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Histopathology biomarker responses in fresh water fish, *Labeo rohita* exposed to Bleaching Powder

[1]Manjit Singh Arora, [2]Atika Anwar Sikilkar, [3*]SangeetaSinha
[1]Associate Professor, [2]Post Graduate student, [3]Assistant Professor
**Corresponding author: Department of Zoology, Nowrosjee Wadia College, Pune-07, Maharashtra, India.*

Abstract: *Fish are regarded as valuable organisms in monitoring aquatic ecosystems. They are established as indicator organisms and widely used in both laboratory studies and ecotoxicology. A range of biomarkers are developed for monitoring effects of xenobiotics on fish. Bleaching Power (Calcium hypochlorite) is commercially used as a laundry detergent and the water used for such process goes into the aquatic environment such as water resources. Quality of the water can be indicated by the fishes living in that environment. The present study was carried out to study gills and liver histopathology in the freshwater fish, labeo rohita which exposed to Different concentration of bleaching powder (2.5, 10, 30 and 50 ppm) for 96 hour. Histopathological changes in gills and liver observed microscopically showed increasingdegrees of damage in the tissues in correlation with the concentration of bleaching powder, while gills and liver ofcontrol groups exhibited a normal architecture. The present study clearly demonstrated that all the treated body organs exhibited significant damage with response; amongst the body organs the liver is an important target organ for bleaching powder toxicity in this species could be possibly used as a model organism for toxicity studies.*

Keywords:*Bleaching Powder, Histopathology, Marker Enzymes, Gill, Liver, Labeo rohita*

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I. INTRODUCTION

Fishes are rich in vitamins (fat-soluble vitamins A, D, and E, and water-soluble vitamins, B complex) and minerals (especially calcium, phosphorus, iron, selenium)(Choo et al., 2003; Salim, 2003; Butchiram et al., 2013). Therefore, fish can provide an important source of nutrients, particularly in those diets which are lacking nutritional constituents (Jawahar et al., 2015). Fishes,the most diverse group of vertebrate fauna are important component of food chain and any effect of toxicant may have adverse effects on histology of fish. Fishes are generally regarded as sentinels of bioindicators for aquatic pollution and indispensable experimental models in eco toxicological studies (Kirk.,1995). One of the most important non-target aquatic organism fish, affected by detergent pollution (Rani et al., 2014).The overall ecosystem which included plants and animals was adversely affected by lots of industrial effluents like heavy metals cadmium, calcium etc. and effluents like detergents (Mizanur eta l., 2013). Beaching Powder is one of them. Bleaching powder also known as calcium hypochlorite $(CaClO)_2$ is an inorganic compound. It is marked as chlorine powder or bleach powder for water treatment and as a bleaching agent. The adverse effect of bleaching powder is widely studied in organism including humans and other mammals (Chowdhury et al., 2012; Reddy., 2013; Rahman et al., 2013). The assessment of eco-toxicological risks caused by pesticides to ecosystems is based on data on the toxicity and effects of pesticide preparation to non-target organisms. Histological changes provide a rapid method to detect effects of irritants, especially chronic ones,in various tissues and organs(Bernet.et al., 1999).

During pathological studies the variations in the histology are exploited for evaluation of physiological state of the animal. Heavy metals causing toxicity in fishes(Adnan.,2017). Fish is good indicator of aquatic contamination because its biochemical stress responses are quite similar to those found in mammals(Santos et al., 2019). Chronic and sublethal toxicities of surfactants to aquatic animals (Michael A Lewis; 1990).Influence of the detergent tide on enzyme activities of freshwater fish, *Labeo rohita*(K Pechiammal and J Vasanthi,2017). Studies on acute toxicity of metals to the fish *Labeo rohita*,(Noor et al., 2006; Adnan et al., 2017).The immunosuppressive effect of alpha-permethrin on Indian major carp,rohu(Nayak et al.,2004).An important consideration for studying the toxicity of detergents on the fingerlings of rohu was the paucity of information on the younger developmental stages which are considered to be more susceptible and vulnerable to toxicants than those of adult stages. The present study was undertaken on the histopathological effects of different concentration of bleaching powder on fingerlings of *Labeo rohita*.

II. MATERIALS AND METHODS

Collection and maintenance of animals

The Indian Major carp, *L. rohita* were procured from Government fish farm, Pune, India. The test organisms were transferred to the laboratory in the plastic bags and were washed with 0.1% $KMNO_4$ solution to get rid of dermal infection. Healthy fingerlings were selected and acclimated in dechlorinated tap water for 15 days; during this period they were fed with oilcake (1 g), thrice a day by dissolving in 10 mL of dechlorinated tap water. Water was replenished 75% on daily basis with routine cleaning of aquaria leaving no faecal matter and unconsumed food. In the present study chlorine free tap water was used which had the following physiochemical characteristics (APHA, 2005); temperature 25 ± 1.0℃, pH 7.4 ± 0.07, salinity 0.25 ± 0.1 ppm, dissolved oxygen 6.5 ± 0.4 mg/L, total hardness 17 ± 0.5 mg/L and alkalinity 36 ± 0.5 mg/L.

Toxicant used

Commercial bleaching powder was purchased from Sigma Aldrich Corporation, USA (CAS no. 7778-54-3, 211389). Stock solution of bleaching powder was prepared by dissolving 1 ml of bleaching powder in appropriate amount of normal tap water.

Acute toxicity test

Into 5 litre plastic tubs containing 1L of test solution, twenty test animals were introduced in a static bioassay system. Experiments were carried out in replicates and a separate control was maintained. The fingerlings were not fed during the period of exposure.

After conducting range finding tests, five different concentrations namely 0, 2.5, 10, 50 and 100PPM were selected to determine the LC50 values. Mortality and behaviour were observed everyday in each concentration.

Measurement of cytotoxic marker enzyme

After removal of organs (Gills and liver), blotted dry with Whatman filter paper. Then 100 mg of each tissue were homogenates (100 mg/2.5 mL, w/v) with 0.25 M sucrose solution in ice cold condition (Hogeboom., 1948). The homogenates were centrifuged for 20 min at 6000 rpm (ice cold condition) and the clear supernatant fluid was removed and used to determine the level of GOT, GPT and LDH activities. GOT and GPT activities were measured according to Reitmen and Franckel (1957) at 505 nm against distilled water. LDH was measured using standard protocol [15] at 340 nm against distilled water. Optical density was measured with the help of a UV-spectrophotometer. Activity of all enzymes was expressed in IU/L.

Microscopy Examination

At the end of exposure period, 5 fish were taken from each replicate tank. The gill arches of the fish wereexcised from both sides. Fish were dissected, the abdominal cavity was operated and the liver was excised quickly and was fixed in Bouin's solution as a histological fixative for 24 h (Tao et al., 1999). According to Humason (1967), the specimens were processed as usual in the recognized method of dehydration, cleared in xylene and finally embedded in paraffin wax before being sectioned at 5 µm using a rotary microtome (Leica RM 2235 Germany). The specimens were stained with hematoxylin and eosin. Finally, the prepared sections were examined and photographically enlarged using light microscopy (Hamilton compound photomicroscope).

Statistical analysis

The mortality (%) data obtained were used to calculate the 24, 48, 72 and 96 hr LC50 values, using a statistical package (Grafpad software). ANOVA was used to compare the LC50 values of bleaching powder to test organisms after 96 hrs. All experiments were repeated at least five times and data presented is average of these replicates. One-way analysis of variance (ANOVA) test associated with the Tukey's test was used to determine the statistical significance of the differences among experimental groups. All the statistical analyses were done using SPSS 17.0 software.

III. RESULTS

Morphometric measurements and behavioural changes

During the study the morphometric measurements of fingerlings of Rohu were also taken. A Morphometric measurement of fingerlings varies in weight (11.4 gm ± 1.3), Length (7.5 cm ± 0.8), Breadth (1.7 cm ± 0.4) and height (1.2 cm ± 0.3). No significant variations were observed on Morphometric measurements in terms of weight, length, breadth and height (Table 1).

When experimental fishes were introduced into water containing bleaching powder at higher concentrations, they started showing discomfort within few minutes and began to move rapidly. Fingerlings of *Labeo rohita* exhibited a variety of behavioural responses like opercular movement was 20-25 times more faster

than controlled, loss of nervous control, try to jump out of media. Body was slimy due to mucus secretion from epithelium of gills. As the concentration of bleaching powder was increased the fishes started swimming towards water surface, very fast movement of operculum hanging vertically erratic swimming loss of equilibrium and bubble formation at the surface increased.

To measure and evaluate the median lethal concentration (LC50)

In the following experiment the rohu fingerling was treated with different concentrations of Bleaching powder ranging from 0 to 50 PPM. At the different concentrations of bleaching powder viz 0, 2.5, 10, 30 and 50 PPM, the mortality were 0, 0, 25, 45 and98percent respectively. Figure 1 shows the mortality rates and LC50's for bleaching powder was 33 PPM.

Quantitation of cytotoxic marker enzyme (LDH, GPT and GOT) under different conditions

On addition of the bleaching powder as a toxicant at different concentrations (0, 2.5, 10, 30, and 50 PPM), the specific activity of LDH was significantly increased with respect to the control in a concentration dependent manner. At the concentration of 50 ppm bleaching powder the activity of LDH (Figure 2 and 3) was significantly increased in liver (186 ± 1.9 IU/L, $p<0.001$) and gills (132 ± 1.4 IU/L, $p<0.001$) as compared to controls (Liver: 98 ±1.1 IU/L; Gills: 63 ±1.7 IU/L). The activities of GOT was also significantly increased at 50 PPM (Liver: 91 ± 1.1 IU/L, $p<0.001$; gills: 74 ± 3.0 IU/L, $p<0.001$) compared to controls (Liver: 46 ± 1.1 IU/L; gills: 26 ± 1.0 IU/L) respectively. Similarly the activities of GPT were also significantly increased in liver and gills tissues at 50 PPM compared to respective controls. (Liver: 93 ± 2.0 IU/L, $p<0.001$; gills: 60 ± 2.0 IU/L, $p<0.001$) compared to controls (Liver: 45 ± 1.0 IU/L; gills: 22 ± 0.9 IU/L)

Histopathology of Gills and Liver tissue

The histopathological analysis showed severe lesions in liver and gills of rohu fingerlings at different concentration of bleaching powder compared to controls.

Histology of liver

The surface of the liver is covered with serous membrane and some connective tissue extends inwards into parenchyma. It is composed of parenchymal cells (hepatocytes) and lattice fibres. Hepatic cells are roundish polygonal, containing clear spherical nucleus. They are located amongst sinusoids forming cord like structures known as hepatic cell cords. Blood sinusoids, are irregularly distributed between the polygonal hepatocytes. Fairly large quantities of lipid glycogen granules were also observed in the cytoplasm (Plate 1, Fig. A).

Histopathology of liver

The changes observed in the liver tissue on exposure to different concentration of bleaching powder included swelling and rounding off of hepatocytes, detachment of cells from each other. Pancreatic acini appeared to have lost its architecture. Cytoplasm of hepatocytes became more basophilic. These changes include degenerated hepatocytes presenting a homogenous cytoplasm and a large central or sub central spherical nucleus (Plate 1, Fig. B-E). The important histopathological changes observed in the bleaching Powder treated groups were pyknotic nuclei and clear cell foci. The liver tissue at 10 ppm of bleaching powder exposure at lowest concentrations revealed vacuolation of hepatocytes, condensation of nuclear chromatin and swelling of hepatocytes (Plate 1). At a high concentration of bleaching powder (50 ppm) of exposure there is extensive vacuolation of hepatic cells (Plate 1, Fig E) with several foci of coagulative necrosis, blood congestion and accumulation of dark granules. Appearance of Blood streaksamongst hepatocytes are a marked change in treated liver.

Histology of gills

Histological study of the gills shows a typical structural organisation of the respiratory lamellae in the untreated fish. There are four gill arches and each arch is composed of numerous gill filaments with two rows of semi-circular secondary lamellae that are aligned along both sides of the primary gill lamellae.The primary gill lamellae consist of centrally placed rod like central axis with chloride cells and with blood vessels on either side. The lamellae are lined by squamous epithelium and many capillaries split by pillar cells run parallel along the surface. Between the lamellae, the filament is lined by a thick stratified epithelium constituted by several cellular types, such as chloride, mucous and pavement cells. (Plate 2, Fig. A).

Histopathology of gills

Bleaching powder exposure has (Plate 2, Fig. B–E) induced noticeable pathological changes in fish gill architecture. The changes include curling of secondary lamellae , a few lamellar capillary aneurism at the tip of the secondary lamellae and desquamated epithelium at different concentration of bleaching powder (Plate 2,

Fig. B - E). At concentration (30 ppm) of bleaching powder gills tissue showed rupture and breakdown of pillar cell system, hyperplasia of epithelial cells and lifting of secondary gill lamellar epithelium. The gills of experimental fish showed extensive oedema of the epithelial cells and blood congestion (aneurism) in many areas of secondary lamellae with complete damage and breakdown of the pillar cell system at 50 ppm concentration of bleaching powder (Plate 2). Shortened and clubbing of ends of the secondary gill lamellae, fusion of adjacent secondary gill lamellae and necrosis in the primary lamellae were well marked.

IV. DISCUSSION

Fish health reflects, and gives a good indication of the health status of a specific aquatic ecosystem. Early toxic effects of pollution are evident on cellular or tissue level before significant changes can be identified in fish behaviour or external appearance. Aquatic vertebrates particularly fish appear to have similar enzyme and receptor systems as in mammalian system (Huggett et al., 2003). By changing and adapting metabolic functions, fish react to environmental toxicants. Changes in the enzymatic activities of aquatic organisms are widely used to demonstrate tissue damage and also diagnosis of fish diseases (Nemcsok and Boross, 1982; Pacheco and Santos, 2002; Jawahar*et al*., 2015). GOT and GPT usually present within cell membranes, cytoplasm and mitochondria. The accumulation or binding of toxicants in these cells lead to damage and disintegration of cells, releasing these enzymes into blood circulation, results in increase in blood serum transaminases during stress conditions (Galina et al., 1992; Malarvizhi et al., 2012). In aquatic monitoring, increased activities of GOT and GPT indicated hepatic tissue damage. Increase in GOT and GPT activity in monocrotophos treated fish Channapunctatus indicates liver damage (Agrahari et al., 2007; Schreiber et al., 2011).

In the present study, the significant increase in GOT and GPT activity in gill and liver during acute treatment indicates that the damage of the organs due to bleaching powder toxicity or the organism tries to mitigate the toxicant induced stress by increased rate of metabolism. However the observed decrease in GOT and GPT activity in gill and liver during acute treatment signifies that detoxification mechanism may not be sufficiently effective to prevent the action of the bleaching powder on the system. LDH enzyme activity can be used as a good indicator of the anaerobic capacity of a tissue, chemical exposure and stress in fish (Rendon-von et al., 2005; Schreck et al., 2016). Elevated LDH activity in gills suggests that the aerobic catabolism of glycogen and glucose has shifted towards the formation of lactate, which may have adverse long-term effects on the organisms (Napierska et al., 2009; Ray et al., 2016a). In the present study (Figure 2 and 3), the elevation of LDH activity in gill, and liver has occurred may be due to the metabolic changes caused by the bleaching powder. Further disruption of respiratory epithelium might have caused tissue hypoxia resulting in a decrease in oxidative metabolism which may be responsible for increase in LDH activity in toxicant stressed animals (Sinha et al., 2018).

Histological analysis appears to be a very sensitive parameter and is crucial in determining cellular changes that may occur in target organs, such as the gills, liver and kidney (Salamat et al., 2016; Das et al., 2018). A histological investigation may therefore prove to be a cost effective tool to determine the health of fish populations, hence reflecting the health of an entire aquatic ecosystem in the bio-monitoring process. In this study, the gill, histology of the fresh water fish, *Labeo rohita* was analysed. Gill hyperplasia has been regarded as a common sign of chronic toxicity caused by various chemical pollutants (Peebuaa et al., 2006; Hadi and Alwan 2012). In the present study, mild to moderate hyperplasia was evident after acute and chronic exposure of *Labeo rohita* due to bleaching powder. Histological observations on gills of control fingerlings showed normal architecture of primary and secondary lamellae. Whereas fingerlings infected with different concentrations of bleaching powder in gills showed fusion and loss of secondary lamellar epithelium. Santos and his group (2019) observed various alternations in gill histopathology in two Native Fish Species from the Hydrographic Douro Basin. These pathological changes may be a reaction to bleaching powder intake or an adaptive response to prevent the entry of the pollutants thorough the gill surface. The observed alterations like proliferation of the epithelial cells, partial fusion of some secondary lamellae and epithelial lifting are defence mechanisms, since, in general, these result in the increase of the distance between the external environment and the blood and thus serve as a barrier to the entrance of contaminants. Similar results were observed (Jabeen et al., 2018) wherein gills and liver tissues showed damage due to metal contaminated sediments exposure.

The parenchymatous hepatic tissue in teleosts, has many important physiological functions and also detoxification of endogenous waste products as well as externally derived toxins, drugs, heavy metals and pesticides (Roberts and Rodger, 200 1; Santos et al., 2019). Due to these reasons, the hepatic cells are damaged severely, on chronic exposure to copper. The liver exhibited several pathological changes including hyperplasia, degeneration of blood vessels, vacuolisation, hypertrophy; pyknotic nuclei, necrosis, and accumulation of blood vessels (Plate 1).Significant changes were observed in the liver tissue at lethal and sublethal concentrations of bleaching powder with marked swelling of the hepatocytes in places with areas of diffuse necrosis (Plate 1,Fig. E). Radhaiah and JayanthaRao (1992) reported moderate cytoplasmic degeneration in hepatocytes, formation of

vacuoles, rupture of blood vessels and appearance of blood vessels amongst hepatocytes and pyknotic nuclei in the liver of Tilapia mossambica exposed to fenvalerate. Sakr et al. (2005) observed histopathological changes induced in the liver after exposing the fish Clariasgariepinus to fenvalerate. Similar results were also observed by Jabeen et al., 2018. Several histopathological symptoms that appeared in fish organs would serve as biomarker responses in bleaching powder toxicity. of fresh water ecology.

V. CONCLUSION

The present study indicates that bleaching powder induced alterations in the marker enzymes activities of the freshwater fish at acute concentration. These alterations can be considered as a tool for biomonitoring of pharmaceutical drug substances in the aquatic environment. However, further studies are needed to understand the risk of bleaching powder using different end points.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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TABLES

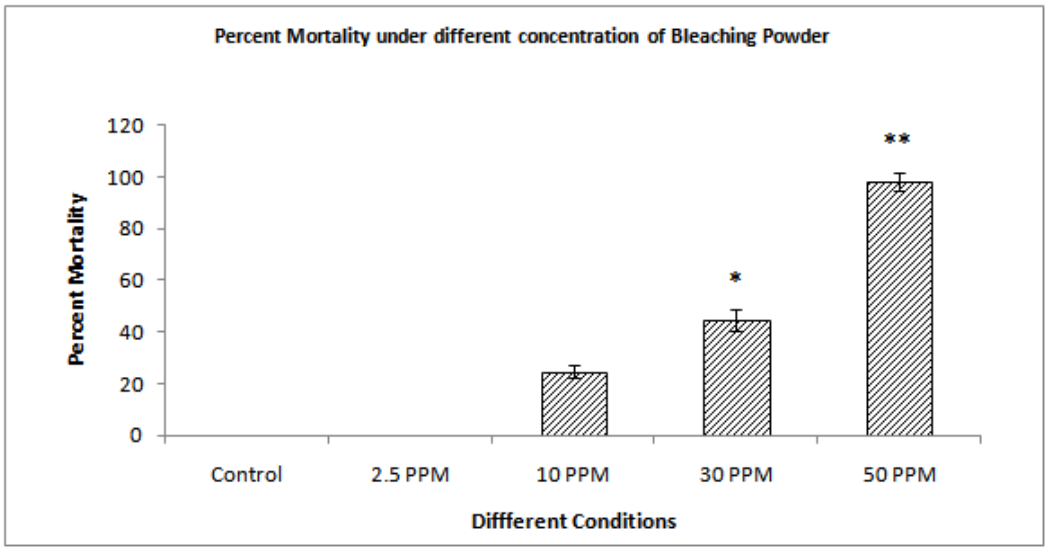
Table 1: Morphometric measurements of fishes

Different Concentrations	Wt. of fingerlings (gms)	Length (cm)	Breadth (cms)	Height (cms)
Control	11.5 ± 1.5	6.5 ± 0.4	1.4 ± 0.2	1.0 ± 0.4
2.5 ppm	10.2 ± 1.2	7.1 ± 0.7	2.1 ± 0.2	1.1 ± 0.2
10 ppm	11.0 ± 0.8	8.4 ± 0.4	1.6 ± 0.4	1.4 ± 0.5
30 ppm	11.5 ± 1.1	7.1 ± 1.2	1.5 ± 1.2	1.4 ± 0.3
50 ppm	12.9 ± 1.4	8.4 ± 1.0	2.1 ± 0.7	1.3 ± 0.2

Values are means ± SE of five individual observations. B.P.: Bleaching Powder.

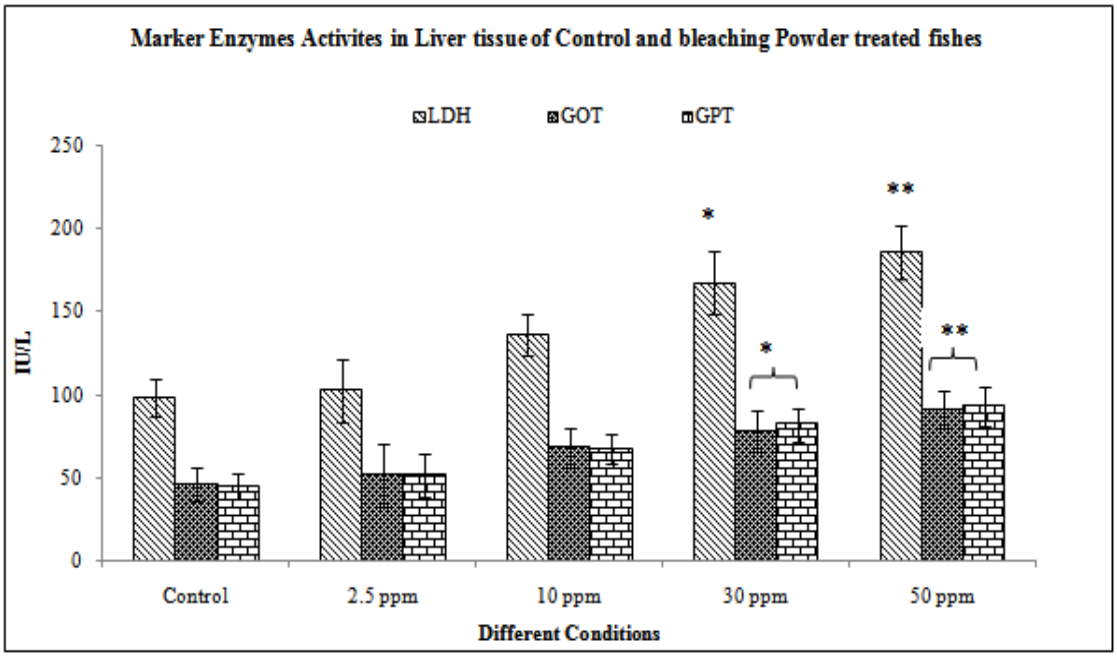
FIGURES

Figure 1: Percent mortality of Labeo rohita fingerlings exposed to different concentrations of bleaching powder



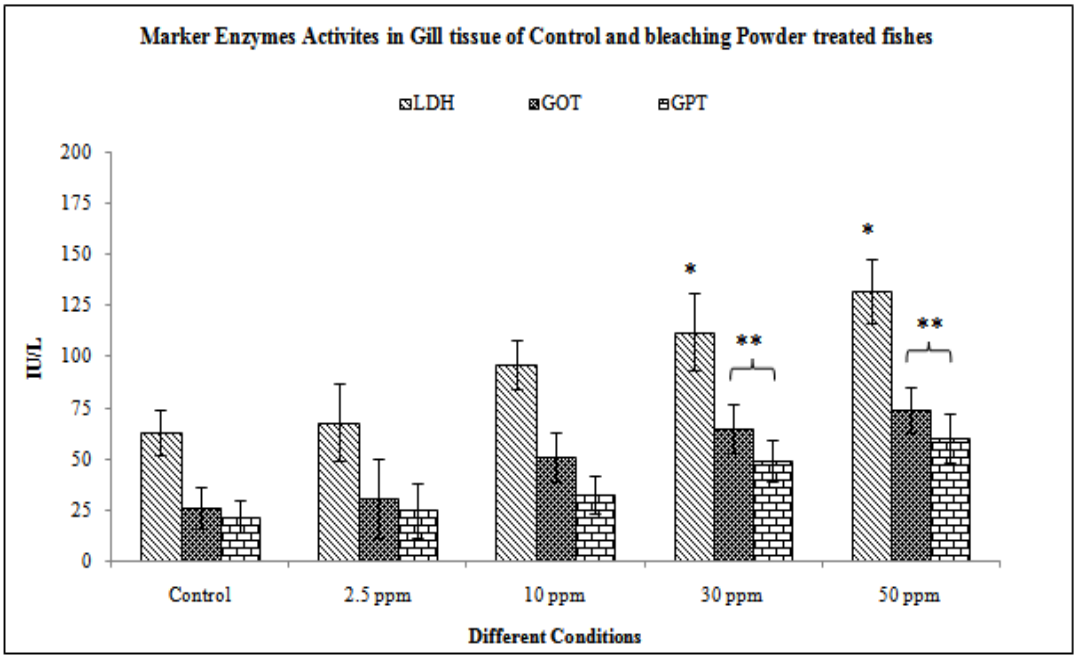
** Statistically significant ($p<0.001$) compared to control group.
* Statistically significant ($p<0.05$) compared to control group.

Figure 2: Effect of bleaching Powder on biomarker enzyme activities in the liver tissues under different conditions



** Statistically significant ($p<0.001$) compared to control group.
* Statistically significant ($p<0.05$) compared to control group.

Figure 3: Effect of bleaching Powder on biomarker enzyme activities in Gill tissues under different conditions



** Statistically significant ($p<0.001$) compared to control group.
* Statistically significant ($p<0.05$) compared to control group.

Plate 1: Histopathology of Liver tissues under different conditions (40 X)

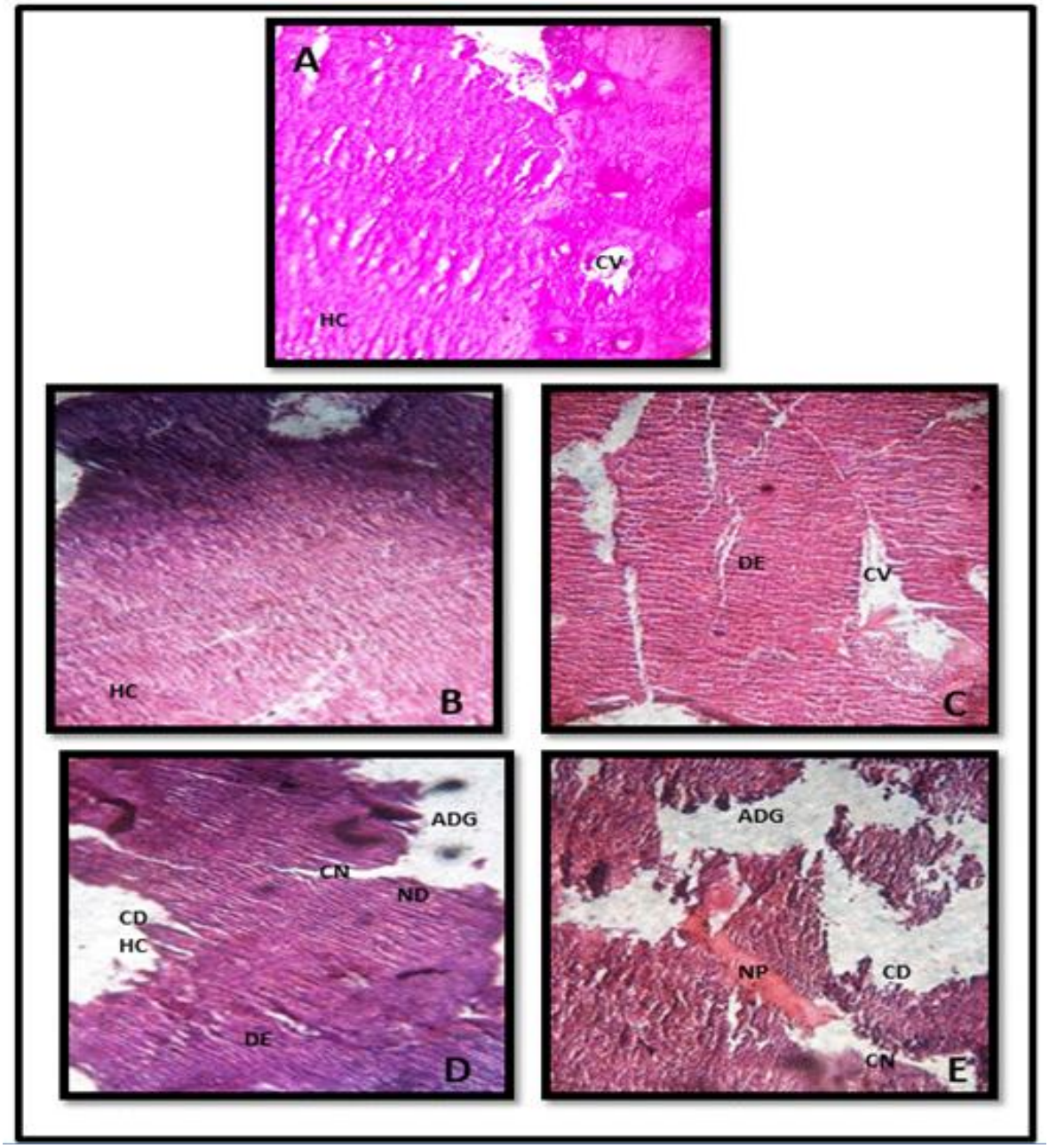


Plate 1 : Histological changes of liver in rohu fingerlings under different conditions. Light micrographs of a paraffinsection stained with Hematoxylin and Eosin (40 X). (A) Control; (B) 2.5 ppm B.P. (C) 10 ppm B.P. ; (D) 30 ppm B.P.;(E) 50 ppm B.P.

Abbreviations used: B.P. - Bleaching Powder; CV- Central Vein; HC – hepatocyte; CD – cytoplasmic degeneration; DE –damaged epithelium; NP – nuclear pyknosis; CV – cytoplasmic vacuolation; ND – nuclear degeneration; ADG –accumulation of dark granules; CN – cellular necrosis

Plate 2: Histopathology of Gill Tissues under different conditions (40 X)

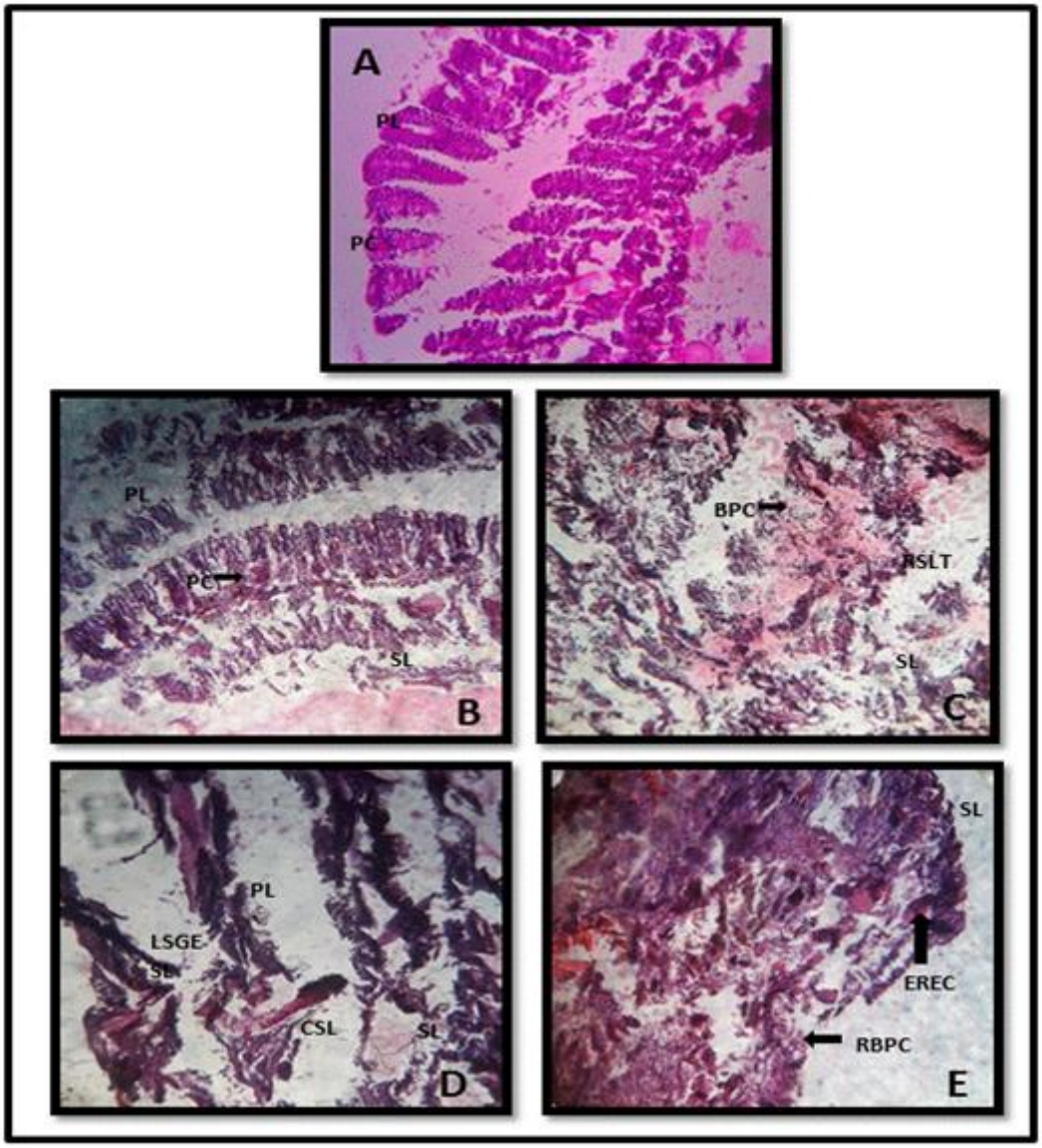


Plate 2 : Histological changes of gills in rohu fingerlings under different conditions. Light micrographs of a paraffin section stained with Hematoxylin and Eosin (40 X). (A) Control; (B) 2.5 ppm B.P. (C) 10 ppm B.P. ; (D) 30ppm B.P.; (E) 50 ppm B.P.
Abbreviations used: B.P. – Bleaching Powder PL – primary lamellae; SL – secondary lamellae; PC – pillar cells; BPC– breakdown of pillar cells; LSGE – lifting of secondary gill lamella epithelium; CSL – curling of secondary lamellae;RSLT – rupture of secondary lamellae tip; RBPC – rupture and breakdown of pillar cell system; EREC – oedema and rupture of epithelial cells

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