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Enhancement effects of microalgae against heavy metals-induced toxicity in *Oreochromis niloticus* fish with emphasis on hematological, immunological and histological aspects

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ABSTRACT

Microalgae can be used as natural feed supplements for fish protection against heavy metal toxicity. The present study investigated the effect of a mixture of heavy metals (Zn, Cu, & Pb) on *Oreochromis niloticus* and the potential protective roles of the microalgae "*Chlorella vulgaris* and *Spirulina platensis*" against heavy metals toxicity for 35 days. Upon the addition of 10 & 15% of *C. vulgaris* and *S. platensis* in fish diets, an improvement was detected in the hematological parameters (RBCs, WBCs, Hb, Ht, MCV, MCH, & MCHC) of fish exposed to heavy metals. Meanwhile, several histopathological alterations were observed in the gills, liver, intestine and muscles of fish as a result of heavy metals accumulation in these orgains. However, a decrease was recorded in the histopathological changes regarding the examined tissues of heavy metals-treated fish upon using *C. vulgaris* and *S. platensis*. In addition, *C. vulgaris* and *S. platensis* showed a marked reduction in the bioaccumulation of Zn, Cu, & Pb in the liver, intestine and muscles. Besides, *C. vulgaris* and *S. platensis* decreased the immunohistochemical expression of the proinflammatory cytokine "tumor necrosis factor alpha (TNF-α)" and increased the expression of the proliferative protein (ki-67) in the examined tissues of the exposed fish. It was deduced that, *C. vulgaris* and *S. platensis* in fish diets provided protection against heavy metals inducing hematotoxicity, immunotoxicity and hisopathological alterations of *O. niloticus* fish.

INTRODUCTION

Water pollution with heavy metals is considered one of the most critical environmental problems **(Briffa *et al.*, 2020)**. Generally, heavy metals are defined as non-biodegradable metallic chemical elements with higher densities compared to water **(Jaishankar *et al.*, 2014)**. Metals pollution in aquatic ecosystems has emerged due to

agricultural, industrial wastes and anthropogenic activities **(Al-Homaidan *et al*., 2014; Ali *et al*., 2019)**. The most popular heavy metals of particular concern are lead (Pb), mercury (Hg), arsenic (As), copper (Cu), cadmium (Cd), zinc (Zn), nickel (Ni), and chromium (Cr) **(Kushwaha *et al*., 2019)**. Lead has serious effects on fish causing hematological, renal, reproductive, and neurological disorders **(Miloskovic, 2013; Hussain *et al*., 2018)**. Copper and zinc are necessary nutrient metals for the growth and physiological functions of many organisms **(Abdel Gawad, 2018)**. Nevertheless, elevated concentrations of these elements can cause eminent health problems for aquatic organisms **(Wadige *et al*., 2017; Delahaut *et al*., 2020)**. In this respect, Cu and Zn are absorbed by fishes due to their high solubility in the aquatic environments, and they are transferred to humans as end users in the food chain **(Ahmed *et al*., 2016)**.

Fish is considered an ideal model to evaluate the toxic effects of heavy metals in aquatic environments **(Sabullah *et al*., 2015; Al-Ghanim *et al*., 2019)**. Hence, the determination of heavy metals concentrations in the fish tissues is very important for human consumers **(Morshdy *et al*., 2021)**. Remarkably, the tilapia is an essential commercial fish in aquaculture worldwide; it represents an important source of animal protein **(Sharawy *et al*., 2017)**. The Nile tilapia (*Oreochromis niloticus*) is the main species of freshwater fishes that inhabit the River Nile in Egypt, and it can concentrate large amounts of heavy metals **(Abdel-Mohsien & Mahmoud, 2015)**. In fact, some heavy metals can induce the upregulation of proinflammatory genes and the histpathological changes of different tissues in fish **(Hermenean *et al*., 2015; Sun *et al*., 2019)**. Consequently, the removal of toxic heavy metals from contaminated water is of prime importance to protect aquaculture and avoid their health hazards. In this regard, numerous techniques have been used to remove heavy metals from wastewater such as reverse osmosis, electrophoresis, ion exchange and chemical precipitation, membrane filtration, etc.. **(Bashir *et al*., 2019)**. However, these techniques have many disadvantages such as incomplete metals removal and high costs **(Jawad *et al*., 2018)**.

Recently, biological treatment has become an alternative method to remove toxic heavy metals from wastewater because it has several advantages such as easy performance, low cost and high efficiency **(Hazarika *et al*., 2015; Razzak *et al*., 2022)**. In this context, many types of microorganisms including algae, bacteria, and fungi have been used for the reduction of heavy metals toxicity **(Mustapha & Halimoon, 2015; Kapahi & Sachdeva, 2019)**. Algae, in particular, act as cleansing and detoxifying phytonutrients against toxic heavy metals **(Ameri *et al*., 2020)**. Additionally, algae are used in fish farms as a good alternative source of proteins, vitamins, and essential fatty acids **(Abbas *et al*., 2020)**. *Chlorella vulgaris* is one of the most common algae reported for the removal of heavy metals from aqueous solutions **(Kumar *et al*., 2020)**. It is considered as a prokaryotic unicellular photosynthetic green microalga **(Goher *et al*., 2016)**. The microalga, *Spirulina platensis*, is another example that can protect aquatic environments through wastewater recycling **(Al-Homaidan *et al*., 2015)**. It is a

photosynthetic filamentous blue-green alga, with a simple structure and a complex composition **(Abd El Hay *et al*., 2019)**.

C. vulgaris and *S. platensis* have high heavy metals binding capacities due to the presence of proteins, polysaccharides and lipid on their cell wall surfaces containing some functional groups, which can act as binding sites for heavy metal ions **(Jaafari & Yaghmaeian, 2019)**. Furthermore, *C. vulgaris* and *S. platensis* have proved their immune-modulating, anti-tumor, and anti-inflammatory properties through the therapeutic control of induced inflammatory diseases **(Mahmoud *et al*., 2018)**. Therefore, the present study was conducted to assess the effects of a mixture of heavy metals (Zn, Cu, & Pb) on the hematological parameters, histological aspects, and immunohistochemical expression of TNF-α and ki-67 of *O. niloticus* fish. In addition, the potential protective role of the microalgae, *C. vulgaris* and *S. platensis*, against the toxic effects of heavy metals was investigated.

MATERIALS AND METHODS

Fish maintenance and rearing

A total number of 500 healthy *O. niloticus* fish, with an average body weight of 32±1 g were obtained from a commercial fish farm in Giza Governorate, Egypt. Fish were transferred in polyethylene bags with aerated water to the Environmental Research Department, Theodor Bilharz Research Institute, Giza, Egypt. Prior to the experiment, fish were acclimated for 2 weeks in glass aquaria (60 L), with dimensions of 70 × 50 × 60cm and provided with 35 liter of dechlorinated tap water (48 h). Fish in the glass aquaria were provided with air pumps and maintained at a temperature of 25°C. The fish were fed twice daily on a commercial fish feed containing 35% crude protein.

Diets preparation

C. vulgaris powder was obtained from a commercial algae culture in Menoufia Governorate, Egypt, while *S. platensis* was purchased as a commercial product from Spiro Tec Company in Egypt. Basal feed (35% crude protein) and dried microalgae; *C. vulgaris* and *S. platensis* were separately mixed with 10% and 15%, respectively. Diets were mixed thoroughly with 100ml of water for 1kg, then pelletized and air-dried for 48h at room temperature. The diets were stored in a refrigerator at 4°C for subsequent use.

Toxicity of heavy metals against *O. niloticus* fish

A series of concentrations was prepared from reagent-grade metals of $CuSO_4.5H_2O$, $Pb(NO_3)_2$, and $ZnSO_4.7H_2O$ (Merck, Darmstadt, Germany), using dechlorinated tap water to determine their toxicity against *O. niloticus* fish after 96h **(APHA, 2005)**. Three replicates were used for each metal (10 fish/ aquarium). After the exposure period, dead fish were counted and the lethal concentrations (LC_{50} & LC_{90}), and sublethal concentrations (LC_5, LC_{10}, LC_{15}, & LC_{25}) were computed using SPSS program version 17.

Experimental design

Approximately, 180 individuals of *O. niloticus* with the afore- mentioned average body weight were exposed to 1/5 LC_5 of a mixture of Zn, Cu, & Pb. The experimental design included six groups of fish: the first group were fed a basal diet without heavy metals (BF–HMs), while the second group were fed a basal diet and exposed to heavy metals (BF+HMs), the third and fourth groups were fed on *C. vulgaris* (CL) at levels of 10 & 15%, respectively, and exposed to heavy metals. The fifth and sixth groups were fed on *S. platensis* (SP) at levels of 10 & 15%, respectively, and exposed to heavy metals. The experiment was carried out in 18 glass aquaria with dimensions of 70 × 50 × 60 cm and filled with 35 L of dechlorinated tap water (48 h). Water temperature was maintained at 25°C to ensure stable experimental conditions. Three aquaria were used for each group and provided with air pumps. Fish in each group were fed twice a day (9:00am and 3:00pm) for 35 days. The amount of feed consumption for each diet was readjusted every 7 days according to the new fish biomass, considering 3 % of live body weight **(NRC, 1993)**. To keep constant heavy metals concentration, water and heavy metals in the glass aquaria were completely replaced every 7 days.

Hematological parameters

At the end of the experimental period, three fish were randomly collected from each aquarium for blood sampling. Blood samples were taken from the caudal vein of fish by sterile syringe using EDTA coating tubes, and the analysis was performed within 24h using ABX Micros 60 instrument. The count of red blood cells (RBCs$\times 10^6$ mm^3) and white blood cells (WBCs$\times 10^3$ mm^3) was measured according to the method of **Stoskopf (1993)**. The concentration of hemoglobin (Hb g/dl) was determined according to the method of **Zinkl (1986)**. While, hematocrit (Ht %) was measured according to the method of **Brown (1980)**. Mean cell volume (MCV; fl), mean cell hemoglobin (MCH; pg) and mean cell hemoglobin concentration (MCHC; %) were calculated according to the equations suggested in the study of **Haney *et al*. (1992)**; Where,

MCV (fl) = Ht (%) × 10/RBCs ($\times 10^6$ mm^3);

MCH (pg) = Hb (g/dl) × 10/RBCs ($\times 10^6$ mm^3), and

MCHC (%) = Hb (g/dl) × 100/Ht (%).

Bioaccumulation of heavy metals in fish tissues

After 35 days of exposure, the concentrations of Zn, Cu, & Pb in the tissues of intestine, liver, and muscles of fish from each group were analyzed. The tissues were dried at 70°C in an electric oven overnight. Afterwards, the dried tissues were weighed and each of which was transferred into clean screw capped glass tubes and digested with the solution of analytical concentrated HNO_3-$HClO_4$ (4:1 v/v) **(FAO, 1983)**. All glass tubes were left for four hours at room temperature, followed by heating at 40-45ºC for one hour in water bath, and then increased to 70ºC until the end of the digestion process. The digested fish tissues were allowed to cool at room temperature. Each digested tissue

was diluted to 25ml with deionized water and filtered in a volumetric flask. Then, the digested tissues were subjected to heavy metal analysis, using an atomic absorption spectrophotometer (Avanta, Australia) at Environmental Research Department, Thoedor Bilharz Research Institute, Giza, Egypt.

Histological examination

Fish samples were removed from the aquaria after the end of the experiment. Fish samples were dried on filter papers, and then dissected to obtain the organs (gills, liver, intestine and muscles). The general morphological characteristics of fish organs, such as size, color and texture were examined. All organs were preserved in 10% neutral buffered formalin for 24h and dehydrated, paraffinized, and then cross-sectioned at 5 microns, followed by staining with Hematoxylin and Eosin for light microscopic examination **(Bancroft & Gamble, 2008)**.

Immunohistochemical examination

The immune-histochemical staining procedures were performed following the method described by **Ronza *et al*. (2015)**. The immunostaining of the protein expressions of TNFα and ki-67 were examined in the gills, liver, intestine and muscles. The paraffin wax blocks of fish tissues were sectioned at 5µm, deparaffinized in xylene, rehydrated in a graded ethanol series, and treated with 3% hydrogen peroxide solution for 10min. Sections were incubated overnight at 4°C in humid chamber with the primary antibodies: TNF-alpha: sc-52746 and ki-67: sc-23900 at dilution of 1:100. The used antibodies are raised against human tissues (Santa-Cruz Biotechnology, USA). Sections were then washed thrice for 5min in phosphate-buffered saline. Finally, staining was developed with diaminobenzidine substrate, and sections were counterstained with hematoxylin, dehydrated with graded ethanol, and mounted. TNF-α and ki-67 stained sections were assessed using light microscope (Scope A1, Axio, Zeiss, Germany). Photomicrographs were taken using a microscope-camera (AxioCam, MRc5, Zeiss, Germany). Positivity was indicated by brownish cytoplasmic staining of cells.

Statistical analysis

Mean and standard deviation of the hematological parameters for each group were performed using one-way analysis of variance (ANOVA) at $P < 0.05$ by Tukey post-hoc test. The data of heavy metals bioaccumulation are presented as mean ± standard error using T-test. The comparison by percentage of TNF-α and ki-67 positive cells between different groups were tested for significance using Chi^2 test. The statistical analyses were performed using SPSS program version 17 (SPSS, Inc., Chicago, IL) for Windows.

RESULTS

Toxicity of heavy metals against *O. niloticus* fish

The results of lethal and sublethal concentrations of Zn, Cu & Pb against *O. niloticus* fish after 96h of exposure are presented in Table (1). The results revealed that

the values of LC_5, LC_{50}, & LC_{90} were 5.9, 17.0 & 37.0 mg L^{-1} for Zn; 0.44, 1.4 & 3.2 mg L^{-1} for Cu; and 1.1, 2.11 & 3.9 mg L^{-1} for Pb, respectively.

Table 1. The toxic effect of Zn, Cu & Pb on *O. niloticus* fish after 96h of exposure

Heavy metal	LC_5	LC_{10}	LC_{15}	LC_{25}	LC_{50}	LC_{90}
Zn	5.9	7.2	8.9	11.0	17.0	37.0
Cu	0.44	0.64	0.8	0.9	1.4	3.2
Pb	1.1	1.3	1.5	1.7	2.11	3.9

Hematological parameters

The results of the hematological parameters of *O. niloticus* fish exposed to a mixture of Zn, Cu & Pb and fed with 10 & 15% of *C. vulgaris* and *S. platensis* after 35 days are illustrated in Table (2). The results demonstrated a significant reduction in the levels of RBCs, WBCs, Hb, Ht, MCV, MCH, and MCHC of *O. niloticus* fish exposed to heavy metals at $P<0.05$, compared to the control group which was fed a basal feed only (BF–HMs). In addition, the results showed that *C. vulgaris* and *S. platensis* increased the levels of the hematological parameters of *O. niloticus* fish exposed to heavy metals. The levels of RBCs, WBCs, Hb, Ht, MCV, MCH & MCHC in the fish group fed CL15+HMs were 1.95±0.08 × 10^6/mm^3, 118.85±7.50 × 10^3/mm^3, 11±0.20 g/dl, 33.8±1.60 %, 173.3±8.71 fl, 56.41±2.57 pg & 32.5±1.78 %, respectively. Moreover, the highest levels of RBCs, WBCs, Hb, Ht, MCV, MCH & MCHC were measured in the fish group fed with SP15+HMs (2.37±0.11 × 10^6/mm^3, 122.95±5.64 × 10^3/mm^3, 13.68±0.21 g/dl, 41.9±1.90 %, 176.8±9.50 fl, 57.7±3.70 pg & 32.6±1.75 %, respectively).

Table 2. Mean values of the hematological parameters of *O. niloticus* fish exposed to a mixture of Zn, Cu & Pb and fed *C. vulgaris* & *S. platensis*

Hematological parameter	BF–HMs	BF+HMs	Experimental diets			
			CL10+HMs	CL15+HMs	SP10+HMs	SP15+HMs
RBCs (× 10^6/mm^3)	1.51^a±0.01	1.44^b±0.01	1.72^c±0.03	1.95^c±0.08	2.12^d±0.07	2.37^d±0.11
WBCs (× 10^3/mm^3)	108.9^a±5.67	99.3^b±4.87	113.7^a±6.52	118.85^c±7.50	119.35^c±5.62	122.95^c±5.64
Hb (g/dl)	8.05^a±0.07	5.96^b±0.06	8.85^a±0.15	11^c±0.20	12.05^c±0.21	13.68^d±0.21
Ht (%)	25.7^a±1.00	22.6^b±0.88	29.2^c±1.35	33.8^c±1.60	37.2^d±1.05	41.9^d±1.90
MCV (fl)	170.2^a±8.40	156.9^b±6.59	169.8^a±8.71	173.3^c±8.71	175.5^c±9.16	176.8^c±9.50
MCH (pg)	53.31^a±2.21	41.39^b±2.07	51.45^a±2.10	56.41^c±2.57	56.84^c±2.63	57.7^c±3.70
MCHC (%)	31.3^a±0.80	26.4^b±0.98	30.3^a±1.23	32.5^a±1.78	32.4^a±1.10	32.6^a±1.75

* The values are presented as mean of all samples in each group ± SD. Means in the same row with different letters differ significantly (P<0.05); while the same letters indicate non significance. The count of red and white blood cells: RBCs×10^6 mm^3 and WBCs×10^3 mm^3, respectively, Hb: hemoglobin concentration (g/dl), Ht (%): Hematocrit, MCV: Mean cell volume, MCH: Mean cell hemoglobin, MCHC: Mean cell hemoglobin concentration.

Bioaccumulation of heavy metals in fish tissues

The accumulation of Zn, Cu & Pb in different tissues of *O. niloticus* fish are shown in Fig. (1). The results showed that high concentrations of the analyzed metals were recorded in all examined tissues of fish (BF+HMs). The concentrations of Zn & Cu in tissues were high in the following order: liver > intestine > muscles, while Pb concentrations were high in the intestine > liver > muscles. Furthermore, heavy metals concentrations in fish tissues were higher than their concentrations in water. Obviously, *C. vulgaris* and *S. platensis*, significantly reduced the accumulation of Zn, Cu, & Pb in the examined tissues of *O. niloticus* fish. The concentrations of Zn, Cu & Pb in the liver of *O. niloticus* fish fed 15% of *C. vulgaris* were 451.87, 2567.34 & 21.95µg/g, respectively. In addition, the concentrations of Zn, Cu & Pb in the liver of fish fed 15% of *S. platensis* were 375.35, 1443.74 & 21.13µg/g, respectively.

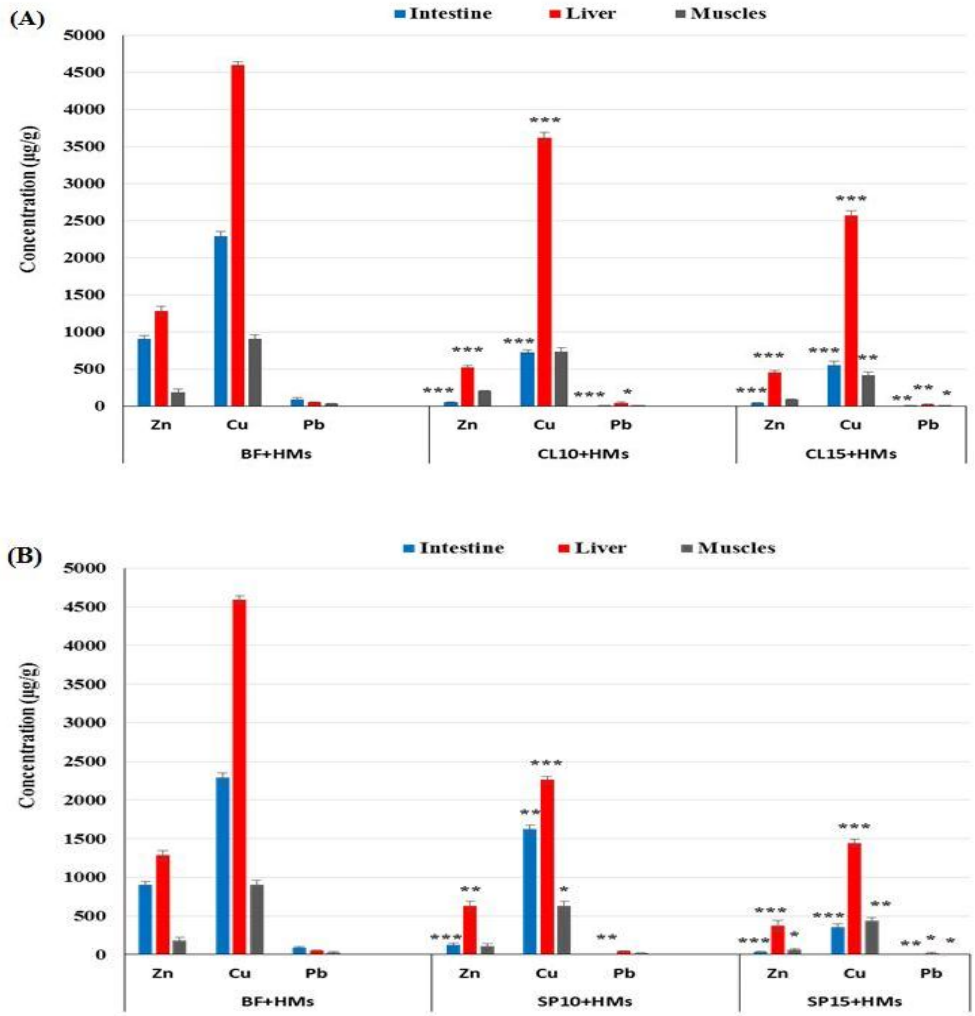

Fig. 1. Heavy metals concentrations (Zn, Cu, & Pb) (Mean ± SE) in intestine, liver, and muscles of *O. niloticus* fish fed (A) *C. vulgaris* & (B) *S. platensis* after 35 days.

* Significant difference at $p < 0.05$, ** Highly significant difference at $p < 0.01$, *** More highly significant difference at $p < 0.001$.

Histological examination

Several histopathological alterations were observed in the gills, liver, intestine, and muscles of *O. niloticus* fish exposed to a mixture of Zn Cu & Pb after 35 days. The gills of control group (BF–HMs) were formed by a filament multilayered epithelium, sectioned by longitudinal capillary axes, which are originated in the lamellae (Fig. 2A). The results showed noticeable pathological changes in the gill structure of fish exposed to heavy metals (BF+HMs), including severe epithelial hyperplasia that fused the secondary lamellae together forming undifferentiated cells (Fig. 2B). On the other hand, the fish fed with 10 & 15% of *C. vulgaris* showed that the features of their gill lamellae were similar to the control group (BF–HMs) (Fig. 2C & D). Additionally, the gill structure of fish fed 10 & 15% of *S. platensis* was similar to the fish of the control group (Fig. 2E & F).

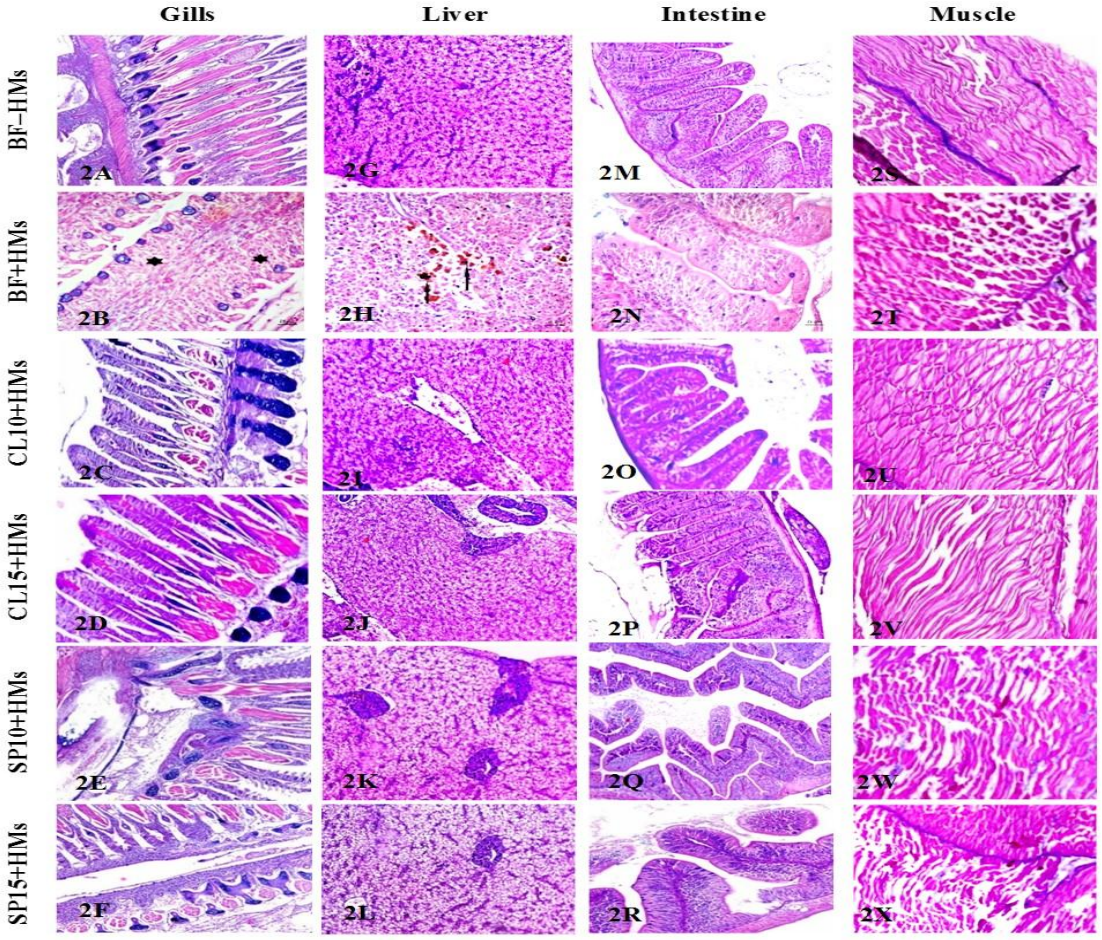

Fig. 2. Photomicrographs (H&E, 200x) of transverse sections in the gills, liver, intestine, and muscles of *O. niloticus* fish: BF–HMs group: fish fed a basal diet without heavy metals (2A,G, M & S), BF+HMs group: fish fed a basal diet and exposed to the heavy metals (2B, H, N & T), CL10+HMs group: fish fed 10% of *C. vulgaris* (2C, I, O & U), CL15+HMs group: fish fed 15% of *C. vulgaris* (2D, J, P & V), SP10+HMs group: fish fed with 10% of *S. platensis* (2E, K, Q &W) & SP15+HMs group: fish fed 15% of *S. platensis* (2F, L, R & X).

The liver of the control group (BF–HMs) displayed normal structure and lipoprotein such as droplets. The normal liver tissue contained strands of the hepatic cells which were large in size, hexagonal in shape, with centrally located nucleus and a homogenous cytoplasm (Fig. 2G). Contrarily, an extensive vacuolation of parenchyma hepatocytes, pyknotic nuclei and a few focal necrosis of the hepatic cells were observed in fish exposed to heavy metals (Fig. 2H). However, the liver of the fish fed *C. vulgaris* and *S. platensis* appeared in a normal structure, with hexagonal hepatic cells and nucleus (Fig. 2I, J, K & L). The intestine of the control group (BF–HMs) showed normal structure formed by tunica mucosa, with a loose connective tissue lamina propria, tunica submucosa, tunica muscularis (inner circular and outer longitudinal smooth muscles) and tunica serosa layers (Fig. 2M). The results showed a damage in the cells of the apex of villi, with irrigular arrangement of goblet cells in the intestine of fish exposed to heavy metals (Fig. 2N). Adversely, the results showed that the villi of mucosa propria were normal, and the goblet cells were arranged in its place in the fish fed with *C. vulgaris* and *S. platensis* (Fig. 2O, P, Q & R). On the other hand, the muscles of the control group (BF–HMs) showed normal structure of fibers (Fig. 2S). Our results showed a degeneration in muscle bundles, accompanied with focal areas of necrosis and a splitting of fibers of fish exposed to heavy metals (Fig. 2T). Obviously, the muscle fibers of fish fed with 15% of *C. vulgaris* showed normal structure, compared to the other diets groups (Fig. 2V).

Immunohistochemical examination

The expressions of TNF-α in the gills, liver, intestine, and muscles of *O. niloticus* fish exposed to heavy metals (Zn, Cu, & Pb) are shown in Table (3) and Fig. (3A-X). The results indicated that TNF-α expression in the liver, intestine, and muscles was not detected in the control group (BF–HMs), while it was found only in 5% of cells of gills. The highest expression of TNF-α was observed in the examined tissues of fish exposed to heavy metals ($P<0.001$). The results showed that no TNF-α expression was observed in the liver, intestine and muscles of fish fed on *C. vulgaris* and *S. platensis*, while low expression of TNF-α was observed in the gills.

Table 3. Percentage of the proinflammatory cytokine; tumor necrosis factor alpha (TNF-α) in the examined tissues of *O. niloticus* fish exposed to a mixture of Zn, Cu & Pb and fed *C. vulgaris* & *S. platensis*

Tissue		Percentage of TNF-α positive cells					
		BF–HMs	BF+HMs	CL10+HMs	CL15+HMs	SP10+HMs	SP15+HMs
Gills	%	5	40***	10	0	20***	20***
	Chi^2 BF–HMs		37.16	2.59	3.28	11.70	11.70
	Chi^2 BF+HMs			22.43	47.53	8.60	8.60
Liver	%	0	30***	0	0	0	0
	Chi^2 BF–HMs		37.69				
	Chi^2 BF+HMs			32.98	32.98	32.98	32.98
Intestine	%	0	30***	0	0	0	0
	Chi^2 BF–HMs		37.69				
	Chi^2 BF+HMs			32.98	32.98	32.98	32.98
Muscles	%	0	30***	0	20***	0	0
	Chi^2 BF–HMs		37.69		24.50		
	Chi^2 BF+HMs			32.98	2.16	32.98	32.98

Comparison by percentage using Chi^2 test "$Chi^2= ([ad-bc]-n/2)^2*n/(a+b)(c+d)(a+c)(b+d)$". *** More highly significant difference compared to the control group (BF–HMs) at $p < 0.001$.

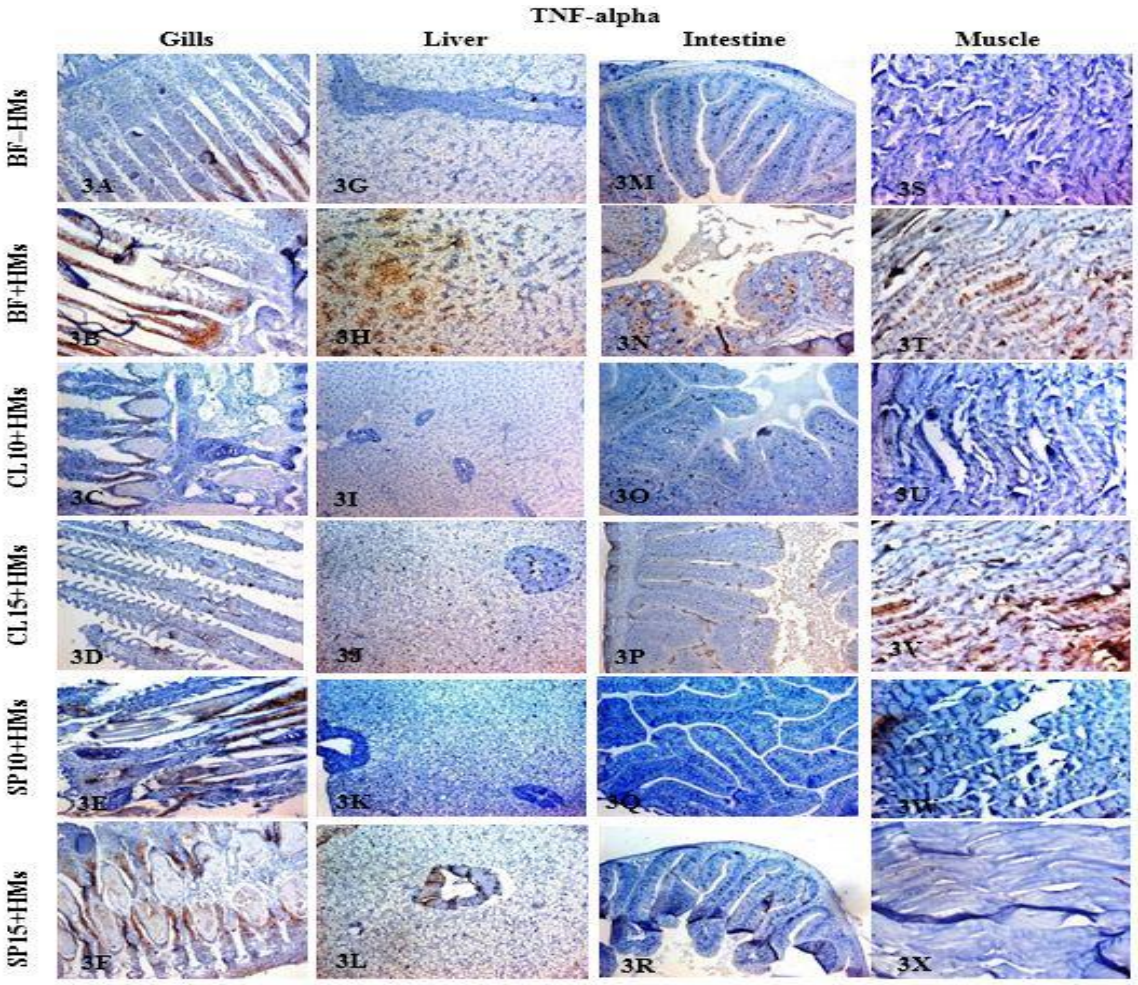

Fig. 3. Immunohistochemical expression of TNF-α (200x) in the gills of *O. niloticus* fish: BF–HMs: positive expression in 5% of cells **(3A)**, BF+HMs: positive expression in 40% of cells **(3B)**, CL10+HMs: positive expression in 10% of cells **(3C)**, CL15+HMs: no expression **(3D)**, SP10+HMs: positive expression in 20% of cells **(3E)** & SP15+HMs: positive expression in 20% of cells **(3F)**. Liver; BF–HMs: no expression **(3G)**, BF+HMs: positive expression in 30% of cells **(3H)**, CL10+HMs: no expression **(3I)**, CL15+HMs: no expression **(3J)**, SP10+HMs: no expression **(3K)** & SP15+HMs: no expression **(3L)**. Intestine; BF–HMs: no expression **(3M)**, BF+HMs: positive expression in 30% of cells **(3N)**, CL10+HMs: no expression **(3O)**, CL15+HMs: no expression **(3P)**, SP10+HMs: no expression **(3Q)** & SP15+HMs: no expression **(3R)**. Muscles; BF–HMs: no expression **(3S)**, BF+HMs: positive expression in 30% of cells **(3T)**, CL10+HMs: no expression **(3U)**, CL15+HMs: positive expression in 20% of cells **(3V)**, SP10+HMs: no expression **(3W)** & SP15+HMs: no expression **(3X)**.

The results of ki-67 expression in the gills, liver, intestine, and muscles of *O. niloticus* fish exposed to heavy metals (Zn, Cu, & Pb) are illustrated in Table (4) and Fig. (4A-X). The results indicated that ki-67 expression was detected in the examined tissues of the control group (BF–HMs). The lowest levels of ki-67 expression were observed in all examined tissues of fish exposed to heavy metals (BF+HMs). Contrarily, the expression of ki-67 was significantly increased in the examined tissues of fish fed on *C. vulgaris* and *S. platensis* ($P<0.001$) compared to the exposed group (BF+HMs).

Moreover, the highest values of ki-67 expression were detected in the most examined tissues of fish fed 10 & 15% of *S. platensis*, compared to 10 &15 % of *C. vulgaris* groups and the control group (BF–HMs).

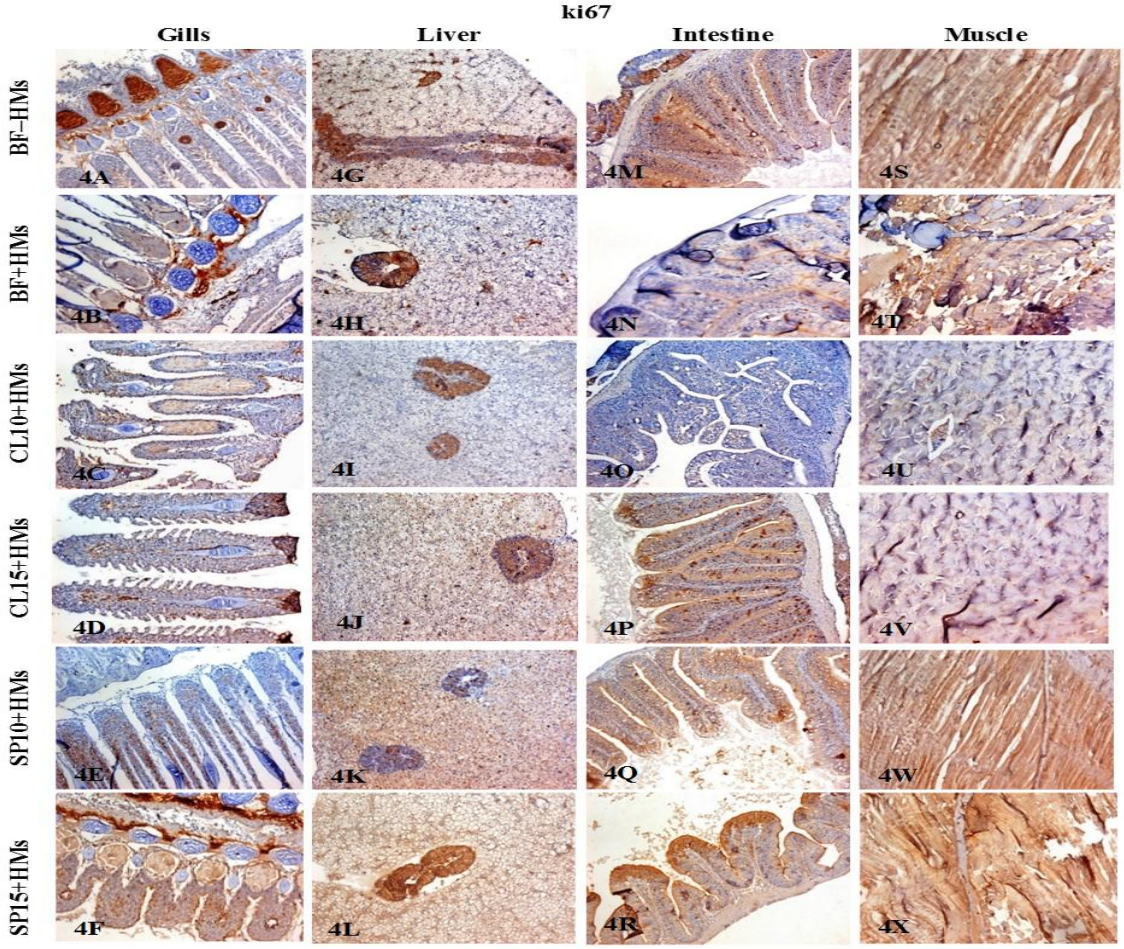

Fig. 4. Immunohistochemical expression of Ki-67(200x) in the gills of *O. niloticus* fish; BF–HMs: positive expression in 20% of cells (4A), BF+HMs: positive expression in 10% of cells (4B), CL10+HMs: positive expression in 20% of cells (4C), CL15+HMs: positive expression in 30% of cells (4D), SP10+HMs: positive expression in 70% of cells (4E) & SP15+HMs: positive expression in 50% of cells (4F). Liver; BF–HMs: positive expression in 15% of the hepatic cells and in wall of vessels (4G), BF+HMs: positive expression in 10% of the hepatic cells and in wall of vessels (4H), CL10+HMs: positive expression in 15% of the hepatic cells and in wall of vessels (4I), CL15+HMs: positive expression in 50% of the hepatic cells and in wall of vessels (4J), SP10+HMs: positive expression in 70% of the hepatic cells and in wall of vessels (4K) & SP15+HMs: positive expression in 50% of the hepatic cells and in wall of vessels **(4L)**. Intestine; BF–HMs: positive expression in 70% of cells **(4M)**, BF+HMs: positive expression in 10% of cells (4N), CL10+HMs: no expression **(4O)**, CL15+HMs: positive expression in 50% of cells **(4P)**, SP10+HMs: positive expression in 50% of cells (4Q) & SP15+HMs: positive expression in 50% of cells **(4R)**. Muscles; BF–HMs: positive expression in 80% of cells **(4S)**, BF+HMs: no expression **(4T)**, CL10+HMs: positive expression in 10% of cells (4U), CL15+HMs: positive expression in 10% of cells (4V), SP10+HMs: positive expression in 90% of cells (4W) & SP15+HMs: positive expression in 90% of cells (4X).

Table 4. Percentage of the proliferative protein (ki-67) in the examined tissues of *O. niloticus* fish exposed to a mixture of Zn, Cu, & Pb and fed on *C. vulgaris* & *S. platensis*.

Tissues		Percentage of ki-67 positive cells					
		BF–HMs	BF+HMs	CL10+HMs	CL15+HMs	SP10+HMs	SP15+HMs
Gills	%	20	10	20	30	70***	50***
	Chi^2 BF–HMs		3.18	0.03	3.23	52.55	21.12
	Chi^2 BF+HMs			4.75	13.78	77.52	40.02
Liver	%	15	10	15	50***	70***	50***
	Chi^2 BF–HMs		0.73	0.04	29.54	64.16	29.54
	Chi^2 BF+HMs			1.65	40.02	77.52	40.02
Intestine	%	70	10***	0	50**	50**	50**
	Chi^2 BF–HMs		72.52	104.64	7.52	7.52	7.52
	Chi^2 BF+HMs			8.53	40.02	40.02	40.02
Muscles	%	80	0	10***	10***	90*	90*
	Chi^2 BF–HMs		130.02	96.18	96.18	4.75	4.75
	Chi^2 BF+HMs			12.74	12.74	167.29	167.29

Comparison by percentage using Chi^2 test "Chi^2= $([ad-bc]-n/2)^2*n/(a+b)(c+d)(a+c)(b+d)$". * Significant difference at $p < 0.05$, ** Highly significant difference at $p < 0.01$, *** More highly significant difference at $p < 0.001$.

DISCUSSION

Aquatic pollution with heavy metals is considered a global issue where it causes health risks to fish and human **(Farouk *et al*., 2020)**. To the best of our knowledge, this is the first study to investigate the enhancement effects of *C. vulgaris* and *S. platensis* on the hematological, immunological and histological aspects of *O. niloticus* fish exposed to a mixture of heavy metals (Zn, Cu, & Pb). The present study indicated that Zn, Cu, & Pb had high toxic effects on *O. niloticus* fish after 96 h of exposure. In toxicology, a 96-hour exposure experiment is the most common procedure to evaluate the toxicity of particular compounds in fish **(Lammer *et al*., 2009)**. Generally, the hematological variables in fish can be used as biomarkers to evaluate the effects of heavy metals **(Al-Asgah *et al*., 2015)**. The present study showed that heavy metals caused a reduction in the levels of the hematological parameters of *O. niloticus* fish compared to those of the control group (BF–HMs). These results are in accordance with **Abdulnabi (2020)** who found that heavy metals caused a drastic reduction in the levels of RBCs and Hb. In the same vein, **Abdel-Warith *et al*. (2020)** recorded a significant decrease in WBCs, Ht, MCH, and MCHC levels of *O. niloticus* fish exposed to heavy metals. Our study also indicated that 10 & 15% of *C. vulgaris* in diets increased the levels of RBCs, WBCs, Hb, and Ht of *O. niloticus* fish exposed to heavy metals. This matches with the findings of **Abbas *et al*. (2020)**, as they reported that the supplementation of *C. vulgaris* in fish diets resulted in a significant increase in RBCs, WBCs, Hb, and Ht levels of tilapia fish.

Furthermore, the results of the hematological parameters proved that the supplementation of *S. platensis* was more effective than of *C. vulgaris* supplementation in

the diets of *O. niloticus* fish exposed to heavy metals. This result is similar to those stated by **Sherif *et al*. (2012)** who found that the supplementation of *S. platensis* in diets of *O. niloticus* fish significantly increased the levels of RBCs, WBCs, and Hb. These results are also in agreement with those obtained by **Simanjuntak *et al*. (2018)** who reported that the supplementation of *S. platensis* in diets of gurami fish (*Osphronemus gouramy*) showed the highest values in RBCs, WBCs, Hb, Ht, MCV, and MCH. The current study indicated that high concentrations of Zn, Cu, & Pb were accumulated in the examined tissues of *O. niloticus* fish. Moreover, the highest concentrations of Zn & Cu were accumulated in the liver tissues compared to the other tissues. This is confirmed by **EL-Shaer and Alabssawy (2019)** who stated that the concentrations of the investigated heavy metals in the liver of *O. niloticus* fish were much higher than the other tissues. Meanwhile, fish liver plays an important role in detoxification of heavy metals from the blood coming from the intestine **(Tayel *et al*., 2018)**. The results revealed that the concentrations of Zn, Cu, & Pb were higher in the examined tissues of fish than in water. A similar trend was observed by **Abd-El -Khalek *et al*. (2012)** who reported that fish accumulate the heavy metals directly from water and their concentrations may ultimately reach many times above those measured in water.

The results also showed that the supplementation of *C. vulgaris* and *S. platensis* in fish diets reduced the accumulation of Zn, Cu, & Pb in the examined fish tissues. The same trend was reported by **James *et al*. (2009)** who proved that dietary supplementation of Spirulina reduced copper accumulation in the tissues of carp (*Cirrhinus mrigala*), and elimination of more copper through feces. According to **Kaoud *et al*. (2012)**, the microalgae ameliorated the toxic effects of heavy metals on *O. niloticus* fish, which indicating their capability to chelate them from the water. **Almomani and Bhosale (2021)** reported that microalgae can remove several types of heavy metals from wastewater at the same time. With regard to our histopathological findings, several alterations were observed in the gills, liver, intestine, and muscles of *O. niloticus* fish exposed to a mixture of Zn, Cu & Pb. In fact, the fish gills are the primary target organs of the pollutants such as heavy metals **(Hermenean *et al*., 2017)**. Our results indicated that heavy metals caused severe epithelial hyperplasia and fusion of the secondary lamellae in the gills of *O. niloticus* fish. Similar findings were reported by **Younis *et al*. (2020)**, who found fusion of the secondary lamellae in the gills of *O. niloticus* fish exposed to heavy metals.

Regarding liver, extensive vacuolation of parenchyma hepatocytes may be due to the accumulation effect of Zn & Cu and the increase of their concentrations in the liver tissues. Similar changes were obtained by **Abiona *et al*. (2019)**, as the vacuoles were observed in the hepatocytes of *O. niloticus* fish exposed to heavy metals. The results also showed damage in the cells of apex of villi in the intestine of fish exposed to heavy metals. These results are in coincidence with the findings of **Maharajan *et al*. (2016)** who reported that some pathological alterations were observed in the intestine of Asian

sea bass, *Lates calcarifer* (Bloch) exposed to copper. These results agreed with **Mahboob *et al*. (2020)** who stated that some histopathological alterations were observed in the muscles of *O. niloticus* fish exposed to heavy metals. Notably, the supplementation of *C. vulgaris* and *S. platensis* through feed seems to be marked beneficial to alleviate the heavy metals induced severe histopathological changes in the examined tissues of *O. niloticus* fish. These results are in line with **Zahran *et al*. (2019)** who revealed that *C. vulgaris* supplementation reduced the histopathological alterations of *O. niloticus* fish exposed to sodium arsenite toxicity. In the same vein, **Khalila *et al*. (2018)** found that Spirulina supplementation improved the histological changes of *O. niloticus* fish in normal conditions.

TNF-α is a pro-inflammatory cytokine which have been applied as a valuable tool for measuring immune responses in fish against stressful conditions **(Attia *et al*., 2020)**. In this study, the expression of TNF-α significantly increased in the examined tissues of *O. niloticus* fish exposed to a mixture of heavy metals. Our results are consistent with those of **Jin *et al*. (2015)** who reported that Zebrafish (*Danio rerio*) exposed to Cd and Cr resulted in a significant upregulation of TNF-α gene expression. Also, **Rajeshkumar *et al*. (2017)** reported that the accumulation of heavy metals exhibited a significant increase in the expression of TNF-α in the gills, kidney, and liver of common carp (*Cyprinus carpio*). In this regard, the higher bioaccumulation of Zn, Cu, & Pb in the examined tissues of *O. niloticus* fish might lead to the induction of inflammation and upregulation of pro-inflammatory TNF-α cytokine, being in agreement with the histopathological damages. In contrast, no expression of TNF-α was detected in the liver, intestine and muscles of the fish exposed to a mixture of heavy metals and fed on *C. vulgaris* and *S. platensis*. The same trend was reported by **Galal *et al*. (2018)**, where a downregulation in the expression levels of TNF-α was observed in the spleen of *O. niloticus* exposed to penoxsulam and fed on 10% of *C. vulgaris*.

Based on our results, the supplementation of *C. vulgaris* and *S. platensis* in fish diets significantly increased the expressions of ki-67 in the examined tissues of *O. niloticus* fish exposed to heavy metals. Our findings were in line with **Khani *et al*. (2017)** who found *C. vulgaris* supplementation in fish diets showed a significant increase in the levels of IgM and lysozyme in Koi carp (*Cyprinus carpio*). In concurrence with our findings, **Ragap *et al*. (2012)** stated that *S. platensis* enhanced the immune response in *O. niloticus* fish. Our study found that *C. vulgaris* and *S. platensis* showed less levels of inflammation (indicated by low expression of TNF-α) and high activated repair indices (denoted by high expression of ki-67) in the examined tissues of *O. niloticus* fish exposed to heavy metals. Overall, the present study showed positive effects of these microalgae which enhanced the immune status of *O. niloticus* fish against heavy metals toxicity. Such enhancement is due to the positive effects of some ingredients of *C. vulgaris* such as carotenoids, phycobilins, fatty acids, polysaccharides, vitamins, and sterols, which is responsible for immune-stimulting effects **(Soontornchaiboon *et al*. 2012)**. Additionally,

the beneficial effects of *S. platensis* is due to the presence of various bioactive components particularly, phycocyanin, essential fatty acids, carotenoids, antioxidant pigments, vitamins and minerals which have immunostimulant and protective effects **(Watanuki *et al*., 2006)**.

CONCLUSION

Our findings have clearly indicated the toxic effects of heavy metals on *O. niloticus* fish. *C. vulgaris* and *S. platensis* supplementation significantly acted as detoxifying and reduced the residues of heavy metals in the examined tissues of *O. niloticus* fish. Also, *C. vulgaris* and *S. platensis* provided protection against a mixture of heavy metals which induced hematotoxicity, immunotoxicity and hisopathological alterations of *O. niloticus* fish. Based on the findings of the present study, it could be concluded that *C. vulgaris* and *S. platensis* have immunostimulant effects on fish and both of them can be used as feed supplements in aquaculture industry.

Ethical statement

The study was approved by the Committee on the Ethics of the Theodor Bilharz Research Institute (TBRI) and institutional guidelines for the care and use of animals.

Conflict of interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analyzed during this study are included in this article and data have not been shared.

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