**Toxicity of Two Therapeutants Hydrogen Peroxide and Formalin in Nile tilapia (Oreochromis niloticus)**

**Abstract:**

The FDA has granted approval for the use of chemicals hydrogen peroxide and formalin in aquaculture. The toxicity of these two compounds was evaluated in the current study for freshwater Nile tilapia (Oreochromis niloticus) weighing 75 ± 2.5g. For 96 hours, ten fish each were subjected in triplicate to concentrations of hydrogen peroxide at 0 ppm, 150 ppm, 250 ppm, 350 ppm, 450 ppm, and 600 ppm. Similarly, for 96 hours, 10 fish each were subjected in triplicate to the following concentrations of formalin: 0 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm, 350 ppm, 400 ppm, and 450 ppm. Every day, the mortality rate was noted. Each chemical's LD50 was determined using the non-linear regression approach. Hematological parameters, such as white blood cells, red blood cell, hemoglobin, and thrombocyte number as well as serum parameters, such as ALT, AST, creatinine, and BUN, and enzymes that inhibit free radicals, such as SOD, CAT, and GSH, were determined following the collection of blood and liver tissue. Following the slaughter fish. The liver, muscle, gills, and heart were collected for histopathology. The LC50 values for formalin and hydrogen peroxide were found to be 231.2 µg/ml and 314.6 µg/ml, respectively. Sublethal concentrations of hydrogen peroxide significantly increased hematological parameters such as total WBC, RBC, hemoglobin, and thrombocyte count, and serum parameters such as ALT, AST, creatinine, and BUN, while antioxidant enzymes such as SOD, CAT, and GSH significantly decreased. Fish exposed to formalin had significantly higher levels of ALT, AST, creatinine, BUN, and significantly lower levels of antioxidant enzymes. Histology of the muscles of fish that were exposed to sublethal concentrations of two chemicals showed hyperplasia, lamellar fusion of the gills, infiltration of hemocytes and muscular atypia in the heart, and separation of muscle fiber bundles in the liver.

Keywords: Therapeutant, Nile tilapia, Hydrogen Peroxide, Formalin

## 1. Introduction

Aquaculture is defined as "the organized cultivation of aquatic creatures, such as finfish, crustaceans, molluscs, and aquatic plants, in freshwater, seawater, brackish water, and inland saline water" (FAO, 2006).As of 2020, the industry is regarded as one of the world's fastest-growing food production systems, accounting for around 52% of worldwide fish and invertebrate production and 97% of total seaweed production. Globally, aquaculture has expanded and intensified at an incredible rate, much exceeding the annual growth rate of terrestrial livestock and dairy production, which reached an all-time high in 2018 (FAO, 2020).

Infectious diseases present a major constraint on aquaculture production, causing high mortality levels and impaired growth due to infection. Antimicrobials (natural or synthetic origin) that are sufficiently non-toxic to the host are generally used as chemotherapeutic agents for the treatment of infectious diseasesand as prophylactic agents in aquaculture(Baticados and Paclibare, 1992;Kumar and Roy, 2017). Additionally, antibiotics are utilized in aquaculture as preventative or therapeutic interventions (Subasinghe *et al.,* 2005). Since antibiotics can be highly costly and accumulate in the tissues of aquatic animals, their use in aquaculture creates public health issues due to the spread of antimicrobial resistance.

In order to control the external infections of farmed fish, FDAhas approvedonly one therapeutic chemical (Formalin) and three Low Regulatory Priority substances (sodium chloride, hydrogen peroxide, and acetic acid) (Singh and Singh, 2018). External bacteria and parasites may be successfully controlled by hydrogen peroxide (Speare & Arsenault, 1997, Arndt and Wagner, 1997; Lumsden *et al*., 1998; Avendaño-Herrera *et al.*, 2006; Bowker *et al.,* 2012). Hydrogen peroxide has been considered as a ‘green’ therapeutants which do not contribute residues to the environment (Lieke *et al.*, 2020). Formaldehyde hydrate (Formalin), which has an electrophilic nature, may react with the functional groups of many biological macromolecules, including proteins, DNA and RNA, polysaccharides, and glycoproteins (Kiernan, 2000; BMA, 2015). Formalin is one of the most applied chemical therapeutant in intensive aquaculture (Francis-Floyd, 1996; Boyd & McNevin 2015). It may be used as a prophylactic measure or with therapeutic purposes and is extremely effective against most protozoan parasites (*Ichthyophthirius* spp., *Costia* spp., *Epistylis* spp., *Chilodonella* spp., *Scyphidia* sp., *Trichodina* spp.) and monogenetic trematodes (*Cleidodiscus* spp., *Gyrodactylus* spp. and *Dactylogyrus*spp. (FDA 1995; Francis-Floyd, 1996; Shao, 2001).

## 2. Materials and Methods:

### 2.1. Experimental animals:

In total, 480 disease-free, healthy fish (*O. niloticus* of size 84.51±22.59 gm) were used in the experimental investigation. These fish were obtained from Perumbavoor, Ernakulam, Kerala, during the second week of August 2022. According to the requirements set forth by CPCSEA for Experimentation on Fishes, 2021, the entire study was conducted in an environment with sufficient

management, operation, care, and maintenance facilities (No. 1174/ac/08/CPCSEA). The experiment was carried out in the wet lab of the Kerala University of Fisheries and Ocean Studies (KUFOS), Panangad, Kochi, Faculty of Fisheries Science, Department of Aquatic Animal Health Management.

### 2.2. Determination of 96hr $LD_{50}$

$LC_{50}$ of $H_2O_2$ andformalin for 96 hr were determined as per test no. 203 of OECD guidelines with some modifications (OECD, 2019). Ten fish were kept in 100Lwater in triplicate for treatments and control. The concentrations of $H_2O_2$ used were 0 ppm, 150 ppm, 250 ppm, 350 ppm, 450 ppm and 600 ppm. The quarantined and acclimatized fish were kept in respective tanks for 48 hours before starting the experiment. After adding respective dose of $H_2O_2$, water quality parameters were recorded. The water quality parameters were maintained optimum by continuous aeration and 50% water exchange. The respective dose of $H_2O_2$was added daily. $H_2O_2$ was provided by Merck life science private limited, Mumbai. No feeding was done. Mortality in each tank was recorded daily.

In the case of formalin, the concentrations used were 0 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm, 350 ppm, 400 ppm and 450 ppm. Feeding was not done. The water quality parameters were maintained optimum by continuous aeration and 50% water was exchanged and added appropriate dose daily.Formalin was provided by Emplura Merck life science private limited, Mumbai. Mortality in each tank was recorded daily.

### 2.3. Blood sampling

After 96 hours of experiment, fishes that survived were anaesthetized by using clove oil (Eugenol) 0.5mL/L. Blood samples were collected using sterile syringes (2.5ml, 24 gauge) from caudal vein. The blood sample were collected with the addition of anticoagulant EDTA (Himedia) (1g EDTA in 10ml of distilled water, pH 8), in plastic K3 EDTA tubes (Microsidd, India) for haematological analysis and without anticoagulant in 1.5ml Eppendorf tubes for serum analysis.

### 2.4. Haematological parameters

The haematological parameter like WBC (white blood corpuscles count), RBC (red blood corpuscles count), HGB (Haemoglobin), PLT(thrombocytes count) were analysed, using haematology analyser (Benesphera, India).

### 2.5. Serum Analysis

The blood sample for serum analysis were allowed to coagulate at room temperature. Subsequently, the samples were centrifuged for 20 min at 3000 rpm. Blood serum was collected and stored at -20°C and used for further analysis. Blood serum concentration of alanine transaminase (ALT), aspartate transaminase (AST), creatinine (CRE) and, blood urea nitrogen (BUN) was measured using Serum Analyzer (Fuji, Japan) making use of the respective slides supplied for use in the equipment.

### 2.6. Antioxidant Enzyme Assay

Antioxidant enzyme activity such as superoxidase dismutase, SOD (Madesh and Balasubramanian, (1998)), catalase CAT (Sinha, 1972), Reduced glutathione (GSH) (Sedlak and Lindsay, 1968) in liver of Nile tilapia were analysed.

### 2.7. Histology

The muscle, liver, gill and heart tissue of Nile tilapia exposed to different concentrations of $H_2O_2$ and formalin were aseptically and carefully removed and fixed in 10% Neutral Formalin Buffer (NBF). The tissues were processed, sections were made and haematoxylin-eosin staining was done (Roberts,2012). The slides were observed under trinocular microscope (ZEISS, Carl Zeiss India) with camera attached (Leica, Germany) using 100X and 200X magnifications.

### 2.8. Statistical analysis

All the data are expressed as mean ± standard deviation (Mean±SD). The data were compared using a one-way analysis of variance (ANOVA). The significance level was set at $P<0.05$. Statistical analysis was performed with the software package SPSS 26. Post hoc tests for comparison of means were also done using the same software.

## 3. Results

### 3.1. Water quality parameters in the acute toxicity test with $H_2O_2$

Water quality parameters were tested after the addition of respective doses of $H_2O_2$ in the treatment tanks. The range of water quality parameters in were optimum and are presented in table 1.

**Table1.Water quality parameters in the acute toxicity test with $H_2O_2$**

| Water Quality Parameter | Control | Treatment |
|---|---|---|
| pH | 7-7.5 | 7-8 |
| Alkalinity | 122-128 ppm of $CaCO_3$ | 122-130 ppm of $CaCO_3$ |
| Hardness | 160-178 ppm of $CaCO_3$ | 157-178 ppm of $CaCO_3$ |
| Dissolved oxygen | 6-9 ppm | 22-28.2 ppm |
| Temperature | 25-29°c | 25-29°c |
| Ammonia | 0.01-0.05 | 0.01-0.05 |

### 3.2. Determination of 96hr $LC_{50}$ of $H_2O_2$ in Nile tilapia

The mean mortality rate obtained for 96 hr acute toxicity tests at various concentrations of $H_2O_2$ are given in table 2. The results when analysed by non-linear regression model, the log $logEC_{50}$ was found to be 2.498 and $LC_{50}$ was found to be 314.6 ppm of $H_2O_2$ (Figure 1).

**Table2.The mortality rates of Nile tilapiatreated with different concentrations of $H_2O_2$.**

| Concentration | Mean Mortality Rate |
|---|---|
| 0 ppm | 0% |
| 150 ppm | 6.7% |

| | |
|---|---|
| 250 ppm | 26.7% |
| 350 ppm | 60% |
| 450 ppm | 83% |
| 600 ppm | 100% |

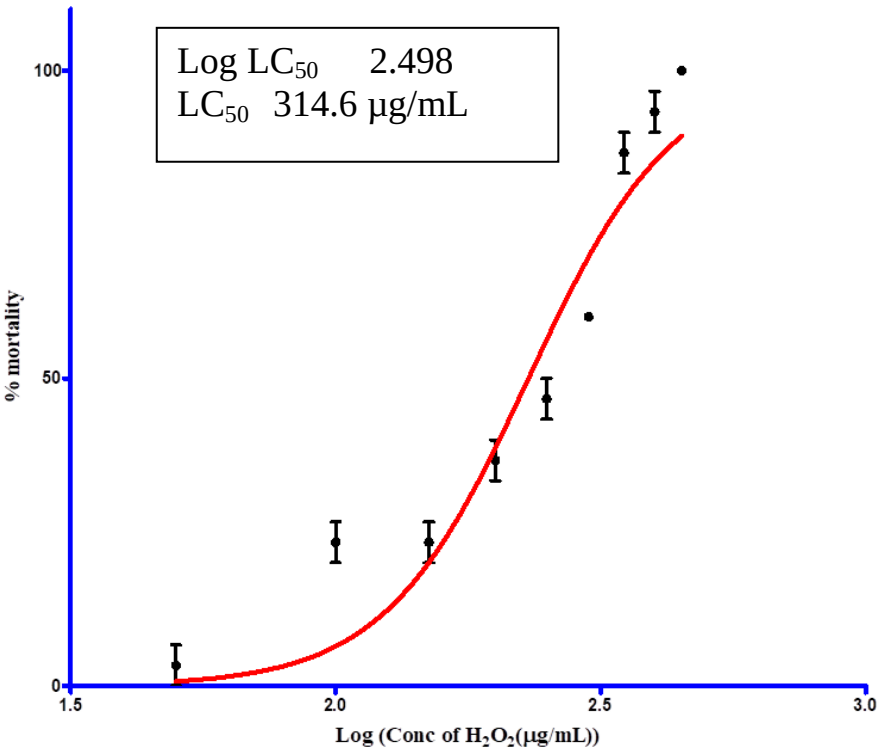



**Fig. 1. $LC_{50}$ of $H_2O_2$ in Nile tilapia**

### 3.3. Haematological, serological and antioxidant enzyme activity analysis of Nile tilapia exposed to $H_2O_2$.

The haematological parameters such as RBC,WBC, HGB and PLTof fish from control and treatment groups are presented in table3. The results obtained in the present study revealed that there is significant ($P \leq 0.05$) difference in haematological parameters due to lethal (≥250 ppm) and sublethal dosage of $H_2O_2$(≤ 150 ppm). It can be observed that all the parameters increased at a dosage of 150 ppm and as the concentration of $H_2O_2$ increased, there is considerable reduction in all the parameters measured.

The serum parameters of *O. niloticus* when exposed to different concentrations of $H_2O_2$ are given in table3. All the parameters significantly increased at a concentration of 250 ppm and beyond, compared to control. The ALT, AST, CRE and BUN of fish from control and treatment groups were presented in table3. The results obtained in the present study revealed that there is significant ($P \leq 0.05$) difference in serological parameters due to lethal and sublethal dosage of $H_2O_2$. It can be observed that

upto 150 ppm of $H_2O_2$ there was no significant increase in these parameters, but at 250 ppm and above, all the parameters significantly increased.

Activity of antioxidant enzymes such as SOD, CAT and GSH in liver of Nile tilapia exposed to different concentrations of $H_2O_2$ were measured. All The values are given in figure2. SOD and GSH are given in primary axis and CAT values ware given in secondary axis. GSH reduced significantly at 150 ppm and below. Catalase level at 150 ppm was similar to that of control, but increased significantly above that concentration. The SOD activity significantly reduced at 450 ppm of $H_2O_2$.

**Table 3. Haematological and serological parameters of Nile tilapia exposed $H_2O_2$**

| **Parameters** | **Control** | **150 ppm** | **250 ppm** | **350 ppm** | **450 ppm** | **F value** | **Sig. value** |
|---|---|---|---|---|---|---|---|
| **RBC ($\times 10^6$/µl)** | $1.65\pm0.28^a$ | $1.87\pm0.23^b$ | $1.26\pm0.27^c$ | $1.33\pm0.13^c$ | $1.23\pm0.11^c$ | 4.858 | 0.019 |
| **WBC($\times 10^3$/µl)** | $164.3\pm10.42^a$ | $173.33\pm5.13^a$ | $165.5\pm8.08^a$ | $131.9\pm9.05^b$ | $120.7\pm12.2^b$ | 18.813 | < 0.001 |
| **HGB (g/dl)** | $8.56\pm1.15+^a$ | $10.76\pm1.24^a$ | $7.23\pm0.64^b$ | $7.36\pm0.75^b$ | $7.53\pm0.80^b$ | 18.813 | 0.005 |
| **PLT($\times 10^3$/µl)** | $58.66\pm4.04^a$ | $64\pm5.46^a$ | $62.66\pm5.8^a$ | $43.33\pm3.05^b$ | $39.66\pm2.51^b$ | 19.863 | < 0.001 |
| **AST (u/l)** | $157\pm13.45^a$ | $141\pm19.6^a$ | $224\pm23.64^b$ | $246.3\pm22.0^b$ | $250.3\pm24.3^b$ | 17.637 | < 0.001 |
| **ALT (u/l)** | $8\pm1.73^a$ | $9.2\pm2.1^a$ | $11.66\pm0.57^b$ | $24\pm1.58^b$ | $26.3\pm1.73^b$ | 37.754 | < 0.001 |
| **CRE (mg/dl)** | $0.16\pm0.03^a$ | $0.16\pm0.04^a$ | $0.23\pm0.03^b$ | $0.26\pm0.01^b$ | $0.34\pm0.03^b$ | 16.179 | < 0.001 |
| **BUN (mg/dl)** | $1.01\pm0.02^a$ | $1.03\pm0.15^a$ | $1.66\pm0.05^b$ | $1.72\pm0.04^b$ | $1.68\pm0.2^b$ | 29.322 | < 0.001 |

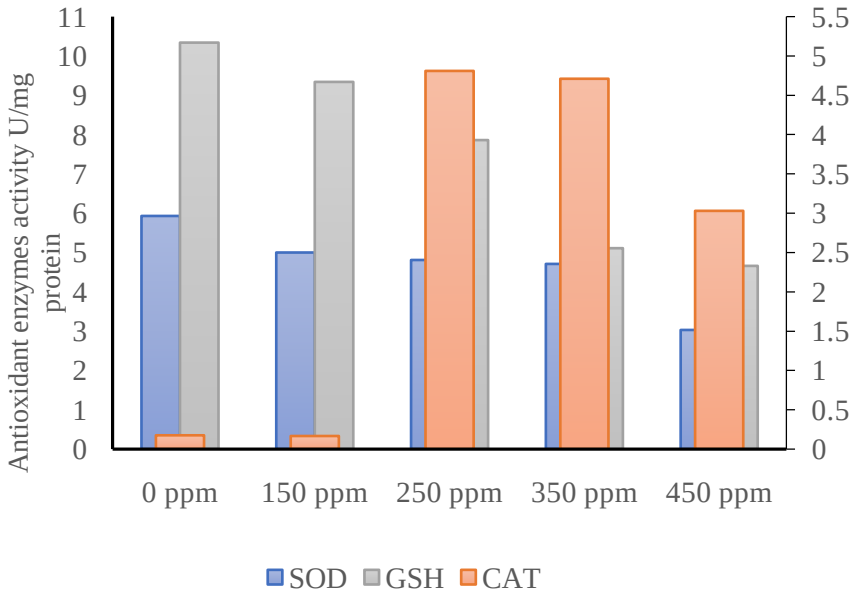



**Fig. 2. Activity of antioxidant enzymes of Nile tilapia exposed $H_2O_2$**

### 3.4. Water quality parameters in the acute toxicity test of formalin in Nile tilapia

The results of the physiochemical characteristics of the rearing water from treatment tanks with different concentrations of formalin are presented in table4. Although addition of formalin results in reduction of oxygen content and hardness, all the values were within optimum range.

**Table4. Water quality parameters in acute toxicity test with formalin**

| **Water Quality Parameter** | **Control** | **Treatment** |
|---|---|---|
| pH | 7-7.5 | 7-8 |
| Alkalinity | 122-128 ppm of $CaCO_3$ | 100-128 ppm of $CaCO_3$ |
| Hardness | 178 ppm of $CaCO_3$ | 115-140 ppm of $CaCO_3$ |
| Dissolved oxygen | 6-9 ppm | 5.2 – 5.6 ppm |
| Temperature | 25-29°c | 25-29°c |
| Ammonia | 0.01-0.05 | 0.01-0.05 |

### 3.5. Determination of $LC_{50}$ of formalin in Nile Tilapia

The mean mortality rate obtained for acute toxicity tests at various concentrations of formalin are given in table5. The results when analysed by non-linear regression model, the log $LC_{50}$ was found to be 2.364 and $LC_{50}$ value of formalin in Nile tilapia was found to be 231.2 ppm (Fig.3).

**Table5. The mortality rate of Nile tilapiais treated with different concentrations of formalin.**

| Concentration of formalin | Mean of Mortality Rate |
|---|---|
| 0 ppm | 0% |
| 50 ppm | 6% |
| 100 ppm | 23% |
| 150 ppm | 23% |
| 200 ppm | 36% |
| 250 ppm | 46% |
| 300 ppm | 60% |
| 350 ppm | 86% |
| 400 ppm | 93% |
| 450 ppm | 100% |

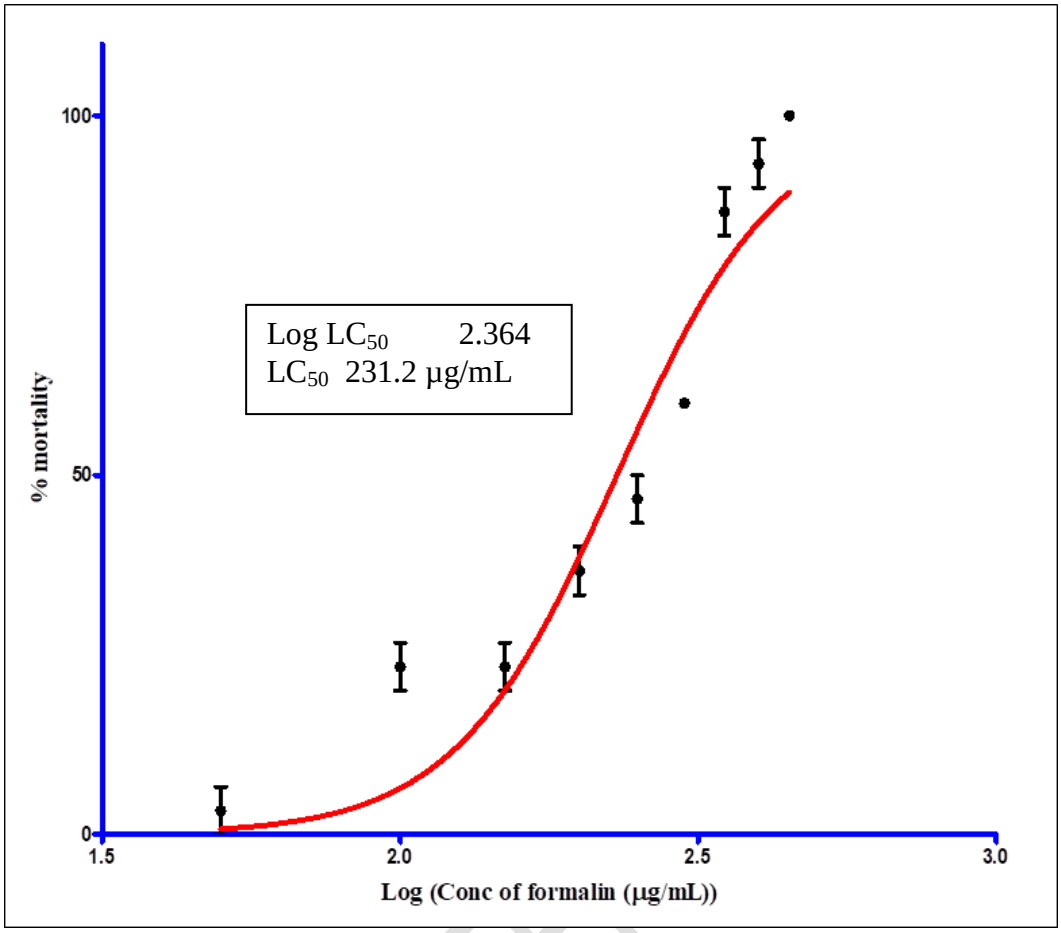



**Fig.3. $LC_{50}$ of formalinin Nile tilapia**

### 3.6. Haematological, serological and antioxidant enzyme activity of Nile tilapia exposed to formalin.

The haematological and serum parameters of *O. niloticus* when exposed to different concentrations such as 0ppm, 50 ppm, 150 ppm and 250 ppm of formalinwere given in table6. All the parameters increased at 150 ppm and then significantly reduced ($P<0.05$) at 250 ppm.The serum parameters as AST, ALT, creatinine, and BUN showed significant difference between the treatments. All the parameters were significantlyincreased at concentrations ≥150 ppm of formalin.

Activity of antioxidant enzymes such as SOD, CAT, and GSH were given in figure 4. SOD and GSH are given in primary axis and CAT values were given in secondary axis. SOD, CAT and GSH significantly reduced with increasing concentration of formalin.

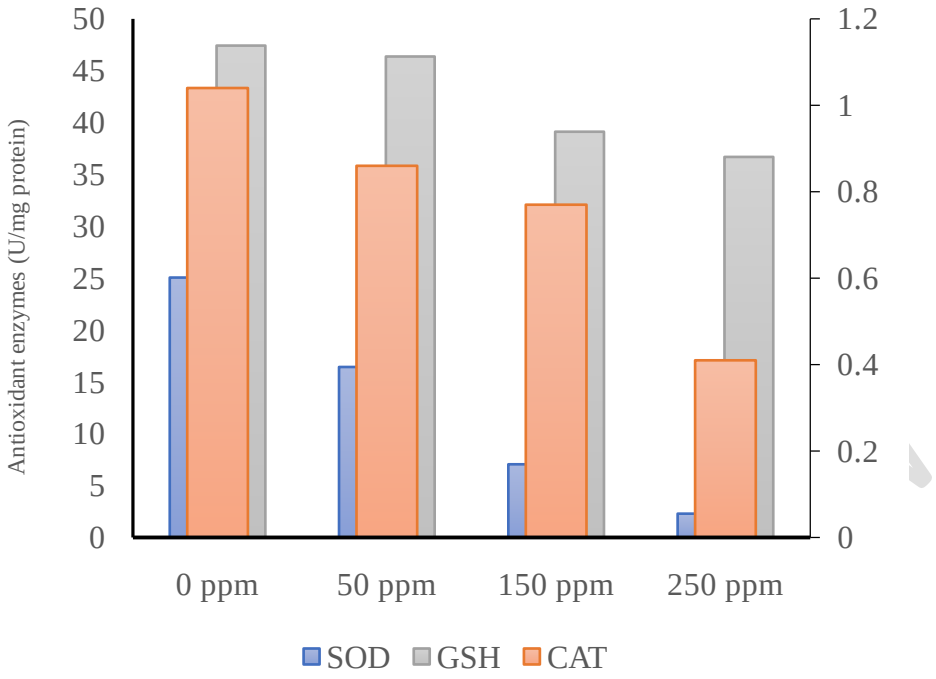



**Fig. 4. Antioxidant enzyme activity in Nile tilapia exposed to formalin**

**Table6. Hematological and Serological parameters of Nile tilapia exposed to formalin**

| **Parameters** | **0 ppm** | **50 ppm** | **150 ppm** | **250 ppm** | **F value** | **Sig. value** |
|---|---|---|---|---|---|---|
| **RBC($\times10^6$/µl)** | 1.36±0.10$^a$ | 1.38±0.10$^a$ | 1.63±052$^b$ | 1.24±0.22$^c$ | 0.922 | 0.003 |
| **WBC($\times10^3$/µl)** | 140.4±2.9$^a$ | 156.1±10.8$^b$ | 202.36±2.3$^c$ | 133.13±14.5$^d$ | 33.692 | < 0.001 |
| **HGB (g/dl)** | 8.4±0.65$^a$ | 8.5±0.26$^a$ | 9.3±0.5$^a$ | 7.7±0.5$^b$ | 5.584 | 0.023 |
| **PLT ($\times10^3$/µl)** | 29.66±3.2$^a$ | 36.66±2.08$^a$ | 63.33±5.7$^b$ | 43.66±3.2$^c$ | 43.291 | < 0.001 |
| **AST (u/l)** | 128.6±16.9$^a$ | 138.33±8.5$^a$ | 159.33±17.38$^b$ | 178.6±32.57$^c$ | 3.470 | < 0.001 |
| **ALT (u/l)** | 10.66±1.15$^a$ | 12.66±2.08$^{ab}$ | 15.66±3$^b$ | 21.66±1.52$^d$ | 16.220 | < 0.001 |
| **CRE (mg/l)** | 1.83±0.23$^a$ | 1.8±0.3$^a$ | 2.9±0.2$^b$ | 3.16±0.35$^b$ | 19.779 | < 0.001 |
| **BUN (mg/l)** | 0.256±0.03$^a$ | 0.33±0.02$^a$ | 0.563±0.06$^b$ | 0.583±0.03$^b$ | 48.531 | < 0.001 |

### 3.7. Histopathology of Nile tilapia treated with $H_2O_2$ and Formalin

The results obtained after haematoxylin and eosin staining of muscle tissue sections at different concentrations of $H_2O_2$ and formalinwere given in figure 5. Figure 5A and 5B represented the muscle tissues of control showing normal structure. At concentration of 150 ppm of $H_2O_2$, presented a marked reduction in the interfascicular space between the muscle bundles and a few focal areas of

necrosis (Fig. 5C). At the lethal concentration of 350 ppm (Fig. 5D), muscle fibres were completely disorganized with increased endomycial distance and vacuolations. At 50 ppm formalin (Fig. 5E), marked reduction in the interfascicular space with infiltration of blood cells was observed. At 250 ppm formalin (Fig. 5F), the muscle fibre bundles were completely disorganized and had loss of architecture and vacuolation.

The H&E-stained liver sections were given in figure 6. The control sections revealed the presence of melanomacrophage centres associated with liver tissue and pancreatic tubules were seen distributed in hepatic tissue (Fig. 6A&B). In the liver tissue exposed to 350 ppm of $H_2O_2$, (Fig. 6C), the melanomacrophage centres were almost absent and the hepatocytes were vacuolated, necrotic with karyorrhexis and pyknosis. Hyperplasia of hepatocytes was also evident. The section of liver of the fish exposed to lethal concentration of formalin (250 ppm) (Fig. 6D) showed that the cells were disoriented, hyperplasic, vacuolated, further the margins of hepatocytes were not distinct and there were necrotic areas with syncytial cells.

The histological section of the gills of the healthy fish (Fig. 7A) showed well-formed secondary lamellae with erythrocytes in the centre. The filament cartilage of the primary lamellae was also well formed (Fig. 7B). In the case of fish exposed to 350 ppm of $H_2O_2$ (Fig. 7C), the secondary lamellae were shrunken with very few blood cells. There was sloughing of secondary lamellae from the tip of the filament. The filament cartilage was degenerated. The gills of fish exposed to 250 ppm of formalin (Fig. 7D) showed extensive infiltration of blood cells and degenerated filament cartilage.

In the control fish, the heart muscle fibres were found highly organized having blood cells (Fig. 8A&B). But in the heart tissue of the fish exposed to 150 ppm $H_2O_2$, the muscle fibres were shrunk (Fig.8C). At 350 ppm, the heart muscle cells were highly disorganized and there was heavy infiltration of blood cells into the interstitial spaces. Haemorrhage and congestion were noted. In the case of fish exposed to 250 ppm formalin, there was vacuolation, infiltration of blood cells and pericarditis (Fig. 8D).

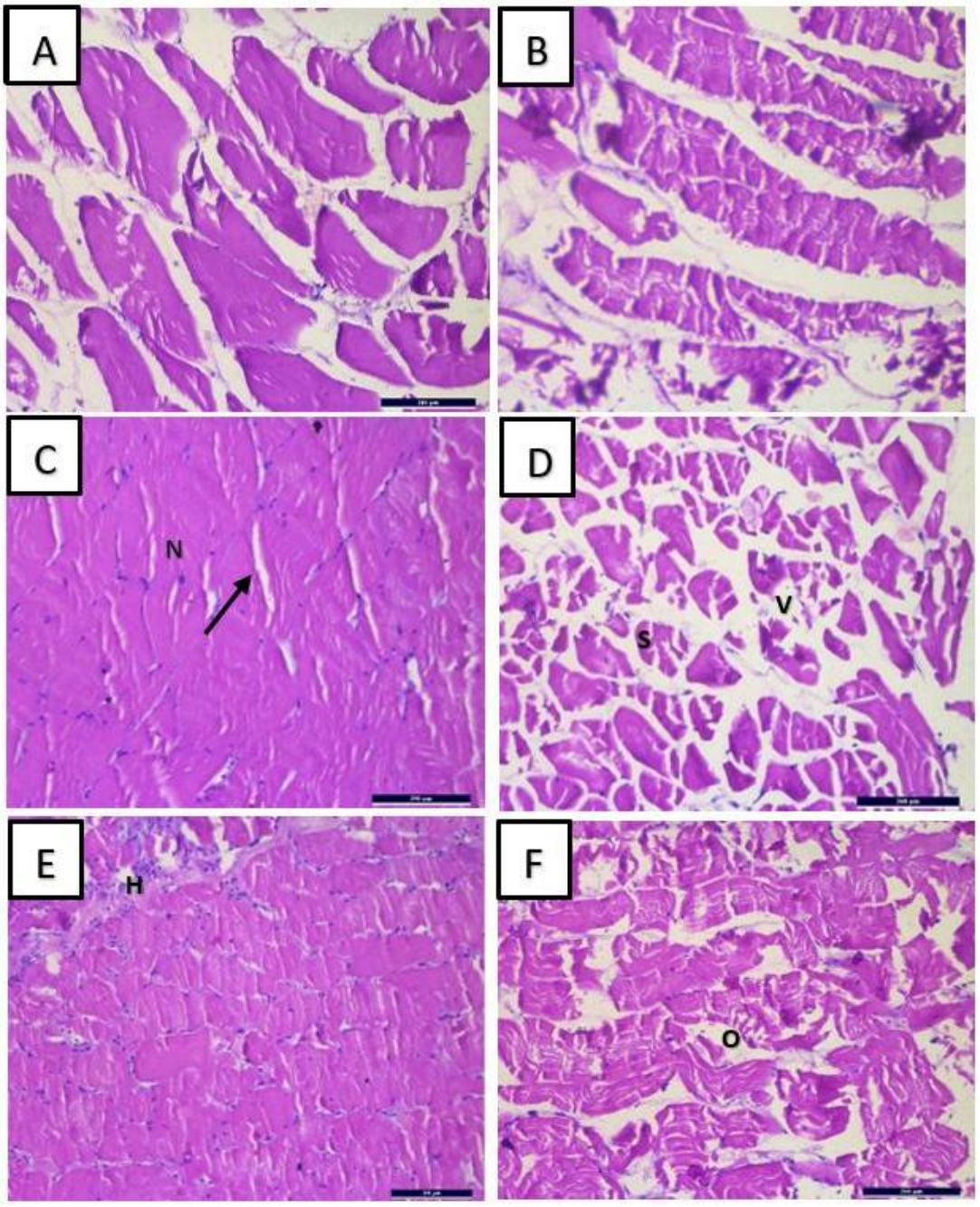



**Fig. 5.Muscle tissue section of Nile tilapia exposed to different concentrations of $H_2O_2$ and formalinafter exposure for 96 hrs (200X)**

A & B: Control; C: At 150 ppm of $H_2O_2$ showing necrosis (N) and decreased space between muscle fibres (arrow); D: At 350 ppm of $H_2O_2$ splitting of muscle fibres (S) and vacuolation (V); E: At 50 ppm formalinshowing infiltration of haemocytes (H); F: At 250 ppm of formalinshowing disoriented muscle fibres (O).

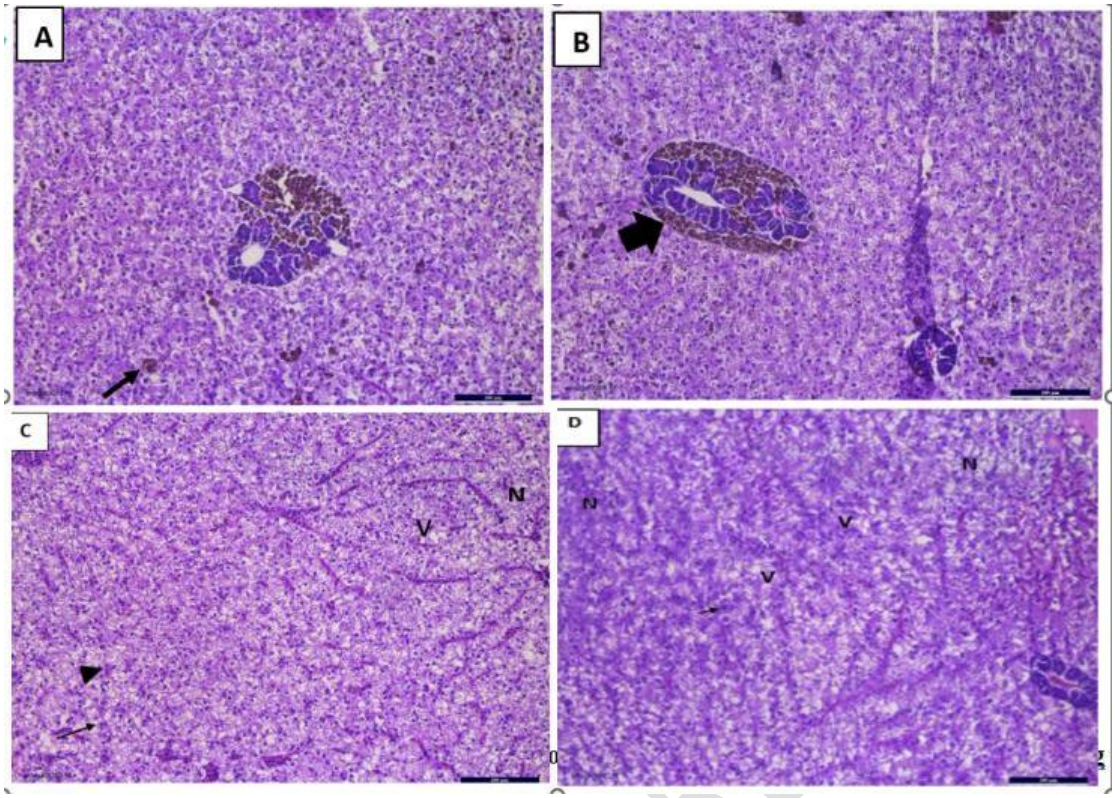
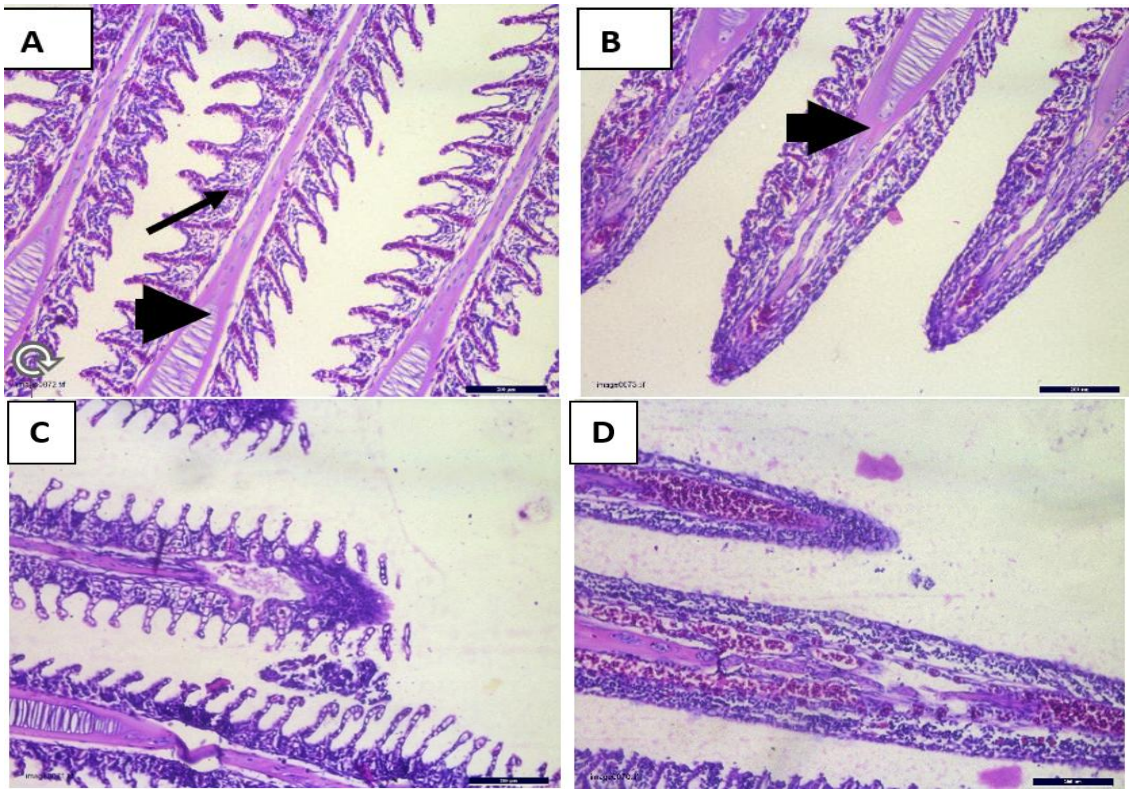



**Fig. 6.Liver tissue section of Nile tilapia exposed to different concentration of $H_2O_2$ and formalinafter exposure for 96 hrs (200X)**

A&B: Control withmelanomacrophage centers (MMC) (thin arrow) and pancreatic tubules (thick arrow). C:At 350 ppm $H_2O_2$, there is absence of MMC, hepatocytes vacuolated (V), areas of necrosis (N), karyorrhexis (arrowhead) and pyknosis (arrow). D:At 250 ppm of formalin, the hepatocytes are disorganized, cell membrane indistinct, syncytial cells (arrow), vacuole formation (V) and necrosis (N).

**Fig.7.Gill tissue section of Nile tilapia exposed to different concentration of $H_2O_2$and formalinafter exposure for 96 hrs (200X).**

A. Gill section of control fish with secondary lamellae with erythrocytes in the centre (thin arrow), B. Well-formed filament cartilage (arrow head). C. Gills of fish exposed to 350 ppm of $H_2O_2$ showing shrinking of secondary lamellae and lack of erythrocytes, sloughing of lamellae, degenerative changes in the filament cartilage. D. Gill lamellae of Nile tilapia exposed to 250 ppm formalin showing massive infiltration of erythrocytes and degenerated filament cartilage.



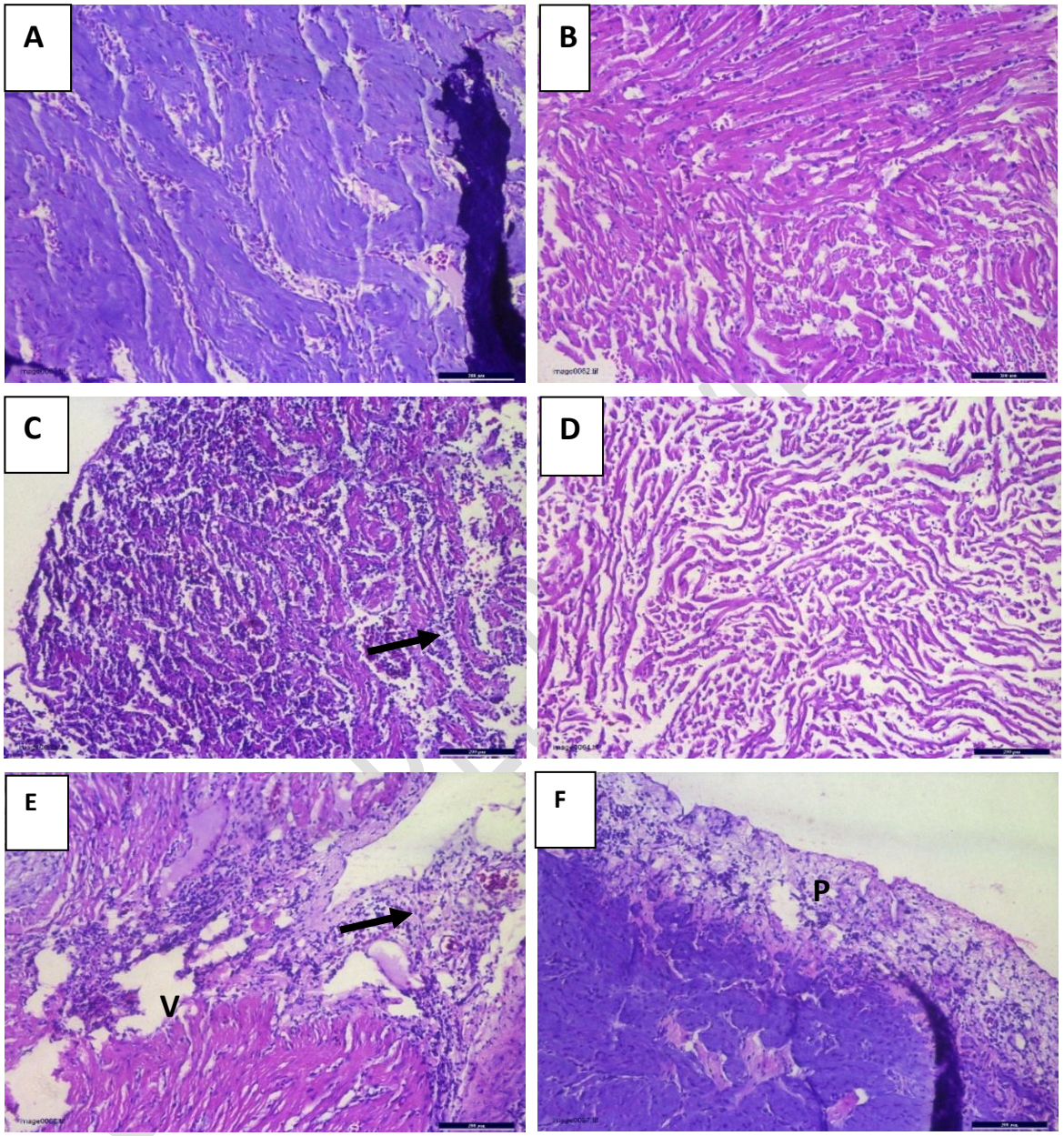



**Fig. 8. Heart tissue section of Nile tilapia exposed to different concentration of $H_2O_2$and formalinafter exposure for 96 hrs (200X)**

A. Bulbus arteriosus; B. Atrium; C. Heart tissue of Nile tilapia exposed to 350 ppm $H_2O_2$ with disorganised muscle fibers (arrow); D. Shrinking of muscle fibers exposed to 150 ppm $H_2O_2$; E. Heart tissue of Nile Tilapia exposed to 250 ppm formalin showing vacoulation (V) and pericarditis (arrow); F. thickening of pericardium/Pericarditis (P).

## 4. Discussion:

Acute toxicity of $H_2O_2$ to several fish species has been determined in various studies and the $LC_{50}$ it was found to vary between 50 to 2010 mg/L (Clayton and Summerfelt 1996; Arndt and Wagner 1997; Rach *et al.,* 1997). Hydrogen peroxide can be toxic to fish, resulting in morbidity due to gill damage or mortality depending on the size of the fish, fish species, water temperature, concentration of the chemical, and exposure duration (Russo and Yanong, 2007). Haematological parameters of like RBC, HGB and HCT have been used as parameters of physiological stress by toxic chemicals in fish (Kawatsu, 1980; Chandrasekar and Jayabalan, 1993; Sampath *et al.,* 2003). At 250 ppm and higher concentrations all the hematological parameters significantly reduced on exposure to $H_2O_2$.This may be due to chemical oxidative stress to the treated fish (Soivio and Oikari 1976; Soivio and Nikinmaa 1981).Higher antioxidant enzyme gene expression after short exposure to sublethal concentrations of hydrogen peroxide have been reported in Atlantic Salmon (Vera and Miguad, 2016). Decrease of antioxidant enzymes due to $H_2O_2$ toxicity via intraperitoneal injection has been reported in *O. niloticus* by Jia *et al.,* (2019). The present study reveals that, at a concentration of 150 ppm of $H_2O_2$, the antioxidant enzyme status is increased, but at toxic levels, there is a marked reduction in the enzymes which may be due to decrease in production of these enzymes due to liver damage or due to excessive production of free radicals due to high stress, which are beingscavenged by these enzymes. The damage in liver and gills may be due to the excessive production of free radicals *via* Fenton reaction as suggested by Ayadi *et al.,* (2015).

Acute toxicity ($LC_{50}$—96 h) to formalin vary widely among fish species (0.1–640.0 mg/L) depending on various factors, as many fish species are sensitive to concentrations near those required for controlling and treating parasites (Tavares-Dias, 2021). According to certain protocols, formalin should be used in brief baths (lasting up to one hour) at concentrations between 150 and 250 ml/$m^3$ or in long baths (lasting 24 hours) at concentrations between 10 and 15 ml/$m^3$ (Thoney and Hargis 1991; Martins 2004). Increase in HGB, hematocrit and immature erythrocytes because of formalin exposure have been reported in carp *C. carpio* and rainbow trout *Oncorhynchu- mykiss* (Smith and Piper 1972; Williams and Wootten 1981; Yamamoto 1991; Kakuta *et al.,* 1991). In the present study various serum parameters of fish such as AST, ALT, CRE and BUN were increased significantly at and above 150 ppm of formalin compared to control and 50 ppm. Increased AST and ALT activity were seen in the blood and liver tissues of fish subjected to various doses of carbaryl, monocrotophos, deltamethrin, diazinon, and malathion for varying lengths of time (Banaee *et al.,* 2011; Karmakar *et al.,* 2021). Decreased SOD activity may be a response of increased oxidative stress. The effect of Formalin on lipid peroxidation which results in the decrease in activity of antioxidant enzymes have been reported (Xu and Chang, 2007). Rainbow trout treated to formalin at various doses showed significant decreases in CAT activity throughout their entire body (Ć°spir *et al.,* 2017). hyperplasia of gill lamellae and fatty degeneration in the liver were observed in silver barb (Chinabut *et al.,* 1988). Kakuta *et al.,* (1991) observed hypertrophy, hydropic degeneration and separation on the epithelial cells of gill lamella after exposed to 280 mg L-1 of formalin for 2 h. These histopathological changes in the gill tissue observed in the present study were qualitatively like those reported for formalin-treated rainbow trout and chinook salmon (Wedemeyer 1971; Smith and Piper, 1972; Wedemeyer and Yasutake, 1974). Mert *et*

*al.*, (2015) has observed secondary lamellar fusion after Formalin exposure along with hyperaemia of gill lamellae, telangiectasis on secondary lamellae bronchitis at a temperature in *O niloticus.* In the present experiment also, gills were found to be severely affected with shrinking, sloughing, and hyperaemia.

## 5. Conclusion

To reduce the use of antibiotics and other chemicals harmful for environment and consumers, there is a need to standardise protocols with FDA approved chemicals like $H_2O_2$ and formalin, to treat fish diseases and as preventive measures, which is cheap on a farmer's perspective and equally effective. However, the standardisation should be done for each species, because toxicity differs in different taxa and also with the life stages. The result of the current study concluded that the therapeutic application of $H_2O_2$ and formalinwith recommended dose 150 ppm and 50 ppm respectively is safe to treat adult *O. niloticus* for parasitic, fungal, and bacterial infections.

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