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RESEARCH ARTICLE

Study of Oxidative Stress and Histo-Biochemical Biomarkers of Diethyl Phthalate Induced Toxicity in a Cultureable Fish, *Labeo rohita*

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A B S T R A C T

Diethyl phthalate (DEP) is a widely used low-molecular weight phthalate which is ubiquitously detected in almost all kind of the environmental matrices. The aim of the present study was to investigate the impact of DEP-induced toxicity on a suite of biomarker responses in *Labeo rohita* (rohu). The median lethal concentration of DEP was found to be 4.38 mg L^{-1} for 96 h. Fingerlings were exposed to a sub-lethal concentration of DEP *i.e.* 0.51 mg L^{-1}($1/10^{th}$ LC_{50}) for a period of 21 day of the experiment. Oxidative stress and histo-biochemical biomarkers were studied using gills, liver, kidney and brain tissues on 7, 14 and 21 day. The results indicated that DEP-exposure has damaged the antioxidant status by inhibiting the activities of enzymatic stress markers (catalase, glutathione-s-transferase, glutathione peroxidase, reduced glutathione) in all the studied tissues of rohu throughout the study period. Moreover, a significant increase in the levels of lipid peroxidation was observed in all the studied tissues in a time-dependent manner. The levels of hepatic-nephric biomarkers (ALT, AST, ALP, Urea and Creatinine) were found to be significantly elevated for DEP-exposed rohu when compared to control ($P<0.05$) throughout the study period. The histopathological marker showed severe lesions in the gills (hypertrophy, fusion and curling of lamellae,), liver (pyknotic nuclei, leukocytes infiltration and vacuolization) and kidney tissues (glomerulus expansion, narrowing and elongation of renal tubules) of DEP-exposed rohu. In conclusion, the above mentioned histo-biochemical parameters could be used as biomarkers for DEP toxicity monitoring in the aquatic ecosystem.

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INTRODUCTION

Biomarkers of exposure are early physiological and biochemical alterations that occur as a response of exposure to a contaminant (Hook *et al*., 2014; Hussain *et al.,* 2018). Oxidative stress is one of the important biomarker of exposure and mostly used for toxicological assessments (Hameed *et al*., 2017; Ghaffar *et al.,* 2018; Jaffar *et al*., 2019). Oxidative stress is caused by excess production of free radicles and reactive oxygen species (ROS) which readily reacts with the cellular macromolecules resulting in their impaired structures and functions (Regoli and Giuliani, 2014; Hameed *et al*., 2017). Enzymatic oxidative stress markers such as catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and non-enzymatic markers like reduced glutathione (GSH), vitamin E, A and heat shock proteins provides defense by neutralizing these ROS and therefore; maintain normal physiological processes of the body (Gauvin *et al*., 2017).

Liver key-functioning enzymes (aminotransferases and phosphatases) are considered as potential biomarkers of effects in toxicological studies showing the state of liver injury *(*Mcgill, 2016; Hussain *et al.,* 2019). Histological alternations in different tissues (gills and liver) have been used as highly sensitive biomarkers for xenobiotic induced toxic effects on the fish health (Paithane *et al*., 2012). Fish are the inhabitant of aquatic enviornment and are first to get exposed to all the chemicals released into the aquatic enviornment therfore; extensively used as bioindicator species in biomonitoring programmes (Oost *et al*., 2003).

Phthalates are esters of phthalic acid and were first synthesized in 1930. Since then they are frequently used in plastic industries for enhancing the durability and elasticity of plastics and their products (Li *et al*., 2017). Diethyl phthalate (DEP) is a low molecular weight phthalate and used as a solvent in plastic industry and in the manufacture of personal-care products like perfumes, shampoo, soap, after shave lotions and cosmetics (Jurewicz *et al*., 2013). DEP has been detected in water (Cheng *et al*., 2019), sediments (Chen *et al.*, 2017) and agricultural soil (Sun *et al*., 2018) throughout the globe.

Fish can absorb phthalates from contaimnated water through gills during respiration, dermal expoure and intake of contaminated food particles and sediments (Bhatia *et al*., 2014). Several *in-vivo* studies showed that DEP exposure caused endocrine disruption (Barse *et al.*, 2007), oxidative stress (Kang *et al.*, 2010), neurotoxic (Ghorpade *et al*., 2002), embryo toxic (Zhou *et al*., 2011), hepatotoxic (Zhang, 2014), hematotoxic (Poopal *et al*., 2017) and histopathlogical effects (George *et al*., 2017) in different culturable fish species.

Labeo rohita (Rohu) is the most prestigious culturable fish species and highly demanded as food fish in Pakistan.Therefore, the present study was conducted to determine the toxic effects of DEP exposure on rohu fingerlings using multiple biomarkers approach.

MATERIALS AND METHODS

Fish procurement and maintenance: Healthy *L. rohita* fingerlings (8.502±0.022 g) procured from a commercial fish seed hatchery, Lahore and were acclimatized to the laboratory conditions prior to the experiment for 15 days in glass aquaria. During the acclimatization, fish were fed on commercial feed at 5% of their wet body weight and also three fourth of the aquaria water was changed on the daily basis. In the present study, the average values for the studied physiochemical parameters of water were recorded as: temperature (27ºC), pH (8.2), dissolved oxygen (5.3 mg L^{-1}), total hardness (148 mg L^{-1}), total alkalinity (380 mg L^{-1}) and electrical conductivity (613 µS cm^{-1}).

Toxicant used: Stock solution of diethyl phthalate (CAS number: 84-66-2; molecular weight: 22.24) was prepared in dimethyl sulfoxide. Various working concentrations of DEP were prepared by dissolving appropriate amount of stock in aquaria water.

Determination of median lethal concentration (LC_{50}) of DEP: Fingerlings were randomly divided into 20 groups (6 fish/group/aquarium). The graded concentrations of DEP (0.5 to 10 mg L^{-1}) with an increment of 0.5 mg L^{-1} were added into each aquarium (50 L tap water). During this period water was replaced daily and fresh toxicant was added for 96 h. Fish were monitored at regular intervals and mortality data was recorded. The median lethal concentration (LC_{50}) of DEP for 96 hours was calculated using probit regression analysis (Faheem and Lone, 2017). The experiment was conducted following OECD standardized guidelines for semi-static bioassays.

Sub-lethal studies: For sub-lethal studies, 0.51 mg L^{-1} of DEP ($1/10^{th}$ of LC_{50}) was added into each glass aquaria and 20 fingerlings per aquarium were introduced. The experiment was done in triplicates. A negative control group without toxicant was also maintained. Water was changed daily in order to avoid the accumulation of fish waste and any leftover feed and renewed by adding the fresh toxicant for 21 days.

Collection of tissues for biochemical assays and histopathology: At 7^{th}, 14^{th} and 21^{st} day of experiment, fish (5fish/group/replicate) were randomly taken out and anesthetized. Fish were dissected humanely and tissues (gills, liver, kidney and brain) were collected. One portion of each tissue was cleaned of extraneous tissues in sodium phosphate buffer (0.1 M, pH: 7.4), snap frozen in liquid nitrogen and stored at -20ºC until further biochemical assays were performed. While, the other portion of tissues (gills, liver and kidney) were preserved in bouin fixative for histological preparations.

Determination of enzymatic and non-enzymatic oxidative stress assays: For performing the oxidative stress assays, each tissue was homogenized in chilled sodium phosphate buffer and centrifuged (4ºC) at 13000 rpm for 30 minutes and the post mitochondrial supernatant was stored in labeled eppendorfs at -20ºC until further biochemical assays were performed. While, the other portion of homogenate was used for estimating the levels of tissue lipid peroxidation. Enzymatic and non-enzymatic oxidative stress assays (CAT, GST, GSH and LPO) were performed by following the protocol of Faheem and Lone (2017). The GPx assay was performed by following the protocol of Mohandas *et al*. (1984).

Determination of hepato-nephric key-functioning enzymes levels: Tissue homogenates were used for estimation of ALT, AST, ALP, Urea and Creatinine. The biochemical assays were performed using commercially available kits (Randox, UK).

Histological preparations: For the histological preparations tissues were processed through the serial grades of ethanol, cleared in xylene and impregnated with wax. Tissues sections were cut at five microns thickness and were stained with hematoxylin and eosin (H&E) by following Ortiz-Ordonez *et al*. (2011). The slides were studied and photographed.

Statistical analysis: The data was analyzed using GraphPad prism version 8. Student's '*t*' test was performed at 95% level of significance to compare sample mean between control and DEP-treated rohu.

RESULTS

Effects of DEP on enzymatic and non-enzymatic antioxidant defense system: The effects of DEP-induced toxicity on antioxidant marker enzymes activities in gills, liver, kidney and brain tissues of rohu are presented in Fig 1-4. A significant decrease in the CAT activity of gills, liver and brain tissues of DEP-exposed rohu was recorded at 7^{th} day of sampling (Fig. 1A). However, CAT activity

was significantly increased in the brain and kidney tissues of DEP-exposed rohu at 14th day of sampling (Fig. 1B). At the final day of sampling, CAT activity was found to be significantly decreased in all the studied tissues of DEP-exposed rohu when compared to their respective control groups (Fig. 1C). There was a significant decrease in the GST activity in gills, kidney and brain of DEP-treated rohu at 7th day of sampling however; GST activity was significantly increased in the liver tissue (Fig. 2A). At 14th and 21st day of sampling, GST activity was significantly decreased in all the studied tissues of DEP-treated rohu (Fig. 2B-C). GPx activity was significantly inhibited in gills and kidney tissues at 7th day of sampling however; increased activities of GPx were recorded for liver and brain tissues (Fig. 3A). GPx activity was found to be decreased in all the studied tissues of exposed rohu on subsequent sampling days (Fig. 3B-C).

The levels of non-enzymatic antioxidant defense markers showed significant alternation in all the studied tissues of DEP-exposed rohu when compared to their respective control groups (Fig. 4). A significant increase in GSH level was observed in all the studied tissues of exposed rohu when compared to the control at 7th day of sampling (Fig 4A). While at 14th and 21st day of sampling, GSH level was significantly decreased in gills, kidney and brain tissues of treated rohu (Fig. 4B-C). A significant increase in lipid peroxidation levels was observed in all the studied tissues of DEP-treated rohu when compared to their respective control groups at each sampling day (Fig 5A-C).

Effect on liver and kidney injury biomarkers: The liver key functioning enzymes levels viz., ALT, AST and ALP in DEP-exposed rohu were found to be significantly altered when compared to control groups. A significant increase in hepatic ALT AST and ALP levels was observed in DEP-treated rohu throughout the study period (Table 1). Urea and creatinine levels were also found to be significantly elevated in DEP-exposed rohu when compared to their respective control groups at each sampling day (Table 1).

Histopathological changes in the gills, liver and kidney: Histological lesions were observed in gills, liver and kidney tissues of DEP-exposed rohu when compared to their control groups (Figs. 6-8). Figure 6A shows normal structure of primary and secondary gills lamellae of control fish while, DEP-exposed fish showed severe alternations in the gills structure such as; curling and fusion of secondary lamellae, hypertrophy, hyperplasia and uplifting of epithelium. Moreover; these gills lesions were seemed to be increased as exposure period prolonged (Fig. 6B-D). The histoarchitecture of liver from control group rohu showed normal size and shape of hepatocytes (Fig. 7A). Severe alternations in the liver such as: pyknotic nuclei, hepatocytes swelling, sinusoidal enlargements, vacuolization, macrophage infiltration and necrosis were observed in DEP-exposed rohu throughout the study period (Fig. 7B-D). The kidney structure of rohu under control group showed normal shape, size of bowman's capsule and renal tubules (Fig. 8A) while, severe lesions (enlargement and narrowing of renal tubules, hypoplastic hematopoietic tissue, hypertrophied nucleus and dilation of glomerulus) were noticed in kidney tissue of DEP-exposed rohu at each sampling (Fig. 8 B-D).

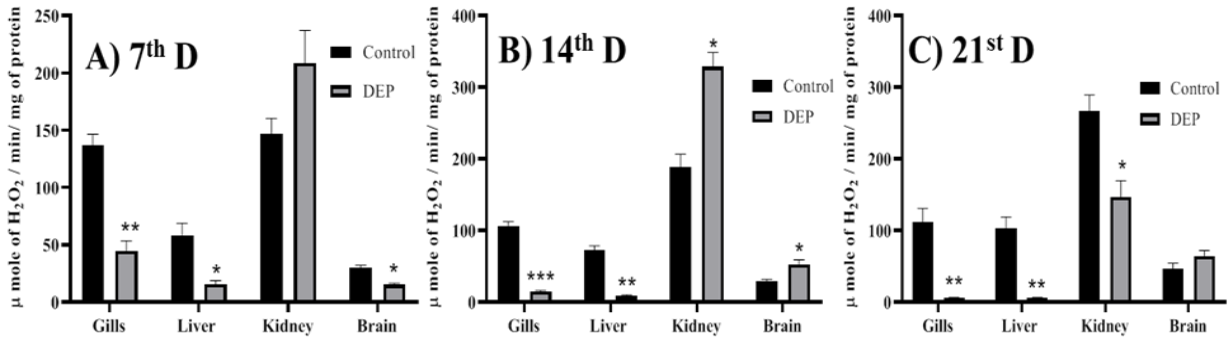

Fig. 1: CAT activity in different tissues of *L. rohita* form control and DEP-exposed groups at 7th (A), 14th (B) and 21st (C) day of sampling. Each value represents the mean±S.E.M (n=5). Columns with different asterisk are significantly different (P<0.05).

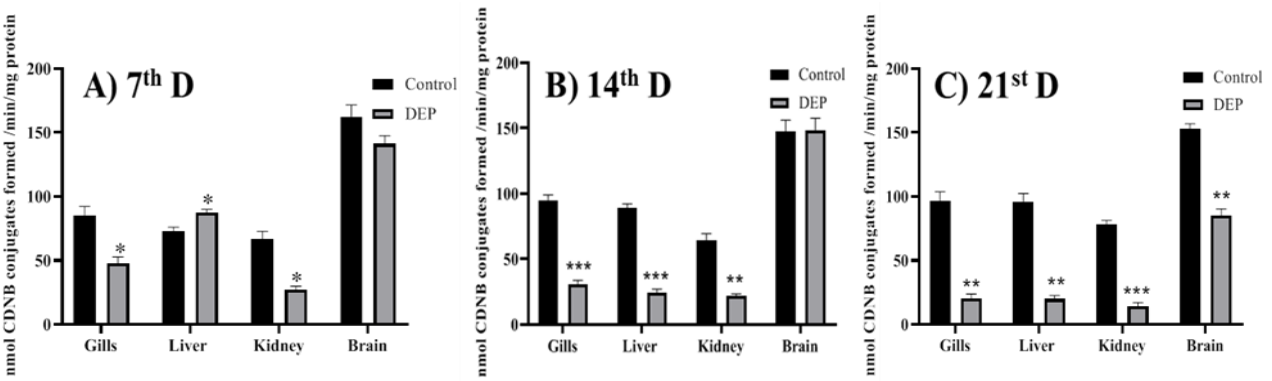

Fig. 2: GST activity in different tissues of *L. rohita* form control and DEP-exposed groups at 7th (A), 14th (B) and 21st (C) day of sampling. Each value represents the mean±S.E.M (n=5). Columns with different asterisk are significantly different (P<0.05).

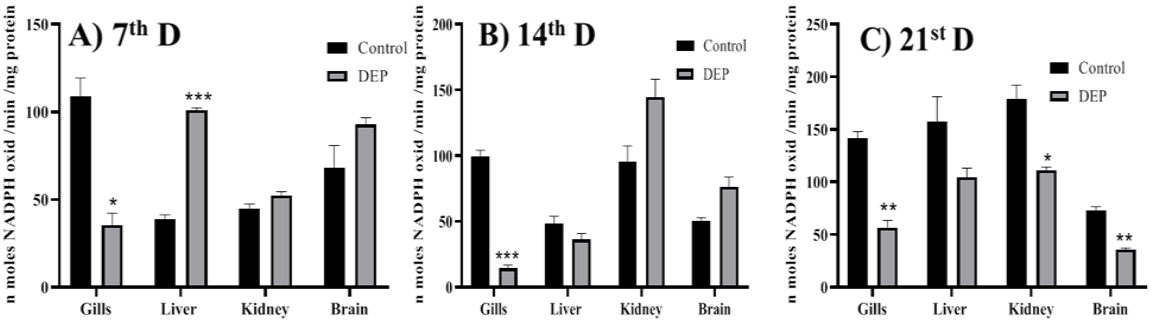

Fig. 3: GPx activity in different tissues of *L. rohita* form control and DEP-exposed groups at 7th (A), 14th (B) and 21st (C) day of sampling. Each value represents the mean±S.E.M (n=5). Columns with different asterisk are significantly different (P<0.05).

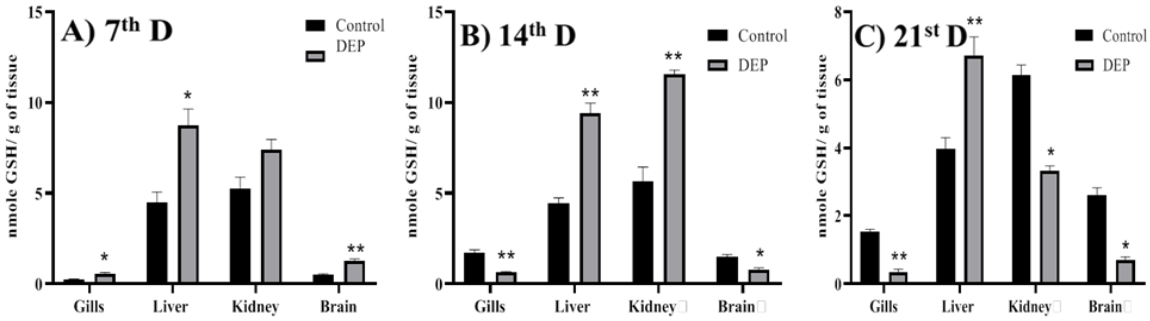

Fig. 4: GSH levels in different tissues of *L. rohita* form control and DEP-exposed groups at 7th (A), 14th (B) and 21st (C) day of sampling. Each value represents the mean±S.E.M (n=5). Columns with different asterisk are significantly different (P<0.05).

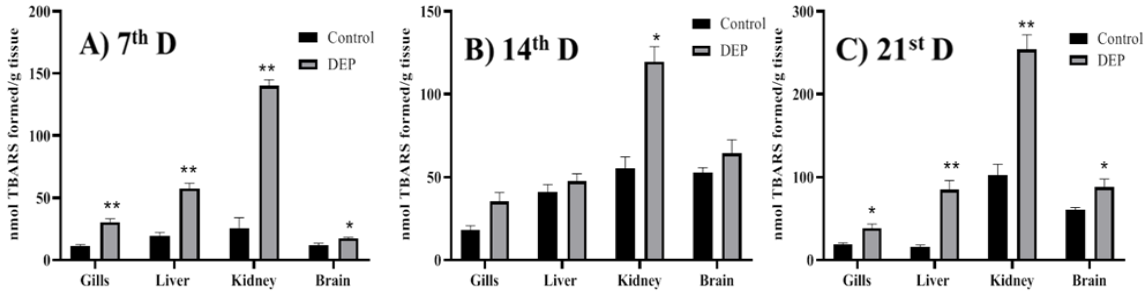

Fig. 5: LPO levels in different tissues of *L. rohita* form control and DEP-exposed groups at 7th (A), 14th (B) and 21st (C) day of sampling. Each value represents the mean±S.E.M (n=5). Columns with different asterisk are significantly different (P<0.05).

DISCUSSION

The present study investigated the impact of a sub-lethal concentration of DEP-induced toxicity on a suite of biomarkers response in some vital organs of *L. rohita* for 21 days. Phthalates are emerging environmental pollutant. Low-molecular weight phthalates are not covalently bounded to their substrate are therefore easily leached out ending up in the environment. Phthalates exposure has been reported to cause oxidative stress in different tissues and plasma of fish elucidated by their excessive ROS production and alternations in their antioxidant enzymes activities (Kang *et al*., 2010; Zhang, 2014).

The results of present study indicated that DEP-exposure has significantly affected the antioxidant defense system of rohu indicated by reduced activities of oxidative stress marker enzymes in the gills, liver, kidney and brain tissues of rohu throughout the study period. CAT is highly sensitive and provides the first line of defense against ROS thereby protecting the organism from oxidative stress (Gauvin *et al*., 2017). In the present study, a significant decrease in the CAT activity was observed in all the studied tissues of DEP-exposed rohu indicating the excess production of ROS than the scavenging ability of this antioxidant enzyme. *Clarias gariepinus* exposed to sub-lethal concentration of DEP (0.01 µg L^{-1}) showed decreased CAT activity in its liver and kidney tissues (Ikele *et al*., 2016). The CAT activity was significantly reduced in the gills and liver tissue of *Cyprinus carpio* exposed to the sub-lethal concentrations (2.65, 5.3 mg L^{-1}) of DEP for 35 days (Poopal *et al*., 2017).

GST is a phase-II biotransformation antioxidant enzyme which is extensively used as oxidative stress biomarker in response to the environmental pollutants induced toxicity (Regoli and Giuliani, 2014). GST activity was primarily increased in the studied tissues of DEP-exposed rohu followed by reduced activity with increased duration of exposure. *Paralichthys olivaceus* juveniles exposed to a sub-lethal concentration of DEP (900 mg Kg^{-1}) showed significant decrease in kidney tissue GST activity (Kang *et al*., 2010). Zhang (2014) reported GST activity was decreased in carp liver tissue exposed to different sub-lethal concentrations of DEP for 20 days. Decreased GST activity might be due to the long term exposure to DEP and decreased GSH levels thus, unbalancing the antioxidant defense system of this fish.

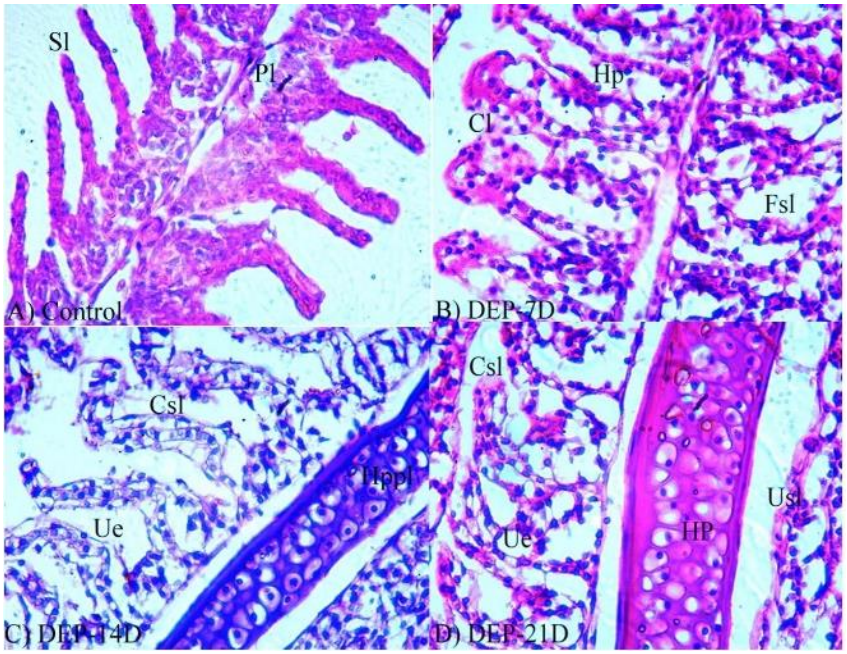

Fig. 6: Gills tissue of *L. rohita* from control (A) and DEP exposed group at 7th (B), 14th (C), and 21st (D) day of the experiment. (Pl) primary lamellae; (Sl) secondary lamellae; (Hp) hyperplasia; (Fsl) fusion of secondary lamellae; (Cl) clubbing of secondary lamellae tips; (Hppl) hyperplasia of primary lamellae; (Ue) uplifting of epithelium; (Csl) curling of secondary lamellae and (Usl) uplifting of secondary lamellae H & E stain, 400X.

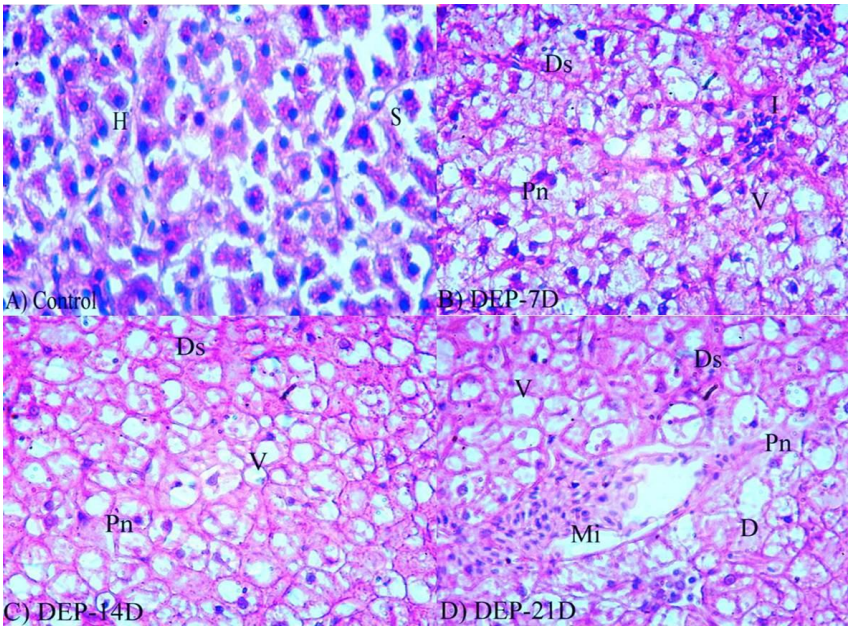

Fig. 7: Liver tissue of *L. rohita* from control (A) and DEP exposed group at 7th (B), 14th (C), and 21st (D) day of the experiment. (H) hepatocytes; (S) sinusoids; (I) infiltration; (Pn) pyknotic nuclei; (Ds) dilated sinusoids; (V) vacuolization; (Mi) macrophage infiltration and (D) degeneration. H & E stain, 400X.

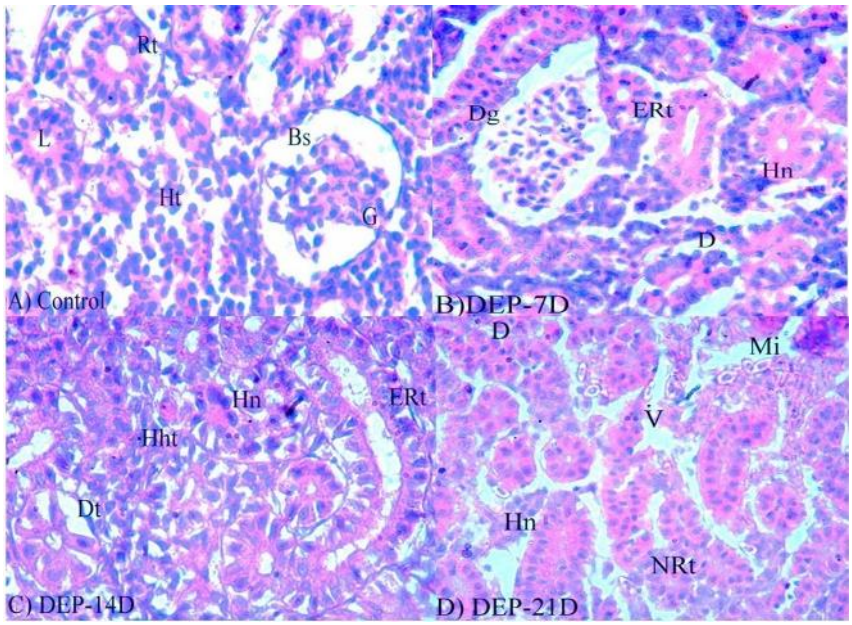

Fig. 8: Kidney tissue of *L. rohita* from control (A) and DEP exposed group at 7th (B), 14th (C), and 21st (D) day of the experiment. (G); glomerulus; (Bs); bowmen's space; (Rt); renal tubules; (Ht) hematopoietic tissue; (L) tubule lumen; (Dg) degenerating glomerulus; (ERt) elongated renal tubules; (D) degeneration; (Hn) hypertrophoied nucleus; (Dt) degenerating tubules; (Hht) hypoplastic hematopoietic tissue; (Hn) hypertrophoied nucleus; (V) vacuolization (MI) macrophage infiltration and (NRt) narrowing of renal tubules. H & E stain, 400X.

Table 1: Impact of DEP-exposure on hepato-nephric key-functioning markers of *L. rohita* (Rohu).

Parameters	Sampling days (D)	Control group	DEP-exposed group
ALT (U/L)	7th	24.83±0.987	40.93±0.823****
	14th	21.94±0.925	42.10±2.32***
	21st	26.01±0.874	45.01±0.508**
AST (U/L)	7th	27.11±1.041	30.42±0.945*
	14th	27.69±1.592	35.84±1.859**
	21st	27.60±1.129	38.12±2.168**
ALP (U/L)	7th	460.0±0.518	743.4±0.216***
	14th	505.5±0.216	836.3±0.110**
	21st	510.3±0.129	877.1±0.470*
Urea (mg/dl)	7th	2.963±0.248	4.961±0.383*
	14th	3.376±0.300	6.133±0.664**
	21st	3.032±0.275	6.684±0.600**
Creatinine (mg/dl)	7th	0.152±0.009	0.224±0.008**
	14th	0.161±0.026	0.251±0.047
	21st	0.152±0.058	0.295±0.034

Values are expressed as mean± S.E.M of 5 fish per group. *=P<0.05, **=P<0.01, ***=P<0.001.

GPx is a major antioxidant enzyme that along with some other antioxidant enzymes (CAT and SOD) catalyzes the reduction of hydroperoxides into the hydroxyl compounds therefore; helps to maintain the oxidant and antioxidant balance in aquatic organisms (Zhou *et al*., 2011). The present study results showed decreased GPx activity in the studied tissues of DEP-treated rohu when compared to the control group. Revath and Chitra (2018) reported long-term exposure to sub-lethal concentrations of diisononyl phthalate (DINP) significantly decreased GPx activity in the gills, liver and muscles tissues of *Oreochromis mossambicus*. GPx activity decreased in liver and kidney tissues of *C. gariepinus* exposed to sub-lethal concentration of DEP (0.05µg L^{-1}) for 21 days (Ikele *et al*., 2016). These finding supports the results of the present study. In our study, inhibition of GPx activity could be ascribed to the overwhelmed antioxidant defense by the excessive hydroperoxides production during the DEP metabolism for a long period following DEP-exposure.

GSH is a thiol containing non-enzymatic antioxidant biomarker which has a pivotal role in the detoxification of xenobiotics by scavenging the ROS (Stein *et al*., 1992). The present study results indicated DEP-exposure has significantly decreased GSH level in all the studied tissues of rohu throughout the study period. In contrast to present study results, elevated GSH level in the liver and kidney tissues of *P. olivaceus* exposed to a sub-lethal concentration of DEP (900 mg Kg^{-1}) for three days was reported (Kang *et al*., 2010). This discrepancy in results may be attributed to the concentration, duration and routes of exposure and also susceptibility of different fish species to DEP.

The imbalance between oxidant and antioxidant defense system initiates the excessive production of ROS, which readily reacts with the unsaturated fatty acids found in the membrane phospholipids therefore; inducing the state of oxidative stress (Gauvin *et al*., 2017). In our study, lipid peroxidation level was significantly increased in all studied tissues of DEP-exposed rohu throughout the study period. Several researchers reported increased lipid peroxidation levels in different tissues of fish exposed to sub-lethal concentrations of DEP (Kang *et al*., 2010; Ikele *et al*., 2016; Poopal *et al*., 2017). All these aforementioned reports are in agreement with our results.

Transaminases are abundantly found in liver tissue and are involved in protein metabolism. Their elevated levels in serum and tissues indicate the state of liver injury and routinely used as biomarker in toxicological studies (Faheem *et al*., 2019). In our study, hepatic ALT, AST and ALP were found to be significantly elevated for DEP-exposed rohu throughout the study period. Similar to our results, several other studies reported elevated levels of ALT, AST and ALP following exposure to the different sub-lethal concentrations of DEP in the serum and tissues of different fish species (Ghorpade *et al*., 2002; Kang *et al*., 2010 and Poopal *et al*., 2017). Furthermore, the results of present study indicated elevated levels of urea and creatinine for DEP-exposed rohu. Hamed and Abdel-Tawwab (2017) reported a significant increase in the serum creatinine and uric acid levels of *O niloticus* exposed to a sub-lethal concentration (1.64 µg L-1) of bisphenol-A for 6 weeks. This report is in agreement with our results.

Gills are most susceptible to the toxicant exposure due to their direct interaction with the environment therefore; histological studies of gills are frequently used as biomarkers (Paithane *et al*., 2012). The results of present study showed severe lesion in gills structure of DEP-exposed rohu. Similar to our results, *C. gariepinus* fingerlings exposed to different sub-lethal concentrations of DEP showed histopathlogical alternations (fusion, rising of the filaments, oedema, uplifting and loss of epithelium) in the gills structure (Obiezue *et al*., 2014).

In the present study, the elevated levels of hepatic ALT, AST and ALP along with histopathlogical lesions of liver tissue further confirms the DEP-induced hepatotoxic effects. Ikele *et al*. (2011) reported *Clarias gariepinus* fingerlings exposed to different sub-lethal concentrations of DEP has severe lesions in liver tissue such as; congestion, fatty changes, pyknotic nuclei and enlargement of sinusoids. Severe histopathlogical lesions (hepatocyte degeneration, necrosis, vacuolization and shrinkage of nucleus) were found in the liver tissue of fish exposed to different concentrations of DEP (Obiezue *et al*., 2014; George *et al*., 2017). All aforementioned reports are supporting the results of present study.

The elevated levels of urea and creatinine in DEP-exposed rohu were further confirmed by histopathlogical lesions found in their kidney tissues. Moreover, these lesions were found to be increased as the experimental duration was prolonged. Ikele *et al*, (2011) reported severe histopathlogical changes (pyknotic nuclei, destruction of renal proximal and distal tubules) in the kidney tissue of *C. gariepinus* exposed to DEP for 30 days. The histo-architecture of kidney tissues of *Channa striatus* showed severe lesions (glomerular distortion, necrosis and atrophy of renal tubules) when get exposed to DEP (George *et al*., 2017). These reports are in agreement with the results of present study.

Conclusions: Results presented here indicated that DEP exposure has elicited oxidative stress, biochemical alternations and histopathological lesions in all the studied tissues of *L. rohita*. Therefore; studied parameters in our study could be used as biomarkers of exposure to DEP-toxicity in ecotoxicological assessments. More research should be done to study the underlying molecular mechanism of DEP toxicity in aquatic organisms.

Authors contribution: ML designed, executed and analyzed the tissue samples in this study under supervision of MF and A. MF analyzed and interpreted the data. MF and A critically revised the manuscript and approved the final version.

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