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Histopathological Changes and Antioxidant Enzymes as Biomarkers for Monitoring the Health of Fish *Cyprinus carpio* L.

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Abstract: The current study focused on monitoring the health of *Cyprinus carpio* (common carp) to assess the health of fish in aquatic environment. The study was designed to include various environmental conditions. Four groups were involved in this study: a control group, a group treated with thyme oil, a group treated with cadmium chloride, and a group exposed to thyme oil and cadmium chloride. The antioxidant levels (glutathione peroxidase and catalase) in gills and muscles were measured at intervals over 30 days. Histological examinations were performed to detect any pathological changes in gills and liver resulting from treatments at 3, 7, 15, and 30 days. Exposure to cadmium chloride induced progressive pathological changes, including gill deformities, lamellar adhesion, and epithelial hyperplasia, as well as hemorrhage, degeneration, and necrosis in the liver. The addition of thyme oil to the fish diet, however, mitigated these alterations in both the gills and liver. The results showed significant variations in GPX1 and catalase activity across treatments. Cadmium chloride increased GPX1 and catalase activity in the liver, gills, and muscles, while thyme oil modulated these effects, with muscle tissue exhibiting the highest activity, especially in cadmium-exposed groups. The results demonstrated that these indicators effectively evaluated fish health, providing effective insights for maintaining fish health in the aquatic environment. It was also observed that good aquatic environmental health is not always associated with high levels of antioxidants as a result of pollution. Periodic monitoring of antioxidant levels is recommended to ensure the health of the aquatic environment.

Keywords: Cellular defense enzyme, *Cyprinus carpio*, Microscopic pathology, Molecular marker

Introduction

A healthy ecological balance heavily relies on factors such as aquatic organisms like fish, as considered a vital natural resource providing protein and lipids (Nur *et al*., 2020). However, pollution is emerging as one of the major environmental concerns that threaten life on Earth. Pollution can affect directly biological molecules and aquatic ecosystems leading to negative effects on the economy unless immediate actions are taken by authorities to

address pollution through a remediation process. Monitoring the health of fish in their natural environments is crucial for those with interests in aquatic systems with stress on the need for improved tools to assess fish health, water quality and pollution (Tort et al., 2011; Huntingford & Kadri, 2014). Regular monitoring is important for evaluating the effectiveness of strategies to enhance aquatic health, productivity, and conversion efficiencies, and addressing conditions that lead to disease through infection, nutritional deprivation, or environmental stress (Lapointe *et al*., 2014). The complicated interaction of harmful factors can have more impacts than their individual effects (Salamat and Zarie, 2012). Therefore, the use of tools for monitoring aquatic systems, such as biomarkers to monitor changes in aquatic health (McCarthy& Shugart1990).

Biomarkers can be described as biological indicators of environmental stress responses (van der Oost *et al*., 2003), that can be used as early warning systems for detecting potentially harmful substances (Meckawy *et al*., 2022), providing useful information on these substances and their potential effects on biological ecosystems. Histological examination is an essential biomarker for evaluating the impacts of environmental stressors on the natural structures of organisms (Nascimento *et al*., 2012; Al-Mamoori *et al*., 2014; Authman *et al*., 2015; Carvalho *et al*., 2020; Alves *et al*., 2022; Abad-Rosales et al., 2022). Other biomarkers, such as antioxidants (Xie et al., 2019; Jung *et al*., 2024), and heavy metal bioaccumulation (Kumar *et al*., 2024), have been used to assess aquatic ecosystem health. However, bioaccumulation, histological alterations, and antioxidant levels are important biomarkers for evaluating the health of aquatic organisms and entire ecosystems. A single biomarker may not provide a complete insight into aquatic ecosystem health (Pinto *et al*., 2009; Viana *et al*., 2013). Pollutants can play roles in delaying the manifestation of histological changes due to their accumulation and their concentration at the time of examination. Antioxidant levels in aquatic ecosystems can be affected by these factors and other factors such as sampling, tissue analysis, and data reporting (Waltham *et al*., 2013). The use of multiple biomarkers can improve general knowledge about changes in aquatic ecosystem health. This study seeks to establish an integrated model for assessing fish health more effectively by investigating the impact of water pollution caused by cadmium and evaluating the protective potential of nutritional supplements like thyme oil. Cadmium was chosen as the pollutant of focus (Mizhir *et al*., 2023), and thyme oil, known for its antioxidant properties, was incorporated into fish feed. The model provides a comprehensive assessment of fish health in polluted environments by analyzing cadmium accumulation, histopathological changes, and antioxidant levels as key health indicators.

Materials & Methods

Experimental fish

150 juvenile common carp (*C. carpio*) with an average weight of 39.37 ± 1.54 g, bred in the laboratory of the Department of Biology at the College of Education Qurna, University of Basrah. Four glass tanks (80 cm x 45 cm x 40 cm, 60 liters) were used to breed fish within a semi-closed system, filled with dechlorinated water, and equipped with electric air pumps to maintain adequate dissolved oxygen levels. The fish were acclimated for 10 days under controlled conditions—temperature at 24 ± 0.5°C, a 12-hour light/dark cycle, dissolved oxygen at 8.42 ± 0.31 mg/L, salinity at 1.076 ± 0.2 g/L, and pH at 7.9 ± 0.24. They have been

fed a commercial diet equivalent to 3% of their body weight bi-daily (Sayed *et al*., 2023). The siphoning process used for maintaining fish includes changing two-thirds of the tank water and ensuring the removal of uneaten food and waste daily. Then, replenishment by dechlorinated water to ensure a clean and stable environment over the study period.

Determination of the LC_{50} of Cadmium Chloride in Fish

The fish were divided into five groups of 12 fish each. Four groups were exposed to different concentrations of cadmium chloride (56.2, 112.4, 168.6, 224.8 mg/L), while the fifth group was not exposed to cadmium chloride, which served as a control. Mortality rates were monitored over 72 hours. Graph Pad Prism V.8 was used to determine the LC_{50} value of cadmium chloride (Hashim et al., 2020).

Exposing Fish to Cadmium Chloride and Thyme Oil

Fish were randomly divided into four experimental groups, 16 fish each: Group1(G1) served as the control, maintained in chlorine-free water and fed a commercial diet with specified protein content; Group 2 (G2) was exposed to an aqueous solution of cadmium chloride ($CdCl_2$), Thomas Baker (India), at a concentration of 16.86 mg/L; Group 3 (G3) received a diet supplemented with thyme oil (SHEER Essence, India) at a ratio of 1:3 thyme oil to feed; and Group 4 (G4) was simultaneously exposed to cadmium chloride at 16.86 mg/L and fed a diet supplemented with thyme oil (1mL of thyme oil to 3 g feed), with prior acclimation to the thyme oil diet for 21 days. All groups were provided with a standardized diet containing 37% protein, 8.84% fat, and 42.64% carbohydrates at 5% of their body weight, twice a day. Samples were collected at 3, 7, 15, and 30 days after treatment, with three replicates. Cadmium concentration levels were measured in the gills and muscles to evaluate the histopathological changes in the gills and liver and analyze antioxidant levels in the gills, liver and muscles.

Examination of Histopathological Changes

Histological examination of liver and gill tissues, collected at 3, 7, 15, and 30-day intervals, was conducted following Humason's (1972) protocol. Fixation using Bowen stabilizer for 24 hours, followed by extensive washing with 50% ethyl alcohol, and subsequent dehydration in a series of alcohol solutions (70%, 80%, 90%, and 100%), each lasting two hours. The clearing phase involved treating the samples with xylene to facilitate infiltration with molten paraffin wax at 56 °C, after which the embedded samples were placed in metal molds filled with clear molten paraffin. Following embedding, the tissues were trimmed and sectioned into 5-7 µm thick slices using a rotary microtome, then mounted on glass slides and stained with hematoxylin and eosin. The stained tissue sections were examined and photographed using a Zeiss imaging microscope equipped with a Leica digital camera, allowing for detailed visualization and documentation of histopathological changes.

Assessment of Antioxidant Enzyme Levels

To assess antioxidant enzyme levels, liver, gills, and white muscles were collected from fish at 7, 15, and 30-day intervals, using specific assay kits for catalase (CAT, Elabscience, Cat. No. E-BC-K031-S) and glutathione peroxidase (GPX1, Elabscience, Cat. No. E-EL-H5410). Protein extraction was carried out via homogenization of 100 mg samples from each tissue, with the addition of 150 µL of extraction buffer and 10 µL of a 2

mM protein inhibitor solution, followed by vortex and a 30-minute room temperature incubation. The homogenate was then centrifuged at 20,000 rpm for five minutes, and the supernatants (100 µL each) were collected for enzymatic assays. For the catalase (CAT) and glutathione peroxidase (GPX1) assays, each supernatant was combined with specific reagents tailored for these analyses, conducted in specialized plates. The plates were processed according to established protocols, and enzymatic activity levels were measured at a wavelength of 450 nm using the Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer.

Statistical analysis

Statistical analysis was conducted using one-way ANOVA with SPSS v19 software. Mean values and standard errors were reported, with significance levels set at $P < 0.05$ for antioxidant assessments and $P < 0.01$ for cadmium chloride accumulation measurements.

Results

LC50 Half-Lethal Concentration

Different concentrations of cadmium chloride (56.2, 112.4, 168.6 and 224.8 mg/L) were exposed to the fish over 72 hours to determine the half-lethal concentration (LC50). The LC50 was 137.46 mg/L, which corresponds to the concentration at which 50% of the fish population died due to cadmium chloride exposure (Fig. 1).

Histological examination showed that the fish gills in the G1 remained healthy without histopathological changes. These include gill arches, primary filaments, and secondary lamellae made of squamous epithelial tissue, with two layers of epithelial cells and a blood capillary, as shown in (Figure 2). The gills of fish treated with thyme oil in G3 showed minor histopathological changes, especially in some secondary lamellae over the 3, 7, 15, and 30 days, as illustrated in (Figs. 3, 4, 5, and 6). The exposure to 16.86 mg/L of cadmium chloride (G2) showed significant pathological changes. Notable histological changes were observed after three days, including the transformation of secondary gill lamellae into shapes resembling an upside-down "L" and the adhesion of some lamellae to each other (Fig. 7). The severity of histopathological changes increased on day 7 with continued deformation and adhesion of the lamellae. It can be also observed an increased division of chloride cells at the bases of the secondary lamellae, manifests as simple hyperplasia confined to the basal quarter. Congestion of capillary blood vessels, and separation of the epithelial tissue layer in certain lamellae (Fig. 8). After 15 days of exposure, the hyperplastic region expanded to the middle of the secondary lamellae (Fig. 9). By day 30, it extended beyond the midpoint along with bleeding in some secondary lamellae filaments (Figs. 10 and 11). However, the histopathological sections of gills from fish exposed to cadmium chloride that were added to dietary supplements (G4) showed that the gills tissue structure was less damaged than fish in the group (G2). After day 3, some morphological changes were observed, such as capillary congestion and other changes in the secondary lamellae (Fig. 12). On day 7, slight hyperplasia, congestion, and separation of the epithelial layer of the secondary lamellae were noted (Fig. 13). By day 15, mild hyperplasia and morphological alterations were present (Fig. 14). By day 30, less severe histological changes, primarily limited to slight hyperplasia and morphological alterations in some secondary lamellae, observed (Fig. 15).

Histological analysis of liver sections from the control group (G1) showed a radial arrangement of hepatocytes around the central

vein, with hepatic sinuses interspersed (Fig. 16) .

No pathological changes in liver tissues that were examined from G3. The tissue's structure was kept intact and consistent melanomacrophages presence during all experiments time (Figs. 17-20). Observation from G2 showed duration-dependent histopathological changes. Significant haemorrhaging was observed and vacuolar degeneration of hepatocytes near the central vein, and central vein congestion after three days (Figs. 21 and 22). These changes persisted after seven days, with the continued presence of melanomacrophages (Figs. 23 and 24). Hepatocyte necrosis was observed after day 15 of exposure to cadmium chloride. On day 30, the abnormalities intensified and affected a larger portion of liver tissue (Figs. 25 and 26) .

Liver tissues appeared mostly normal after three days of exposure in G4 with melanomacrophages present (Fig. 27). At day 7, minor inflammatory changes were noted including localized blood cell infiltration and the continued presence of melanomacrophages (Fig. 28). After 15 days (Figs. 29 and 30) and after 30 days of exposure of combined treatment (Figs. 31 and 32), liver tissue remained largely normal with only isolated portions showing degenerative hepatocytes and clusters of melanomacrophages.

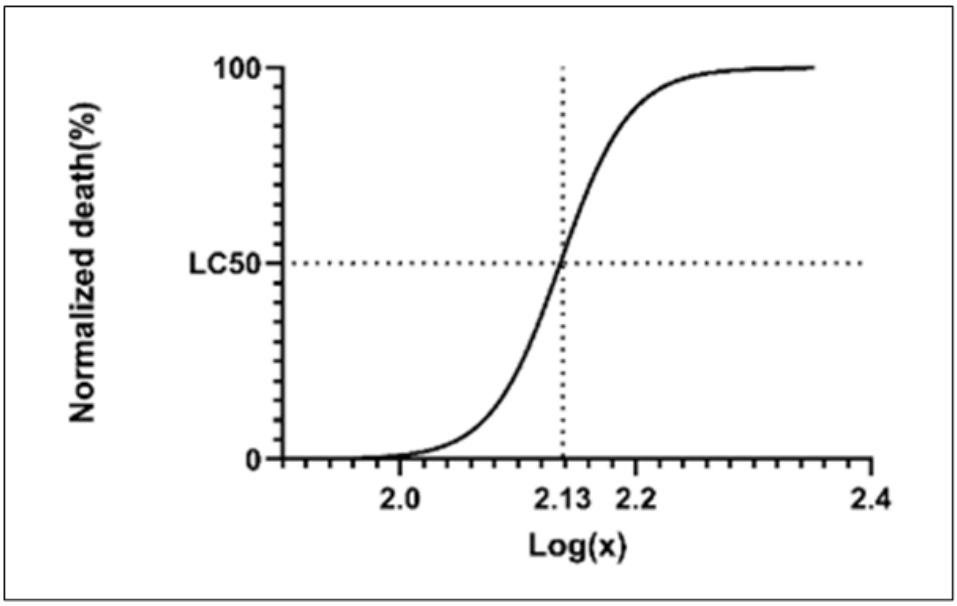

Fig. (1): LC50 concentration of cadmium chloride in common carp within 72 hours

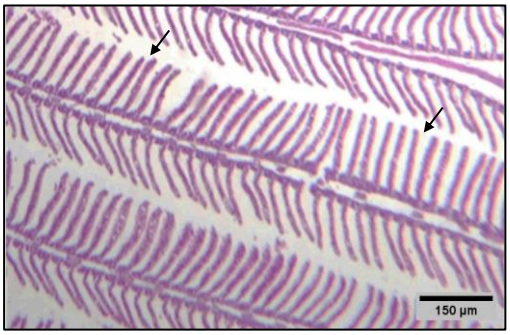

Fig. 2: Histological section of fish gill tissue from the control group, illustrating the secondary gill lamellae (arrows), stained with Hematoxylin and Eosin (H &E).

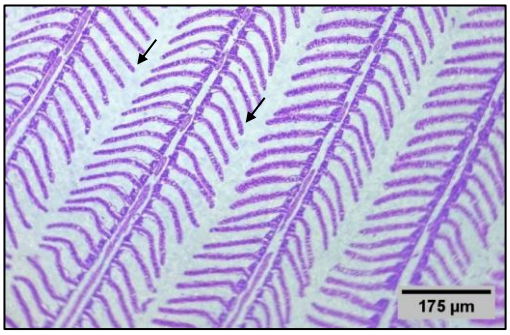

Fig. 3: Histological section of fish gill tissue from the group treated with thyme oil alone after 3 days of treatment, showing the integrity of the secondary gill lamellae (arrows). Stained with Hematoxylin and Eosin (H&E).

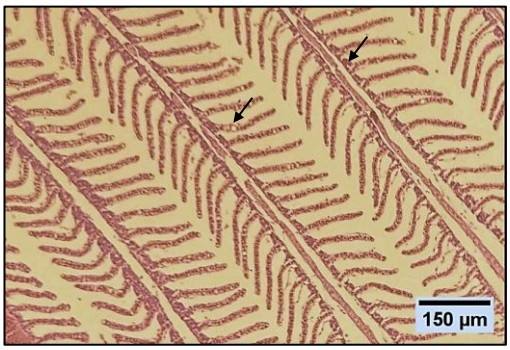

Fig. (4): Histological section of fish gill tissue from the group treated with thyme oil alone after 7 days of treatment, showing slight changes in the shapes of some lamellae (arrows), stained with Hematoxylin and Eosin (H & E).

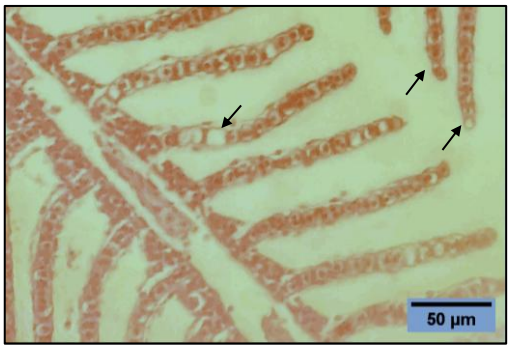

Fig. (5): Histological section of fish gill tissue from the group treated with thyme oil alone after 15 days of treatment, showing slight changes in the shapes of some secondary gill lamellae (arrows), stained with Hematoxylin and Eosin (H & E).

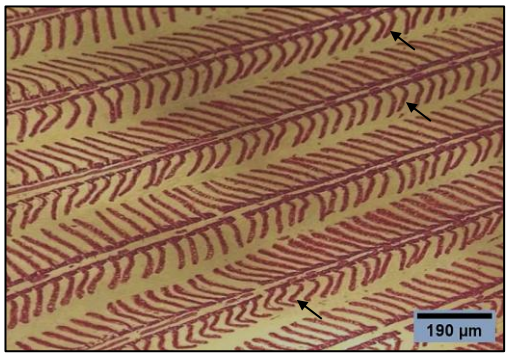

Fig. (6): Histological section of fish gills from the group treated with thyme oil after 30 days of treatment, showing slight changes in the shapes of a few secondary gill lamellae (arrows), stained with Hematoxylin and Eosin (H & E).

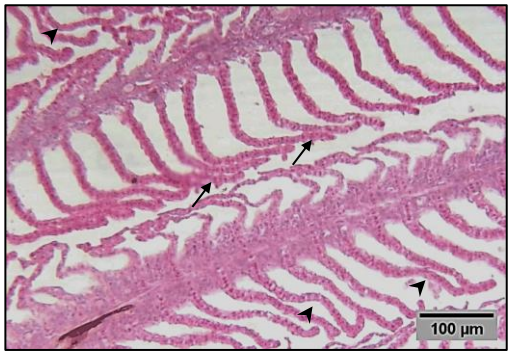

Fig. (7): Histological section of fish gills from the group treated with 16.86 mg/L of cadmium chloride after 3 days of treatment, showing adhesion of secondary gill lamellae (arrows) and changes in lamellar shapes (arrowheads), stained with Hematoxylin and Eosin (H & E).

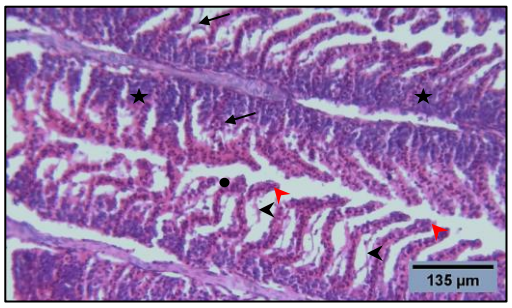

Fig. (8): Histological section of fish gill from the group treated with 16.86 mg/L of cadmium chloride after 7 days of treatment, showing adhesion of secondary gill lamellae (black arrow), hyperplasia (asterisk), detachment (arrowhead), shape change (red arrow), and decongestion (circle), stained with Hematoxylin and Eosin (H & E).

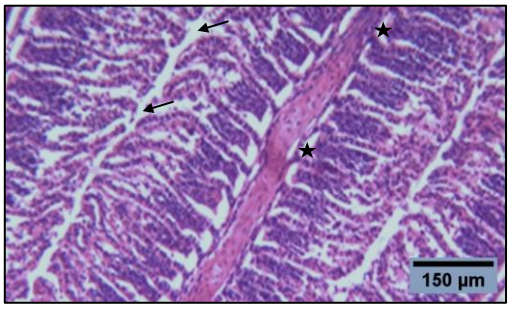

Fig. (9): Histological section of fish gill tissue from the group treated with 16.86 mg/L of cadmium chloride after 15 days of treatment, showing hyperplasia (asterisk) and changes in the shapes of secondary gill lamellae (arrows), stained with Hematoxylin and Eosin (H & E).

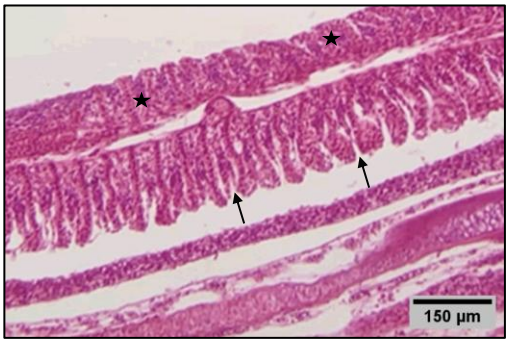

Fig. (10): Histological section of fish gill tissue from the group treated with 16.86 mg/L of cadmium chloride after 30 days, showing capillary vascular congestion in secondary gill lamellae (arrows) and hyperplasia (asterisk), stained with Hematoxylin and Eosin (H & E).

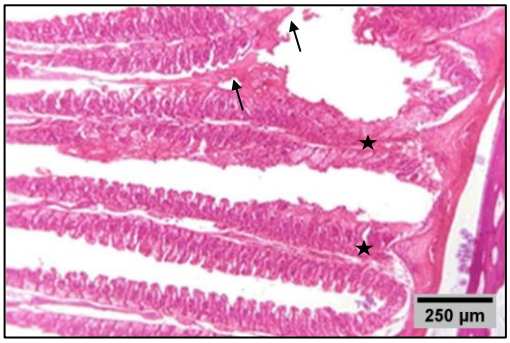

Fig. (11): Histological section of fish gill tissue from the group treated with 16.86 mg/L of cadmium chloride after 30 days, exhibiting severe bleeding in the secondary gill lamellae (arrows) and hyperplasia of the secondary gill lamellae (asterisks), stained with Hematoxylin and Eosin (H&E).

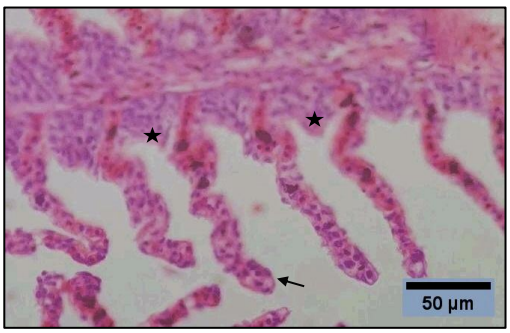

Fig. (12): Histological section of fish gills from the group treated with 16.86 mg/L of cadmium chloride and thyme oil after 3 days, showing congestion and changes in the shapes of secondary gill lamellae (arrow) and the onset of hyperplasia (asterisk), stained with Hematoxylin and Eosin (H & E).

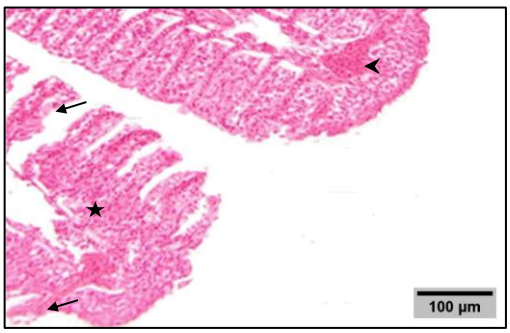

Fig. (13): Histological section of fish gills from the group treated with 16.86 mg/L of cadmium chloride and thyme oil after 7 days, showing detachment of the epithelial tissue layer from the gill lamellae (arrow), simple hyperplasia (asterisk), and congestion in some secondary gill lamellae (arrowhead), stained with Hematoxylin and Eosin (H & E).

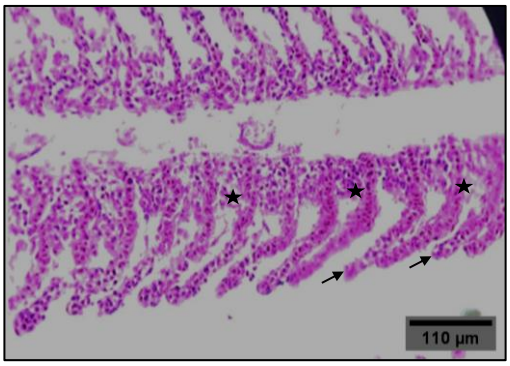

Fig. (14): Histological section of fish gills from the group treated with 16.86 mg/L of cadmium chloride and thyme oil after 15 days, demonstrating changes in the shapes of some secondary gill lamellae (arrows) and hyperplasia (asterisk), stained with Hematoxylin and Eosin H & E.

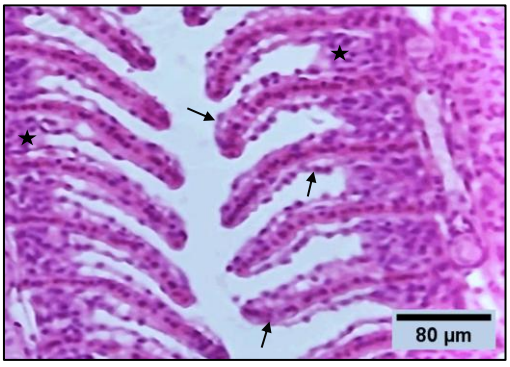

Fig. (15): Histological section of fish gills from the group treated with 16.86 mg/L of cadmium chloride and thyme oil after 30 days, showing slight hyperplasia of the secondary gill lamellae (asterisk) and minor changes in their shapes (arrows), stained with Hematoxylin and Eosin (H & E.)

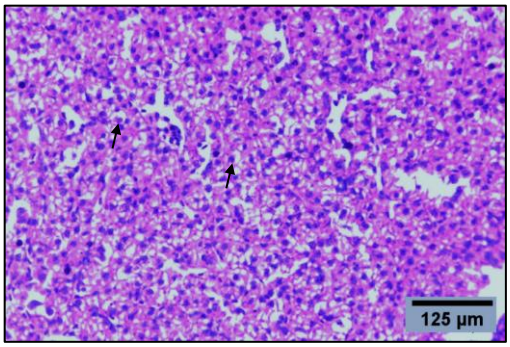

Fig. (16): Histological section in liver tissue Fish Control Group, Hepatocytes (arrow), Hematoxylin and Eosin (E & H).

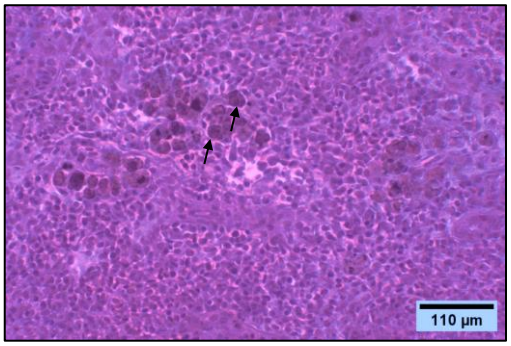

Fig. (17): Histological section in the liver tissue of the fish group treated with thyme oil only after 3 days of treatment, showing the integrity of the tissue with the presence of melanomacrophage (arrow), Hematoxylin and Eosin E & H.

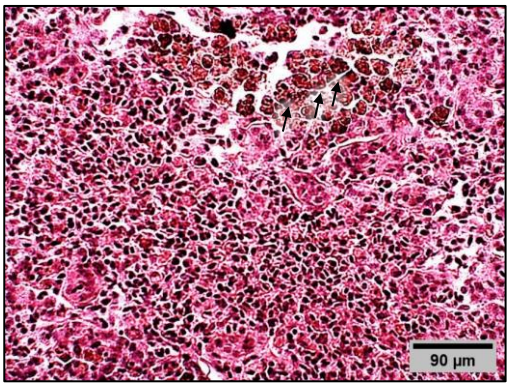

Fig. (18): Histological section in the liver tissue of fish group treated with thyme oil after 7 days of treatment, showing the normal tissue of the liver, cluster of melanomacrophage cells (arrows), Hematoxylin and Eosin (E & H).

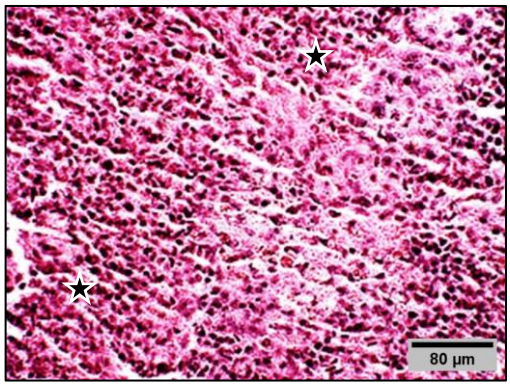

Fig. (19): Segment in the liver tissue of the fish group treated with thyme oil after 15 days Liver tissue safety (asterisk), Hematoxylin and Eosin (E & H).

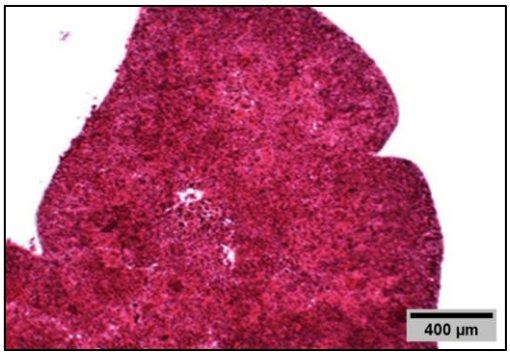

Fig. (20): Histological section in the liver tissue of the treated group with thyme oil only 30 days of treatment, the liver tissue looks normal, Hematoxylin and Eosin (H & E).

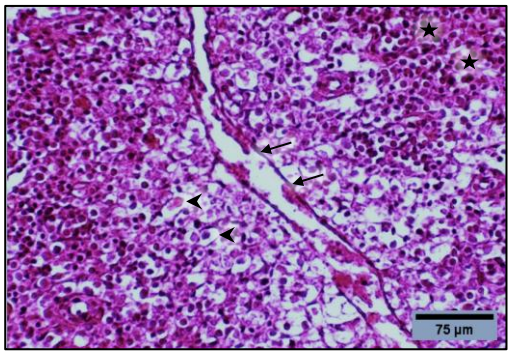

Fig. (21): Histological section in the liver tissue of the treated group with a concentration of 16.86 mm/l of cadmium chloride after 3 days of treatment, showing bleeding in most areas of the liver (asterisk), central vein congestion (arrow), degeneration of hepatocytes (arrowhead), Hematoxylin and Eosin (E & H.)

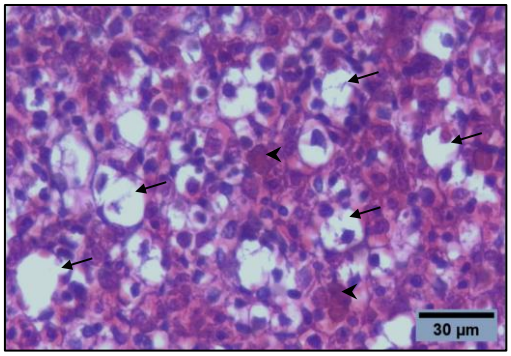

Fig. (22): Section in the liver tissue of fish group treated with a concentration of 16.86 mg / l of cadmium chloride after 3 days of treatment, showing vacuole degeneration (arrow), hepatocytes with affinity for acid pigment (arrowhead), Hematoxylin and Eosin

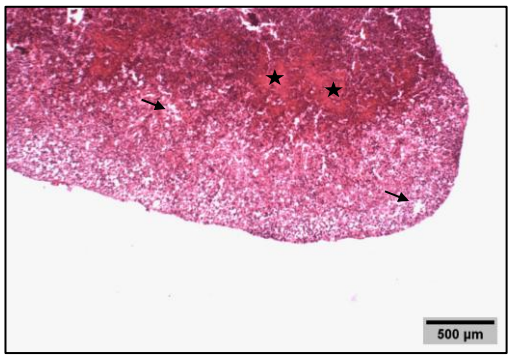

Fig. (23): Section in the liver tissue of the fish of the treated group at a concentration of 16.86 mg/L of cadmium chloride after 7 days of treatment, showing bleeding in most areas of the liver (asterisk), degeneration of hepatocytes (arrow), Hematoxylin and Eosin (E & H).

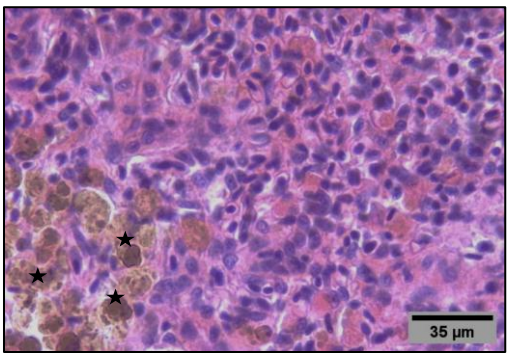

Fig. (24): Section in the liver tissue of the treated group with a concentration of 16.86 mg/L of cadmium chloride after 7 days of treatment, showing the clusters of melanomacrophage (asterisk), Hematoxylin and Eosin (E & H).

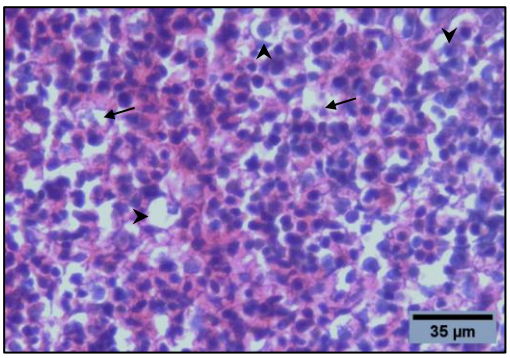

Fig. (25): Section in the liver tissue of the fish of the treated group at a concentration of 16.86 mg/L of cadmium chloride 15 days of treatment, hepatocyte necrosis (arrow), hepatocyte degeneration (arrowhead), Hematoxylin and Eosin (E & H).

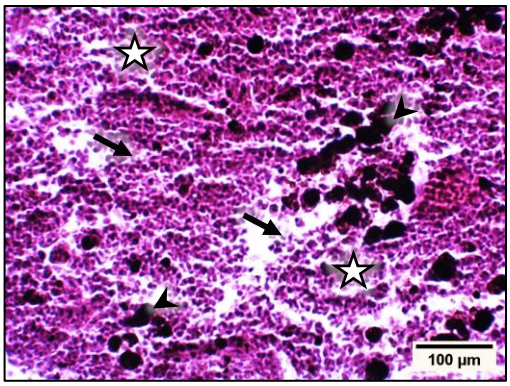

Fig. (26): Section in the liver tissue of fish group treated with a concentration of 16.86 mg / l of cadmium chloride 30 days of treatment, presence of melanomacrophage (arrowhead), hepatocellular atrophy (asterisk), cell necrosis (arrow), Hematoxylin and Eosin (E & H).

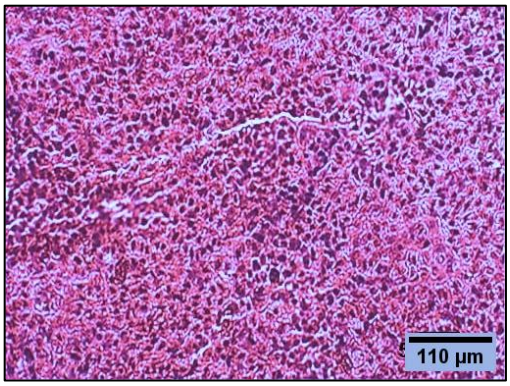

Fig. (27): Section in the liver tissue of fish of the treated group at a concentration of 16.86 mg / l of cadmium chloride with thyme oil after 3 days of treatment, shows the integrity of the hepatic tissue, Hematoxylin and Eosin E & H).

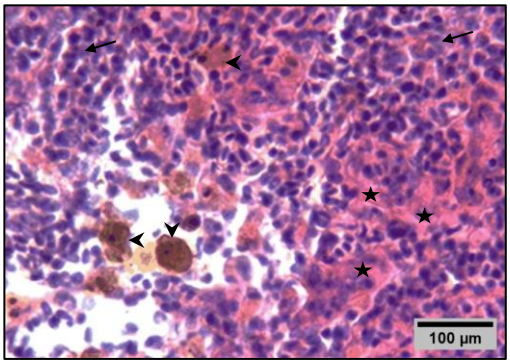

Fig. (28): Section in the liver tissue of the fish group treated with a concentration of 16.86 mg/L of cadmium chloride with thyme oil after 7 days of treatment, showing infiltration of blood cells (asterisk), normal hepatocytes (arrow), cluster of melanomacrophage cells (arrowhead), Hematoxylin and Eosin (H & E).

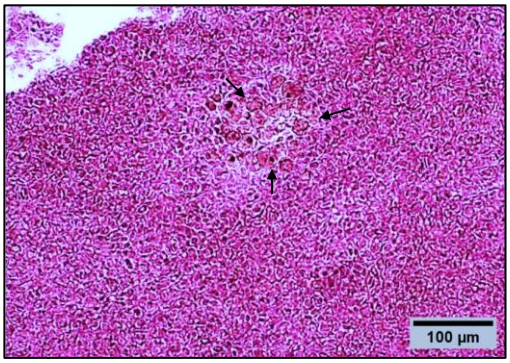

Fig. (29): Section in the liver tissue of fish group treated with a concentration of 16.86 mg / l of cadmium chloride with thyme oil after 15 days of treatment, clusters of melanomacrophage cells (arrows), Hematoxylin and Eosin (H & E).

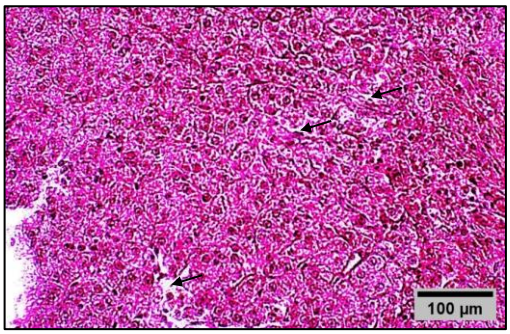

Fig. (30): Section in the liver tissue of the treated fish group at a concentration of 16.86 mg/L of cadmium chloride with thyme oil after 15 days of treatment, the tissue appears normal with degeneration of some hepatocytes (arrow), Hematoxylin and Eosin (H & E).

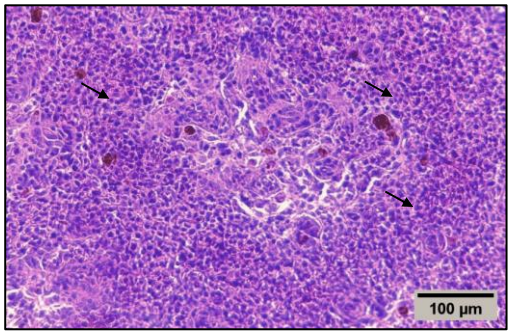

Fig. (31): Segment in the liver tissue of the treated group with a concentration of 16.86 mg / l of cadmium chloride with thyme oil after 30 days of treatment, normal liver cells are observed (arrow), Hematoxylin and Eosin (H & E).

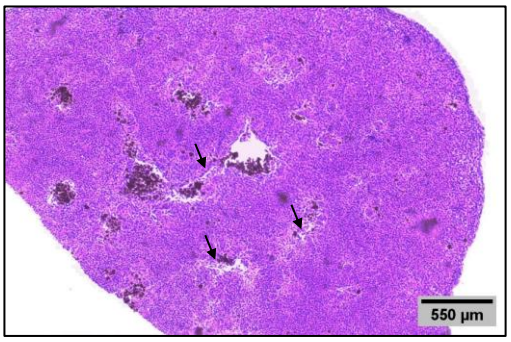

Fig. (32): Segment in the liver tissue of fish group treated with a concentration of 16.86 mg / l of cadmium chloride with thyme oil after 30 days of treatment, presence of melanomacrophage clusters (arrow), Hematoxylin and Eosin (H & E).

Antioxidants

I. Protein expression of the Glutathione peroxidase 1 (GPX1)

The activity of glutathione peroxidase 1 (GPX1) was evaluated in the gills, liver, and muscles of C. carpio at 7, 15, and 30 days across three treatments: thyme oil (G2), cadmium chloride (G3), and a combination of thyme oil and cadmium chloride (G4). The results showed significant variations in enzyme activity among all examined groups.

I.1 In liver across treatments

GPX1 enzyme activity in the liver of fish in all examined groups (G2, G3, and G4) increased significantly ($P \leq 0.05$) beyond normal levels; however, all three groups responded differently over time. In G2 (treatment with cadmium chloride) and G4 (thyme oil and cadmium chloride together treatment), the level of GPX1 enzyme went up significantly ($P \leq 0.05$) after 15 days of treatment. In G3 (the thyme oil treatment) GPX1 levels increased significantly ($P \leq 0.05$) just after 7 days of treatment (Fig. 33).

I.2. In the liver overtime

The activity of the GPX1 enzyme in the liver over time showed distinct patterns. Thyme oil treatment demonstrated a significant increase ($P \leq 0.05$) in enzyme activity at both 7 and 30 days compared to the other groups. However, during the 15 days of treatment, the levels of GPX1 activity were similar to the activity in group G1 (Fig. 34).

I.3. In gills across treatments

The activity of the GPX1 enzyme in the liver over different treatment groups demonstrated varying patterns over time. Both combination of cadmium chloride and thyme oil (G4) treatment showed significant increases in enzyme activity on the 7 and 30 days ($P \leq 0.05$). The thyme oil treatment (G3) showed an increased enzyme activity on day 7 and day 30. These levels were significantly different from those observed on day 15 ($P \leq 0.05$) (Fig. 35).

I.4. In gills overtime

The activity of GPX1 in gills showed a significant increase in enzyme levels in G3 and G4 just after 7 days compared to the control group (G1) and G2 ($P \leq 0.05$). However, on day 15, the enzyme activity decreased but was still significant ($P < 0.05$) in G3 and G4. By the day 30 days, enzyme activity elevated again in all treated groups (Fig. 36).

I.5. In muscles across treatments

The results showed that GPX1 activity increased in fish muscles in all groups after 30 days. Cadmium chloride group treatment showed the highest GPX1 activity just after 7 days, while the thyme oil treatment group (G3) and the combined treatment group (G4) exhibited the highest enzyme levels at day 15 ($P \leq 0.05$) (Fig. 37).

I.6. In muscles overtime

GPX1 enzyme activity was significantly higher ($P \leq 0.05$) in all treatments, except the thyme oil treatment group, which remains similar to the control group up to 30 days (Fig. 38).

The G4 group showed the highest GPX1 enzyme activity after 15 days, while G2 group exhibited the highest enzyme activity after 30 days, both were significantly different ($P \leq 0.05$) (Fig. 38).

I.7. Level of GPX1 activity among fish organs in one period and one treatment

The comparison of the GPX1 enzyme activity in the liver, gills, and muscle tissue over time and treatments show that the enzyme in muscle is at higher level than liver and gills, especially in groups G2 and G4. The results also showed the enzyme activity in the gills was higher than enzyme activity in the liver after 30 days. Additionally, the Thyme oil treatment group G2 notably increased enzyme activity in the liver at all times except at 15 days, but the enzyme activity was the highest in muscle tissue (Figs. 39–41).

II. Estimation of catalase enzyme expression in common carp

The activity of the catalase (CAT) enzyme in the liver, gills, and muscles was examined in common carp. The results revealed significant variation in enzyme activity among the treated at three different time points (7, 15, and 30 days) .

II.1. In liver across treatments

The results showed the catalase enzyme activity was increased, notably when fish were treated with cadmium chloride (G2) after 7 and 30 days; while the thyme oil group (G3) demonstrated the highest enzyme activity after just 7 days, but a significant decrease at 15 and 30 days. The combined treatment group (G4) showed an initial increase in enzyme activity after 7 days, then decreased after 15 days, and stabilized on day 30 (Fig. 42) .

II.2. In the liver overtime

On day 7, the results revealed the catalase enzyme activity increased significantly ($P \leq 0.05$) in the thyme oil group (G3). The enzyme activity was similar in thyme oil (G3) and the combined treatment (G4) after 15 days. The activity was similar in all treated groups after 30 days ($P \leq 0.05$) (Fig. 43C) .

II.3. In gills across treatments

CAT enzyme activity in the gills increased significantly in all treated groups throughout the study. Notably, enzyme levels were very different between the treatment groups. However, the enzyme activity in group G2 decreased and the level was similar to the control group after 15 days (Fig. 44).

II.4. In gills overtime

The results demonstrated that the cadmium chloride in G2 significantly increased ($P \leq$

0.05) enzyme activity after 7 days, while the thyme oil-treated group and the combined treated group showed a significant increase in enzyme activity. The CAT activity decreased significantly ($P \leq 0.05$) in the cadmium chloride group after 15 days, while enzyme levels in the thyme oil treated group and the combined treated group were similar to the 7-day levels. All treated groups demonstrated a significant increase ($P \leq 0.05$) in enzyme activity after 30 days (Fig. 45C) .

II.5. In muscles across treatments

The results showed the activity of enzymes in fish muscles increased significantly ($P \leq 0.05$) in groups G3 and G4 compared to groups G1 and G2 throughout (Fig. 46A). Group G3 had the highest enzyme activity just after 7 days but gradually decreased at days 15 and 30 (Fig. 46 B). Group G4 showed the highest enzyme activity after 15 days (Fig. 46C) .

II.6. In muscles over time

The results indicated a significant increase ($P \leq 0.05$) in the enzyme activity in muscles after 7 and 15 days in the G3 and G4 groups. However, an increase in enzyme activity was observed only in the G4 group after 30 days (Fig. 47) .

II.7. Level of CAT enzyme activity among organs at the same point.

The results showed that the G2 group showed a significant increase in enzyme activity in the gills after both 7 and 30 days, compared to the muscle and liver. Higher enzyme activity was observed in the liver after 30 days. All organs showed a decrease in enzyme activity after 15 days. Conversely, groups G3 and G4 showed an increase in enzyme activity in all organs compared to the control group G1 over time. Enzyme levels were varying among members for each time, except for a decrease in muscle enzyme activity observed in the G3 group after 30 days (Figs. 48–50).

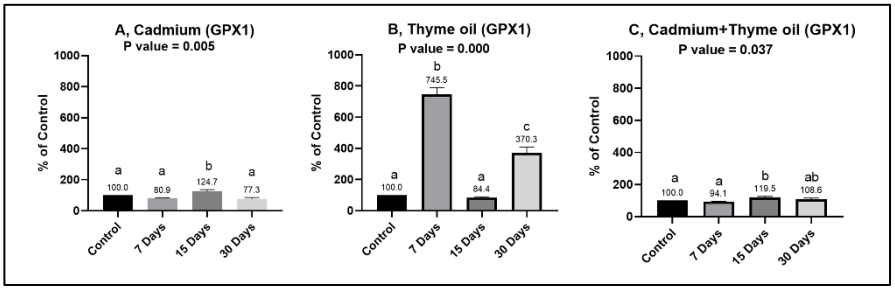

Fig. (33): shows the activity of the enzyme Glutathione peroxidase1 (GPX1) in picograms/ml in the liver of common carp during the periods (7, 15 and 30) days of the experiment for the treatments, A group G2, B the group G3, C the group G4.

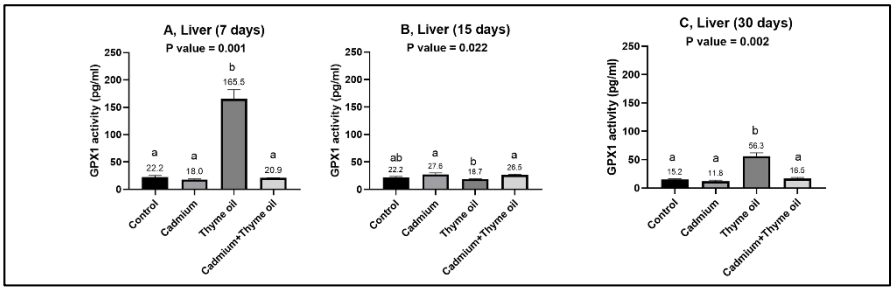

Fig. (34): shows the activity of the enzyme Glutathione peroxidase1 (GPX1) in picograms/ml in the liver of common carp for the three treatments, A 7 days, B 15-day period, C 30-day period.

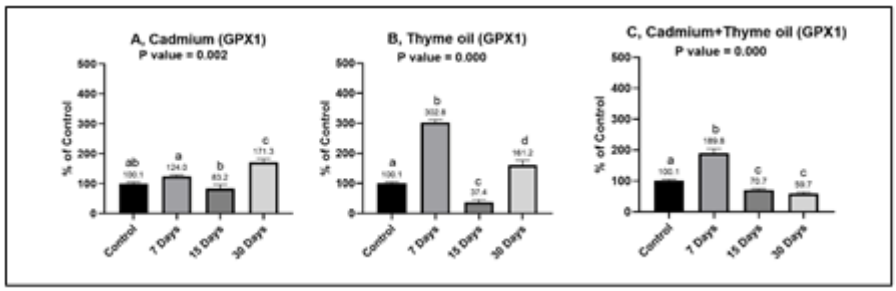

Fig. (35): shows the activity of the enzyme Glutathione peroxidase1 (GPX1) in picograms/ml in the gills of common carp during periods (7, 15 and 30) days of the experiment for treatments A, the group G2 B, the group G3, C, the group G4.

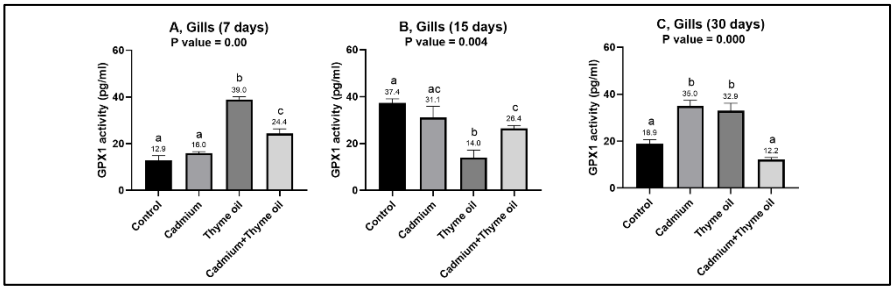

Fig. (36): shows the activity of the enzyme Glutathione peroxidase1 (GPX1) in picograms/ml in the gills of common carp for the three treatments, A 7 days, B 15-day period, and C 30-day period.

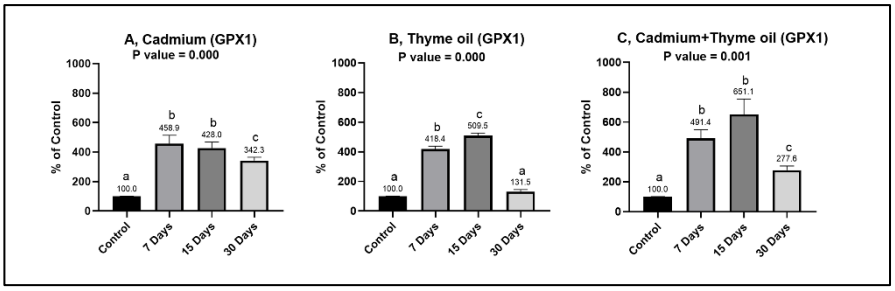

Fig. (37): shows the activity of Glutathione peroxidase1 (GPX1) enzyme in picograms/ml in the muscles of common carp during periods (7, 15 and 30) days of the experiment for treatments A group G2, B the group G3, C the group G4.

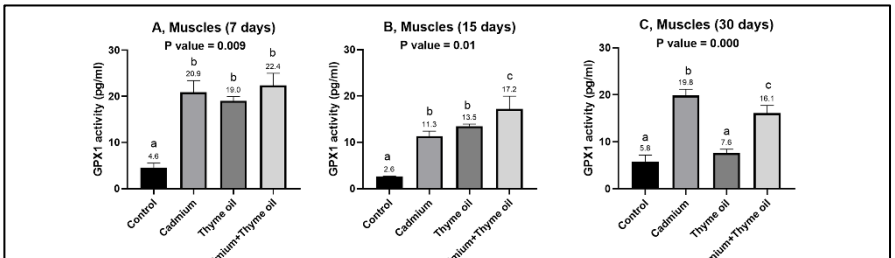

Fig. (38): shows the activity of the enzyme Glutathione peroxidase1 (GPX1) in picograms/ml in the muscles of common carp for the three treatments, A 7 days, B 15-day period, C 30-day period.

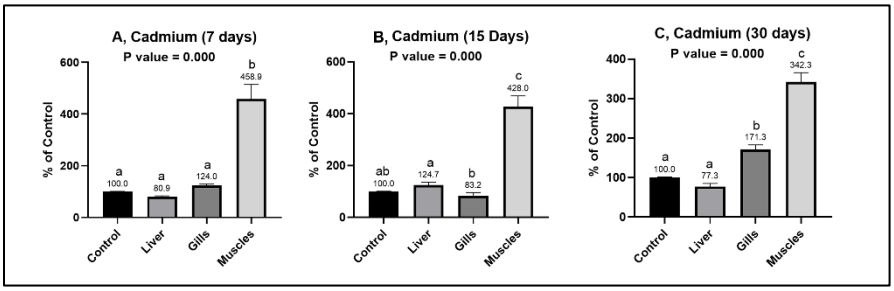

Fig. (39): shows the activity of the Glutathione peroxidase1 (GPX1) in picograms/ml in the organs of common carp (liver, gills, and muscles) for the group G2 during the experimental periods, A 7 days, B 15-day period, C 30-day period.

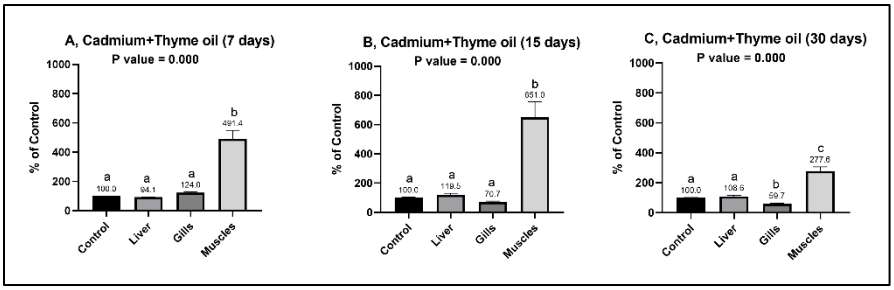

Fig. (40): shows the activity of the enzyme Glutathione peroxidase1 (GPX1) in picograms/ml in the organs of common carp (liver, gills, and muscles) for the group G4 during the experimental periods, A 7 days, B 15-day period, C 30-day period.

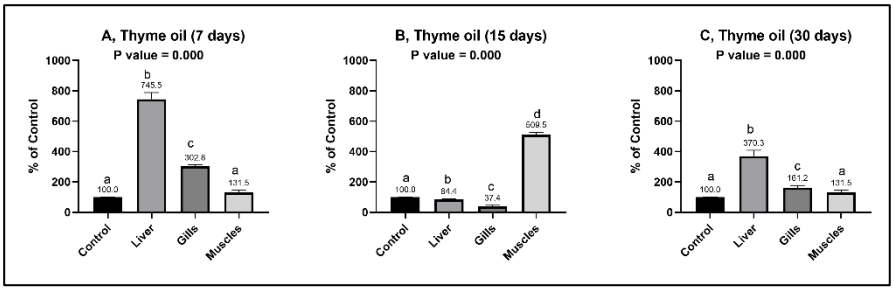

Fig. (41): shows the activity of the enzyme Glutathione peroxidase1 (GPX1) in picograms/ml in the organs of common carp (liver, gills and muscles) for the group G3 during the experimental periods, A 7 days, B 15-day period, C 30-day period.

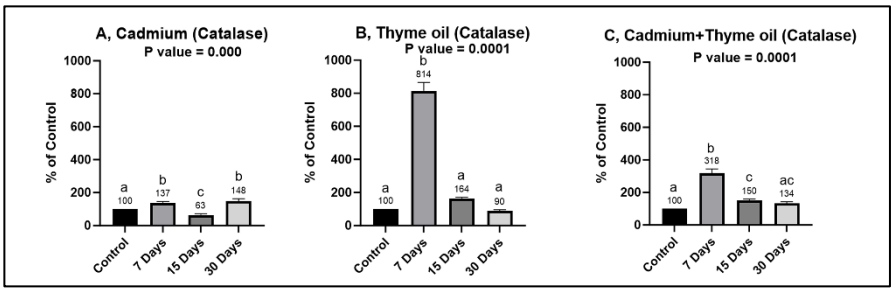

Fig. (42): shows the activity of the enzyme Catalase (CAT) in picograms/ml in the liver of common carp during the periods (7, 15 and 30) days of the experiment for the treatments, A the group G2, B the group G3, and C the group G4.

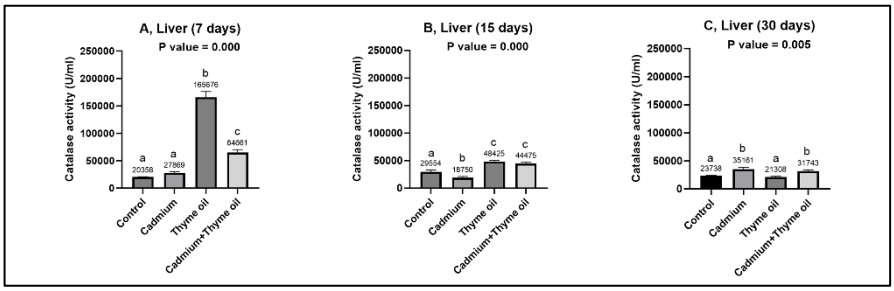

Fig. (43): shows the activity of the enzyme Catalase (CAT) in picograms/ml in the liver of common carp for the three treatments, A 7 days, B 15-day period, and C 30-day period.

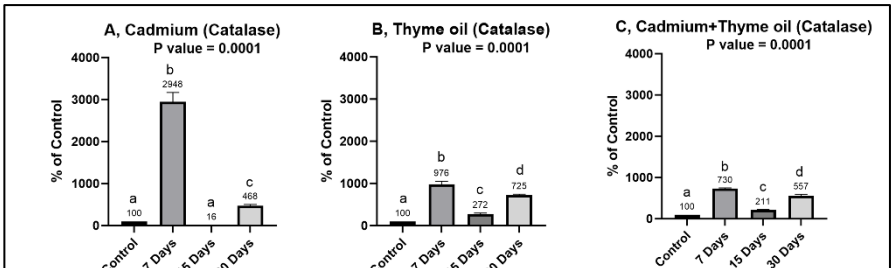

Fig. (44): shows the activity of the enzyme Catalase (CAT) in picograms/ml in the gills of common carp during periods (7, 15 and 30) days of the experiment for treatments A, the group G2, B, the group G3, and C the group G4.

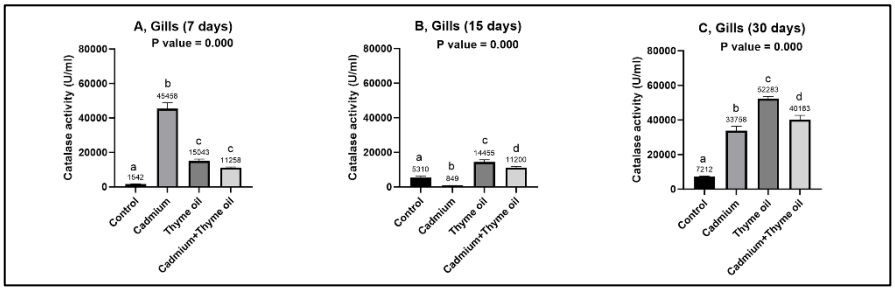

Fig. (45): shows the activity of the enzyme Catalase (CAT) in picograms/ml in the gills of common carp for the three treatments, A 7 days, B 15-day period, and C 30-day period.

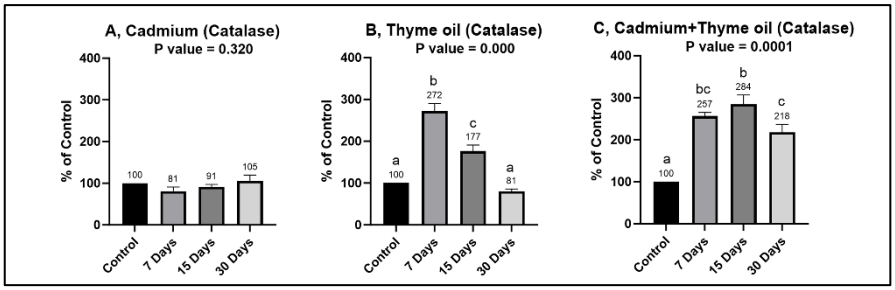

Fig. (46): shows the activity of Catalase (CAT) enzyme in picograms/ml in the muscles of common carp during periods (7, 15 and 30) days of the experiment for treatments A, the group G2, B the group G3, C the group G4.

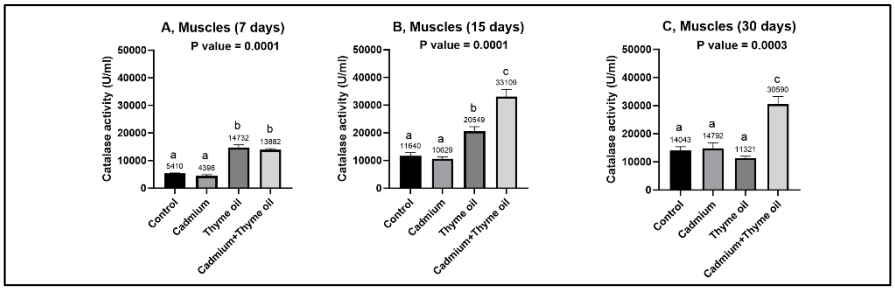

Fig. (47): shows the activity of the enzyme Catalase (CAT) in picograms/ml in the muscles of common carp for the three treatments, A 7 days, B 15-day period, and C 30-day period.

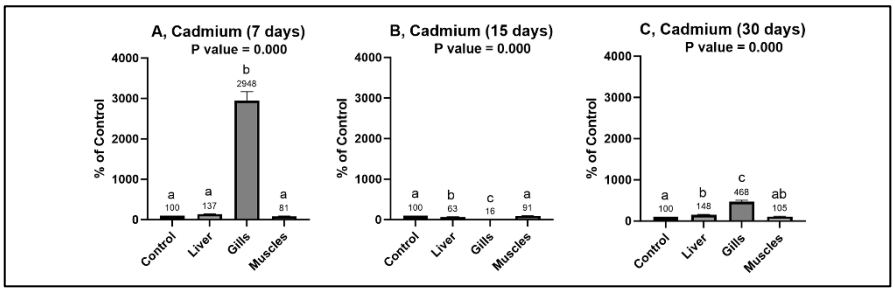

Fig. (48): shows the activity of the enzyme Catalase (CAT) in picograms/ml in the organs of common carp (liver, gills, and muscles) for the group G2 during the experimental periods, A 7 days, B 15-day period, and C 30-day period.

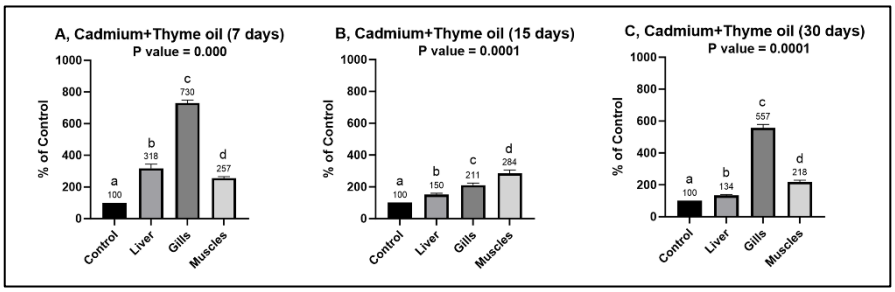

Fig. (49): shows the activity of the enzyme Catalase (CAT) in picograms/ml in the organs of common carp (liver, gills, and muscles) for the group G4 during the experimental periods, A 7 days, B 15-day period, and C 30-day period.

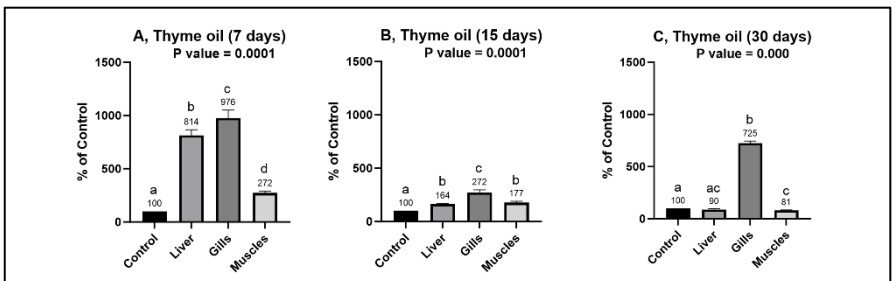

Fig. (50): shows the activity of the enzyme Catalase (CAT) in picograms/ml in the organs of common carp (liver, gills and muscles) for the group G3 during the experimental periods, A 7 days, B 15-day period, C 30-day period.

Discussion

Lethal concentration 50 (LC 50)

The results indicated that the LC50 of cadmium chloride in common carp fish is 137.46 mg/ L. This value is consistent with previous studies that mentioned values between 120.2 and 173.7, but with other fish such as *Rita rita*, and *Clarias gariepinus* (Osisiogu and Aladesanmi, 2019; Muley *et al*. 2000) However, a study reported 49.5 mg/L LC50 concentration for cadmium chloride in *Trichogaster (Colisa) fasciata* over 96 hours (Roy *et al*. 2022). The LC50 varies depending on several external and internal factors. The external factors include the type of element, chemical properties, water components, environmental conditions and the metabolic activity of the toxic substance (Kargin and Cogun, 1999; Verep *et al*., 2016; Canli *et al*., 2001). The internal factors include species, age, size, and any factor that may affect the resistance of fish to cadmium toxicity, (Canli *et al*., 2001).

Histopathological changes

The results of the histopathological study revealed some changes in the liver and gills of fish that were treated with cadmium chloride at a concentration of 16.86 mg /L. Gills are vital for respiratory gas exchange and play essential roles in osmoregulation and acid-base balance (Fernandes & Mazon, 2003; Fernandes *et al*., 2007). They are highly responsive to environmental stressors (Benli *et al*., 2008). Due to their large surface area, they are effective tools for biomonitoring (Bernet *et al*., 1999; Sweidan *et al*., 2015). The results showed the most changes in gills after exposure to cadmium chloride, a common response to pollutants, playing as a defense mechanism against harmful pollutants (Ortiz *et al*., 2003). Pollution stimulates mucous cells in gill lamellae to increase secretion for protection (Diaz *et al*., 2001). Mucus, which are glycoproteins, may change due to hypoxia caused by the hyperplasia of secondary gill lamellae. This change could explain the adhesion of the gill lamellae (Strayer and Rubin, 2012). Cadmium can replace Ca+2 in cytoskeletal proteins (microtubules, microfilaments, and actin), causing epithelial separation of the secondary gill lamellae (Levine *et al*., 1994; Abdel-Gawad *et al*., 2016; Dar *et al*., 2024). This separation leads to an increase in the distance between the capillaries and the surrounding polluted environment, leading to reduction in the harmful effects of environmental pollutants on secondary gill lamellae (Hinton & Lauren, 1990). Moreover, this separation leads to physiological changes in functions such as excretion, absorption, and exchange, which can affect fish health and increase the harmful effects of pollutants (Ahmed *et al*., 2014). These changes have impacted taking oxygen from water, leading to systemic hypoxia (Nowak, 1992) .

In agreement with previous studies, the intensity of hyperplasia increased with prolonged exposure to cadmium (Al-Ali, 2009; Ahmed *et al*., 2014; Carvalho *et al*., 2020). This pathological change is a defense mechanism in fish, but it can also lead to hypoxia (Nowak, 1992; Strayer & Robin, 2012). Long-term exposure to cadmium with continued hyperplasia may eventually lead to neoplasia and cancer (Al-Ali, 2009).

Vascular congestion may occur in fish tissues as a result of exposure to toxic pollutants in aquatic environments. This

congestion, caused by increased blood viscosity, affects blood flow. Factors released by endothelial cells may play a role in controlling blood flow. The increasing blood flow typically occurs in damaged areas (Atamanalp *et al*., 2008). Vascular dilation may occur due to damage in pillar cells, which support the capillaries in gills. Damage in these cells results in damage in the structure that leads to congestion (Crijns *et al*., 2020), and this leads to increased permeability. Ultimately, this causes bleeding and leakage of red blood cells (Slauson & Cooper, 2002). The liver, responsible for detoxification through biotransformation, is vulnerable to damage because of pollutants and toxins effects (Camargo & Martinez, 2007; Ardeshir *et al*., 2022). Vacuolar degeneration occurs due to an imbalance in osmotic pressure, which is related to the accumulation of water within the cells (Kumar *et al*., 2003). The inhibition of cellular protein synthesis may trigger this phenomenon. A glycolysis results in a decrease in adenosine triphosphate (ATP), which leads to a decrease in cellular pH (Al-Ali *et al*., 2011). This decrease in cellular pH may be due to the accumulation of water, fatty substances, or polysaccharides in the cell.

Vacuoles may be filling the cell cytoplasm due to a malfunction in the cell's vital functions, such as metabolism, leading to cell atrophy (Stevens *et al*., 2009). Exposure to toxic substances may cause degenerate cells or cell death due to a lack of blood or oxygen (Kumar *et al*., 2003). Toxins entering a fish's body can cause swelling in liver cells by affecting cell membrane permeability (Stevens *et al*., 2009; Ibrahim *et al*., 2021). Cadmium primarily produces free radicals that target the cell membrane, causing these symptoms (Jones, 2024). Clusters of pigmented phagocytic cells are found in liver tissue called melanomacrophage centers (MMCs). They are also found in other tissues, such as the spleen and kidney head. (Steinel & Bolnic, 2017; Ardeshir *et al*., 2022; Passantino *et al*., 2024). Our study found that melanopacrophages in livers near degenerated areas may link to an increase in number and size due to exposure to pathogens, environmental stress, and immune response (Roberts *et al*., 2010; Steinal & Bolnic, 2017; Ardeshir *et al*., 2022). Moreover, this study revealed there was no connection between melanopacrophages and fish health, and this is in agreement with previous studies (Herraez & Zapata, 1987; Tsujii & Seno, 1990; Passantino *et al*., 2024). The presence of melanopacrophages may be linked to other physiological conditions related to endocrine glands (Ardeshir *et al*., 2022), or with infection by microorganisms (Tsujii & Seno, 1990; Robert *et al*., 2010).

The study discovered that thyme oil, a nutritional supplement, effectively reduced the severity of histopathological changes in the G4 group because thyme oil has active compounds such as phenols, flavonoids, and alkaloids (Luz *et al*., 2021; Amen *et al*., 2024). These compounds are capable of scavenging free radicals (Xu *et al*., 2019; Liu *et al*., 2022). However, minor histopathological changes in the G4 group are likely due to an inadequate dosage of thyme oil in the feed that prevents major histopathological changes.

Histological examination of fish organ tissues is crucial for assessing the impact of pollutants on marine life and evaluating the effectiveness of anti-pollution measures. By analyzing tissue structure and function in fish exposed to pollutants, researchers can determine the health implications for both. Therefore, histological examinations are essential for addressing the negative effects of toxic pollutants on marine life and the health of aquatic environments.

Antioxidants

Fat is the main component of fish feed that is greatly affected by oxidation. Antioxidants can be used to maintain the stability of fats and prevent their deterioration caused by oxidation. The defense mechanism of a fish's body consists of enzymatic and non-enzymatic antioxidants that play an essential role in combating free radicals and protecting biomolecules and body tissues (Ighodaro & Akinloye, 2018). Exposure to pollutants, especially heavy metals like cadmium, can lead to increased levels of oxidative stress in tissues (Pruski & Dixon, 2002). Cadmium acts as an oxidizing agent due to its ability to generate free radicals, which can cause damage to cells and tissues (Liu *et al*., 2022). In response to the increase in oxidative stress, the body's defense system increases the production of antioxidant enzymes (Cuypers *et al*., 2010). These enzymes can convert harmful free radicals into compounds that are harmless to cells and tissues and restore the body's oxidative balance (Valavanidis *et al*., 2006). This explains the elevated levels of oxidative enzymes such as catalase and glutathione peroxidase as a defensive response to limit the damage caused by oxidative stress resulting from exposure to cadmium.

Cadmium is known to generate free radicals that lead to oxidative stress in cells. When cells are stressed by cadmium, they quickly respond and produce antioxidants to restore balance (Jones, 2024). A series of cellular signaling processes are involved in producing metalloproteins (Finkel, 2011). It was found during this study that treating fish with cadmium chloride led to increased antioxidant levels. Similarly, antioxidant levels increased also when fish were treated with thyme oil or a combination of thyme oil and cadmium chloride. The reason for this is that thyme oil contains active compounds such as terpenes, phenols, and flavonoids that enhance the production of antioxidant enzymes (Kumar & Pandey, 2013; Ghafarifarsani *et al*., 2022; Amen *et al*., 2024). These processes strengthen the body's defenses against oxidative damage. On the other hand, free radicals induce the production of antioxidants by activating cellular signals (Selvaraj *et al*., 2015; Sun & Shahrajabian, 2023) .

The results of this study were consistent with previous studies that demonstrated the effects of thyme oil in generating antioxidants (Honcharenko *et al*., 2018; Hoseini and Yousefi, 2019; Mirghaed *et al*., 2020; Yousefi *et al*., 2022; Ghafarifarsani *et al*., 2022; Jasim *et al* .,2023). Other studies have shown the ability of thyme oil to decrease free radical levels (Aldosary *et al*., 2021; Lawrence *et al*., 2023), and others have shown increasing antioxidants (Lorenzo *et al*., 2019; Khalil *et al*., 2023). Additionally, thyme oil itself acts as an antioxidant (El-Guendouz *et al*., 2019).

It has been indicated that an increase in GPX1 and CAT enzyme levels in fish organs that are treated with cadmium can generate free radicals (Hu *et al*., 2022). Free radicals signal cells to produce metalloproteins (Mahmoud *et al*., 2017) that stimulate cellular signaling pathways to produce antioxidant enzymes (Choi *et al*., 2007). However, long-time exposure to oxidative stress and continued production of free radicals can eventually lead to the accumulation of these radicals and put cells in an oxidative stress state (Choi *et al*., 2008). This confirms the importance of the cell-enhancing factors in food to control harmful oxidizing agents. The effect of thyme oil GPX1 enzyme levels on fish gills was not clear with a long-time exposure to cadmium; however, it appears to have a significant impact during the 7 days. This effect was also indicated in the combined treatment of cadmium and thyme oil. Thyme

oil increased GPX enzyme levels when presented alone in fish feed (Hoseini & Yousefi, 2019). This explains the fluctuation in GPX levels during treatment time, the enzyme levels were high on days 7 and day 30, with a decrease indicated on day 15.

This was also observed in the liver and muscles of fish treated with thyme oil alone. It was observed that the levels of GPX enzyme in fish muscles exposed to thyme oil increased from 7 to 15 days of treatment, but they returned to normal after 30 days. The reason for this variation in regulation may be attributed to the difference in the nature and function of the cells and their metabolic regulation (Mahboub *et al*., 2022).

Conclusions

Thyme oil protects against cadmium pollution by increasing the activity of antioxidant enzymes such as catalase (CAT) and glutathione S-transferase (GST). Furthermore, the combination of histopathological analyses and monitoring antioxidant levels serve as a reliable marker for assessing fish health. Studying pathological tissue changes and antioxidant enzyme levels over time provides valuable information on fish health. However, it was observed that an increase in antioxidant levels at specific times does not always indicate good health in fish, as pollutants can elevate antioxidant levels as an initial defensive reaction. Therefore, a comprehensive evaluation of fish health should be conducted over successive periods to achieve an accurate assessment.

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Contributions of authors

Ali Abd Allateef Al-Ali supervised the preparation and writing of the manuscript, including the formulation of the results, the diagnosis of histopathological changes, and the implementation of the study methodology. Sarah T. Al-Saray was responsible for the acquisition, analysis, and accurate annotation of the histological sections. Sajad A. Abdullah contributed to revising the manuscript and critically reviewing its scientific content and linguistic accuracy. Anaheed A. Mohammed developed and designed the research plan and established the methodological framework of the study. All authors read and approved the final version of the manuscript for submission.

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Conflicts of Interest

The authors declare no competing interests.

Ethical approval

All relevant national and international standards for the care and use of animals were complied with.

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التغيرات المرضية النسيجية والإنزيمات المضادة للأكسدة كمؤشرات حيوية لمتابعة الحالة الصحية لأسماك الكارب *Cyprinus carpio* L

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المستخلص :ركزت الدراسة الحالية على متابعة الحالة الصحية لأسماك الكارب الشائع (Cyprinus carpio) لتقييم صحة الأسماك في البيئة المائية. صُمِّمت الدراسة لتشمل ظروفًا بيئية مختلفة، حيث تضمنت أربع مجموعات: مجموعة ضابطة، ومجموعة عولجت بزيت الزعتر، ومجموعة عُرّضت إلى كلوريد الكادميوم، ومجموعة تعرضت لكل من زيت الزعتر وكلوريد الكادميوم. جرى قياس مستويات مضادات الأكسدة (الغلوتاثيون بيروكسيداز والكاتالاز) في الخياشيم والعضلات على فترات زمنية خلال 30 يومًا. كما أُجريت الفحوصات النسيجية للكشف عن أي تغيّرات مرضية في الخياشيم والكبد الناتجة عن المعاملات عند الأيام 3، 7، 15، و30. أدى التعرض لكلوريد الكادميوم إلى تغيّرات مرضية متقدمة، شملت تشوّهات في الخياشيم، والتصاق الصفائح الخيشومية، وفرط تنسّج الظهارة، إضافةً إلى حدوث نزيف، وتنكس، ونخر في الكبد. في المقابل، أسهمت إضافة زيت الزعتر إلى غذاء الأسماك في التخفيف من هذه التغيرات في كل من الخياشيم والكبد. أظهرت النتائج وجود اختلافات معنوية في نشاط إنزيمي GPX1 و كاتالاز بين المعاملات المختلفة. إذ أدى كلوريد الكادميوم إلى زيادة نشاطهما في الكبد والخياشيم والعضلات، بينما عمل زيت الزعتر على تنظيم هذه التأثيرات، وكانت أنسجة العضلات الأعلى نشاطًا، ولاسيما في المجموعات المعرضة للكادميوم. أوضحت النتائج أن هذه المؤشرات كانت فعّالة في تقييم صحة الأسماك، وقدمت رؤى مهمة للحفاظ على صحة الأسماك في البيئة المائية. كما لوحظ أن سلامة البيئة المائية الجيدة لا ترتبط دائمًا بارتفاع مستويات مضادات الأكسدة الناجمة عن التلوث. وعليه، يُوصى بإجراء مراقبة دورية لمستويات مضادات الأكسدة لضمان صحة البيئة المائية.

الكلمات المفتاحية: الإنزيمات الدفاعية الخلوية؛ الكارب (*Cyprinus carpio*)؛ التغيرات المرضية المجهرية؛ المؤشرات الجزيئية.