



Multiple biomarkers based appraisal of deltamethrin induced toxicity in silver carp (*Hypophthalmichthys molitrix*)

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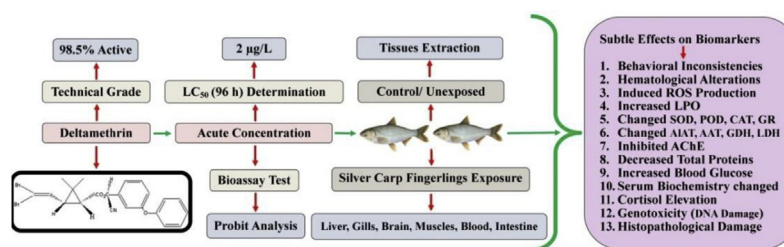
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HIGHLIGHTS

- Exposure to the acute concentration of deltamethrin induced oxidative stress in silver carp.
- Oxidative stress led to different biochemical and histopathological toxicities in silver carp.
- Alterations in behavior, hematology, and serum biochemistry were also observed.
- This study will broaden the ideal toxicity markers used to characterize oxidative stress development.

GRAPHICAL ABSTRACT



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ABSTRACT

Deltamethrin (DLM) is α -cyano (type II) synthetic pyrethroid. DLM exposure leads to strong neurotoxic effects and a number of complex toxicological syndromes. The current study assessed DLM mediated oxidative stress, behavioral, hematological, histopathological, and biochemical toxic effects on silver carp (*Hypophthalmichthys molitrix*). Exposure to an acute concentration (2 µg/L) of DLM resulted in different behavioral inconsistencies and a time-dependent significant ($P < 0.05$) change in the hematology and serum biochemistry of silver carp. A significant ($P < 0.05$) increase in the activities of reactive oxygen species, lipid peroxidation, and antioxidant enzymes whereas a significant decrease in total protein contents in the liver, gills, brain, and muscle tissues were observed. DLM exposure increased the activities of metabolic enzymes in the gills, muscles, and liver of silver carp. A significant ($P < 0.05$) increase in DNA damage in peripheral blood erythrocytes was evident. DLM exposure led to a time-dependent significant ($P < 0.05$) increase in the whole-body cortisol and blood glucose level, while a significant decrease in acetylcholine esterase activity in the brain, liver, and muscle tissues. Different histopathological changes in the liver, gills, brain, and intestine were observed, however, no significant change in the gross anatomy and morphometric parameters of the fish was observed. The current study provides valuable information for devising better strategies regarding environmental management, chemicals' risk assessment, biodiversity conservation, and monitoring of the aquatic organisms. DLM was concluded to be highly toxic to fish. The extensive use of DLM should be prohibited or allowed under strict

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environmental laws; otherwise, it might lead to the extermination of the susceptible wildlife, such as commercially very valuable but nearly threatened silver carp.

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1. Introduction

Pesticides refer to a group of compounds used to suppress or control the activities of target organisms (Velki and Hackenberger, 2013; Fahad et al., 2015). Pesticides contribute considerably to human welfare by protecting crops against pests and reducing vector-borne diseases. However, some pesticides persist in the environment for very long and lead to various adverse effects with diverse toxicological endpoints in the exposed non-target organisms (Ullah and Zorriehzahra, 2015). A number of pesticides are regulatorily restricted to use. Yet, the uncontrolled uses of pesticides are continuously increasing due to their broad spectrum activities, easy availability, and low cost. Pesticides are categorized into various types and classes, based on their active ingredients, uses, and mechanisms of action (Ullah et al., 2018a).

Pyrethroid is a synthetic analog of pyrethrin, which is extracted from the dried flower of *Chrysanthemum* plants. Synthetic pyrethroids (SPs) form a diverse class of pesticides having more than a thousand insecticides. SPs were introduced in the 1970s as a novel group of insecticides (Vani et al., 2012). The applications of SPs are continuously increasing due to a decrease in the use of organophosphates (James et al., 2017). SPs are used in industries, agriculture, households, and medicine/public health purposes (Hoiyńska-Iwan et al., 2018). SPs are quite powerful, broad spectrum, and environment-friendly due to their weak aqueous mobility, low volatility, and less persistence in the environment (Ullah, 2015). On account of their easy degradability, higher efficacy, and less toxic to mammals and birds (Velíšek et al., 2006), SPs are prioritized over carbamate, organophosphorus, and organochlorine insecticides (Fetoui et al., 2008). However, fish are very sensitive to SPs because of having insufficient hydrolytic enzymes and strong neurotoxic effects of SPs on them (Ensibi et al., 2013; Arslan et al., 2017). Research on toxicological effects of SPs on aquatic organisms received much attention recently because of their severe toxicity to them, especially fish.

Deltamethrin (DLM) is a type II (α -cyano) SP but can simultaneously demonstrate some effects of type I pyrethroids (Breckenridge et al., 2009; Tan and Soderlund, 2010). DLM was introduced in 1974 and is widely employed due to its easy disintegration, higher efficacy, and photostability (Kumar et al., 2015). Moreover, it is less toxic to mammals and birds, reasonably toxic to amphibians, and highly toxic to invertebrates. DLM is employed to control pests (mites, beetles, weevils, and ants) of various crops (vegetables, soybeans, corn, cereals, and cotton), to protect stored crops, as well as in public health programs to control disease vectors (Haverinen and Vornanen, 2016). DLM flows into water bodies through runoffs from agricultural practices, domestic wastes, public health programs, and industrial effluents. It kills as well as adversely affects the survival of different aquatic organisms including fish for being highly toxic to them (Pimpão et al., 2008; Assis et al., 2009). DLM exposure induces oxidative stress that ultimately leads to the death of the fish at an acute concentration. However, at sublethal concentrations, it compromises the immunity of the fish, disturbs their normal behavior and body systems (physiology, biochemistry, etc.), and make them susceptible to secondary infections. The half-life of DLM in surface waters is observed to be several days on account of high evaporation and

absorbability, and its occurrence in the real-world freshwater environment is observed to be ranging from 10 ng/L to 24 μ g/L (Ensibi et al., 2014).

To monitor the toxicological effects of pesticides on non-target aquatic organisms, different parameters including oxidative stress stimulation, behavioral inconsistencies, genotoxicity induction, histopathological damage, hematological disturbances, enzymatic alterations, and hormonal profiles are observed (Ullah and Zorriehzahra, 2015). These toxicological endpoints are convenient tools to monitor environmental quality, assess chemical risks and safety, investigate threshold values, derive permissible concentrations, and evaluate organismal vulnerabilities to specific chemicals or toxicants such as pharmaceutical drugs, heavy metals, pesticides, etc. These parameters work as key biomarkers for measuring the interactive effects of environmental xenobiotics on biological systems. The increment or decrement in these biomarkers indicates the potential effects of the toxicants on the exposed organisms. The changes in the biomarkers display the alterations in the biochemical processes and metabolism of the fish and subsequently reveal the mechanism of the toxicities/hostile effects of pollutants.

The toxicological impacts of DLM are widely studied on different organisms. A plethora of well-documented research on humans indicated that DLM exposure led to an increase in caspase-9 activity (DNA fragmentation), increased sodium and potassium influx, and ultimately led to apoptosis/cell death, etc. (Hossain and Richardson, 2011; Hsu et al., 2012; James et al., 2017). DLM exposure led to neurotoxic effects in rats (Tan and Soderlund, 2010), immunotoxic effects such as ROS generation, caspase-3 activation, increase in apoptotic cells etc. in mice (Kumar et al., 2014; Nieradko-Iwanicka and Borzęcki, 2015; Tewari et al., 2018a, 2018b), disturbed the trans-epithelial transport of ions in rabbits (Hoiyńska-Iwan et al., 2018), inhibited AChE and CES, and disturbed the activities of antioxidant enzymes in earthworm, *Eisenia andrei* (Velki and Hackenberger, 2013), and damaged the ultrastructure of midgut of the insect, *Callibaetis radiates* (Santos et al., 2018). DLM exposure led to different toxic effects on birds, such as decreased body weight of *Gallus gallus domesticus* in a dose-dependent manner (Chandra et al., 2013), and increased plasma uric acid, creatinine, activities of creatinine phosphokinase, lactate dehydrogenase, and aspartate aminotransferase while decreased globulin, total protein, alkaline phosphatase, and AChE in *Coturnix japonica* (Hamidipoor et al., 2015). DLM exposure decreased eggs production and survival rates, increased glucose concentration in hemolymph and AMP deaminase while inhibited LDH, SDH, and OAT in the snail, *Helisoma duryi* (Bakry et al., 2011). DLM exposure disturbed amphibians, such as inhibited acid phosphatase and activated AChE in *Xenopus laevis* (Aydin-Sinan et al., 2012), and led to neurological effects (such as spasmodic contractions) in *Physalaemus gracilis* (Macagnan et al., 2017).

The toxic impacts of DLM on different aquatic organisms are also well studied. DLM exposure mediated toxicities in crustaceans, such as increased respiratory metabolism in *Porcellio scaber* (Unkiewicz-Winiarczyk and Gromysz-Kałkowska, 2012), and up or down-regulated 39 proteins responsible for metabolic processes, stimulus-response, antioxidant activities, receptor activities, structural molecular activity, binding, catalytic activity, and apoptosis in *Daphnia magna* (Toumi et al., 2014; Ren et al., 2017).

DLM exposure significantly reduced the swimming velocity and disturbed AChE, ACh, LDH, and antioxidant enzymes in prawn, *Palaemon serratus* (Oliveira et al., 2012). DLM exposure resulted in oxidative damage at the cellular and genomic level in freshwater mussels, *Unio tumidus* (Charissou et al., 2004), and increased LPO while decreased catalase and glutathione in the gills and digestive glands of *Unio elongatulus eucirrus* (Köprücü et al., 2008).

DLM exposure led to different toxicological endpoints in different species of fish including *Ancistrus multispinis* (Assis et al., 2009), *Puntius chrysopterus* (Pawar et al., 2009), *Catla catla* (Vani et al., 2011), *Cyprinus carpio* (Ensibi et al., 2013), *Sparus aurata* (Guardiola et al., 2014), *Anabas testudineus* (Devi and Gupta, 2014), *Pangasius hypophthalmus* (Hedayati and Tarkhani, 2014), *Cirrhinus mrigala* (David et al., 2015), *Oreochromis niloticus* (Abdel-Daim et al., 2015; Cengiz et al., 2017), *Labeo rohita* (Suvetha et al., 2015a, 2015b), *Carassius carassius* (Haverinen and Vornanen, 2016), *Colossoma macropomum* (dos Santos Cunha et al., 2018), and *Channa punctatus* (Singh et al., 2018). However, research regarding DLM induced toxicities on silver carp is still scanty, more specifically multi-biomarkers based studies. The current study was aimed to study the toxic effects of DLM on silver carp (*Hypophthalmichthys molitrix*, Valenciennes 1844). Silver carp were selected considering its high commercial value, a higher rate of consumption, as well as its continuously vanishing wild population. The study was multi-biomarkers based, involving behavior, hematology, reactive oxygen species (ROS), lipid peroxidation (LPO), antioxidant enzymes, metabolic enzymes, blood glucose level, serum biochemistry, total protein contents, acetylcholine esterase (AChE), whole body cortisol, DNA, and histomorphology of different tissues of silver carp.

2. Materials and methods

2.1. Test animal, acclimatization, and handling

Healthy and uniform sized fingerlings (6.4 ± 1.3 cm and 8.9 ± 1.2 g) of silver carp (*Hypophthalmichthys molitrix*) were procured from the hatchery and transported through live hauling method to the laboratory (in closed-system). The fish was carefully transferred to the fiberglass tanks after cautious conditioning to avoid stress or damage. The fish and the aquaria were pretreated with KMnO_4 and acclimatized for two weeks. The aquaria were siphoned off for removing wastes and feed remains daily to avoid stress to fish. A photoperiod of 12:12 h was maintained. Different water parameters including temperature, hardness, pH, and ammonia were regularly investigated using pH meter, DO meter, and checker meter (Horiba). The dead fish were removed as quickly as possible to avoid water quality deterioration.

During the study period, the water temperature ranged from 22.9°C to 24.5°C , dissolved oxygen 6.3–7.5 mg/L, and ammonia was under 0.25 ppm. Total hardness ranged from 164 to 181 mg/L, pH 7.0–7.7, and conductivity 245–299 $\mu\text{S}/\text{cm}$.

2.2. Test chemical

The technical grade (98.5% active ingredient) formulation of DLM (Jiangsu Yangnon Chemical Group Co., Ltd.) was used during the study (Table S1). A stock solution was prepared while the required amount of dilutions was used during the study. For evaluating lethal concentration (LC_{50}), five concentrations (1, 2, 3, 4, and 5 $\mu\text{g}/\text{L}$) were prepared; however, the acute concentration (2 $\mu\text{g}/\text{L}$) was used during the study.

2.3. LC_{50} determination

For evaluating the LC_{50} , the fish were exposed to different concentrations (1, 2, 3, 4, and 5 $\mu\text{g}/\text{L}$) of DLM in semi-static bioassay method. Fish survival and mortality was keenly observed and recorded by counting the fish number per aquarium at regular intervals (12 h). The dead fish were immediately removed out of the aquaria to avoid water contamination. The LC_{50} was calculated via probit mortality values (95% CI).

2.4. Experimental design

The experiment was carried out in a semi-static closed system. Healthy, uniform sized and active fish (irrespective of the gender) were stocked at the density of 1.5 g/L. The water was changed and the acute concentration of DLM was restored on a daily basis, after capturing fish from each group for biochemical, hematological, and histopathological analysis. The captured specimens were anesthetized using MS222 (60 mg/L), the tissues were dissected out (over icebox), and stored for analysis at -20°C . The biochemical parameters including reactive oxygen species (ROS), protein contents, different antioxidants [catalase (CAT), peroxidase (POD), glutathione reductase (GR), and superoxide dismutase (SOD)] were assayed in liver, brain, gills, and muscles, whereas, metabolic enzymes [Alanine Aminotransferase (AlAT), Aspartate Aminotransferase (AAT), Lactate Dehydrogenase (LDH), and Glutamate Dehydrogenase (GDH)] were assayed in gills, liver, and muscles tissues. Blood of the specimens was collected (via caudal veins) for hematological study, glucose concentration, serum biochemistry, and DNA damage analysis. For gross anatomical and morphometric changes the total body weight (g), total body length (cm), standard length (cm), body depth (cm), and weight (g) of the liver, gills, and intestine of the fish were weighed. The tissues were transferred to neutral buffered formalin (NBF) for further histopathological studies.

2.5. Behavioral study

The fish from both the groups were keenly observed in both the control and treated groups for behavioral inconsistencies and alterations. Different behavioral parameters including movement, equilibrium, operculum beat, air gulping, jumping, hypo activeness, hyper activeness, abrupt swimming, aggression, increased operculum beats, motionlessness, vertical position adaptation, fast swimming, and increased surface activity were profoundly studied.

2.6. Hematology

The blood samples were collected via severing the caudal peduncle. Before studying the hematological indices, the blood was checked for any parasitic infestation through placing a drop on a clean slide and examining under a microscope to ensure the use of infection-free blood for the study. To avoid coagulation, EDTA (1.26 mg/0.6 ml) was used, which is observed to be more reliable for hematological studies than heparin and sodium citrate (Faggio et al., 2014). The hematological parameters including WBCs, RBCs, Hb, PCV, MCV, MCH, and MCHC were assayed by following David et al. (2015).

2.7. Biochemical study/assays

On account of the small size of the brain and liver of the fish, three samples were pooled together to acquire requisite weight for analyzing the biochemical effects. The biochemical parameters including ROS, LPO, total protein contents, blood glucose,

antioxidant enzymes including CAT, SOD, GR, and POD in liver, gills, brain and muscles, metabolic enzymes including AAT, GDH, LDH, and AIAT in liver, muscles, and gills, and DNA damage in peripheral blood erythrocytes were evaluated by following (Ullah et al., 2018b), AChE in brain, muscle, and liver tissues by following (Bibi et al., 2014), and the whole body cortisol was assayed by following Zuberi et al. (2014).

2.7.1. Reactive oxygen species (ROS)

The Reactive oxygen species (ROS) was evaluated through 2',7'-dichlorofluorescein diacetate (DCF-DA). The pooled tissues (0.1–1 g wet wt.) samples were incubated in 100 ml of DCF-DA (10 μ M) in a water bath in methanol (37 °C; 30 min). The fluorescence of DCF was observed at 525 nm (emission) and 488 (excitation) wavelengths through spectrofluorometer. For fluorescence value, the standard (0.500 nM DCF) curve was used.

2.7.2. Lipid peroxidation (LPO)

For assaying LPO, 1 ml reaction mixture was procured by mixing 0.2 ml supernatant with 0.02 ml of 100 mM ferric chloride, 0.2 ml of 100 mM ascorbic acid, and 0.58 ml of 0.1 M phosphate buffer (pH 7.4). The mixture was incubated for an hour in a water bath (37 °C), and 1 ml of 10% trichloroacetic acid was added for stopping the reaction. Thiobarbituric acid (1 ml) was added to the tubes and boiled in a water bath for 20 min, followed by cooling down through the ice bath, and centrifugation at 2500 \times g (10 min). After a minute, change in the solution's absorbance was recorded at 535 nm using a spectrophotometer.

2.7.3. Antioxidant enzymes

For assessing the activities of catalase, 0.1 ml enzyme extract, 2.5 ml of 50 mM phosphate buffer (pH 5), and 0.4 ml of 5.9 mM H₂O₂ were mixed. The absorbance change was observed after a min at 240 nm for CAT through spectrophotometer. For assaying peroxidase activities, 0.1 ml enzyme extract was mixed with 0.1 ml of 20 mM guaiacol, 0.3 ml of 40 mM H₂O₂, and 2.5 ml of 50 mM phosphate buffer (pH 5). The absorbance change was observed at 470 nm for the POD. For superoxide dismutase assay, the reaction mixture was procured by mixing 0.3 ml supernatant with 1.2 ml of 0.052 mM sodium pyrophosphate buffer (pH 7), and 0.1 ml of 186 μ M phenazine methosulphate. The reaction was initiated by adding 0.2 ml of 780 μ M NADH and was stopped with 1 ml of glacial acetic acid after a min. The amount of chromogen formed was recorded at 560 nm through spectrophotometer. Glutathione was assayed by procuring 2.0 ml of the solution by mixing 1.475 ml of 0.1 M phosphate buffer (pH 6.5), 0.025 ml of 1 mM CDNB, and 0.2 ml of 1 mM of reduced glutathione. The absorbance change was observed through spectrophotometer at 340 nm.

2.7.4. Total protein contents

For analyzing protein contents, the tissues (90 mg) were homogenized in phosphate buffer and centrifuged (4 °C, 10,000 rpm) for 20 min. A stock solution of the standard bovine serum albumin (BSA) was procured (15 mg BSA and 15 ml distilled water). Solution 1 was prepared by mixing 50 ml 0.1N NaOH and 50 ml 2% Na₂CO₃ solutions. Solution 2 was prepared by mixing 10 ml 2.37% sodium potassium tartrate and 10 ml 1.56% CuSO₄ solutions. The 2 ml commercial reagent was mixed with 2 ml water to prepare Folin-Ciocalteu reagent. For procuring alkaline solution, 2 ml of solution 2 was mixed with 100 ml of solution 1. Then, 0.1 ml of thawed sample mixed with an alkaline solution (1 ml) was incubated for 10 min, followed by mixing 1:1 Folin-Ciocalteu phenol reagent with each tube (vortexed to mix properly), and incubation (30 min). The OD (optical density) was observed via a spectrophotometer

(595 nm). BSA standard curve was used for calculating protein concentration.

2.7.5. Blood glucose

The level of blood glucose was assessed using a blood glucose meter. The strip having blood drop from the collected specimen of both the groups (control and treated) was inserted into the glucometer. The results for the detected glucose level were shown on the screen of the meter.

2.7.6. Serum biochemistry

No anticoagulant was added to the blood samples for serum biochemical analysis. The procured blood was kept on ice for an hour, followed by centrifugation (3000 rpm, for 10 min) for serum isolation. The samples were stored (–80 °C) for further analysis of different biochemical parameters including total protein, albumin, total bilirubin, urea, chloride, potassium, sodium, inorganic phosphate, and cholesterol by following Qadir et al. (2014).

2.7.7. Acetylcholinesterase (AChE) assay

The tissues (90 mg) were homogenized in 0.1 M phosphate buffer (pH 7.5) and then centrifuged for 10 min (3000 rpm), followed by further centrifugation of the supernatants for 10 min (3000 rpm). The final product was collected and used in the assessment of AChE activity through acetylcholinesterase assay kit.

2.7.8. Whole-body cortisol

The thawed samples were homogenized with PBS (phosphate buffer saline), ultrasonicated for approximately 60 s using sonicator, followed by decanting the contents into a separating funnel, and finally extracting hormone using diethyl ether. The ether was evaporated at 45 °C under nitrogen's gentle stream. The final extract was stored (–80 °C) and was analyzed through cortisol kit.

2.7.9. DNA damage

The process of comet assay/SCGE was carried out as a three-layer procedure, as performed by (Ullah et al., 2016a). After SCGE, two slides were procured for each group and were used for further analysis during fluorescent microscopy. The slides, stained with Acridine orange, mounted on an epifluorescent microscope were observed keenly. The captured digital images were scored through CASP Lab (Version 1.2.3 beta 1). Hundred randomly chosen cells were observed through CASP, and fifty cells were scored for each of the two slides. The DNA damage was quantified by studying different parameters including olive tail moment (OTM), tail moment (TM), percent DNA in the tail (%TDNA, percentage), and tail length (TL, μ m).

2.7.10. Metabolic enzymes

Homogenates of the tissues (2% w/v) were prepared in 0.25 M solution of ice-cold sucrose, then centrifuged for 15 min (1000 \times g), and the supernatants were collected for analyzing the activities of the enzymes. For AAT, the incubation mixture was prepared by mixing phosphate buffer, L-aspartic acid, α -ketoglutarate, and the supernatant (enzyme source). The mixture was incubated (30 min, 37 °C), then the ketone reagent was added to stop the reaction, followed by keeping it at lab temperature for 20 min and adding NaOH. The developed color was read against blank reagent (545 nm) through spectrophotometer. For AIAT, 2 ML incubation mixture was prepared by mixing phosphate buffer, D1-alanine, α -ketoglutarate, and supernatant (enzyme source). The solution was incubated (30 min, 37 °C), then 2,4-dinitrophenyl-hydrazine was added to stop the reaction, and then NaOH was added. The developed color was recorded at 545 nm.

For GDH, a solution mixture was prepared by mixing phosphate

buffer, sodium glutamate, INT, NAD, and the enzyme source. The 2 ml mixture was incubated for 30 min, followed by reading the enzyme activity at 340 nm. For LDH, the solution mixture was prepared by mixing buffer, lactic acid, sodium azide, and plasma. The solution was incubated at 37°C (2 min), followed by addition of the color reactive [NAD⁺ (1.2 mmol/L), INT (0.64 mmol/L), phenazine (0.26 mmol/L), and sodium azide (1.23 mmol/L)]. Again incubated for 5 min (37°C), followed by adding HCl to stop the reaction, keeping the tubes at room temperature for 5 min, and reading the absorbance at 500 nm using a spectrophotometer.

2.8. Histomorphological examination

The collected fish samples and extracted tissues were weighed and measured for gross morphometric and anatomical parameters [total body length, total body depth, standard body length, total body weight, gills weight, liver weight, and intestine weight were recorded], following by transferring the tissues to NBF for preservation and further analysis. Eosin and hematoxylin stains were used for staining the slides. The stained slides were mounted with Canada balsam and coverslips and were kept in the incubator overnight. Xylene was used to remove extra Canada balsam of the slides. The slides were studied for both the groups under a light microscope having a fit digital camera. The procured photomicrographs were studied for histopathological alterations.

2.9. Statistical analysis

The data, expressed as mean \pm SE, was analyzed through one-way analysis of variances (ANOVA), followed by LSD (least significant difference) for testing the homogeneity of variance (multiple variance analysis) in Statistix (Version X). The *P*-value (*P* < 0.05) less than 0.05 was considered to be significant statistically.

3. Results

3.1. LC₅₀ (96 h) appraisal of DLM

Different concentrations, percent mortality, log concentrations, and probit kill values are shown in Table S2. Mortalities were observed to be concentration and time-dependent. Log concentrations were plotted against percent mortality and Probit kill, which resulted in curves formation (Figures S1). The analysis showed 50% mortalities of the exposed fish after 96 h at 0.301 log concentration, which demonstrated 2 µg/L of DLM to be acute (LC₅₀) concentration against silver carp.

3.2. Effects of DLM on behavior

The behavioral alterations appeared after about 3 h of DLM exposure. DLM exposure led to time-dependent different behavioral inconsistencies in the treated group (Table S3). The fish were observed to exhibit jumping initially with loss of balance, and abrupt and fast swimming within first 24 h; hyperactivity, aggression, and increased surface activity were observed till 48 h, while their operculum beats decreased and they became sluggish after 48 h. After 72 h, the fish were observed to be hypoactive, gathering at the corner of the aquarium, sometimes motionless, and occasionally adapted vertical position before death. However, no such signs were observed in fish of the control group.

3.3. Effects of DLM on hematology

The data regarding hematological parameters including White blood cells (WBCs), Red blood cells (RBCs), Hemoglobin (Hb),

Packed Cell Volume level (PCV), Mean Corpuscular Volume level (MCV), Mean Corpuscular Hemoglobin level (MCH), and Mean Corpuscular Hemoglobin Concentration level (MCHC) are shown in Table S4. No change was observed in these parameters in the control group/unexposed fish after 24, 48, 72, and 96 h. However, DLM exposure led to a time-dependent significant increase in the level of WBCs, MCV, and MCH, whereas, a significant decrease in the level of RBCs, Hb, PCV, and MCHC was observed after 24, 48, 72, and 96 h.

3.4. Effects of DLM on biochemical biomarkers

DLM exposure led to a significant increase in the production of ROS in the tissues (liver, muscles, brain, and gills) of silver carp (Fig. 1A). A significant time-dependent increase was observed in the activity of LPO in muscles, gills, brain, and liver of the fish after exposure to DLM (Fig. 1B). Exposure to DLM significantly reduced the total proteins in the liver, gills, muscles, and brain of silver carp (Fig. 1C). A linear and significant increase in the activities of the antioxidant enzymes (SOD, POD, GR, and CAT) was observed in the tissues (gills, liver, muscles, and brain) of silver carp after exposure to DLM (Fig. 2). DLM exposure resulted in a linear increase with time in the level of blood glucose in the DLM treated group (Figure S2). Potassium, cholesterol, total bilirubin, and urea significantly increased while sodium, total protein, albumin, chloride, and inorganic phosphate significantly decreased in the serum of DLM exposed silver carp (Fig. 3). A significant inhibition of AChE activity was observed in the brain, liver, and muscles tissues of silver carp after exposure to DLM (Fig. 4). Exposure to DLM led to an increase in the whole-body cortisol of silver carp (Fig. 5). A time-dependent linear increase in the level of DNA damage was observed in peripheral blood erythrocytes of silver carp after exposure to an acute concentration of DLM. No DNA damage was observed in unexposed fish, however, a significant increasing trend (*P* < 0.05) in DNA damage was observed in the DLM exposed fish in terms of tail moment (TM), percentage of DNA in the tail (%TDNA), tail length (TL), and olive tail moment (OTM) (Fig. 6). DNA strand breaks were evident in fluorescent photomicrographs of the DLM exposed silver carp (Figure S3). A significant linear increase was observed in the activities of metabolic enzymes (AAT, ALAT, LDH, and GDH) in the liver, muscles, and gills of silver carp after exposure to the acute concentration of DLM as compared to the unexposed fish/control group (Figure S4).

3.5. Effects of DLM on histopathology, and gross anatomy and morphometry

DLM exposure resulted in different histopathological alterations in the liver (congestion, increased sinusoidal spaces, sinusoidal dilation, necrosis, hepatic cells' disorientation, pycnosis, inflammatory cells' accumulation, fibrosis, cellular shrinkage, and hemosiderosis), gills (disruption of gill arch, atrophy of lamellae, accumulation of calcium, fusion and folding of lamellae, lamellae disruption, goblet cells desquamation, epithelial cells desquamation, epithelial disruption, detachment and degeneration of lamellae, and necrosis), brain (haemorrhage, neuronal degeneration, discoloration, spongiosis, and infiltration), and intestine (necrosis, separation of the basement membrane, and disruption, eruption, shredding of the mucosal cells, and an increase in the goblet cells) of silver carp (Fig. 7, Table S5). However, no significant difference was observed in gross morphometric and anatomical parameters including total body length, standard body length, total body weight, body depth, and weight of the liver, intestine, and gills (Figure S5). The severity of the histopathological damage was linearly increasing with time; the histopathological changes observed

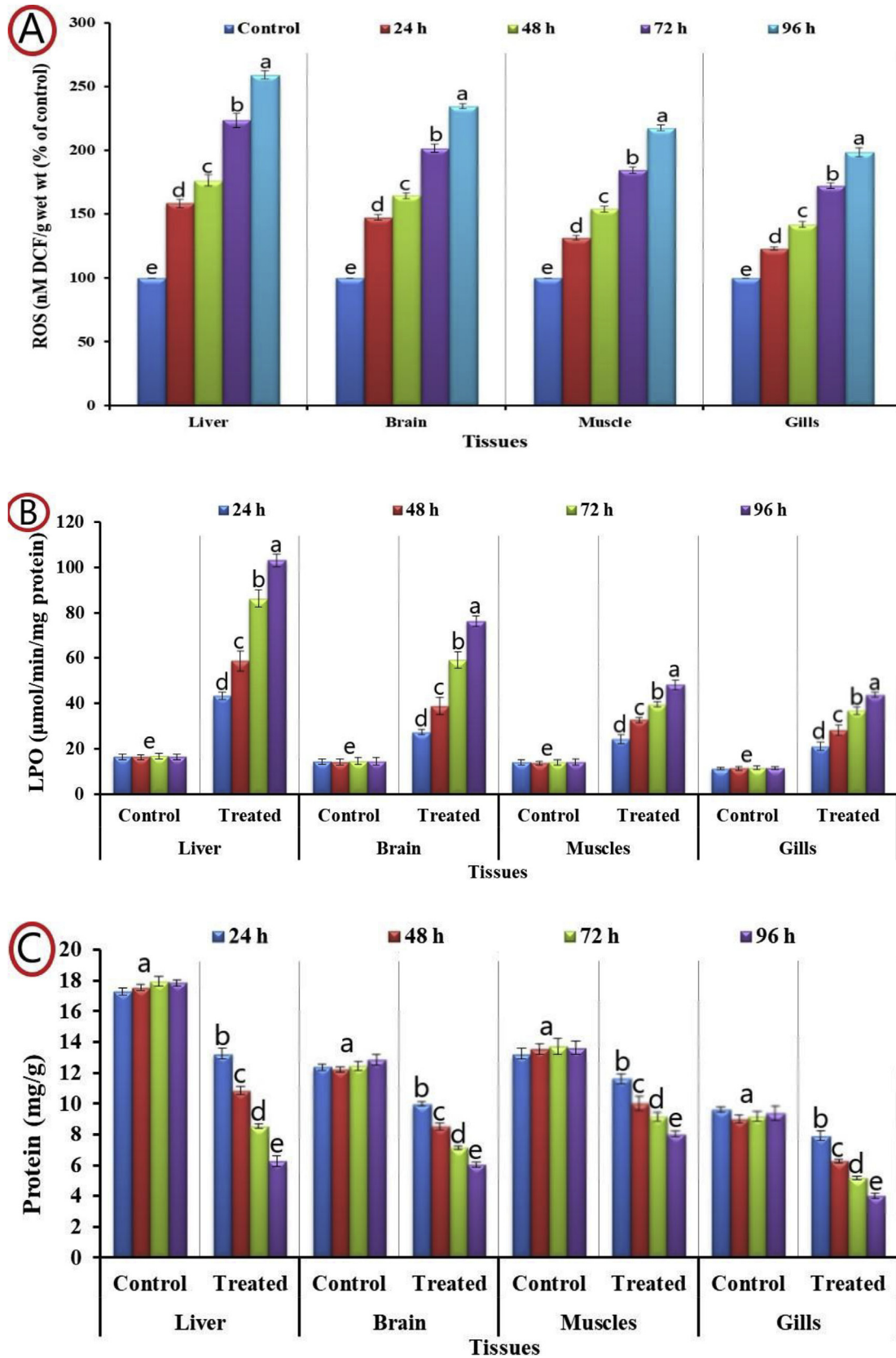


Fig. 1. (A) ROS (nM DCF/g wet wt. (%age of the control)), (B) LPO ($\mu\text{mol}/\text{min}/\text{mg}$ protein), and (C) total protein (mg/g) contents in the liver, brain, muscle, and gills of *Hypophthalmichthys molitrix*. Data presented as Mean \pm S.E. (n = 9). Means with different superscripted letters are significantly different ($P < 0.05$). (ANOVA followed by LSD test).

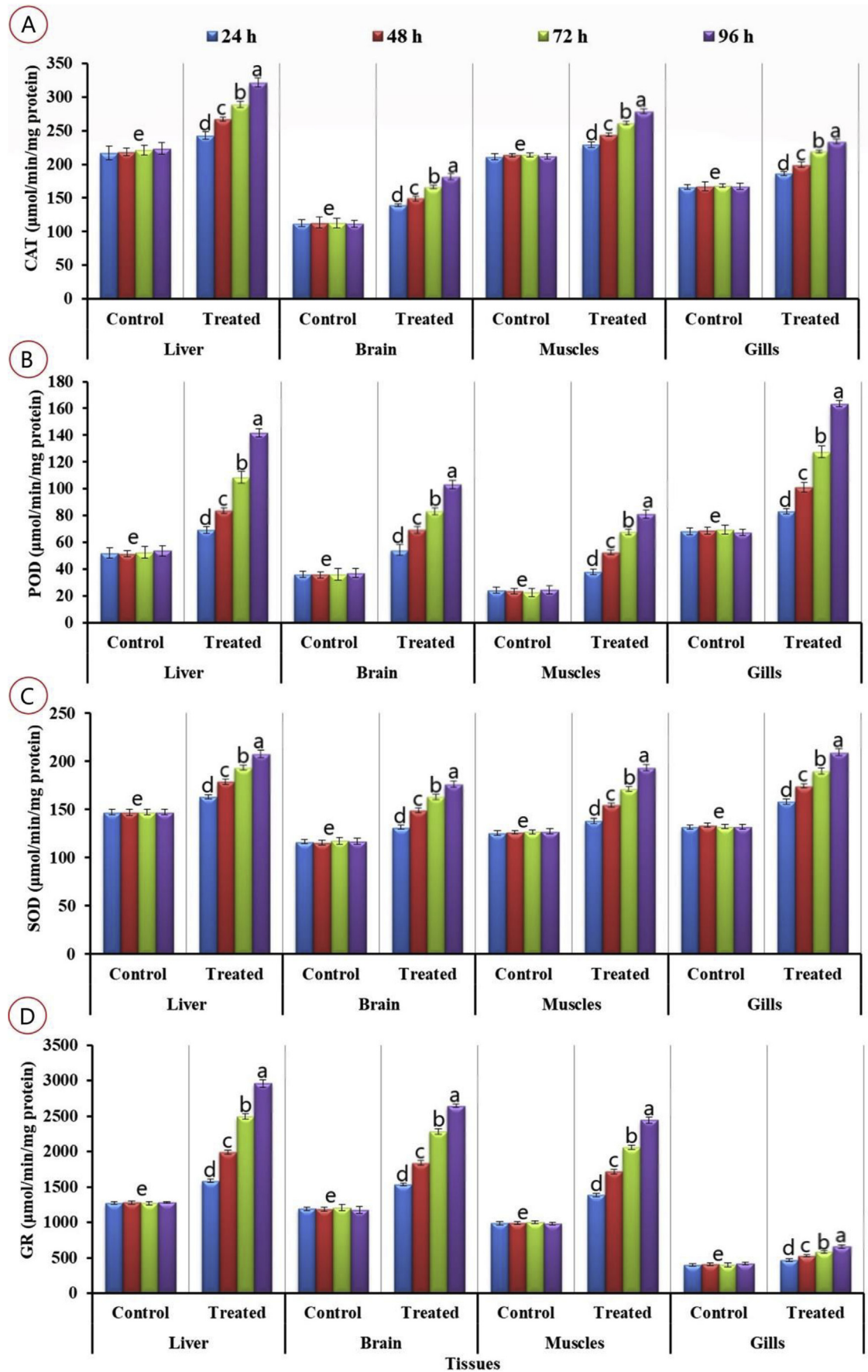


Fig. 2. Activities ($\mu\text{mol}/\text{min}/\text{mg}$ protein) of antioxidant enzymes ((A) CAT, (B) POD, (C) SOD, and (D) GR) in liver, brain, muscles, and gills of *Hypophthalmichthys molitrix* at different time intervals. Data presented as Mean \pm SE (n = 9). Means followed by different letters are significantly different ($P < 0.05$). (ANOVA followed by LSD test).

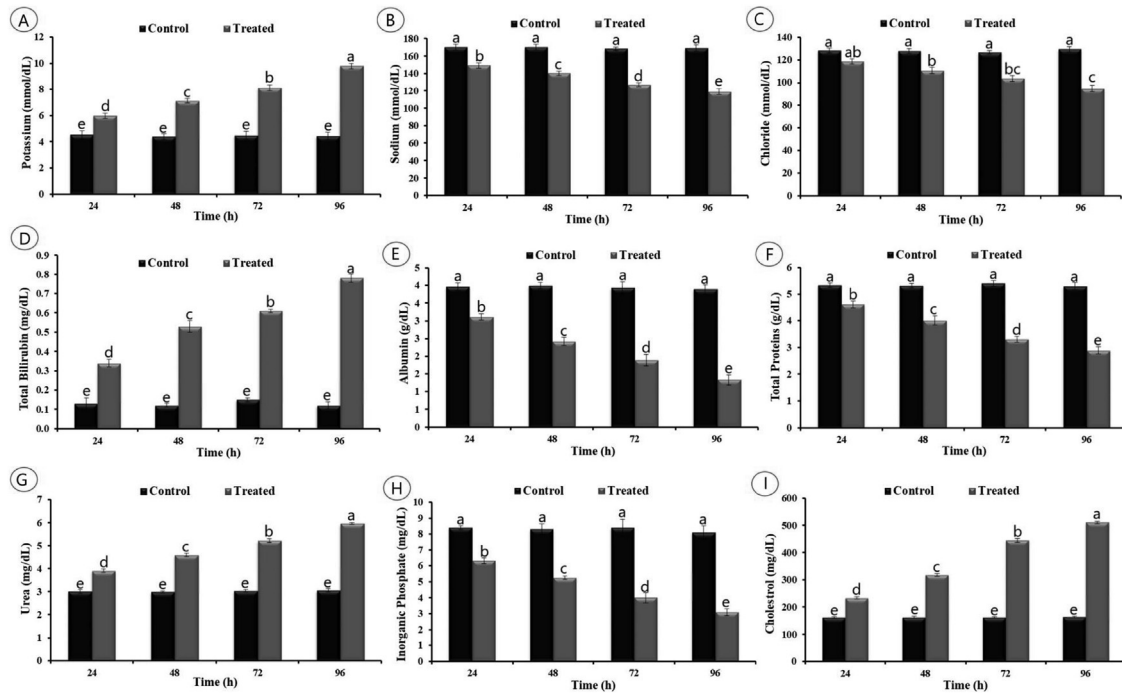


Fig. 3. Serum Biochemistry ((A) Potassium, (B) Sodium, (C) Chloride, (D) Total Bilirubin, (E) Albumin, (F) Total Protein, (G) Urea, (H) Inorganic phosphate, and (I) Cholesterol) in the control and DLM treated *H. molitrix*. Data presented as Mean \pm S.E. (n = 6). Means with different superscripted letters are significantly different ($P < 0.05$). (ANOVA followed by LSD test).

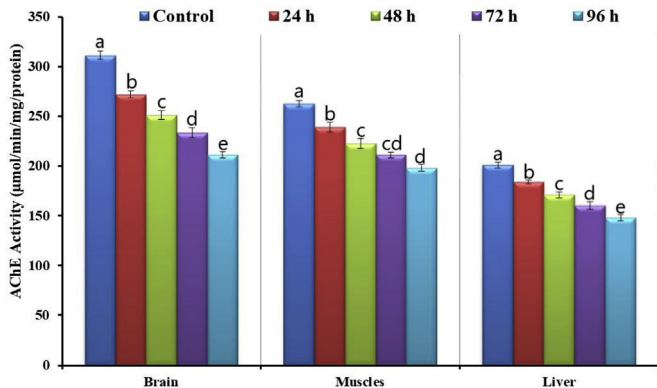


Fig. 4. AChE activities ($\mu\text{mol}/\text{min}/\text{mg}/\text{protein}$) in the brain, muscles, and liver tissues of *Hypophthalmichthys molitrix* in control and DLM treated groups. Data presented as Mean \pm S.E. (n = 9). Means with different superscripted letters are significantly different ($P < 0.05$). (ANOVA followed by LSD test).

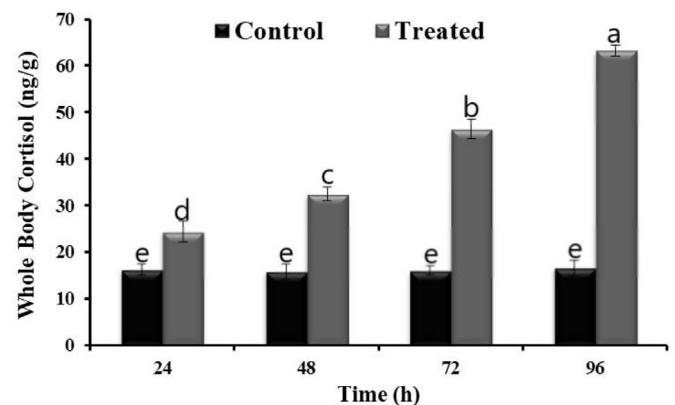


Fig. 5. Whole body cortisol (ng/g) in the control and DLM treated *H. molitrix*. Data presented as Mean \pm S.E. (n = 6). Means with different superscripted letters are significantly different ($P < 0.05$). (ANOVA followed by LSD test).

were more severe after 96 h than after 24 h.

4. Discussion

The advancement in commerce, agriculture, and industries are coupled with a linear increase in the use of different chemicals such as pesticides, heavy metals, fertilizers, etc. These chemicals saturate the air and flow into the water. Exposure to over permissible limits of these chemicals is associated with mortalities, various diseases, and diverse genetic alterations and disorders in non-target organisms including humans, terrestrial, and aquatic animals (Ullah et al., 2016b). Among these chemicals, pesticides make a large portion. Pesticides pose a serious threat to the functionality and integrity of the biological systems in the exposed humans and organisms, such as birds, amphibians, fish, etc. Fish has emerged as a model

organism for assessing chemicals risks and safety, environmental monitoring, and ecotoxicological studies due to their ability of bio-accumulating and metabolizing various contaminants including pesticides. Studies on the pathophysiological effects of pesticides on fish in the laboratory provide information regarding the impacts of residual pesticides on them. Moreover, laboratory studies are also conducted to elucidate mechanisms of toxicity of different pesticides. The current study was undertaken to assess the toxic impacts of the acute concentration of DLM on silver carp. The overall flowchart of the experiment, sampling, studied parameters, and results, in brief, are presented in Figure S6.

4.1. Acute toxicity of DLM to silver carp

The toxicity of pesticides depends on their carrier contaminant

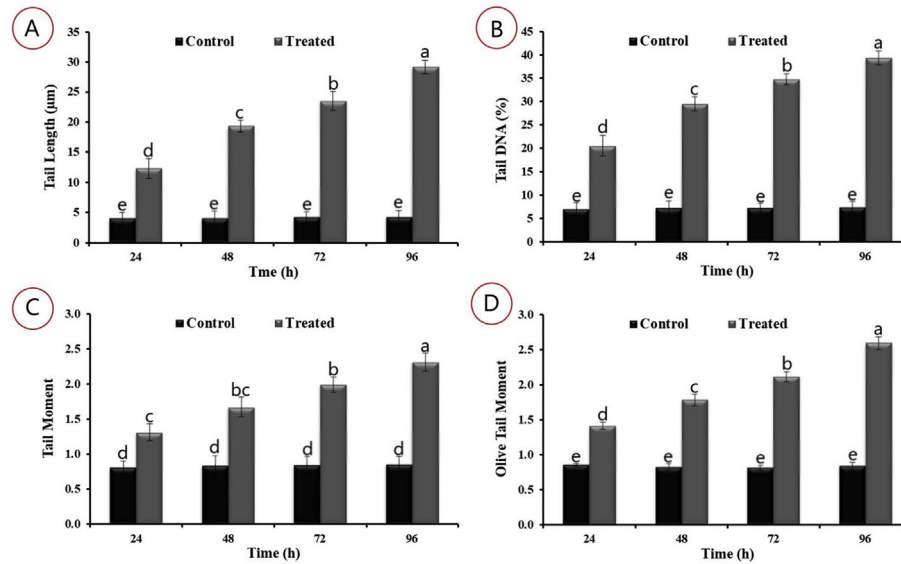


Fig. 6. (A) Tail length (μm), (B) Tail DNA (%), (C) Tail moment, and (D) Olive tail moment of the comets in peripheral blood erythrocytes of silver carp at different time intervals in control and treated groups after exposure to LC_{50} of DLM. Data presented as mean \pm S.E. ($n = 6$). Means with different superscripted letters are significantly different ($P < 0.05$). (ANOVA followed by LSD test).

and active or inert ingredients, as well as the pH, temperature, and other physico-chemical parameters of the ambient water (Ullah et al., 2016a). The severity of the toxic effects of pesticides also depends upon the size, sex, age and health of the fish (Ullah, 2015). Fluctuation in temperature is observed to be a major reason for differences in severity of the toxic effects of pesticides on fish. A negative correlation between the acute toxicity of DLM and temperature is reported (Unkiewicz-Winiarczyk and Gromysz-Kalkowska, 2012), whereas, a positive association between temperature and severity in toxicity of cypermethrin (another SP) against *Danio rerio* is reported (Uddin et al., 2018). An increase in the toxic effects of SPs on the reproduction of *Salmo salar* in the cold aquatic environment during spawning season is also reported (Singh et al., 2018). Similarly, a positive association between pesticides toxicity and body weight of the fish is also reported. However, in the current study, no association in the toxicity of DLM with temperature and body size of the fish was observed.

The acute concentration of DLM against silver carp displayed the severity of DLM (as a highly toxic SP) to silver carp. The reported LC_{50} of DLM against other fish species included $14.6 \mu\text{g/L}$ against *Oreochromis niloticus* (El-Sayed et al., 2007), $4.84 \mu\text{g/L}$ against *Catla catla* (Vani et al., 2011), $2.6 \mu\text{g/L}$ against *Brycon amazonicus* (de Moraes et al., 2013), 0.38 mg/L against *Labeo rohita* (Suvecha et al., 2015a), $4.7 \mu\text{g/L}$ for *Salmo trutta fario* (Karatas, 2016), and $7.33 \mu\text{g/L}$ against *Channa punctatus* (Singh et al., 2018). The differences in the toxicity of the same pesticides against different fish species are attributed to the differences in the tolerance and susceptibility of the species in association to accumulation, excretion, and biotransformation of the pesticide.

4.2. Behavioral response

The alterations in the behavior might be associated with the inhibition of the AChE, which is usually active at both neuromotor and neural junctions in the muscle tissues. The inhibition of neuromuscular AChE led to the blockage of neural transmission and subsequent increase of ACh (acetylcholine) at nerve endings, which in turn resulted in these behavioral inconsistencies. The behavioral inconsistencies including hyperactivity, loss of balance, increased

swimming rate, and body twitches in the DLM exposed fish were probably because of the caudal bending, which in turn impacted the swimming pattern that led to imbalanced swimming and equilibrium loss. The fish became sluggish and motionless after 48 h of DLM exposure, which might be due to an increased level of inhibition of AChE activity and increase in ACh that consequently desensitized nicotine ACh receptors leading to muscular weakness (David et al., 2013).

4.3. Hematological parameters

Hematology is widely employed in ecotoxicological and chemical risk assessments as a major biomarker of stress in fish (Brum et al., 2014). Fish take pesticides through gills leading to their stomach-intestine. Subsequently, the pesticides get transported to other organs and tissues of the fish through blood. The blood is continuously exposed to the pesticides once they are taken into the body, therefore, the hematological parameters (WBCs, RBCs, Hb, etc.) and the tissues producing blood cells (hematopoietic tissues) face different destructive and toxic effects. The stress induced in fish is due to the alteration of the exogenous and endogenous origins of pesticides, and these alterations are instantaneously observed in the blood parameters (Özok et al., 2018). The exposed fish restore normal metabolism during stress through homeostatic mechanisms, however, the stress factors are not sufficient for the end-stage metabolism (Duran and Talas, 2009).

Reduction in RBCs, Hb, PCV, and MCHC after exposure to DLM may be attributed to the inhibition of erythropoiesis or hemopoiesis, and destruction of RBCs in the hematopoietic tissues indicating anemia. The reduction in the RBCs might also be attributed to hypoxia that led to RBCs destruction or decrease in RBCs genesis due to low- or no-availability of Hb in the cellular medium (Ullah et al., 2015). The significant change in the hematological parameters of silver carp might be associated with the failure of the hematopoietic system, or osmoregulatory dysfunction after exposure to DLM (Vani et al., 2011). The WBCs (lymphocytes, monocytes, granulocytes, and thrombocytes) are associated with the defense mechanism. Lymphocytes aid antibodies production, while granulocytes and monocytes remove the debris of the injured

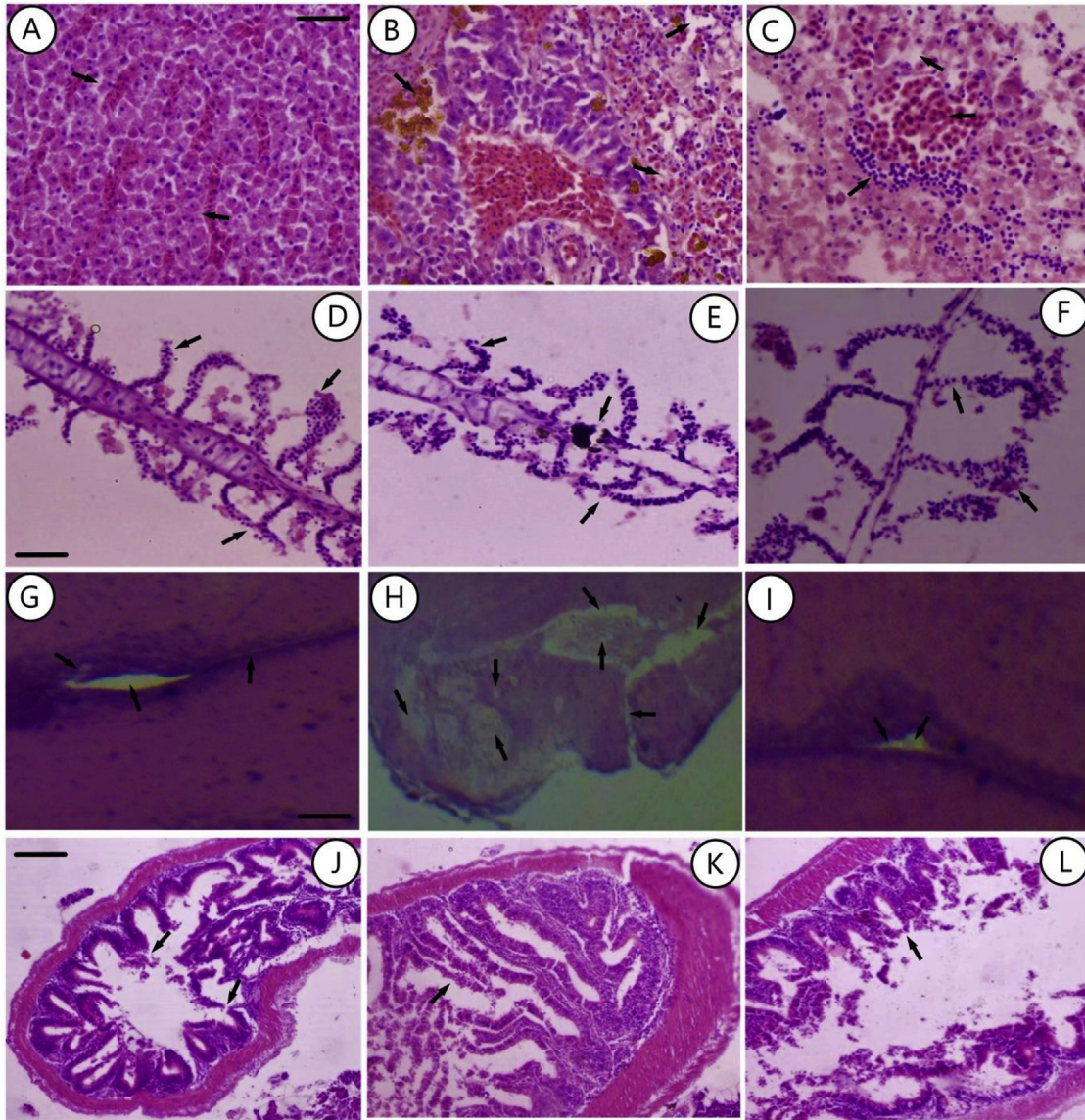


Fig. 7. Histopathological micrographs (scale bar: 300 μ m) of the tissues of DLM intoxicated *H. molitrix*. A ($\times 40$), B ($\times 100$), and C ($\times 100$) show liver [(A) shows congestion with mild dilation of the sinusoidal spaces, (B) shows severe congestion with sinusoidal dilation, vacuolation, and hemosiderosis, and (C) shows congestion, accumulation of inflammatory cells around blood vessel, and cellular shrinkage], D ($\times 40$), E ($\times 40$), and F ($\times 40$) show gills [(D) shows epithelium disruption with fusion and folding of secondary lamellae, (E) shows calcium accumulation, and folding and fusion of the secondary lamellae, and (F) shows detachment and degeneration of the secondary lamellae]. G ($\times 40$), H ($\times 40$), and I ($\times 40$) show brain [(G) shows severe spongiosis, infiltration, and neuronal degeneration, H shows neuronal degeneration, discoloration, infiltration, and spongiosis, and (I) shows severe spongiosis and neuronal degeneration]. J ($\times 40$), K ($\times 40$), and L ($\times 40$) show intestine [(J) shows disruption of the intestinal mucosal cells, (K) shows shredding of the mucosal cells and increase in goblet cells, and (L) shows severe necrosis].

cells. The increase in WBCs in the current study might be associated with the stimulated immune system of the fish in response to tissue damage or compensatory responses of the lymphoid tissue to the circulating lymphocytes. The changes in the hematological parameters in the current study are congruent to the findings of previous research studies on various fish species after exposure to DLM including *Oreochromis niloticus* (El-Sayed et al., 2007) and *Cirrhinus mrigala* (David et al., 2015).

4.4. Oxidative stress

SPs mediate oxidative stress through various mechanisms in fish, primarily measured by monitoring the production of reactive oxygen species (ROS) leading to various modifications at the cellular and molecular level such as modification of lipids, proteins,

DNA, and antioxidants (Lushchak, 2011). ROS damages membranes by making them leaky, which results in different physiological instabilities, necrosis, and finally apoptosis. DLM exposure led to a time-reliant linear increase in ROS production. Pesticides induce oxidative stress through ROS overproduction and subsequent increase in the LPO in different tissues of the exposed organisms (Saxena et al., 2011). The increased activities of antioxidant enzymes and LPO suggested the severe oxidative hazard (overproduction of the free radicals) of DLM that causes injuries to different tissues and neuronal damage. The highest ROS production was observed in the liver followed by the brain. This might be due to the central role of the liver in the detoxification of DLM and the presence of the oxidizable substrates in the brain tissues (Galal et al., 2014).

LPO is the direct reaction of oxygen with lipids. LPO form free

intermediate radicals that generate semi-stable peroxides. LPO is the outcome of the mechanism of free radicals, leading to degradation of phospholipids and loss of membrane's integrity, which in turn induces different toxic effects. The elevation of LPO in the current study indicated damage to the lipids in the tissues (brain, liver, muscles, and gills) of silver carp after exposure to DLM. Moreover, the increase in LPO activity also indicated the production of oxidants at a higher rate, as LPO demonstrates the equilibrium between the production of oxidants and their elimination by antioxidants. The increased LPO was consistent with the increased activities of antioxidant enzymes during the current study. The results of the current study are in correspondence with the previous studies on different species of fish exposed to DLM [*Oreochromis niloticus* (Abdel-Daim et al., 2015), *Channa punctata* (Bhattacharjee and Das, 2017), and *Danio rerio* (Parlak, 2018)].

4.5. Antioxidant enzymes

The activities of antioxidant enzymes are widely assessed as an early sign of intoxication or stress in fish. SPs mediated oxidative stress and modulated activities of the antioxidant enzymes are well reported in vertebrates, specifically in fish. DLM exposure elevated the activities of the antioxidant enzymes in the tissues of silver carp, which might be linked with the production of excessive free radicals. The increase in the activity of CAT is associated with the reduction of H_2O_2 to oxygen and water, as during detoxification the level of hydrogen peroxide increases normally (Shadegan and Banaee, 2018). CAT deters oxidative stress and aids in cellular homeostasis. Similarly, the elevated activity of SOD is associated with the detoxification of the superoxide radicals (O_2^-). Both these antioxidants provide a primary line of defense under stress (Lushchak, 2014). The increase in the activity of GR in the current study might be associated with the defensive role of GR pathways for efficient regulation of ROS production and reducing/ending oxidative stress. The compensatory response of GR with POD, CAT, and SOD inhibit the bioaccumulation of the free radicals and their analogs in the tissues of fish under stress (Ullah et al., 2014).

4.6. Total protein contents

Proteins perform different key functions in fish, such as making the functional and structural cell components, nitrogenous metabolism source, and an energy source under chronic stress. DLM might negatively affect oxygen availability, suppressed oxidative metabolism through inhibition of energy production, and generated hypoxia leading to respiratory distress on account of lactic acid accumulation. Fish change the aerobic pathway of respiration to anaerobic pathway in order to serve energy demand under stress (Ullah et al., 2014). As fish have a limited amount of carbohydrates, proteins serve as an alternate energy source to fulfill the energy demand. Therefore, the decrement in the protein contents in the tissues of silver carp after exposure to DLM might be associated with protein degradation to fulfill the increased demand of energy for metabolic purposes or weakened/damaged machinery of protein synthesis under stress.

4.7. Blood glucose

Blood glucose level is a widely employed biomarker in toxicological and chemical risk assessment studies. The increase in blood glucose indicated that the fish became hyperglycemic after DLM exposure. The increased blood glucose might be due to the effected gluconeogenesis or glycogenolysis. Moreover, the respiratory metabolism is depressed under stress, so, the release of the hyperglycemic hormones (glucocorticoids and catecholamine)

degraded the glycogen and glucose, which leaked out into the blood and subsequently resulted in hyperglycemia (Uddin et al., 2018).

4.8. Serum biochemistry

Serum ions are sensitive biomarkers and are widely employed in ecotoxicological and chemical risk assessment studies. The level of serum ions alter very quickly under stress due to the reduction in intestinal fluid absorption, decreased extrusion of bronchial ions, and morphological alterations of the cells. In the current study, the decreased sodium might be associated with the flow of sodium into the nerves, which was displayed by the inhibition of AChE activities (attributed to sodium inflow in a higher rate, giving rise to multiple nerve impulses, which consequently lead to the release and accumulation of ACh). The level of potassium increased while the level of chloride decreased, which might be associated with the instability in the permeability of the membranes under stress. Alterations in the hydromineral balance might be associated with the impaired osmoregulation or the effects of DLM on the active transport processes, metabolism, and/or endocrine system (Firat et al., 2011). Moreover, the changes in these ions might be linked with the injury to the gills and kidneys of silver carp, as these tissues are involved in ions exchange between the ambient water and the fish. During the current study, histopathological damage to the gills of silver carp was observed after DLM exposure, which might result in a decreased activity of Na^+/K^+ ATPase that controls ion regulation. In addition, the disruption of gill epithelium was observed, which might lead to an increase in water influx, salt efflux, and epithelium permeability and consequently decreased the concentration of sodium and chloride ions, and plasma osmolality (Monteiro et al., 2005).

The decreased level of albumin (hypoalbuminemia) and total proteins (hypoproteinemia) might be linked with the impairment of kidney and liver of the fish after DLM exposure. Moreover, the decrease in the total proteins in the blood serum might be associated with the alterations in the metabolism or synthesis of protein/free amino acid. In addition, reduction in blood protein might be attributed to increased degradation or proteolytic activity, and/or reduced protein synthesis. The increase in the level of urea might be associated with the gills dysfunction because urea is mainly defecated by gills in fish, not by kidneys (Hassan et al., 2018), which was obvious in the pathological study of the gills. An increase in the level of cholesterol (hypercholesteremia) was observed which might be associated with the failure of the liver and kidney leading to the release of cholesterol at a higher level into the blood. Moreover, it might also be associated with the altered permeability of the hepatic cells. In addition, the pesticides accumulation in the liver disrupted the metabolism of lipids and increased the cholesterol level in the serum (Firat et al., 2011).

4.9. AChE

The inhibition of the AChE leads to nerve impulses, making nerves permeable to sodium and subsequently allowing inflow of sodium into the nerve. DLM might delay the gated sodium channel to close, allowing the heavy flow of sodium that result in continuous and multiple nerve impulses instead of the usual single impulse. The impulses release ACh (a neurotransmitter; which stimulates other nerves), leading to the accumulation of ACh within the nerve synapses, which ultimately result in decreased cholinergic transmission and a number of different neurotoxic effects. These effects include convulsions, opercular movement, and surfacing in fish (Singh et al., 2018). The AChE activity was inhibited by DLM exposure in the tissues of silver carp. The highest inhibition

was observed in the brain of the exposed fish, which might be the basic reason behind the behavioral inconsistencies of the fish, more specifically erratic swimming, equilibrium loss, hyperactivity, and adapting vertical positions. The changes in the swimming pattern might be associated with the inhibition of the activity of neuro-muscular AChE leading to desensitization of nicotine ACh receptors and consequently to muscular weakness after DLM exposure. The results of the current study are congruent with the previous studies on *Catla catla* exposed to DLM (Vani et al., 2011), and *Channa punctatus* exposed to DLM and triazophos (Singh et al., 2018).

4.10. Whole-body cortisol

Cortisol is a corticosteroid hormone. Its structure is highly conserved among vertebrates and is considered as an excellent biomarker for indicating the functional alterations in HPA (hypothalmo-pituitary-interrnal axis) (Kumar et al., 2016). Cortisol plays the key role in organizing fuels (glucose, fatty acids, and lipids) to maintain homeostasis under stress. Organisms require more energy to cope with the stress (Özok et al., 2018). Increase in the activity of cortisol in the current study is corresponding with the increase in glucose level in the blood of the DLM exposed silver carp. It might be associated with the increased demand for energy or to overcome the abrupt change in the hydro-mineral-metabolism under DLM stress. The bidirectional interaction of immune and endocrine systems is obligatory to maintain life under stress while the major end product of stress on physiology is the secretion of cortisol, which regulates energy metabolism. So, the higher activity of cortisol might also be due to the activated interaction between immune system and pituitary-interrenal axis under stress to eliminate DLM; hence, the stress axis released cortisol as an adaptive response. The result of the current study is consistent to previous studies revealing a significantly increased level of cortisol in different fish species after exposure to different SPs pesticides, such as *Labeo rohita* exposed to DLM (Suvetha et al., 2015b), and *Alburnus tarichi* exposed to cypermethrin (Özok et al., 2018).

4.11. DNA damage

Comet assay is employed as a simple, versatile, sensitive, and reliable technique to evaluate the DNA damage *in vitro*, *in vivo*, and *in situ* in response to different pollutants in different fish tissues including liver, gills, and blood (Galindo et al., 2010; Ullah et al., 2016b). However, blood was used to assess DNA damage in the current study, based on its easy collection, and easy processing. Almost 97% of the fish blood is comprised of RBCs, which make fish blood as a widely utilized tissue for evaluating DNA damage through comet assay (Ullah et al., 2017). Pesticides exposure leads to excessive production of ROS and free radical metabolites which consequently lead to DNA disruption/Genotoxicity/DNA damage upon interaction with the DNA. The DNA damage in the current study was initiated after the interaction of the DNA with the DLM or its metabolites in the treated group. The linear increase in the DNA damage might be associated with the clastogenic effect of DLM. The increase in DNA damage with time can be associated with the linear increase in the production of ROS. ROS break DNA either directly or through hydroxyl and hydrogen peroxide ions, consequently leading to DNA having oxidized bases (Ullah et al., 2016b). DNA damage is the outcome of cellular lesions after the failure or weakening of the antioxidant enzymes system to neutralize ROS. The DNA damage was detected as an increase in the studied parameters in the current study. The tail length shows the DNA fragments' size during the unwinding step of SCGE. The tail DNA percentage shows

the percentage of DNA dragged out of the head. OTM shows the distance between the center of the head and the center of the tail and is used to assess the amount of damaged DNA.

4.12. Metabolic enzymes

DLM accumulated on the biological membranes, generated free radicals, increased LPO, and consequently damaged the tissues of silver carp. Aminotransferases (AT) is sensitive to environmental change; therefore, it is widely employed as a handy biomarker in environmental monitoring, chemical risk assessments, and ecotoxicological studies. AT is employed to assess cell inflammation and necrosis. AAT and AIAT are employed to evaluate the injury to the solid tissues of the exposed models (Ullah et al., 2018b). So, the increased activities of AIAT and AAT in the current study indicated that DLM impaired the functions of the cells and injured the tissues of silver carp. LDH (enzyme from glycolysis) was studied to observe the integrity of the cells and intensity of the metabolic process. During stress, the damage to cellular membranes leads to substantial leakage of LDH from the cells (Tkachenko et al., 2013). The increase in LDH was corresponding to the increased activities of AT, which damaged the tissues and subsequently elevated the LDH leakage out of cells (Maksymiv et al., 2015).

4.13. Histomorphology

Histopathological examination is a useful and powerful biomarker for evaluating the toxic effects of chemicals/toxicants/pollutants, deleterious/abrupt environmental change, and environmental stressors. Histopathology emerged as an important biomarker in ecotoxicological studies on account of being rapid and applicable to different tissues of the fish including liver, gills, brain, intestine, and kidney (Ullah et al., 2018b). Histopathology is more sensitive as compared to single biochemical response for evaluating the health of the organism because the histological alterations demonstrate a transition of biological organization from lower-level biochemical effect (at the individual level) to higher-level effects across the population. No significant changes were observed in the gross anatomical and morphometric parameters of silver carp after exposure to DLM, which might be attributed to the shorter duration of the experiment. The results regarding slight but no significant change in the anatomical and morphometric parameters are congruent to previous studies on *Ctenopharyngodon idella* exposed to endosulfan (Hasan et al., 2015), and *Labeo rohita* exposed to malathion (Ullah et al., 2018b). The histopathological damage clearly displayed the acute toxic effects of DLM on fish, which could possibly lead to severe pathophysiological toxicity and ultimately result in death.

Gills are involved in a number of extremely important functions such as respiration, excretion, acid-base balance, and osmoregulation. Gill is the primary organ of contact of fish with the environment and all toxicants in it. Therefore, the morphology of gills is a key indicator in ecotoxicological and environmental monitoring studies. The changes observed in the gills of DLM exposed silver carp were the disruption of gill arch, lamellae and epithelium, necrosis, atrophy of lamellae, accumulation of calcium, desquamation of epithelial and goblet cells, detachment and degeneration of lamellae, and fusion and folding of lamellae. The severe histopathological alterations induced by DLM in the gills of silver carp might be associated with the higher lipophilicity of DLM. The severity of DLM against several fish species is well documented, which might be due to the higher absorption rate of absorption of DLM through gills. The liver is the center for detoxification of almost all types of chemicals/toxicants including pesticides (Ullah et al., 2018b). The severe changes observed in the liver

(congestion, increase in sinusoidal spaces, sinusoidal dilation, fibrosis, necrosis, hemosiderosis, the disorientation of hepatic cells, pycnosis, accumulation of inflammatory cells, and cellular shrinkage) demonstrated liver as the primary organ of DLM detoxification. The histopathological alterations in liver and intestine are a typical biomarker of stress in fish (Glover et al., 2007). The changes observed in the intestine of silver carp after exposure to DLM were disruption and shredding of mucosal cells, the eruption of the intestinal mucosa, necrosis, a higher number of goblet cells with cellular infiltration, and separation of the basement membrane. The increase in the goblet cells might be associated with the activation of the defense system against DLM. The goblet (highly-polarized secretory) cells play a key protective role under stress. The goblet cells produce and secrete different mediators such as TEF3 (trefoil factor 3 – cell migration and apoptosis inhibition) and MUC2 (mucin – mucus layer formation (lubrication, limiting the passage of molecules into the mucosa) and defense against enteric pathogens) (Hasan et al., 2015). The changes observed in the brain were haemorrhage, discoloration, infiltration, neuronal degeneration, and spongiosis, which might be associated with the lipophilicity of DLM and efficient absorption/accumulation of DLM by the brain of silver carp.

The results are congruent with previous studies. A number of investigators reported the induction of different types of lesions in different fish species intoxicated with different SPs, such as DLM exposed *Oreochromis niloticus* (El-Sayed and Saad, 2008), cypermethrin exposed *Danio rerio* (Shi et al., 2011), DLM and diazinon exposed *Pangasius hypophthalmus* (Hedayati and Tarkhani, 2014), cypermethrin exposed *Tor putitora* (Ullah et al., 2015) and DLM exposed *Cyprinus carpio* (Stara et al., 2015).

5. Conclusion

The results revealed 2 µg/L DLM to be LC₅₀ (for 96 h) against silver carp, indicating DLM as severely toxic to silver carp. At acute concentration, DLM exposure led to behavioral inconsistencies, alterations in hematological parameters, induced oxidative stress (measured in terms of increase in ROS production, LPO, and activities of antioxidant enzymes including SOD, POD, CAT, and GR), and increased the activities of metabolic enzymes (AAT, AIAT, LDH, and GDH) in brain, gills, liver, and muscle tissues of silver carp. The level of whole-body cortisol and blood glucose increased while total protein decreased in liver, brain, gills, and muscle tissues. DLM exposure inhibited AChE activity in brain, muscles, and liver, while increased the level of DNA damage in peripheral blood erythrocytes. Exposure to DLM resulted in altered serum biochemical profile such as increased the level of potassium, cholesterol, urea, and total bilirubin, however, decreased the level of sodium, chloride, albumin, total protein, and inorganic phosphate. The acute concentration of DLM resulted in different severe histopathological changes in the liver, brain, intestine, and gills of silver carp. The current study clearly concluded and classified DLM to be highly deleterious to fish. Therefore, its extensive and injudicious use should be regularly and strictly monitored. DLM use should be allowed under strict environmental regulations. Otherwise, as observed in the current study, DLM can lead to mass mortalities and consequently to the extinction of the highly economical and widely consumed fish species, such as the nearly threatened silver carp at very little concentration.

Conflicts of interest

The authors report no conflicts of interest in this work.

Ethical statement

The research was conducted according to the ethical standards. All applicable institutional, national, and/or international guidelines for the care and use of animals were followed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2018.09.145>.

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