectrophotometer (340 nm).

Glutathione Peroxidase (GSH-Px, nmol of GSH oxidized/ min/ mg protein) was assayed by following Mohandas et al. (1984). The reaction solution was procured after mixing 0.1 M phosphate buffer (1.49 ml, pH 7.4), 1 mM sodium azide (0.1 ml), glutathione reductase (1 IU/ml, 0.05 ml), 1 mM GSH (0.05 ml), 1 mM EDTA (0.1 ml), 0.2 mM NADPH (0.1 ml), 0.25 mM H2O2 (0.01 ml), and 10% homogenate (0.1 ml) in a total volume of 2.0 ml. NADPH disappearance was recorded at 25 °C (340 nm).

Glutathione-s-transferase (GST, μmol of chloro-2,4-dinitrobenzyne conjugated formed/min/mg protein) was assayed by following Habig et al. (1974). The reaction solution was prepared by mixing 0.1 M phosphate buffer (1.475 ml, pH 6.5), 1 mM 2,4-Dinitrochlorobenzene (CDNB, 0.025 ml), 1 mM reduced glutathione (0.2 ml), and 10% homogenate (0.3 ml) in a total volume of 2.0 ml. The change in the absorbance was recorded (340 nm).

Glutathione content (GSH, μmol/ g tissue) was assayed by following Jollow et al. (1974). The mixture was prepared by mixing 0.1 M phosphate buffer (1.475 ml, pH 6.5), 1 mM CDNB (0.025 ml), 1 mM reduced glutathione (0.2 ml), and 10% homogenate (3 ml) in a total volume 2.0 ml. The change in absorbance was recorded (340 nm).

The activities of Aspartate Aminotransferase (AAT, mg protein/ h) and Alanine Aminotransferase (AlAT, mg protein/ h) were assayed by following Bergmeyer (1965). For AAT, briefly, homogenate of the liver (2% w/v) was prepared in ice-cold sucrose (0.25 M) solution, centrifuged at 1000 × *g* (15 min) and the collected supernatant was used for assaying enzyme activity. The incubation mixture (2.0 ml) was prepared by mixing phosphate buffer (Na2HPO4 + NaH2PO4, 100 μ moles, pH 7.4), L-aspartic acid (100 μ moles), α-ketoglutarate (2 μ moles), and the supernatant (0.5 ml) as the enzyme source. It was incubated for 30 min (37 °C), and the reaction was stopped by adding ketone reagent (1 ml, 0.001 M, 2,4-dinitrophenyl hydrazine solution in 1 N HCl). The contents were kept at lab temperature (20 min), and then NaOH (10 ml, 0.4 N) was added to it. The color developed was read using spectrophotometer (545 nm) against the blank reagent.

For AlAT, briefly, the incubation mixture (2 ml) was prepared by mixing Dl-alanine (100 μ moles), phosphate buffer (100 μ moles, pH 7.4), α-ketoglutarate (2 μ moles), and the supernatant of the homogenate (0.5 ml – 2% w/v prepared in ice-cold sucrose (0.25 M) solution) as enzyme source. The reaction solution was incubated for 30 min (37 °C), and the reaction was stopped by adding 2,4-dinitrophenyl hydrazine solution (1 ml – prepared in ketone reagent (1 N HCl)). The color developed after NaOH addition, as mentioned for AAT earlier. The optical density was recorded using spectrophotometer against a reagent blank (545 nm).

Glutamate Dehydrogenase (GDH, mg protein/ h) was assayed by following Lee and Hardy (1965). The solution mixture (2 ml volume) was prepared by mixing 100 μmol phosphate buffer (pH 7.4), 75 μmol sodium glutamate, 2 μmol INT (2,4-iodophenyl-3-(4-nitrophenyl)-5-phenyltetrazolium chloride), 0.1 μmol NAD, and 0.5 ml enzyme source (prepared in 0.25 M sucrose solution). The mixture (2 ml) was incubated (30 min) and then the enzyme activity was measured using spectrophotometer (340 nm).

Lactate Dehydrogenase (LDH, mg protein/h) was assayed by following Franciscato et al. (2011). The mixture solution was prepared by mixing 200 mmol/L buffer (pH 8.2), 260 mmol/L lactic acid, 7.7 mmol/L sodium azide, and plasma (25 ml), and was incubated for 2 min (37 °C). Then the color (1.2 mmol/L NAD +, 0.64 mmol/L INT, 0.26 mmol/L phenazine, and 1.23 mmol/L sodium azide) reactive was added. The mixture was incubated at 37 °C (another 5 min), and the reaction was stopped by the addition of 200 mmol/L HCl. The tubes were kept at room temperature (5 min) and the absorbance was recorded (500 nm).

2.6. Alkaline Single Cell Gel Electrophoresis (SCGE) assay/comet assay

The standard protocol of Singh et al. (1988), with minor modification (Klaude et al., 1996), as performed by Pandey et al. (2011) was adopted for evaluating DNA damage induced by Malathion in the liver cells of rohu at acute concentration. The comet assay was performed by following different steps, such as: preparing microscopic slides having cells in agarose; membrane lysis for releasing DNA; forming single strand DNA via contact (pH 13); electrophoresis; DNA staining; followed by procuring images from the slides through a fluorescent microscope and then comet scoring.

Cell suspension (15 μL), mixed with LMPA (low melting point agarose, 0.5%, 85 μL), and layered on frosted slides (coated with 1% normal agarose (200 μL)), were again layered with 100 μL LMPA after gel solidification, and were kept submerged in lysing solution at 4 °C (overnight). The slides (side by side positioned in gel electrophoresis (horizontal units)), dipped in fresh alkaline buffer (cold) were left for 20 min (4 °C) in the same solution to unwind DNA and single-strand breaks formation (from alkali-labile sites). Then the alkaline electrophoresis (300 mA, 15 V(0.8 v/cm)) was carried out in the same alkaline buffer at 4 °C (20 min). The slides were neutralized gently in tris buffer (0.4 M, pH 7.5).

The procured images from the slides, mounted on epifluorescent microscope (400 ×, Nikon AFX-1, Optihot) and stained with Acridine orange, were scored (100 random selected cells, 50 for each replicated slide) using Comet Assay Software Project (CASP) Lab (Version 1.2.3 beta 2) for DNA damage quantifying parameters: Tail length (TL, μm), Tail Moment (TM), Percentage of Tail DNA (%TDNA, %), and Olive Tail Moment (OTM).

2.7. Histomorphological examination

A total of 6 fish from each group were selected and their morphometric and anatomical (total body length, weight, standard length, and body depth) parameters were recorded. After dissecting the fish, the liver samples were collected and weighed. Then, the tissues were transferred to NBF. The tissues were preserved and slides were prepared by following Rosety et al. (2005). The slides, stained with eosin and hematoxylin, mounted with Canada balsam, having coverslips were kept overnight in an incubator. Xylene was used to remove extra Canada balsam of the slides. The slides for both control and treated groups were studied and photomicrographs were procured under digital

camera fit light microscope (OPTIKA B-350).

2.8. Statistical analysis

The results (Mean ± S.E.) were analyzed by ANOVA (analysis of variance), followed by multiple variance analysis through least significant difference (LSD) in Statistix (V 8.1), considering P-value < 0.05 as statistically significant.

3. Results and discussion

3.1. Toxicity evaluation

The present study revealed 5 µg/L Malathion to be LC_{50} against rohu, which is very much lower than 15 mg/L, previously reported by Thenmozhi et al. (2011) and a bit higher than 9.0 µL/L, reported by Patil and David (2009). Change in LC_{50} of the same chemical against different or same species, or the LC_{50} of different chemicals against same or different fish species is due to the specification, formulation, and stereochemistry of the pesticides or their active molecules (Ullah, 2015). The isomers of the pesticides may vary, which make their precise toxicity to be different. Most of the time research is conducted to explore and compare the toxicity of different pesticides. The pesticides having single isomer are more toxic than the ones having different isomers combination in their formulation (Ullah, 2015).

The toxicity of pesticides is also associated with the carriers of the contaminants as well as inert or active ingredients (Ullah et al., 2014). For fish, toxicity also depends on temperature, health, size, weight, and age of the fish (Farah et al., 2004). The temperature was ranging from 24.4 °C to 25.9 °C, and no association between LC_{50} and temperature was observed. However, Kumaraguru and Beamish (1981) observed an inverse correlation between LC_{50} and temperature. Moreover, research revealed different pesticides to be more toxic in winter as compared to summer, such as Singh et al. (2010) observed tenfold difference in the LC_{50} value at 10 °C, 15 °C, and 20 °C for 96 h. According to WHO (1992), there does exist an inverse relationship between body weight and pesticide toxicity.

3.2. Malathion induced ROS generation, oxidative stress, and toxic effects on protein metabolic and antioxidant enzymes

The intracellular ROS was estimated through DCF-DA (non-fluorescent stain), that was converted into florescent DCF by cellular oxidants via oxidation. A linear increase was observed in the level of ROS in the liver of rohu after exposure to Malathion in a time-dependent manner, as compared to the fish in control group (Fig. S2). The generation and increase of ROS level demonstrated Malathion induced oxidative stress, which was confirmed by a linear increase in the activities of ROS scavenging enzymes to avoid or reduce ROS induced/mediated Hepato-toxicity and cellular damage.

Malathion exposure led to a significant ($P < 0.05$) reduction in the total protein contents whereas an increase was observed in the activity of LPO (Table 2), which clearly demonstrated the oxidative stress induced by Malathion in rohu. A significant increase ($P < 0.05$) was observed in the activities of AAT, AlAT, GDH, and LDH in the treated groups (Table 3). Malathion exposure also led to a statistically significant increase ($P < 0.05$) in the activities of CAT, SOD, POD, GSH, GR, GST, and GSH-Px (Table 4).

A change in the protein contents in response to pollutants is a key parameter, indicating the susceptibility of the organ by altering their functions. The change in protein contents demonstrated the intoxication induced by Malathion, which disturbed the normal cells functioning and consequently altered the basic biochemical mechanisms in fish (Thenmozhi et al., 2011). The reduction in protein contents showed the severity of the stress induced by Malathion in the liver of rohu. The results were in congruence with the results obtained for *Oncorhynchus mykiss* (Velisek et al., 2008), *Tor putitora* (Ullah et al., 2014), and *Labeo rohita* (Ullah, 2015; Ullah et al., 2016a) after exposure to different pesticides.

Table 2
Effect of Malathion exposure on protein contents (mg/g) and lipid peroxidation (µmol/ min/ mg protein) in the liver of rohu. Data are represented as Mean ± SE (n = 9). Means followed by different letters are significantly different ($P < 0.05$). (ANOVA followed by LSD test).

Time (h)	Protein Content		LPO	
	Control	Treated	Control	Treated
24	16.52 ± 0.38^{e}	13.26 ± 0.61^{a}	12.67 ± 1.63^{e}	22.75 ± 1.74^{d}
48	16.24 ± 0.59^{e}	11.05 ± 0.52^{b}	12.35 ± 1.35^{e}	31.94 ± 2.31^{c}
72	16.78 ± 0.48^{e}	8.63 ± 0.34^{c}	13.01 ± 1.88^{e}	44.15 ± 3.31^{b}
96	16.45 ± 0.34^{e}	6.47 ± 0.17^{d}	13.23 ± 1.49^{e}	61.17 ± 3.16^{a}

LPO is considered as the highly derived deleterious attacks by free hydroxide radical (itself produced from H_2O_2 through Fenton reaction (Ullah, 2015)), resultant of oxidative damage to tissues or organs (Pandey et al., 2008; Dabas et al., 2014). The elevated LPO in the current study reflected that Malathion caused lipid Peroxidation due to the excessive production of free radicals, subsequently leading to oxidative stress that ultimately induced molecular and clastogenic damage (Raisuddin and Jha, 2004; Jha, 2008).

Oxidative stress is one of the potential reasons, leading to tissues injury. It is commonly believed that oxidative stress induces toxic effects (Kan et al., 2012). Reactive oxygen species (ROS) damages membranes at increased levels as observed in the current study, which makes them leaky and ultimately leads to necrosis and cell death or apoptosis. Pesticides induce oxidative stress in fish through different mechanisms. However, the oxidative stress induced is monitored through ROS-induced modifications of cellular constituent including modified proteins, lipids, DNA, and high or low molecular mass antioxidants (Lushchak, 2011).

LPO involves the direct reaction of lipids and oxygen, for forming free intermediate radicals and producing semi-stable peroxides. The mechanism of pathological free radical leads to lipid peroxidation along with phospholipids degradation and loss of integrity of the membrane, an important factor of toxicity induction (Afaq, 2010). The increase in LPO activity indicated damage to lipids. The results of the current study is in correspondence with the result of the earlier studies on different fish species exposed to different pesticides including cypermethrin (Ullah et al., 2014; Ullah, 2015), endosulfan (Ullah et al., 2016b), roundup (Lushchak et al., 2009), tattoo (Kubrak et al., 2012), and sencor (Maksymiv et al., 2015).

The activities of AAT, GDH, AlAT, and LDH are extensively assessed for evaluating the toxicological effects of chemicals. The marked increase in the activities of protein metabolic enzymes might be due to the hepatic injury induced by Malathion exposure. Being lipophilic in nature, Malathion got accumulated on biological (hydrophilic lipid bilayer) membranes generating free radicals that led to an increase in LPO and consequently in hepatic damage. Our results are in congruence with the results of Dubey (2012), Aita (2014), and Ullah (2015).

Aminotransferases are very sensitive to pollutants, hence considered as one of the best indicators for monitoring and assessing the necrosis and cell inflammation in the liver. In the current study, the activities of AAT and AlAT increased which might be associated with the higher release of the enzymes due to the injury to hepatocytes and impaired function of the liver cells. Our results are in congruence with Han et al. (2013), Ullah (2015), and Maksymiv et al. (2015). Other studies on various animals also reported the same findings (Chiali et al., 2013). The results from this section clearly showed that Malathion induced stress in rohu, which led to the injury of the solid tissues, among which the liver might have been the first that got affected.

LDH is assessed in toxicological studies because of being a terminal

Table 3
Effect of Malathion exposure on the activities of protein metabolic enzymes (mg protein/ h) in the liver of rohu. Data are represented as Mean ± SE (n = 9). Means followed by different letters are significantly different ($P < 0.05$). (ANOVA followed by LSD test).

Enzymes	Control	Treated			
		24 h	48 h	72 h	96 h
AAT	1.97 ± 0.09^{e}	2.26 ± 0.16^{d}	2.51 ± 0.21^{c}	2.83 ± 0.32^{b}	3.12 ± 0.27^{a}
AlAT	4.71 ± 0.34^{e}	5.86 ± 0.29^{d}	6.51 ± 0.47^{c}	7.84 ± 0.71^{b}	8.66 ± 0.12^{a}
GDH	0.26 ± 0.006^{e}	0.32 ± 0.003^{d}	0.41 ± 0.001^{c}	0.48 ± 0.002^{b}	0.53 ± 0.001^{a}
LDH	0.21 ± 0.003^{e}	0.36 ± 0.002^{d}	0.52 ± 0.001^{c}	0.67 ± 0.001^{b}	0.81 ± 0.007^{a}

enzyme from glycolysis. It is a very useful biomarker for depicting the metabolic process intensity and cellular integrity during stress condition (Monteiro et al., 2007). Being a very abundant enzyme, LDH leaks out of the cells substantially and are often evaluated for finding the integrity of the cells under stress (Maksymiv et al., 2015). Our result of elevation in LDH activity was in congruence with the results of Maksymiv et al. (2015) and Ullah (2015), but Velisek et al. (2009) observed a decrease in the activity of LDH in *Cyprinus carpio* after metribuzin treatment, while some researchers reported no change in LDH activity after long-term exposure to simazine (Oropesa et al., 2009; Velisek et al., 2012).

The increase in the activities of antioxidant enzymes indicated an attempt of the fish to remove oxyradicals. The increased level of these enzymes, such as increased level of GST, detoxify and eliminate toxic xenobiotics and ROS (Li, 2009). A very similar result has been reported by Kabila et al. (1999) in *Macrobrachium malcolmsonii* after heavy metals exposure, Ullah et al. (2014) in *Tor putitora* and Ullah (2015) in *Labeo rohita* after exposure to Cypermethrin, and Ullah et al. (2016a) in *Labeo rohita* after exposure to endosulfan.

Living cells have the defense system of the antioxidant enzymes to neutralize ROS. The system consists of different enzymes such as SOD, GR, GSH-Px, GST etc., forming a high molecular mass antioxidant group, which acts on low molecular mass antioxidants under rest condition to maintain a steady ROS state/level. Under stress, antioxidant enzymes help the cell to cope with the elevated ROS level, which results from an imbalance between ROS elimination and generation (Lushchak, 2011; Sies, 2014). During the current study, the increase in ROS level was due to its higher production as compared to elimination, and that's why a simultaneous elevation in the activities of antioxidant enzymes was observed.

The SOD activity increased time-dependently, which can be correlated with the production of higher concentration of superoxide anions. SOD catalyzes the conversion of superoxide anion radicals to H_2O_2 and molecular oxygen, thus protecting the cell against oxidative damage induced by superoxides (Vutukuru et al., 2006). An increase was observed in the activity of CAT because it is responsible for scavenging H_2O_2 primarily. So, the CAT activity might have increased on account of increase in the production of free radicals. CAT protects the biological system against ROS by catalyzing H_2O_2 conversion to molecular oxygen and water (Romeo et al., 2000). The CAT activity got increased in the current study, indicating an increase in the scavenging capability of the fish against H_2O_2. The activity of GSH-Px also increased, because it is also responsible for defending tissues against H_2O_2. It might also be linked with the effective GSH-Px response on account of the higher renovation of the epithelium (Dabas et al., 2014).

GR plays a significant role in the cellular antioxidant protection. It catalyzes the oxidized glutathione conversion back to reduced state (Lushchak, 2012). On its prominent role as an antioxidant protecting component, it is used as a potential biomarker in animals under oxidative stress development (Cazenave et al., 2006). In the current study, the activity of GR increased which is in positive correlation with the increase in GSH. The results obtained are in congruence with previous studies on *Channa punctatus* exposed to atrazine (Nwani et al., 2010), *Tor putitora* exposed to Cypermethrin (Ullah et al., 2014), *Labeo rohita* exposed to Cypermethrin (Ullah, 2015) and endosulfan (Ullah et al., 2016a) while other reported decrease in the activity of GR in *Cyprinus carpio* in response to terbutryn (Velisek et al., 2011) and in *Carassius auratus* in response to sencor (Maksymiv et al., 2015). Elia et al. (2002) and Stara et al. (2012) reported no change in the activity of GR in *Lepomis macrochirus* and *Cyprinus carpio* in response to atrazine and simazine respectively.

GSH has a central role in modulating oxidative stress induced LPO, working as a reducing substrate in oxidative reactions (Stohs et al., 2000). Malathion exposure increased the GSH content in the hepatocytes of rohu. According to some researchers, GSH provides secondary protection against induced oxidative stress by sustaining a reduced state of the cell (Tort et al., 1996). The time-dependent increase in the GSH in the current study might be the primary protective response of rohu against Malathion mediated time-dependent increase in oxidative stress.

GSH-Px and GST are GSH dependent enzymes. Both play a key role in providing protection to the tissues/organs against oxidative stress (Hayes and Strange, 1995). GSH-Px protects the cells against oxidative stress optimally by scavenging ROOH or H_2O_2 and is considered as complementary to CAT (Halliwell and Gutteridge, 1999). GST catalyzes the conjugates of xenobiotics to tripeptide glutathione (Hermes-Lima,

Table 4
Effects of Malathion exposure on the activities of antioxidant enzymes (GSH-Px (nmol of GSH oxidized/min/mg protein), GSH (μmol/g tissue), POD (μmol/min/mg protein), SOD (μmol/min/mg protein), CAT (μmol/min/mg protein), GR (μmol/min/mg protein), GST(μmol of chloro-2,4-dinitrobenzyne conjugated formed/min/mg protein)) in the liver of rohu. Data are represented as Mean ± SE (n = 9). Means followed by different letters are significantly different ($P < 0.05$). (ANOVA followed by LSD test).

Enzymes	Control	Treated			
		24 h	48 h	72 h	96 h
GSH-Px	1.96 ± 0.14^{e}	2.37 ± 0.35^{d}	3.21 ± 0.29^{c}	3.96 ± 0.31^{b}	4.31 ± 0.47^{a}
GSH	1.07 ± 0.007^{e}	1.87 ± 0.16^{d}	2.66 ± 0.22^{c}	3.41 ± 0.17^{b}	3.97 ± 0.11^{a}
POD	49.17 ± 5.25^{e}	67.17 ± 8.18^{d}	83.23 ± 5.24^{c}	107.12 ± 4.34^{b}	133.88 ± 9.65^{a}
SOD	143.25 ± 6.14^{e}	162.24 ± 4.56^{d}	176.44 ± 4.32^{c}	196.23 ± 3.56^{b}	219.42 ± 5.23^{a}
CAT	243.23 ± 8.64^{e}	268.93 ± 6.22^{d}	284.24 ± 8.87^{c}	304.89 ± 5.82^{b}	325.43 ± 9.65^{a}
GR	1227.21 ± 14.33^{e}	1553.27 ± 12.56^{d}	1936.51 ± 9.73^{c}	2271.23 ± 8.67^{b}	2718.21 ± 23.74^{a}
GST	1583.25 ± 17.49^{e}	2201.74 ± 18.83^{d}	2747.63 ± 21.74^{c}	3565.21 ± 23.32^{b}	4107.29 ± 29.17^{a}

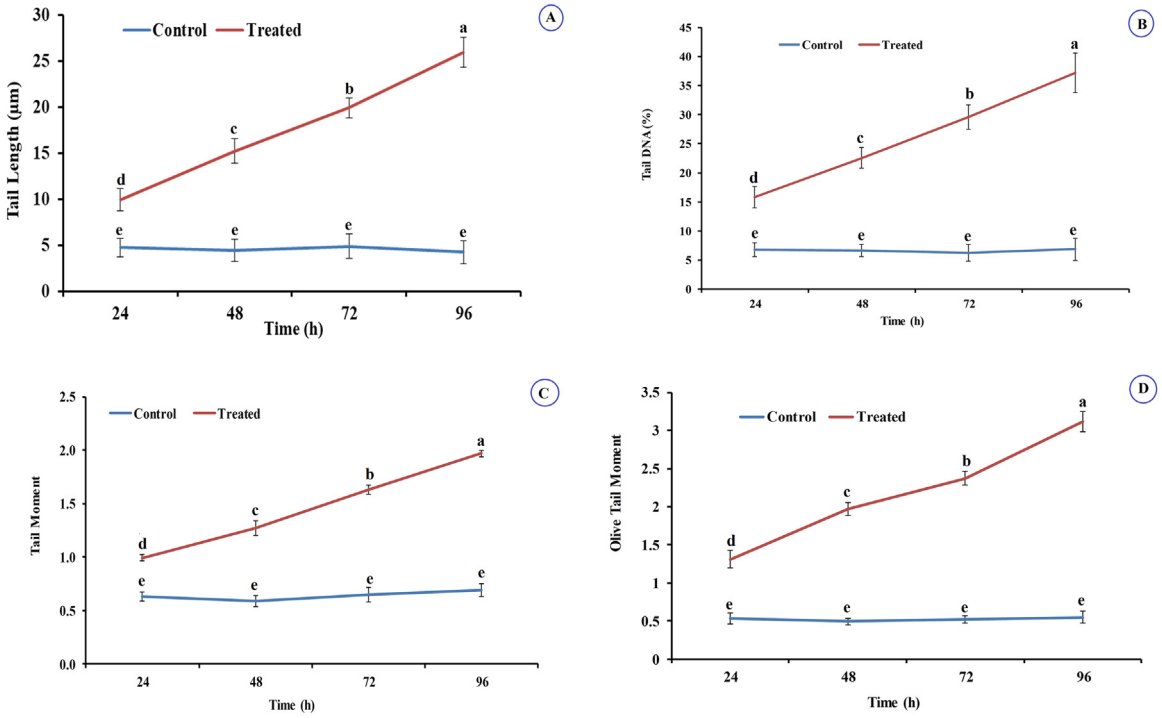

Fig. 1. DNA damage induced in the liver of rohu at different time intervals after exposure to Malathion in terms of (A) tail length (µm), (B) percentage of DNA in the tail (%), (C) tail moment, (D) olive tail moment. Data are represented as Mean ± SE (n = 9). Readings having different letters are significantly different ($P < 0.05$). (ANOVA followed by LSD test).

2004). The activities of GSH-Px and GST increased time-dependently with a timely increase in the GSH, which shows a positive correlation between both these enzymes and GSH. The activities of these enzymes increased in order to resist Malathion induced toxicity and to protect the system from oxidative stress (Dabas et al., 2014). The increase in the activities of GST and GSH-Px observed in the current study is in conformity with some previous studies (Elia et al., 2002; Ullah, 2015; Ullah et al., 2016a) while some reported reduction in the activity of GST (Xing et al., 2012) and GSH-Px (Ballesteros et al., 2009; Stara et al., 2012; Maksymiv et al., 2015), however, Velisek et al. (2012) observed no change in the activity of GST.

3.3. Malathion induced DNA damage

The DNA damage was assessed using tail length, tail DNA percentage, tail moment, and olive tail moment (Fig. 1). The current study showed that Malathion induced a significant ($P < 0.05$) DNA damage in the liver of rohu (Fig. 2), in terms of the aforementioned parameters. The extent of DNA damage was observed to be on a linear increase with time.

The DNA damage was assayed through Alkaline SCGE. It works on the principle that intact DNA migrates slower while damaged DNA migrates much faster towards the anode, which results in the formation of the tail and shape the cells like comets. The comets are formed because, during electrophoresis, the DNA move freely and the tranquil loops of DNA are dragged out of the nucleus/head of the cell (Ullah et al., 2016c). The length of the tail demonstrates the DNA migrated out of the nucleus/head, thus longer the tail length, higher the extent of DNA damage. The smaller DNA fragments migrate farthermost, therefore the length of the tail is primarily demonstrated by the size of DNA fragments, produced during the unwinding step of the comet assay (Kumaravel and Jha, 2006). Percentage of DNA in tail shows DNA moved out of the nucleus in percentage and is considered as a suitable parameter for evaluating DNA damage (Kilemade et al., 2004). Olive tail moment demonstrates distance from the center of the head to the center of the tail and is used for the degree of DNA damage assessment

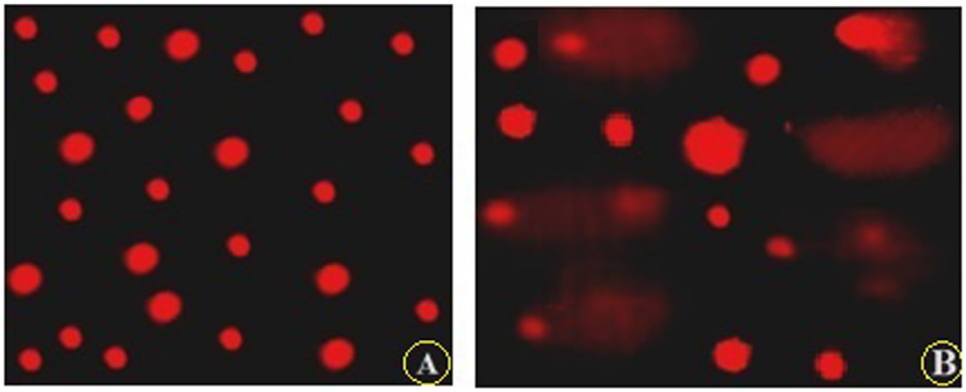

Fig. 2. Fluorescent photomicrograph (40×) of cells in the liver of rohu, after 96 h of exposure to LC_{50} of Malathion using SCGE (stain: Acridine orange). (A) Intact DNA in Control fish, (B) Comets formed in treated fish after exposure to Malathion.

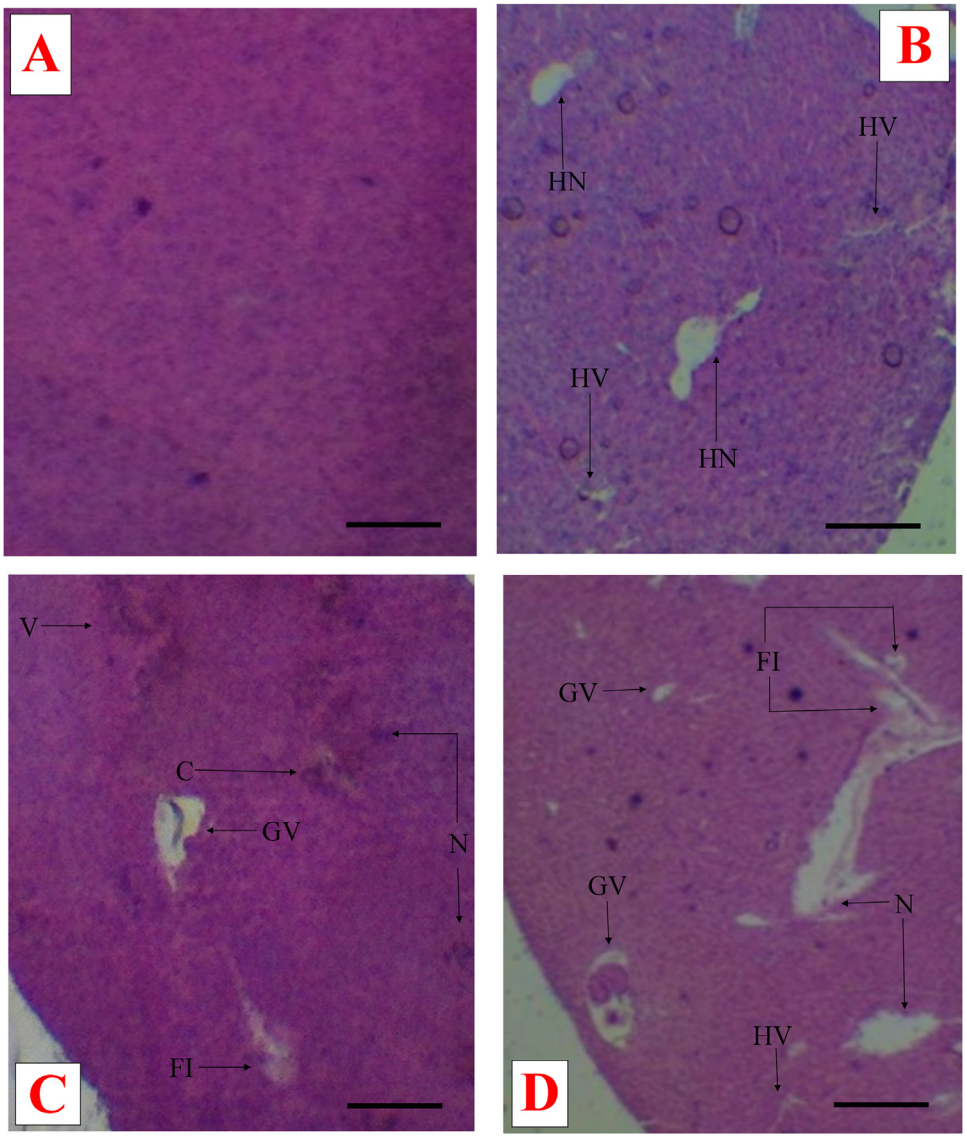

Fig. 3. Histo-micrographs (Magnification ×40; Scale Bars: 500 μm) of the liver of rohu: (A) showing normal structure of the liver (control group), while (B), (C), and (D) showing morphological changes after exposure to Malathion including hepatic necrosis (HN), fatty infiltration (FI), hemorrhage vacuolation (HV), glycogen vacuolation (GV), and congestion (C).

(Singh et al., 1988).

ROS is the outcome of the biotransformation of xenobiotics, which is highly toxic to fish. ROS can break DNA via H_2O_2 and OH^-, which ultimately results in the oxidized DNA bases (Akcha et al., 2003). The fish is protected by the antioxidant enzymes system against ROS but when ROS production surpasses the fish defense systems, it ultimately leads to DNA damage and cellular lesions (Cavalcante et al., 2008; Jha, 2008). Increase in ROS production leads to DNA damage (Ullah et al., 2017). During the current study, an increase was observed in ROS level in a time dependent manner, which led to a simultaneous time dependent oxidative DNA damage.

The results of the current study are in conformity to the previous research studies on genotoxic and mutagenic effects of different organophosphorus pesticides (Das et al., 2006; Rao et al., 2006; Ganguly et al., 2010; Ullah et al., 2016c). The finding reveals Malathion as an inducer of genotoxicity at acute concentration. A serious concern regarding potential threats from Malathion to the genetic materials of the aquatic organisms is clearly displayed in the current study. Therefore, it should be employed carefully in order to avoid any genetic change or unfavorable outcomes for the non-target organisms including fish.

3.4. Malathion induced histopathological damage

Histopathological studies gained importance in ecotoxicology because of being rapid and has been established as a reliable parameter for toxicological, and risks and safety assessment studies. Histological slides are prepared to evaluate the tissue-specific toxic effects of the chemicals or pollutants such as pesticides. Hence, to evaluate the toxic effects of Malathion on the liver of *Labeo rohita*, histopathological slides were prepared and studied for different morphological alterations. Normal appearance of the hepatocytes was observed in the liver of rohu from the control group having a polygonal shape, granulated cytoplasm, and central nuclei. Malathion exposure led to different severe morphological alterations in the liver of rohu, which is considered as a typical biomarker of generalized stress to the fish (Glover et al., 2007). The disparaging changes observed were cellular swelling, hepatic necrosis (HN), fatty infiltration (FI), hemorrhage vacuolation (HV), glycogen vacuolation (GV), and congestion (C) (Fig. 3). These hostile modifications in the liver of rohu can lead to severe physiological problems and ultimately death of the fish.

A slight but non-significant difference was observed for gross

anatomical and morphometric parameters (body length, weight, depth, standard length, and liver weight) between control and treated groups (Fig. S3). The results are in congruence with previous studies. Hasan et al. (2015) exposed grass carp to different acute concentrations of endosulfan and did not observe any significant changes in the anatomical and morphometric parameters.

The liver detoxifies harmful substances including pesticides during metabolism in fish and other vertebrates. However, pesticides beyond a certain concentration lead to disturbance of the normal regulating mechanisms in the liver that consequently leads to different morphological alterations, such as Malathion led to different severe histopathological changes in the current study including HN, FI, HV, GV, and C. The current results are in congruence with previous research studies revealing different histopathological changes induced by pesticides in the liver of different fish species including *Salmo salar* (Glover et al., 2007), *Heteropneustes fossilis* (Joshi et al., 2007), *Clarias gariepinus* (Velmurugan et al., 2009a), *Cirrhinus mrigala* (Velmurugan et al., 2009b), *Oreochromis niloticus* (Benli and Özkul, 2010), *Cyprinus carpio* (Banaee et al., 2011), *Tor putitora* (Ullah et al., 2015), *Ctenopharyngodon idella* (Hasan et al., 2015), etc. The current study demonstrated liver as a central organ of detoxification in fish such as HN might have appeared on account of extra stress on liver cells during Malathion detoxification, while FI and GV indicated fat accumulation and imbalance between substance release and rate of synthesis in hepatocytes (Petal and Bahadur, 2011).

4. Conclusion

The result of the current study revealed Malathion as a potent hepatotoxic pesticide against fish. Moreover, the indices including ROS level, protein content, lipid peroxidation, antioxidants enzymes, protein metabolic enzymes, DNA damage, and histopathology can be used as potential biomarkers for biomonitoring and risk assessment of chemicals in aquatic organisms. The indiscriminate and injudicious use of pesticides should be avoided and strictly prohibited, as these might lead to the population decline of the wild aquatic organisms. Further research on the hormonal profile, cholinergic receptors, the involvement of oxidative damage, as well as their molecular cross-talk is suggested for fully elucidating the mode of action of Malathion. This will also provide key information for devising preventive strategies to reduce the potentially hazardous effects of Malathion on fish species.

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Conflict of interest

The authors declare no competing interest/conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.06.002.

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