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RESEARCH ARTICLE

Di(2-ethylhexyl)phthalate-induced histopathological changes in gill and liver of freshwater fish, *Oreochromis mossambicus* (Peters, 1852)

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Abstract

Di(2-ethylhexyl)phthalate (DEHP), a low molecular weight phthalate was exposed to the freshwater fish, *Oreochromis mossambicus* for 96 h. Acute toxicity of DEHP was evaluated by exposing the contaminant at six different concentrations as 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm, 60 ppm/ L along with positive (propylene glycol was used as vehicle) and negative control groups. Fishes when exposed to DEHP showed aggressive behaviour, restlessness and loss of equilibrium immediately after exposure and it continued only for a day of treatment. Body weight of the animal and the weights of gill and liver tissues remained unchanged throughout the experiment. The main histopathological changes observed in fish gill after exposure to DEHP were upliftment and blebbing of lamellar epithelium, erythrocyte infiltration, aneurysim, loss of primary and secondary lamellae, shortening and curling of secondary lamellae, and complete destruction of gill lamellae in concentration-dependent manner. Liver of control fish exhibited a normal architecture while the fish exposed to DEHP showed cytoplasmic degeneration followed by vacuolization, erythrocyte infiltration, elongated or absence of hepatic nucleus, necrosis and bile stagnation. These changes were prominent and their effects could be observed at different increasing concentrations. The results suggest that acute exposure to DEHP altered the histoarchitecture of gill and liver in *Oreochromis mossambicus*.

INTRODUCTION

Di(2-ethylhexyl)phthalate (DEHP) is a plasticizer used for polyvinyl chloride (PVC) products. It was estimated that the global production of DEHP is about 1 to 4 million tonnes per year. DEHP are released into the environment during production, transport, storage, formulation and processing of PVC and non-polymers. Therefore, DEHP is directly distributed and released into air and waste water, from sewage sludge and solid wastes. Approximately 97% of DEHP is used as plasticizers in polymers, mainly PVC and between 2-3% is used in non-PVC polymers while 2% is used for non-polymer applications. DEHP is known to bioaccumulate in aquatic organisms and it break down in the presence of other chemicals to produce mono(2-ethylhexyl)phthalate (MEHP) and 2-ethylhexanol (European Chemical Bureau, 2008).

Humans are exposed to DEHP through the direct use of consumer products and indirect environmental exposure or due to occupational exposure. For the general population, food is considered as the major source of DEHP exposure and DEHP in food might originate from the environment, food processing or food packaging (Schecter et al., 2013). Children are additionally exposed to DEHP because of mouthing of toys and also human breast milk can be a source of DEHP exposure for nursing babies (Main et al., 2006).

Several consistent short-term and long-term studies have been reported on the effects of DEHP on aquatic organisms. However, there are no studies indicating the effects of DEHP on aquatic animals only exposed through water, and at concentrations below the water solubility. Few studies had reported that there were no possible adverse effects to the aquatic organism after exposure to DEHP below the level of water solubility i.e., at 0.003-1.3 mg/l at room temperature (Staples et al., 1997). In the present study DEHP was dissolved in propylene glycol, an organic solvent as a vehicle to suspend the compound. The effects of DEHP at several concentrations up to maximum solubility range in propylene glycol were used in the present study to evaluate the adverse acute effect of DEHP on freshwater fish, *Oreochromis mossambicus*.

Materials and Methods:

Freshwater fish, Oreochromis mossambicus weighing 3.5 ± 0.75 g and length 5.5 ± 1.5 cm were collected from a fish farm, Safa Aquarium, Kozhikode, Kerala. Fishes were transported to the laboratory with least disturbance and were acclimatized in glass aquarium tanks to the laboratory conditions prior to experiments. Each well-aerated tanks (40 L capacity) were provided with good lighting system was dechlorinated at regular intervals.

The physico-chemical features of the tap water were estimated as per APHA (1998). Water temperature in the test ranged from 28 ± 2°C during the experiment, oxygen saturation of water ranged between 70 and 100 %, pH is 7.6 which were monitored using a standardized measures.

Di(2-ethylhexyl)phthalate (DEHP; CAS No. 117817) of 99.7% purity was obtained from Sigma Aldrich chemical Co., USA. Propylene glycol, an organic solvent was used to dissolve DEHP, where 16 µl of 1 M propylene glycol was sufficient to dissolve 60 ppm of the toxicant by sonication at 50 Hz for 15 minutes with 30sec pulse interval. Therefore, in the present study based on the solubility in vehicle solution DEHP at 60 ppm/ L was taken as a maximum concentration. Experiments were carried out with 10 animals per group maintaining 3 groups of animals for 96 h as follows:

Group I – Positive control: Propylene glycol (16 µl/ L; 1 M).

Group II – Negative control: Toxicant/ solvent-free water.

Group III – DEHP-treated. It has six subgroups at different concentrations of DEHP as 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm, 60 ppm/ L dissolved in propylene glycol.

Each treatment tanks were covered with monofilament nets to prevent the animals from jumping out of test solutions. Mortality and the behaviour of fishes were recorded throughout the study.

At the end of the treatment the body weights of animals in each treatment groups were recorded along with the weights of gill and liver. Data are presented as mean ± SD for ten animals per group. Tissues were preserved in 10% buffered formalin for 24 hours and processed to prepare sections of 4 to 6 microns thickness. The sections were stained with haematoxylin and eosin and mounted in DPx. The structural alteration of gill and liver tissues were observed under light microscope and compared with those of control tissues. Photomicrographs were taken using Catcam shot camera fitted to the Carl Zeiss Axioscope 2 Plus Trinocular Research Microscope.

Results:

Animals when exposed to DEHP at six different concentrations (10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm and 60 ppm/ L) for 96 h did not caused treatment related mortality throughout the study. However, behavioural modifications were noticed in DEHP-treated animals as evidenced by initial stress in swimming behavior. Failure of orientation while swimming, lack of feeding, engulfment of air, hitting on side walls, bottom-dwelling and immobile posture followed by loss of balance were noticed for 12 h after DEHP treatment. After 24 h of DEHP exposure black pigmentation developed throughout the body of treated animal and it retained throughout the end of the experiment (96 h).

The body weights of animal and the weights of liver and gill remained unchanged when compared to the control groups (Table 1). Histopathological observations revealed the normal architecture of control gill (Figure A), however, DEHP exposure at 10 ppm/ L showed damage in gill epithelium, degeneration of gill arches and secondary lamellae and erythrocyte infiltration (Figure B). DEHP at 20 ppm/ L showed absence of gill arches, curling of secondary lamellae and erythrocyte infiltration (Figure C). Aneurysm and deformed gill lamellae was observed at 30 ppm/ L concentration of DEHP exposure (Figure D) whereas at 40 ppm/ L concentration showed enlarged primary lamellae and absence of secondary lamellae (Figure E). Fishes when treated to 50 ppm/ L concentration of DEHP reported to have shortened primary lamellae, erythrocyte infiltration and shortened and curly secondary lamellae (Figure F). Administration of DEHP at 60 ppm/ L showed degenerated gill lamellae, gill arches and a complete necrosis (Figure G).

The histology of control fish liver revealed normal typical paranchymatous polygonal hepatocytes with a central spherical nucleus (Figure 1). Fishes treated to DEHP at 30 ppm/ L onwards showed parenchymatous degeneration, bile stagnation and severe necrosis with other modifications as vacuolization, elongated nucleus and few regions with enucleated hepatocytes were noticed (Figure 2).

Discussion:

Phthalates have an important role in the production of plastics and other materials that have many versatile uses in industry, medicine and in consumer products. In view of more recent scientific research, there are raising concerns regarding the possible environmental and health effects of such plasticizers and their risks of exposure are being kept under close review by several national and international bodies. DEHP is the most abundantly produced phthalate products and are released widely into the environment. DEHP that reaches aquatic environment may cause adverse effects on food chains of aquatic organisms. In the present study acute toxicity tests on the freshwater fish, *Oreochromis mossambicus* was done as a preliminary step for the assessment of the environmental risks of DEHP on aquatic systems.

Behaviour of the fishes is monitored continuously throughout the study. Control fishes during the experiment maintained in well-aerated water was found very active and showed immediate response even to a slight disturbance and their behavior was normal showing movements in well co-ordinated manner. It is well known that fishes exposed to contaminated environment undergo stress which is shown by the altered behavioural pattern. It includes changes in locomotor, feeding and aggressive behaviour and these may be the attempts by the fishes to escape or adjust to the stress condition (Asifa and Chitra, 2015).

DEHP-exposed fishes showed initial stress in swimming behavior. Swimming is one of the most frequently used behavioural traits on fish ecotoxicological research because impaired swimming has severe consequences on feeding, orientation and reproduction (Little and Finger, 1990). Hyperactivity that was noticed initially after DEHP exposure may be due to the stress induced by the toxicant. Hyperactivity is considered as a primary and principal sign of failure of nervous system due to exposure to contaminant which also affects physiological and biochemical activities (Matsumura, 1975). Failure of orientation while swimming, lack of feeding, engulfment of air, hitting on side walls, bottom-dwelling and immobile posture followed by loss of balance were noticed for 12 h after DEHP treatment. The disruption of the functioning of nervous system of fish might be the cause of slow and lethargic swimming, erratic swimming and loss of equilibrium (Pal and Konar, 1989). After 24 h of DEHP exposure black pigmentation developed throughout the body of treated animal and it continued up to 96 h of treatment. Therefore, the study of fish behavior is considered as a selective tool where fishes act as a bio-indicator because they are very sensitive to the change in their environment and play an important role to monitor aquatic contamination.

DEHP did not alter the body weights and also the weights of liver and gill of fish when compared to the control groups and this would indicate that the toxicant did not cause systemic toxicity at acute exposure. Similarly fishes treated with propylene glycol did not cause any behavioural changes and also the body weights or organ weights like that of negative control group (Revathy and Chitra, 2015).

Histological study is a noteworthy and relevant parameter to understand the extent to which the changes that occurred in the structural organization of organs due to exposure to toxicants. Gill is an important organ for respiration, osmoregulation, acid-base balance and nitrogenous waste excretion and they are readily exposed to contaminants which often cause pathology to fish (Mallatt, 1985). In the present study gills showed a typical structural organization of the lamellae in the control fish whereas treatment of DEHP at 10 ppm/ L concentration showed damage in gill epithelium, degeneration of gill arches and secondary lamellae and also erythrocyte infiltration. Absence of gill arches, curling of secondary lamellae and erythrocyte infiltration were observed at 20 ppm/ L concentration. Aneurysm, deformed gill lamellae, enlarged primary lamellae and absence of secondary lamellae were some modifications observed at 30 and 40 ppm/ L concentrations. Exposure to DEHP at 50 ppm/ L reported to have shortened primary lamellae, erythrocyte infiltration and shortened and curly secondary lamellae and at 60 ppm/ L showed degenerated gill lamellae, gill arches and a complete necrosis. Aforementioned structural damage incurred to the gill tissues are due to the exposure to DEHP and such tissue damages were noticed earlier in our laboratory when treated to diisononyl phthalate (DINP), one of the high molecular weight phthalate plasticizers (Revathy and Chitra, 2015).

DEHP-induced histopathological alterations were also evaluated in liver tissues. Control hepatocytes showed normal architecture with clear, monotonous cytoplasm and spherical nucleus. However, histopathological investigations of DEHP-treated fishes at 30 ppm/ L concentration onwards indicated several alterations in liver tissues including degenerated cytoplasm, bile stagnation, cytoplasmic vacuolization, elongated nucleus, a few regions with enucleated hepatocytes and complete necrosis. It is therefore clear that acute exposure to DEHP could result in disorganization of hepatocytes as noticed by destroying the cellular membranes and consequently leads to

necrosis of liver cells. Similar observations have been observed during the acute exposure of DINP to the freshwater fish, *Oreochromis mossambicus* (Revathy and Chitra, 2015). Therefore, to conclude the cautious observations of the preliminary investigation disclose that acute exposure to DEHP induced structural modifications in gill and liver tissues of the freshwater fish, *Oreochromis mossambicus*.

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Table 1 Effect of DEHP on the body weight and tissue weights of the fresh water fish, *Oreochromis mossambicus*

DEHP	Body weight (g)	Weight of gill (mg)	Weight of liver (mg)
Control	3.41 ± 0.14	123 ± 0.07	51.5 ± 0.003
10 ppm/ L	3.285 ± 0.48	120 ± 0.05	50.5 ± 0.006
20 ppm/ L	3.5 ± 0.5	125 ± 0.03	52 ± 0.002
30 ppm/ L	3.565 ± 0.29	124 ± 0.06	52.5 ± 0.004
40 ppm/ L	3.79 ± 0.49	126 ± 0.04	53.5 ± 0.005
50 ppm/ L	3.765 ± 0.61	123 ± 0.05	52.5 ± 0.003
60 ppm/ L	3.66 ± 0.56	124 ± 0.04	53 ± 0.004

Data are expressed in Mean ± SD for 10 animals per group.

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Figure A Photomicrograph showing normal architecture of control gill in *Oreochromis mossambicus* (X100; H & E)

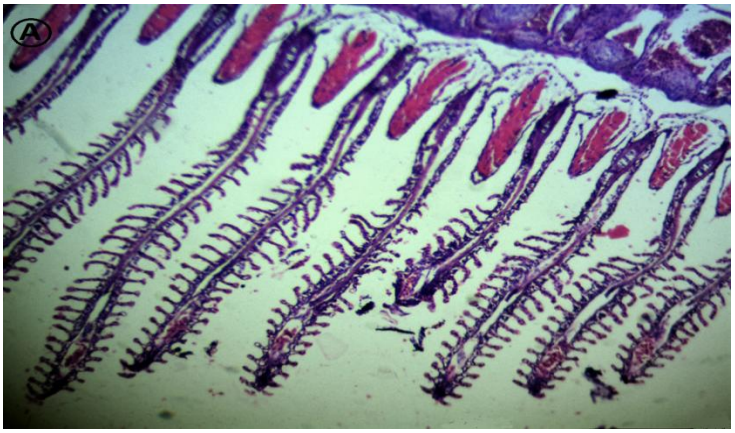

Figure B Photomicrograph of DEHP-treated gill (10 ppm/ L for 96 h) in *Oreochromis mossambicus* (X100; H & E); ED – epithelial damage, EI – erythrocyte infiltration, Right arrow – degeneration of gill arches, Up arrow – degeneration of secondary lamellae

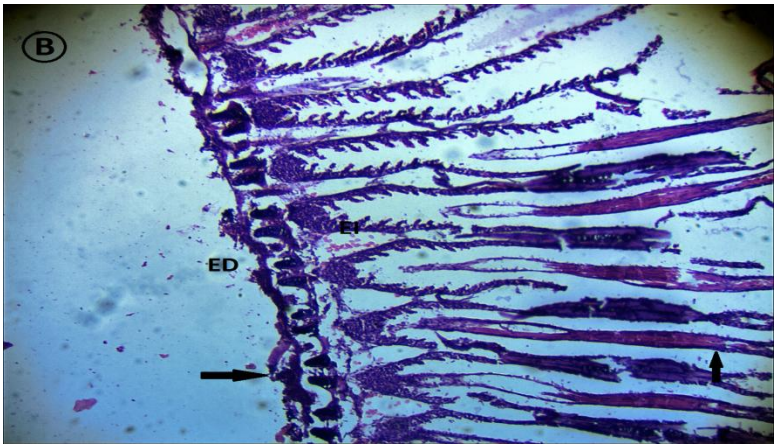

Figure C Photomicrograph of DEHP-treated gill (20 ppm/ L for 96 h) in *Oreochromis mossambicus* (X100; H & E); EI – erythrocyte infiltration, Right arrow – curling of secondary lamellae, Asterisk (*) – absence of gill arches

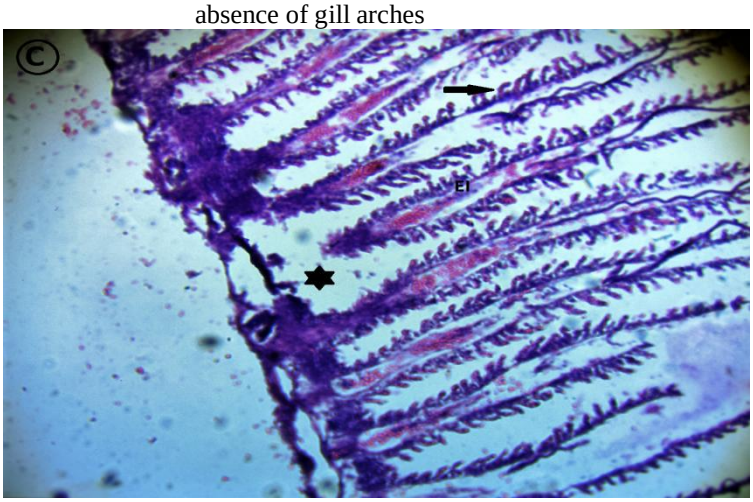

Figure D Photomicrograph of DEHP-treated gill (30 ppm/ L for 96 h) in *Oreochromis mossambicus* (X100; H & E) showing aneurysm; D – deformed gill lamellae

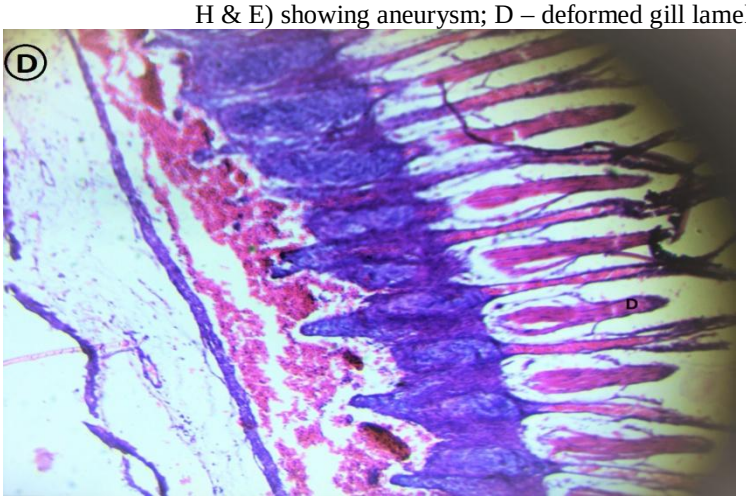

Figure E Photomicrograph of DEHP-treated gill (40 ppm/ L for 96 h) in *Oreochromis mossambicus* (X400; H & E); P - enlargement of primary lamellae, S -absence of secondary lamellae

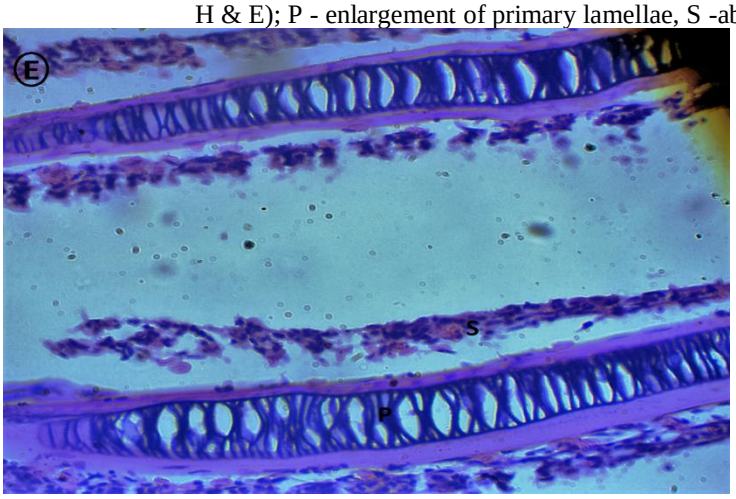

Figure F Photomicrograph of DEHP-treated gill (50 ppm/ L for 96 h) in *Oreochromis mossambicus* (X100; H & E); E – erythrocyte infiltration, P – shortened primary lamellae, S – shortened and curly secondary lamellae

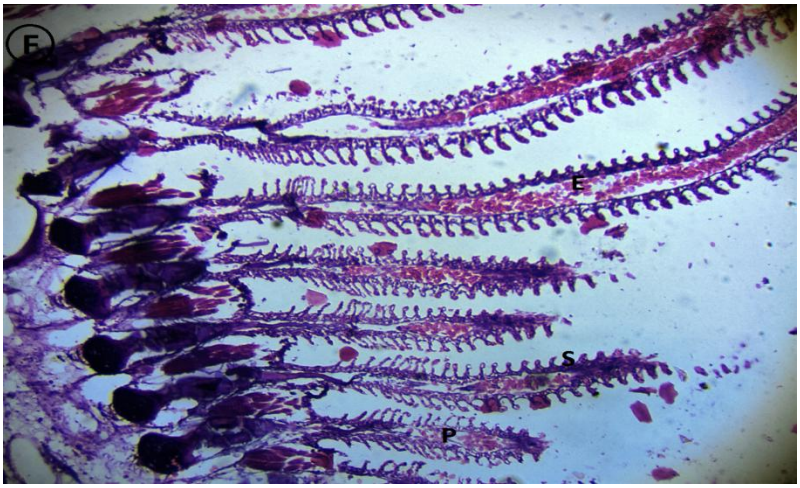

Figure G Photomicrograph of DEHP-treated gill (60 ppm/ L for 96 h) in *Oreochromis mossambicus* (X100; H & E); N – necrosis

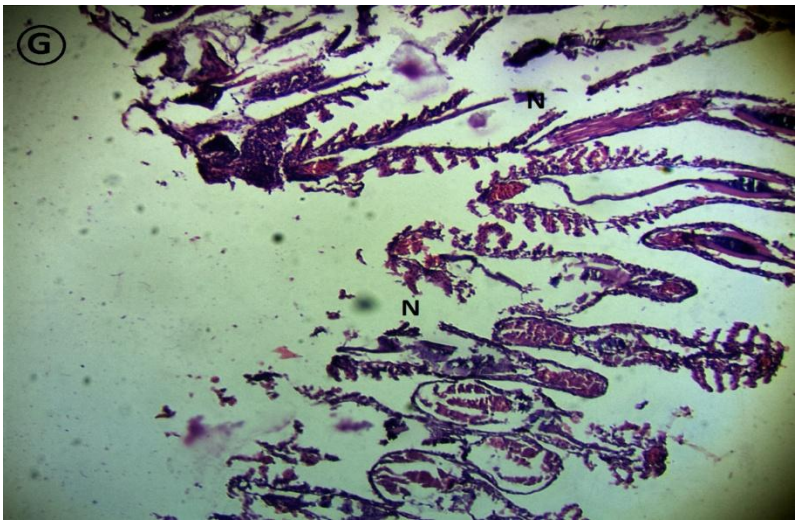

Figure 1 Photomicrograph showing normal architecture of control hepatocyte in *Oreochromis mossambicus* (X400; H & E)

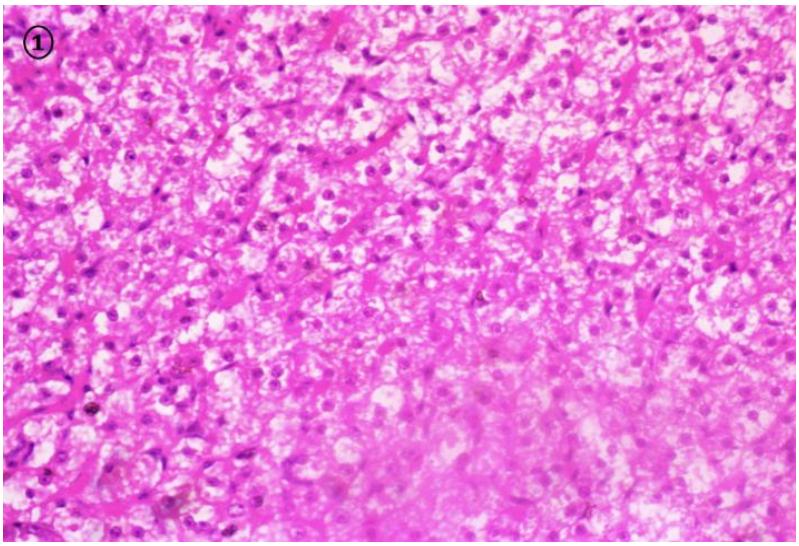

Figure 2 Photomicrograph of DEHP-treated liver (30 to 60 ppm/ L for 96 h) in *Oreochromis mossambicus* (X100; H & E) showing necrosis and bile stagnation