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Effect of Beetroot (*Beta vulgaris* L.) Extract on Enhancing the Immune Status and Antioxidant Enzymes of the Nile tilapia (*Oreochromis niloticus*) Exposed to Lead

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ABSTRACT

The prospective effect of beetroot extract (*Beta vulgaris* L.) to resist the toxic effect of lead (Pb) and enhance the immune status and antioxidant enzyme of the Nile tilapia (*Oreochromis niloticus*) was studied. Fish specimens were divided into 4 groups: C (control group), Pb (group subjected to lead), T1, and T2 (groups injected intraperitoneal with 100 and 150mg/ kg of beetroot extract 2 weeks before being subjected to Pb, respectively). The effects were addressed on the humoral immune parameters, (antiproteases, lysozyme total protein, and globulin) and antioxidant enzymes [Glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) activities], as well as some biochemical parameters. Pb accumulation was studied in gills, liver, and muscle tissues. The results revealed a significant enhancement in some immune parameters viz. a lysozyme and total protein in T1 groups compared to other groups, especially with the Pb group which showed the lowest significant values ($P< 0.05$). The groups injected with beetroot extract showed the lowest significant values ($P< 0.05$) of Pb accumulation in tissues compared to Pb group. While, T1 group recorded a significant increase ($P< 0.05$) in hepatic protein content, GST, SOD, CAT, and POX, respectively, compared to the Pb group. Moreover, Pb group showed a significant decrease compared to the control group. Most of the biochemical parameters, including glucose, cholesterol, AST, and uric acid recorded the highest values in Pb group compared to the other groups, but without significant difference. The albumin value of T1 group showed the highest significant values compared to other groups. To conclude, the injection of the Nile tilapia with 100mg/ kg of beetroot extract may contribute to protecting fish from the harmful effects of lead by enhancing the immune system and antioxidant enzymes, and improving the general fish health.

INTRODUCTION

Recently, aquaculture stands out as a rapidly expanding sector in the agricultural industry, with a global reach. Regrettably, this industry encounters several challenges, and among them, water pollution emerges as a significant and perilous issue. The primary sources of this pollution include urban industrial wastewater as well as agricultural and

domestic sewages **(Osman & Kloas, 2010; Osman *et al*., 2012)**. Water pollution creates numerous stressors that impact both the immune and physiological systems in fish, thereby elevating their vulnerability to infectious diseases. The use of antibiotics and other chemotherapeutics for disease control can potentially contribute to the emergence of pathogen strains resistant to treatment, environmental pollution, and the accumulation of residues in fish, causing serious health problems either for fish or for humans when consumed **(Awad *et al.*, 2013)**. Therefore, there is a notable inclination toward utilizing medicinal plants and their active components to strengthen the immune and physiological systems of cultivated fish, ultimately increasing their ability to resist infectious diseases **(Awad, 2010)**. Previous studies using plant immunostimulants in fish diets demonstrated an enhancement in the immune status and the detoxification process of pollutants, and hence reduced the harmful effect of aquatic pollutants **(Abbas & Awad, 2016; Abbas *et al.*, 2016; Veisi *et al.*, 2021)**.

With a rapidly growing population, expanding industrial activities, and urbanization, Egypt faces challenges related to heavy metal pollution in its aquatic ecosystems **(Ahmed *et al.*, 2023)**. Lead (Pb), a toxic heavy metal, enters water sources through various anthropogenic activities, such as industrial discharge, agricultural runoff, and urban waste disposal **(El-Moselhy *et al.*, 2014)**. Once entered into aquatic ecosystems, lead can bioaccumulate in organisms, posing risks to both aquatic life and human health **(Mitra *et al.*, 2022)**. The toxic effects of lead (Pb) on fish include impaired growth, reproduction, and neurological function, as well as increaseing the susceptibility to diseases and mortality **(Abdelzaher, 2022; Abdelzaher *et al.*, 2022)**. For example, spotted snakehead fish (*Channa punctatus*) exposure to sub-lethal concentration of lead acetate caused a suppression in the immune parameters **(Paul *et al.*, 2014).**

Beet (*Beta vulgaris* L.) is a flowering plant belonging to the family Chenopodiaceae. It is mainly cultivated in the Mediterranean, Europe, and Asia Minor and has been used in folk medicines **(de Oliveira *et al.*, 2023)**. Beetroot is rich in pigments like betaxanthins and betacyanin, belonging to the betalain family. This group comprises water-soluble nitrogen-containing pigments derived from betalamic acid. Numerous studies highlighted betalains as health-protective compounds in beetroots, with connections to antioxidative, anti-inflammatory, and antitumor effects **(Clifford *et al.*, 2015)**. Previous investigations demonstrated the efficiency of beet molasses in reducing the negative effects and enhancing the immune responses of common carp after being exposed to copper **(Veisi *et al.*, 2021)** and titanium oxide **(Sheikh Veisi *et al*., 2022)**. The only influence of beetroot on fish health was conducted on growth, where a dietary meal of beetroot revealed an elevation in the growth performance of snow trout (*Schizothorax richardsonii*) after 2 months of a feeding trial **(Jha *et al.*, 2012)**. However, no investigation was carried out regarding their immune status. Therefore, the current study was concerned with investigating the possible influence of beet (*Beta vulgaris*) root

extract to reinforce the immune system in the Nile tilapia (*Oreochromis niloticus*) and to force the harmful effect caused by lead (Pb). Lead has been chosen as a model of toxic heavy metal which affects the ecosystem.

MATERIALS AND METHODS

Plant extract

The beet (*Beta vulgaris* L.) roots were obtained from the local Egyptian market. Plant specimen was identified by Dr. Abdelghafar Abu-Elsaoud (Faculty of Science, Suez Canal University, Ismailia, Egypt). The root was squeezed by an electronic squeezer, and the juice was dried by laboratory bench-top vacuum freeze dryer before being stored in the fridge until used.

Experimental design and fish sampling

Approximately, 84 Nile tilapia (*Oreochromis niloticus*) with an average weight 25± 3g were obtained from a commercial fish farm in Cairo, Egypt. The fish were acclimatized for 15 days in dechlorinated tap water for 2 weeks in 50L aquaria with a constant aeration at 27± 2°C, pH ranging between 7.0 & 7.5, and dissolved oxygen fluctuating between 6.2 and 6.9mg/ L. Fish were fed twice daily during acclimatization and experimented with a commercial diet (27% protein ration) at a rate of 2% of the fish body weight. Moreover, fish were randomly divided into 4 groups, each with 21 fish (7 fish per replicate). The first two groups (T1 and T2) were injected intraperitoneally with 0.1ml of 100 and 150mg/ kg of fish with a beet extract dissolved in 0.9% of NaCl, respectively. The second two groups (C and Pb) remained without injection. After 2 weeks of injection, T1, T2 and Pb groups were exposed to LC_{50} of $PbCl_2$ (0.36mg/ L) for 3 weeks. The LC_{50} was determined according to **Abdelzaher *et al.* (2022)**. The control fish group was reared in normal water without lead (Pb). The study was ethically approved by the National Research Center of Animal Care Committee, and the regulations for the care of animals in research were followed.

Blood was obtained from the caudal vein of fish after exposure to anesthesia (3-amino benzoic acid ethyl ester, Sigma-Aldrich) using a 3ml capacity syringe. Subsequently, the blood samples were placed in Vacuettes without heparin and left to clot for 2 hours at 4˚C, followed by centrifugation at 1100x g for 25 minutes at 4˚C. The resulting serum was collected and stored at -20˚C until needed.

Humoral immune parameters

1- Lysozyme activity

The turbidimetric assay for lysozyme was conducted according to the method of **Parry *et al.* (1965)**. Thus, 40µl of serum was added to two ml of a suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich, 0.2mg/ ml in 0.05M sodium phosphate buffer, pH 6.2). The assay was carried out at 25˚C, and absorbance was measured after 0.5 and 4.5min at 530nm on a spectrophotometer. A unit of lysozyme activity was defined as the sample amount causing a decrease in the absorbance of 0.001/ min.

2- Antiproteases activity

The anti-trypsin activity was carried out according to **Ellis (1987)** and **Lange *et al.* (2001)**. Briefly, 20µl of serum and 20µl standard trypsin solution (Sigma-Aldrich, 5mg/ ml) were incubated for 10min at 22˚C. Then, 200µl of 0.1M PBS (PH 7.2) and 250µl of azocasein solution (20 mg/ ml PBS) were added to the mixture before incubation at 22˚C for 1 hour. The reaction was stopped with 500µl of 10% (v/v) of trichloro acetic acid (TCA). The mixture was incubated for 30min at 22˚C before centrifugation at 6000x g for 5min. Then, 100µl of the supernatant was transferred to a 96 microwell flat bottom plate containing 100µl of 1N NaOH/ well. The spectrophotometer measured the absorbance at 410nm. The positive control (100%) was created by substituting the serum with a buffer. In the negative control, both serum and trypsin were replaced with a buffer. The percentage inhibition of trypsin activity was determined by comparing it to a positive control sample.

3- Total protein, albumin and globulin

Total protein **(Cannon *et al.*, 1974)** and albumin (**Tietz, 1995**) were estimated using commercial biochemical kits (Bio-diagnostics, Egypt). Globulin was obtained by subtracting albumin concentration from total protein concentration. Each biochemical parameter was calorimetrically analyzed according to its manufacturer's instructions using an Agilent Cary UV-Vis spectrophotometer.

Pb accumulation analysis

Fish samples were dissected and the gills, liver, and muscle tissues were excised and freeze-dried. Tissues were cleaned, rinsed in double deionized water, and blotted on filter paper. Then, 0.5g of each tissue was placed in a clean screw-capped tube and digested according to the method of **Finerty *et al.* (1990)**. Concentrated nitric and perchloric acid (AR grade) in a 5:5 ratio was used in Teflon beakers on a hot plate at 50°C for about 5 hours until the organic matter decomposition took place. The digested solutions were cooled to the room temperature, filtered and diluted to a final volume of 50ml with deionized distilled water. The obtained solutions were then analyzed by using

inductively-coupled plasma mass spectrometry (ICP-MS) (model ELAN 9000, Perkin Elmer ICP-MS, USA) to measure Pb concentrations.

Antioxidant enzymes

A. Determination of enzyme activity

After dissecting the fish, liver specimens from each group were directly immersed in liquid nitrogen and homogenized in phosphate buffer saline solution (pH 7.5). Then, specimens were centrifuged in a cooling centrifuge at 9000g for 30min, and the supernatants were collected. The protein content was determined according to Bradford **(Bradford, 1976)**. Different enzyme activities (GST, SOD, CAT, and POX) were determined using a spectrophotometer of JASCO, Tokyo, Japan, as follows:

1-Glutathione S-transferase activity (GST) was determined according to **Habig *et al*. (1974)**. The assay mixture contained 0.1M potassium phosphate buffer (pH 6.5), 1mM CDNB in ethanol, and 1mM GSH in a total volume of 1ml. The increase in absorbance at 340nm was monitored against a blank solution for 3min at 25ºC. The product extinction coefficient was considered as 9.6mM/ cm. One GST unit is equivalent to the enzyme amount which conjugates 1µmole CDNB in 1min.

2- Superoxide dismutase activity (SOD) was determined according to the method of **McCord and Fridovich (1969)**. SOD activity assay was based on the SOD ability to inhibit the reduction of cytochrome C by scavenging the superoxide anion formed by xanthine-xanthine oxidase system. The reaction mixture assay is 1.0ml buffer 20mmol/ L potassium phosphate (pH 7.8), containing 0.1mM EDTA, 0.01mM cytochrome C, and 0.05mM sodium xanthine. The reaction was initiated by adding 21 units of xanthine oxidase, which react with sodium xanthine (substrate) producing superoxide anion, making a reduction to cytochrome C at 550nm. One unit activity of SOD is the amount which causes 50% reaction inhibition for a reduction rate of cytochrome C.

3- Catalase activity (CAT) was determined according to the method of **Aebi (1984)** by using hydrogen peroxide decomposition assay. Activity units (U) of CAT are defined as the number of H_2O_2 moles that decomposed per min. A final volume of 1ml of the reaction mixture contained 50mM hydrogen peroxide (H_2O_2) in 50mM phosphate buffer (pH 7.0). The decrease in the absorbance of hydrogen peroxide was monitored at 240nm at 25°C for 2min using an extinction coefficient of 39.4M/ cm .

4- Peroxidase activity (POX) was determined according to method of **Gulcin and Yildirim** (**2005**) by using guaiacol oxidation method. The reaction mixture consisted of 8µ moles of H_2O_2, 60µ moles guaiacol, 150µ moles sodium acetate buffer, and pH of 5.6. The enzyme activity was determined by measuring the difference in absorbance (0.01) at 470nm at 25°C for 3min.

B. Polyacrylamide gel electrophoresis antioxidant activity staining

Hepatic protein and different antioxidant enzymes activities were determined using native gel electrophoresis (native 7% PAGE) according to **Smith (1976)**, and stained as follows:

1- GST activity staining on PAGE

Activity staining of GST on PAGE was determined according to **Ricci *et al.* (1984)**. After electrophoresis, the gel was placed in a 0.1M K-phosphate buffer pH 6.5, containing 4.5mM GSH, 1mM CDNB, and 1mM NBT. It was incubated at a 37°C for 10 minutes, then the gel was washed with water and incubated at room temperature in 50ml of 0.1M Tris-HCl buffer (pH 9.6) containing 3mM PMS. Blue insoluble formazan appeared on the gel surface in about 3- 5 min, except in the glutathione transferase area. Gels were washed with water and placed in 1M NaCl.

2- SOD activity staining on PAGE

The SOD activity was stained on PAGE as described by **Weisiger and Fridovich (1973)**. The gel was immersed in a reaction mixture containing 50ml of 0.1M Tris-HCl (pH 8.6), 20mg Nitro blue tetrazolium (NBT) and traces of phenazine methosulfate (PMS). The gel was exposed to daylight, and SOD activity emerged as achromatic areas on a blue background.

3- CAT activity staining on PAGE

The CAT activity was stained on PAGE, as suggested by **Harris (1977)**. The gel was incubated in 3% H_2O_2 for 15min after electrophoresis and rinsed with clean water before submerging in a 1:1 solution containing 2% potassium ferricyanide and 2% ferric chloride.

Biochemical analysis

The biochemical analysis, including serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) **(Reitman & Frankel, 1957)**, glucose concentration **(Caraway & Watts, 1987)**, total cholesterol **(Ellefson & Caraway, 1976)**, and uric acid and creatinine **(Tietz, 1995)** were determined using commercial biochemical kits (Bio-diagnostics, Egypt) according to the manufacturer's instructions, using an Agilent Cary UV-Vis spectrophotometer (100/300 Series).

Statistical analyses

The data underwent analysis using one-way analysis of variance (ANOVA). If variances were detected among treatments, means were compared using Tukey's test with the assistance of Minitab statistical software (Minitab, Coventry, UK). Significance was established at a level of $P< 0.05$.

RESULTS

Humoral immune parameters

Although there was no significant difference in antiprotease activity (Fig. 1) among the tested groups, a slightly higher value was recorded in T1 group compared to the others. Similarly, T1 group showed the highest significant difference of lysozyme activity (Fig. 2) compared to T2 and Pb groups ($P< 0.05$). However, Pb group showed the lowest significant values and lysozyme activities. In addition, T1 group reported the highest significant values ($P< 0.05$) of total protein (Fig. 3) compared to T2 and Pb groups. Contrast to other assays, C group showed the highest globulin value (Fig. 4), followed by T1 group (without significant difference).

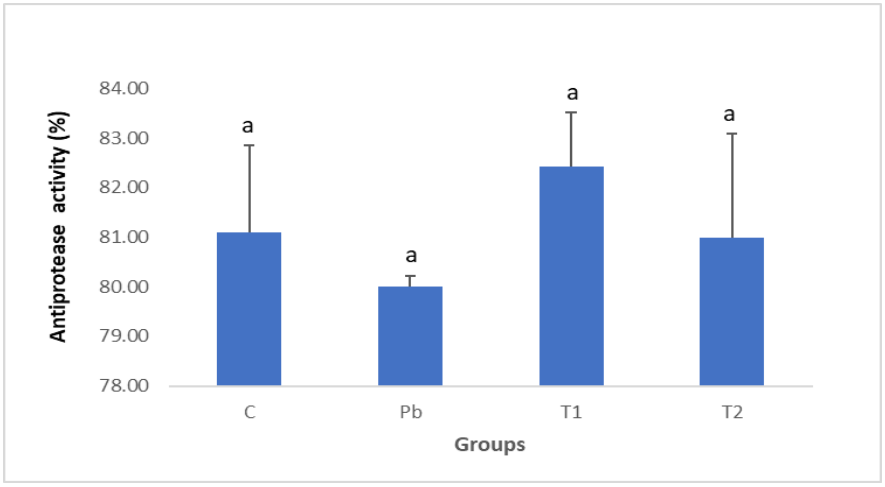

Fig. 1. Antiprotease activity of four groups of the Nile tilapia; C (control group), Pb (group subjected to Pb), T1 and T2 (groups injected with 100 and 150mg/ kg of beetroot extract before subjected to Pb, respectively). Groups sharing the same letter did not show significant differences ($P> 0.05$). The bars represent the mean values with standard error (mean ± S.E.)

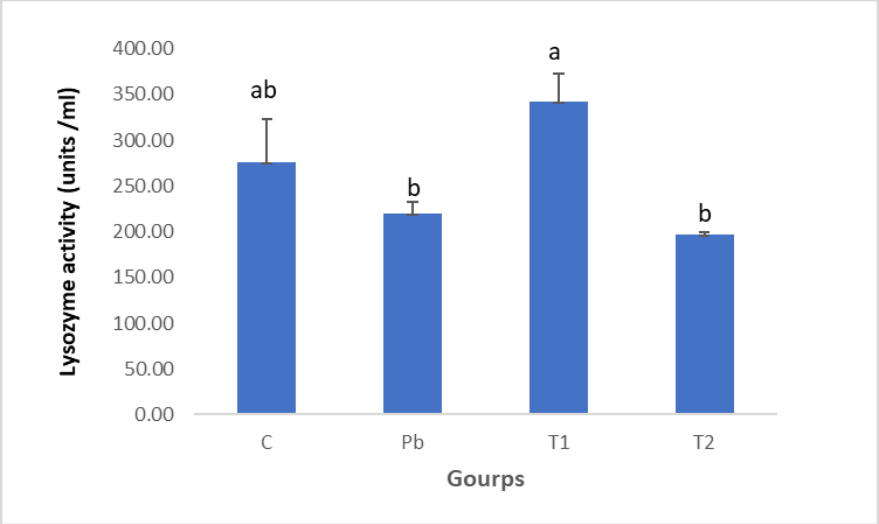

Fig. 2. Lysozyme activity of four groups of the Nile tilapia; C (control group), Pb (group subjected to Pb), T1 and T2 (groups injected with 100 and 150mg/ kg of beetroot extract before subjected to Pb, respectively). Groups sharing the same letter did not show significant differences (P> 0.05). The bars represent the mean values with standard error (mean ± S.E.)

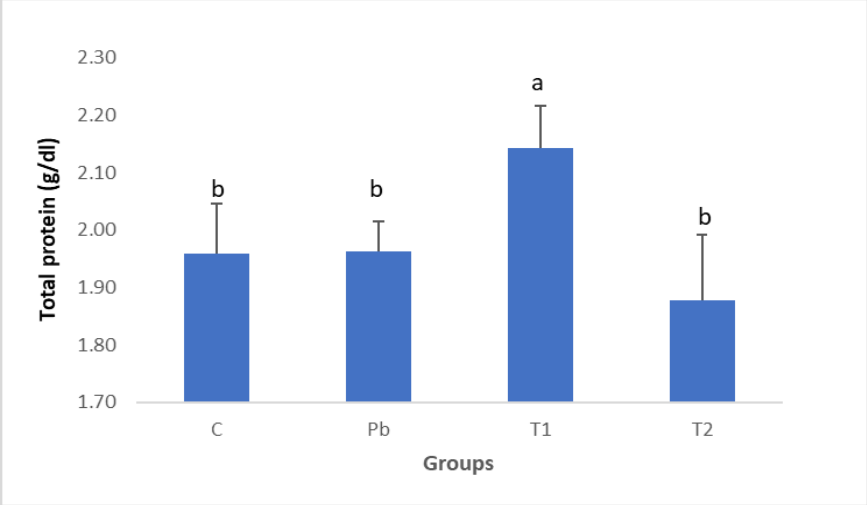

Fig. 3. Total protein of four groups of the Nile tilapia; C (control group), Pb (group subjected to Pb), T1 and T2 (groups injected with 100 and 150mg/ kg of beetroot extract before subjected to Pb, respectively). Groups sharing the same letter did not show significant differences (P> 0.05). The bars represent the mean values with standard error (mean ± S.E.)

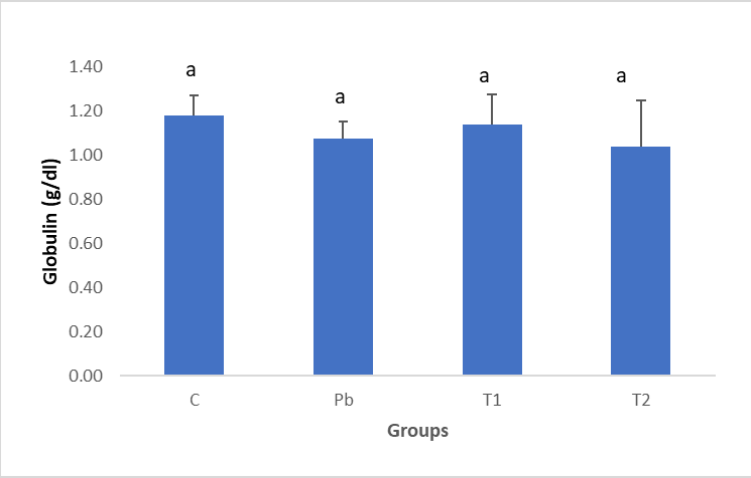

Fig. 4. Globulin of four groups of the Nile tilapia; C (control group), Pb (group subjected to Pb), T1 and T2 (groups injected with 100 and 150mg/ kg of beetroot extract before subjected to Pb, respectively). Groups sharing the same letter did not show significant differences ($P > 0.05$). The bars represent the mean values with standard error (mean ± S.E.)

Pb accumulation analysis

Statistical analysis demonstrated a significant increase in Pb concentration ($P < 0.05$) in the Pb group in both the gills and liver compared to the control, T1, and T2 groups, as illustrated in Table (1).

Table 1. Pb concentrations (µg/ g wet wt.) in different Nile tilapia tissues of experimental groups

Tissue	Control	Pb	T 1	T 2
Gills	0.001 ± 0.001^{b}	0.04 ± 0.014^{a}	0.006 ± 0.001^{b}	0.007 ± 0.004^{b}
Liver	0.001 ± 0.001^{b}	0.011 ± 0.002^{a}	0.003 ± 0.001^{b}	0.002 ± 0.001^{b}
Muscles	<DL	0.001 ± 0.001^{a}	<DL	<DL

Data are expressed as mean ± standard deviation.
<DL = values were below the detection limit, 0.001ppm for Pb.

Total hepatic protein and antioxidant enzymes

The present results revealed a significant ($P < 0.05$) decrease in the total hepatic protein value, GST, CAT, and POX in fish group that was subjected to lead (Pb group) compared to the control group (Table 2). Concerning fish group that was injected with 10mg/ Kg of beetroot extract (T1 group), there was a significant increase ($P < 0.05$) in the hepatic protein content, GST, SOD, and CAT compared to the Pb group; while, those values are still lower than those of the control group. Regarding T2 group, which received 15mg/ Kg of beetroot extract, there was insignificant changes in antioxidant enzyme activities compared to the Pb group.

Table 2. Total hepatic protein and antioxidant enzymes in the liver of the Nile tilapia injected with beetroot extract and subjected to lead

	C	**Pb**	**T1**	**T2**
Hepatic protein (mg/ml)	9.52 ± 0.49 [a]	3.52 ± 0.93 [b]	7.75 ± 0.30 [a]	6.69 ±1.93 [ab]
GST (unit/ml)	0.39 ± 0.28 [a]	0.01 ± 0.01 [c]	0.26 ± 0.18 [b]	0.11 ± 0.08 [c]
SOD (unit/ml)	1367.05 ± 966.65 [ab]	936.69 ± 662.34 [b]	1753.13 ±1239.65 [a]	1348.05± 953.22 [ab]
Catalase (unit/ml)	863.53 ± 24.08 [a]	519.49 ± 51.61 [c]	736.24 ± 48.16 [ab]	679.47 ± 70.53 [bc]
POX (unit/ml)	1.70 ± 0.35 [a]	0.45 ± 0.11 [b]	1.33 ± 0.52 [ab]	0.99 ± 0.24 [ab]

Data are expressed as mean ± standard deviation.
The values in the same row with different letters reflect significant differences between groups ($P < 0.05$).

The visualization of the protein and antioxidant enzyme activities on the polyacrylamide gel is a powerful tool for comparison between groups, protein, and enzymes results. The biochemical data are confirmed by electrophoretic analysis of total hepatic protein and antioxidant enzymes. Electrophoretic analysis of hepatic protein pattern shows that the protein band of Pb group (lan2) exhibited a marked lower intensity than the control group (lan1) band intensity, while (lan3) band intensity of T1 group remained more or less similar to the control band (lan1), and T2 group (lan4) protein band intensity was slightly lowered compared to control band (lan1) intensity (Fig. 6a). The electrophoretic analysis of catalase activity pattern compared to control band (lan1) showed that CAT activity band intensity of Pb group (lan2) decreased sharply, while CAT activity band intensity of T1 group (lan3) and T2 group (lan4) were almost the same of (lan1) control band (Fig. 5b). Electrophoretic analysis of glutathione S-transferase activity pattern showed an obvious high decrease in GST activity band intensity (lan2) of Pb group compared to control band (lan1). T1 group (lan3) GST activity band intensity was almost near to control band (lan1) intensity, while T2 group (lan4) GST activity band intensity was slightly less than control band (lan1) intensity (Fig.5c). Electrophoretic analysis of superoxide dismutase activity showed a marked decrease in SOD activity band intensity of Pb group (lan2) compared to control band (lan1) intensity, while T1 group (lan3) SOD activity band intensity was increased compared to (lan1) control band intensity, and T2 group (lan4) SOD activity band intensity was almost the same of (lan1) control band intensity (Fig. 5d). Total hepatic protein, GST, CAT and SOD were investigated by the analysis on 7% native PAGE, revealing a decrease in fish group that was subjected to lead toxicity (Pb group) compared to the control group. While in the group injected with 10mg/ Kg of beetroot extract before subjection to lead (Pb group), there was an increase in hepatic protein content, GST, SOD, CAT respectively compared to the Pb group.

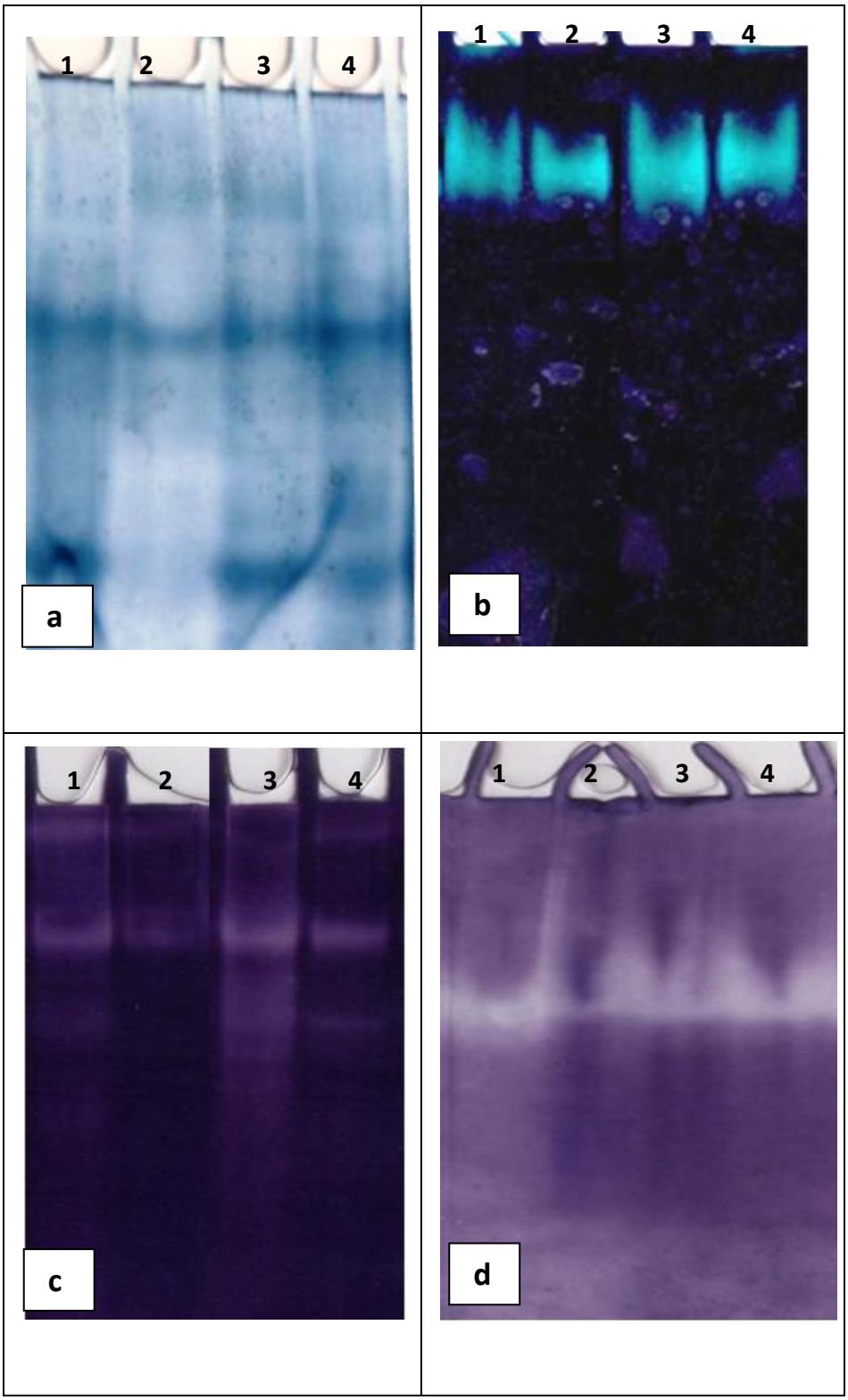

Fig. 5. Electrophoretic analysis on 7% native polyacrylamide gel of **(a)** hepatic protein pattern, **(b)** hepatic catalase activity pattern, **(c)** hepatic Glutathione S-transferase activity pattern, (**d)** hepatic superoxide dismutase activity pattern of control group (lan1), Pb group (lan2), T1 group (lan3) and T2 group (lan4)

Biochemical analysis

Generally, no significant differences were detected between the values of biochemical parameters ($P > 0.05$). The Pb group recorded the highest values compared to the other groups in most biochemical parameters. In addition, T1 group reported the highest significant value ($P < 0.05$) of total protein (Fig. 3) and albumin (Table 3).

Table 3. Biochemical analysis of the Nile tilapia injected with beetroot extract and subjected to lead

	C	**Pb**	**T1**	**T2**
Albumin (g/dl)	0.78 ± 0.07 [b]	0.89 ± 0.13 [b]	1.01 ± 0.07 [a]	0.84 ± 0.1 [b]
Glucose (mg/dl)	61.40 ± 5.08 [a]	63.47 ± 4.98 [a]	58.68 ± 0.85 [a]	62.19 ± 0.55 [a]
Cholesterol (mg/dl)	136.26 ± 7.28 [a]	138.00 ± 16.13 [a]	131.14 ± 4.29 [a]	129.32 ± 4.60 [a]
AST (U/l)	45.36 ± 11.09 [a]	56.71 ± 5.67 [a]	51.94 ± 5.74 [a]	40.62 ± 0.74 [a]
ALT (U/l)	20.42 ± 2.85 [a]	29.51 ± 2.27 [a]	26.70 ± 1.54 [a]	30.39 ± 0.54 [a]
Creatinine (mg/dl)	0.55 ± 0.06 [a]	0.51 ± 0.04 [a]	0.45 ± 0.02 [a]	0.51 ± 0.01 [a]
Uric acid (mg/dl)	4.43 ± 0.18 [a]	5.05 ± 0.13 [a]	4.57 ± 0.18 [a]	5.03 ± 0.40 [a]

Data are expressed as mean ± standard deviation.
The values in the same row with different letters reflect significant differences between groups ($P < 0.05$).

DISCUSSION

Immune and physiological systems share a common focus on an organism's ability to withstand various environmental stressors, including viral and bacterial infections, as well as exposure to xenobiotics. Several studies have established a mutually supportive connection between biotransformation and immune systems in numerous fish species **(Reynaud *et al*., 2008)**. On the other hand, contamination with heavy metals, especially lead (Pb), has a specific impact on the immune system of fish. Investigations have shown that lead exposure can cause immunosuppression in fish **(Paul *et al*,. 2014; Dai *et al*., 2018)**.

Plant products serve as a valuable source of bioactive molecules, with certain compounds identified as promising treatments due to their ability to modulate immunity and prevent or control diseases in fish **(Awad & Awaad, 2017)**. The plant material when used as food additive in fish has the ability to reduce the toxicity of pollutants and increase the immune status **(Abbas & Awad, 2016; Abbas *et al*., 2016)**. The immune status in fish can be evaluated by measuring some humoral immune parameters (antiprotease activity, lysozyme activity, total protein, albumin and globulin). Among these parameters, antiproteases are considered as essential components in the defence mechanisms of different organisms, as they actively control and hinder the functions of potentially harmful proteases. These antiproteases function by either binding to the active sites of proteases or by effectively 'trapping' the proteases, preventing protein hydrolysis **(Laskowski & Kato, 1980)**. The Nile tilapia exposed to Benzo-a-Pyrene (BaP) after being administrated with mustard extract and mustard crude showed resistance to the

harmful effect of pollutants and an enhancement in the immune parameters (antiprotease activity, lysozyme activity, and total protein) **(Abbas *et al.,* 2016)**. Furthermore, injecting catfish with a low dose of a leek extract (5mg/ kg) protects against BaP harmful effects by boosting the immune parameters (antiproteases, myeloperoxidase, total protein, albumin and globulin) **(Abbas & Awad, 2016)**. Our study showed an insignificant increased antiprotease activity in the group injected with 100mg/ kg (T1) of beetroot extract compared to other groups. The lowest activity recorded in the Pb group compared to other groups (even it is not significant) could reflect the immunosuppression effect.

Lysozyme plays a crucial role in the host defence mechanisms against infectious diseases by opsonin and activates the complement system and phagocytes **(Grinde, 1989)**. Countless plants showed an active effect via enhancing the immune system in fish which has been proven by increasing the immune parameters like lysozyme. For example: Rainbow trout fed for 2 weeks with plant immunostimulants, like tetra (*Cotinus coggyria*) **(Bilen *et al.,* 2011)**, and black cumin oil (*Nigella sativa*) **(Awad *et al.,* 2013)** showed an increasing lysozyme activity. The current study revealed a significant elevation ($P< 0.05$) in lysozyme activity in the group injected with 100mg/ kg (T1) of beetroot extract compared to other groups which reflects the efficiency of this group in resisting the toxicity of lead. Similar observations have been reported in the Nile tilapia where the mustard extract showed efficiency as an immuno-modulator to resist the dramatic effect of BaP and enhance immune parameters, such as lysozyme activity, antiprotease activity, and total protein **(Abbas *et al.,* 2016)**. Serum proteins serve various functions, such as regulating water balance in fish **(Wedemeyer & Yasutake, 1977)**, and acting as protective agents through acute phase proteins, which restrict the spread of infectious agents **(Gerwick *et al.,* 2002)**. Consequently, the heightened concentrations of total protein observed in fish fed with immunostimulants are often attributed by researchers to the enhancement of the non-specific immune response. An increase in total protein levels of the Nile tilapia was observed after being fed diets supplemented with plant immunostimulants, including 0.1% mixture of yellow leader, (*Astragalus membranaceus*) and Japanese honeysuckle (*Lonicera japonica*) **(Ardó *et al.,* 2008)**. Furthermore, **Awad *et al.*** (**2019**) found that feeding rainbow trout with hala extract diets, particularly at the highest dose (2%), significantly enhanced various immune parameters (total protein, antiprotease, myeloperoxidase content and bactericidal activity), in addition to resisting the infection with *Yersinia ruckeri*. Our investigation reported high total protein and albumin values in the group injected with 100mg/ kg (T1) of beetroot extract compared to other groups. This observation could be ascribed to the efficiency of this dose in resisting the harmful effect of lead and enhancing the immune status of fish. Previous study revealed that the administration of beet molasses can reduce the suppression effects of copper nanoparticles on the immune status of common carp (*Cyprinus carpio*); whereas, 2% of beet molasses resists the negative effect of copper and increases lysozyme, total protein, albumin, and globulin **(Veisi *et al.,* 2021)**.

Our results deduced that injection with beetroot extract has a notable effect on reducing lead accumulation in key organs such as the gills, muscles, and liver. The ability of beetroot to reduce Pb accumulation in fish organs can be attributed to several factors. Among them, beets contain compounds such as organic acids and polyphenols, which have chelating properties, meaning they can bind to heavy metals like lead and form stable complexes that are less readily absorbed by fish tissues **(Shahidi & Ambigaipalan, 2015; Gulcin & Alwasel, 2022)**. Additionally, the high antioxidant content of beets may help mitigate the oxidative stress induced by lead exposure, thereby reducing its toxic effects on fish organs **(Gurer 2000; Patra *et al.*, 2011)**.

Antioxidant enzymes represent an important defence mechanism of fish against the oxidative stress produced by heavy metal pollution **(Coelho *et al.*, 2011)**. In the present study, exposure of the Nile tilapia to lead toxicity in Pb group caused a significant reduction in the total hepatic protein and CAT, GST, and POX activities. Another comparable study by **Salaah *et al.* (2022)** revealed the negative impact of lead toxicity on the antioxidant enzymes in the liver, gills, and muscles of the Nile tilapia. Pb can displace zinc in cysteine chemical structure causing impairment in antioxidant levels, since cysteine represents an important amino acid in the synthesis of antioxidants **(Hodson *et al.*, 1977)**. Interestingly, the injection with beetroot extract in our study before exposure to Pb toxicity neutralized the negative effect of Pb and caused significant activation of antioxidant enzymes especially in the T1 group which received the lower dose (10mg/ kg) of the extract. Such finding could be attributed to the presence of several flavonoid compounds in beetroot extract that trigger its high antioxidant and radical-scavenging properties **(El-Beltagi *et al.*, 2022)**. Similar previous studies in fish revealed the positive effect of using plants either as extract or crude to resist the harmful effect of heavy metal and increase the antioxidant enzyme activities. For example, the use of 0.5 and 1% of onion either extract or crude in the Nile tilapia diet, along with the exposure to cadmium at the same time, showed resistance to the toxicity effect and enhancement in the antioxidant enzymes, especially GST and SOD **(Elgendy *et al*,. 2023)**. Moreover, catfish injected with 5mg/ kg of leek extract before being exposed to BaP revealed an elevation in GST activity compared to the control **(Abbas & Awad, 2016)**. In fact, plants are rich with many nutrients which have a powerful effect on enhancing the antioxidant enzymes. In this context, using 1% extract of Moringa oleifera in the Nile tilapia diet for 30 days showed a significant increase in SOD, CAT and POX activities **(Elgendy *et al.*, 2021)**. Additionally, feeding the Nile tilapia with 0.5 and 1% of onion extract of crude for 45 days enhanced the activity of the antioxidant enzyme (SOD, CAT, GST and POX) **(Younes *et al.*, 2021)**.

Biochemical analyses are good biomarkers that reflect fish health, especially with using new immunostimulants. ALT and AST represent important biomarkers that reflect liver function and their leakage in the blood reveals hepatocyte damage due to heavy metals toxicity **(Authman *et al.*, 2013)**. An increase in cholesterol and glucose levels reflects the

physiological response of fish towards heavy metal pollution and their need for excess energy to withstand the stress conditions **(Roohi *et al.*, 2017)**. Uric acid and creatinine levels in serum are traditional biomarkers that are used to check the kidney function **(Abdel-Tawwab *et al.*, 2013)**. Despite the insignificant changes in biochemical parameters, there were increases in glucose, cholesterol, AST, and uric acid in Pb group, reflecting the toxic effect of Pb on many physiological functions. With long-term exposure, these effects may lead to dangerous effects in fish. Similarly, **Abdelzaher *et al.* (2022)** revealed high values of ALT, AST, urea, creatinine, glucose, and cholesterol by increasing the duration and concentration of Pb exposure.

On the other hand, the fish injected with beetroot extract (T1& T2) neutralized the side effects of Pb exposure by slightly improving the biochemical analysis compared to the fish in Pb group. Pre-studies that used medicinal plants and their active compounds as feed additives in fish have similar effects on improving the physiological status of fish and mitigating the harmful effect of heavy metal pollution on fish. Thus, oral doses of curcumin (40 and 80mg/ Kg fish) applied to catfish for 3 weeks neutralized the iron toxicity effects on AST and ALT enzymes **(Abbas *et al.* 2019)**. In addition, feeding the Nile tilapia on fenugreek extract at concentrations of 1 and 3%, while it is exposed to cadmium, resulted in a reduction in some biochemical parameters, such as ALT, AST, and uric acid **(Abbas *et al.*, 2019)**. Moreover, feeding on zeolite as a natural clay for 45 days mitigated the toxic effect of lead acetate on the Nile tilapia and decreased cholesterol, glucose, AST, ALT, and creatinine levels **(Abbas *et al.*, 2021)**.

CONCLUSION

In conclusion, our results revealed that injecting the Nile tilapia with beetroot extract (especially with 100mg /kg) before exposure to lead neutralized the side effects of lead and improved the health status of fish, including immune response, antioxidant, and biochemical analysis. Thus, the current study could suggests using 100ml/ kg of beetroot extract to safeguard the fish against lead's adverse impacts. Future investigations would focus on the effects of the active ingredients of beetroot extract on fish's immune status and general health status.

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