



***Quillaja saponaria* Improved Growth Performance, Hemato-biochemical Profile and Protects Nile Tilapia (*Oreochromis niloticus*) Against Pb and Cd-Induced Oxidative and Inflammatory Tissues Damage**

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Abstract

THIS study aimed to estimate the diminishing effect of *Quillaja saponaria* (QS) on Nile tilapia exposed to heavy metals and its effect on growth performance, hemato-biochemical profile, oxidative response and tissue damage. Fish (N=90, average weight: 61.74 ± 4.72 g) were randomly distributed in triplicates at a rate of 6 fish per 60 L aquarium. Fish exposed to cadmium (Cd) and lead (Pb) mixture with 1.31 mg/L and 58.4 µg/L, respectively and received QS with 150, 300, 500 mg/kg fish feed. Pb and Cd significantly decreased growth performance and feed utilization. Metals deteriorated haemato-biochemical parameters by decreased Hb, HTC and RBCs, increasing WBCs, increasing the levels of ALT, AST, ALP, bilirubin, urea, triglycerides, glucose and total lipids and decreasing total protein, globulin and HDL. Observed increase of MDA levels and decrease SOD, GSH and ChE% activities were recorded in metals- exposed fish. Challenge with metals revealed up-regulated effect of liver CYP19 gene expression. QS enhanced fish growth performance while exposure to metals. QS restored the normal haemato-biochemical indices compared to the control. QS reduced the effect of metals on oxidative status enzymes (decreased MDA values and increased SOD, GSH and ChE%) activities. QS achieved the balance of cell proliferation and apoptosis of CYP19 and GPx gene expressions. In conclusion, QS has diminished effect against Cd and Pb negative impacts on fish. QS concentrations 150, 300 and 500 mg/kg fish feed enhanced growth performance, haemato-biochemical indices, hindered the oxidative stress and achieved cell balance of proliferation and apoptosis.

Keywords: Cadmium, *Quillaja saponaria*, *Oreochromis niloticus*, heavy metals, oxidative status.

Introduction

Heavy metals (HMs) are widespread contaminants of water affecting aquatic ecosystem [1, 2]. Although they are common in the nature, the anthropogenic sources as industries, mining, agriculture, domestic wastes and sewage increase their levels [3, 4]. These activities release a big quantities of HMs into the environment causing metal pollution [5]. HMs are non-biodegradable and persistent substances in the environment [6,7]. The aquatic environment is considered a habitat for aquatic species and a reservoir of potential persistent elements [8].

Although different physico-chemical and biological mechanisms found in the aquatic ecosystems fit to remove or decrease adverse toxic substances' effects, Bioavailability of metal ions inside the living cell may induce alterations in development, growth, reproduction, behaviour of organisms and/or death of injured cells [9, 10]. Cadmium (Cd) and lead (Pb) are a significant group of environmentally risky metals if present [11]. They have a worldwide specific concern [12]. Cd and Pb can incorporate into the food chain through water or food consumption to levels that affect their physiological state and cause great threats to their beings in the food chain [13, 7].

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Being fish one of desired human food, it becomes an indirect source of HMs arriving the human body [14]. Accumulation of metals in fish organs is subjective to some factors as surrounded environmental conditions (Dissolved Oxygen, temperature, salinity, pH etc.), biological factors (species, sex, size and age) and feeding sources [15, 16,14,17]. Effects differ according to the metal species and water mineralization degree [18]. Metal toxicity can affect blood composition processes and injures vital tissues [19]. Influences on fish may include growth inhibition, reduction of reproduction rates, hampering antioxidant capacity, and boosting sensitivity to pathogens [20, 21]. The problem occurs through the production of reactive oxygen species because of accumulation of metals causing oxidative stress and carcinogenesis in addition to behavioral, biochemical, and molecular changes [22].

As good and safe fish quality demand increases, several plant extracts have been broadly involved in fish diet for enhancement of fish growth performance and improvement their diseases resistance [23]. One of the widespread plant extracts is saponin. Saponin is found in diverse of plants which have hydrophobic aglycone. The aglycone may be a steroid or triterpenoid in nature on the way to which one or more sugar chains are involved [24]. Triterpenoid saponins are found in the soapbark tree, *Quillaja saponaria*, which is a conventional medicinal tree native to the Andes region - Colombia, and naturally formed glycosides. *Quillaja saponaria* (QS) is mostly formed by plants and existed in bacteria as well some lower marine organisms [25, 26]. It possesses strong biological activity partly owing to the presence of Quillaic acid [27]. In the past, saponins were mainly considered as a detergent source [28, 29, 30]. Nowadays, they are approved in human diet due to their widespread existence in food additives [25]. Saponins are studied for their role as food constituents in 187 countries [29]. QS is known to its uses as antibiotic and antimicrobial activities [31]. They are one known constituents in plant having a wide biological applications on organisms [32]. QS has a wide application in aquaculture. Extensive research has been approved the effective role of QS as a natural growth promoter when added into the aquafeeds [33,27, 24,34]. It helps enhancing the fish immune-defense mechanism [35, 34], reducing fish mortality [31], protecting against oxidative stress [35]. Elkaradawy et al., [26] revealed the ability of QS in improving water quality and the fish performance. Dietary QS could potentially replace hazardous synthetic androgens in producing all-male populations or reducing female's fertility if fed to tilapia at an early age [25]. Some studies recorded enhancement of antioxidant

enzymes of fish received the diet supplemented with QS [26, 34]. There is no available data on the impact of dietary supplementation of QS against HMs induced toxicity in fish.

The modern aquaculture practices are mainly depending on the intensive and super-intensive farming systems for aquatic species [36]. Worldwide, the aquaculture faces the challenge to optimize its production through sustainable practices [37]. Incorporation metal ions into farmed fish to that level which altering their physiological functions affect the health of consumers and threatens the fish production industry [38]. Nile tilapia is one of the most economically important fish species cultivated in the world [39]. It has the ability to fed on different types of foods, survive in stressed environments and has strong immune system [40]. It is considered a sentinel species of environmental quality [39] and commonly used as an excellent bio-indicator for assessment of HMs contamination and their risks for consumers [41, 19,42,35]. The aim of this study was to evaluate the QS effects on growth performance, haematology, antioxidant capacity, biochemical profile, oxidative status and the health of gills, brain, liver and spleen of Nile tilapia exposed to mixture of HMs.

Material and Methods

Ethical approval

The protocol of this study is reviewed by the institutional committee for the use of aquatic animals in research, faculty of Aquatic and Fisheries Sciences, University of Kafrelsheikh.

Preparation of synthetic heavy metals

Synthetic mixtures of Cd and Pb in the present study, prepared as previously recorded levels measured by Abdel-Tawwab et al. [43] in drainage polluted water (1.31 mg/L and 58.4 µg/L for Cd and Pb, respectively). Lead nitrate Pb (NO₃)₂ and cadmium chloride (CdCl₂·2.5H₂O) (Sigma-Aldrich Chemicals, USA) used to prepare stock solutions. Desirable concentrations were first dissolved in distilled water. Throughout the experimental period, pre-prepared solutions were added during the aquarium water exchange each 2 days from freshly prepared stock solutions for prevention of concentration changes caused by adsorption and evaporation processes [44]. All the other chemicals were of analytical grade.

Diets preparation, fish management, and study design

Nile tilapia (*Oreochromis niloticus*) was obtained from private fish farm (Kafr El-Sheikh governorate, Egypt). Fish were kept for adaptation in indoor tanks

for two weeks for the laboratory condition (12 – 12h light-dark photoperiod cycle). Fish (N=90, average weight: 61.74 ± 4.72 g) were randomly distributed in triplicates at a rate of 6 fish per 60 L aquarium. The tested experimental groups were (1) C-G; control group, fish fed on the basal diet, (2) HM-mix.; pre-prepared heavy metals' mixture (mixture Cd and Pb solution) added into the water and fish received a basal diet, (3) QS₅₀₀; fish were fed on the basal diet containing *Quillaja Saponaria* (QS) extract (ENVIRO QS®- Delacon Biotechnik GmbH, Austria) with concentration 500 mg/kg fish diet reared in water containing HMs, (4) QS₃₀₀; fish were fed on the basal diet containing 300 mg QS /kg fish diet reared in pre-prepared HMs containing water, (5) QS₁₅₀; fish were fed on the basal diet containing 150 mg QS /kg fish diet reared in pre-prepared HMs containing water. QS powder was gently added to the basal diet after the diet components were minced to obtain a homogenous mixture and stored at -20 °C in opaque plastic containers. The basal diet was 30/6, protein/lipids ratio, with 18.73 MJ kg⁻¹ gross energy. Each aquarium was supplied with compressed air via air-stones (using aquarium's air pump) and a mechanical filter (to collect the fish waste). Fish were fed diets twice daily at 8:00 am and 14:00 pm for 40 days. Daily fish mortalities were recorded for each aquarium. Water parameters were recorded daily in each aquarium. Total ammonia nitrogen (TAN) was measured using a portable colorimeter (Milwaukee-Mi 405), Dissolved oxygen (DO) using OxyGuard handy Polaris dissolved oxygen, pH and temperature using pH meter (HANNA- HI98191- PH meter). Water quality was under the natural conditions (temperature was 26.95 ± 1.7 °C, pH 7.63 ± 0.51 , TAN 0.04 ± 0.01 mg/L and DO 5.32 ± 1.62 mg/L) and all parameters were represented as mean $\pm$ SEM) [45].

Fish growth performance and feed utilization efficiency

To calculate Fish feed utilization and growth performance, at the end of the 40-days trial period, the fish were collected and anesthetized using clove oil (50 ml/L of water, Merck, Germany). Each fish was weighed separately using digital balance to get the final weight. Growth evaluation variables were measured as described by Abozahra et al. [46] and Ghalwash et al. [45] as follows: weight gain (WG) = ($W_1 - W_0$), specific growth rate (SGR, % body weight/day) = $100 \times [(\ln W_1 - \ln W_0)/t]$. Feed conversion ratio (FCR) was calculated as FI (g)/WG (g). Fish survival (%) = $100 \times [\text{final number of fish} / \text{initial number of fish}]$. Where W_0 is the initial weight, W_1 is the final weight (g), L is the final fish length and t is the experimental period in days.

Blood collection and serum separation

At the end of the trial, fish feeding was stopped 24 h proximately prior to blood sampling. From the fish caudal vein, blood samples were taken using heparinized syringe containing EDTA (Ethylenediamine tetra acetic acid) as an anticoagulant and gathered in vacuum tubes (3 replicate/aquarium) [47]. For blood serum collection, the assembled blood was collected without anticoagulants and centrifuged at the room temperature at $300 \times g$ for 15 min and the supernatant serum was stored in plastic Eppendorf tubes at -20 °C [48].

Haematological analyses

Red blood cells (RBCs) count, white blood cell (WBCs) count, Haemoglobin content (Hb), Haematocrit (HTC), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) using an automatic blood cell counter (Exigo- Vet., Boule Medical AB Inc., Stockholm, Sweden) [49]. For the other leucocytes count check, from each blood test, two thin films of blood were regulated on previously dry, pre-cleaned microscope slide for staining using a modified Wright's stain. From each stained slide, a total of 100 cells were considered using $\times 100$ oil immersion lens for investigation of lymphocytes, monocytes, Neutrophils and Eosinophils percentages (Exigo- Vet., Boule Medical AB Inc., Stockholm, Sweden) [50].

Serum biochemical analysis

Aspartate aminotransferase (AST), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) activities were measured colorimetrically at 540 nm wavelength [51]. Serum total proteins (TP) were estimated at the wavelength of 540 nm as illustrated by Doumas et al. [52] and albumin and bilirubin were measured at a wavelength 550 nm according to Dumas and Biggs, [53]. The globulin content was calculated by subtracting albumin from TP. Total lipids were evaluated as described by Zöllner, N. and Kirsch,[54]. Serum triglyceride (TG) were estimated using GPO- PAP and the total cholesterol (TC) using CHOD- PAP (commercial clinical kit) [55]. Creatinine was calorimetrically approximate according to Heinegård and Tiderström,[56]. Urea nitrogen and glucose were determined using Bio-Diagnostic Company kits (Bio-Diagnostic) and High density lipoprotein (HDL) was measured using commercial kits (Biodiagnostic Co., Egypt) [57].

Antioxidants status

Serum collected samples were used to estimate the oxidative stress assay. The activities of enzymes

catalase (CAT) and superoxide dismutase (SOD), malondialdehyde (MDA), were evaluated using ELISA kits (Inova Biotechnology, China) at the wavelength 450 nm using the microplate ELISA reader [58]. Glutathione (GSH) activity was determined colorimetrically, using commercial kits (Biodiagnostic Co., Cairo, Egypt), in accordance with the method described by Habig *et al.*[59]. Cholinesterase (ChE) activity were determined according to Brodeur *et al.*[60].

Total RNA extraction, cDNA synthesis and real-time quantitative PCR assay

Total RNA was obtained from 50 mg of Nile tilapia livers with Trizol (iNtRON Biotechnology) following the manufacturer's instructions. The integrity of RNA was authorized by ethidium bromide stained 2% agarose gel electrophoresis. The concentration and purity of RNA were estimated using a Nanodrop BioDrop spectrophotometer (Biochrom Ltd, Cambridge CB23 6DW, UK) based on the A260/A280 nm ratio. Two µg of RNA sample were reverse transcribed using ABT 2X RT Mix cDNA synthesis kit according to manufacturer's Protocol. Gene expression profiling was performed in Rotor Gene-Q (Qiagen-Germany) using gene-specific primer sequences for the amplification of Cytochrome (*CYP19*) and glutathione peroxidase (*GPx*) as represented in Table 1. The amplification reaction was done using ABT 2X qPCR Mix (SYBR) kit. The reaction volume was 20 µl consisting of 10µL SYBR Green, 0.6 µL of forward and reverse specific primer, 1 µL of cDNA template, and nuclease-free water to make the final volume 20 µL. The PCR program was carried out with the following conditions: activation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at the primer-specific temperature for 15 seconds, and extension at 72°C for 25 seconds. This was followed by a melt curve analysis to assess the specificity of amplification at 72°C to 95°C. All genes were tested in triplicates. CT values for each sample were determined and incorporated in "fold change" ($2^{-\Delta\Delta CT}$), calculation based on Livak and Schmittgen, [61], and mRNA expressions for each sample were normalized against the beta actin as a housekeeping gene.

Histopathological procedures

Specimens from liver, spleen brain and gills were taken immediately from the dissected fish. Samples were placed in a 10% prepared buffered formaldehyde solution for fixation. Specimens were fixed in paraffin wax then 3-4µm sections were cut by Leica Rotary Microtome (RM 2145, Leica Microsystems, Wetzlar, Germany). Slides were deparaffinized by running them in xylene to alcohol

to water for histological staining. Each slide was stained using hematoxylin and eosin (H&E) stain according to Suvarna and Layton, [64]. for an investigation of the selected fish tissues and observation of the spreading, occurrence, and invasion of the inflammatory cells. The prepared tissue sections were examined using Leica ICC50W Image Analyzer, microscope at 10X, 20X and 40X magnification.

Statistical Analysis

After testing of data for normality and homogeneity and the normality was assessed using analysis of the residuals, a statistical analysis was performed using GraphPad Prism 6 (GraphPad Prism v6.0, San Diego, CA, USA). Data were evaluated for significant differences between the experimental groups with one- way analysis of variance (ANOVA) followed by Tukey's post hoc test. Differences were confirmed at the 5% probability level. The obtained data were expressed as the mean $\pm$ standard error of the mean (SEM).

Results

Growth performance and feed utilization efficiency

Quillaja Saponaria dietary supplementations with different concentrations (150, 300, 500) revealed significant inhibition of the metals' negative effect on fish growth performance as recorded in Table 2. Challenge using Cd and Pb mixture effect negatively on WG, WG%, SGR and FCR. Fish groups received QS showed enhanced growth performance. In presence of metals, All QS groups displayed significant decrease of FCR ($P < 0.05$) to be close to the growth and feed utilization of the control group. Survival rate didn't display differences ($P > 0.05$).

Haematological parameters

Hemoglobin, HTC and RBCs are found significantly lower ($P < 0.05$) in Nile tilapia exposed to metals' mixture in comparing with the control and QS dietary supplementations. WBCs counts increased significantly in fish exposed to HM challenge ($P < 0.05$), but supplementations with QS diminish the negative effect, especially QS₁₅₀. Lymphocytes, Neutrophils, Monocytes and Eosinophils displayed nonsignificant differences between groups ($p > 0.05$) as summarized in Table (3).

Serum biochemical profile

Exposure to Cd and Pb mixture caused significant deterioration in most of measured biochemical parameters (ALT, AST, total protein, globulin, ALP, urea, triglycerides and total lipids, HDL, glucose, bilirubin), ($P < 0.05$) as illustrated in Table (4). Fish received QS supplementations in groups QS₃₀₀ and

QS₁₅₀ achieved significant enhancement of serum biochemical indices ($P < 0.05$) in comparing with other groups.

Oxidative status

Quillaja Saponaria with its different concentrations accomplished significant enhancement of the fish oxidative status. The effect of Cd and Pb mixture and assimilation with QS supplementations on the oxidative status of Nile tilapia are shown in Figure (1). SOD and GSH showed significant decrease when fish exposed to metals but adding QS enhanced the harmful effect of metals on these enzymes ($P < 0.05$). HMs increased values of MDA ($P < 0.05$). QS ability diminishing HMs impacts mirror on decreasing MDA levels in QS₅₀₀, QS₃₀₀ and QS₁₅₀ groups. In addition, significant restoration ($P < 0.05$) observed on ChE% activity when fish received QS feed additives.

Gene expression assay

Exposure of Nile tilapia to metals displayed exhibited up-regular effect of liver *CYP* gene expression (4.3-fold increase) but there wasn't significant change in liver *GPx* gene expression as represented in Figure 2. Treatment with QS supplementations showed different manners on tested genes. QS₅₀₀ succeeded in up-regular liver *GPx* gene by 20-fold increase and down-regular *CYP* gene expression (0.3-fold change) in comparison with the C-G and HM-mix group.

Discussion

Any deviation of the water quality of fish pond may cause stress on cultured species and impact the fish health and its growth performance [7]. Our findings verify the thought that toxicological effect of metals on fish tissues are intervened across the stimulation of oxidative stress besides animal cells apoptosis. In the present study, exposure of Nile tilapia to a combination of Cd and Pb which accumulate and injure various organs, inhibited fish growth performance. There are no previous studies on the defeated effect of QS on fish exposed to HMs. This work showed significant depletion of Nile tilapia growth performance and feed utilization when exposed to metals. But fish fed with QS supplements (in presence of metals) showed restoration of their feed rate. QS with the different concentrations (150, 300, 500 mg/kg) were observed to have the ability to stimulate Nile tilapia growth, as described before by Levavi-sivan et al.[65] and Stadtlander [66]. Elkaradawy et al.[26] and Elkaradawy et al.[67] recorded improvement in the final body weight (FBW), WG, WGR of Nile tilapia received QS compared with the control. Our results agree with Merrifield and Guroy, [68] who fed striped catfish

with different QS concentration supplements and found that WG, SGR and FCR significantly higher in groups fed QS. Although Deng et al.[34] informed that high doses of saponins may decreased growth values, QS₅₀₀ group showed the highest growth performance. Also, Rezaei,[32] found SGR, WG and food efficiency of *Amatitlania nigrofasciata* were observed in male fed with 700 mg/kg QS and 300 mg/kg female. Improvement of growth performance of fish fed QS because of the amount of saponins which increase permeability of the cell membrane. It has been theorized that this could permit for enhance nutrient absorption [27, 68]. Exposing to chronic stress lowers the ability of the organism to trigger the defense mechanism, producing dysfunctional reaction, following growth inhibition. Hence, enhancing food absorption ability could improve FCR, and raise growth and feed efficiency [69].

Haematological indices are used more and more as indicators of the hazard response in fish to the aquatic toxicology [70]. Exposure of Nile tilapia to metals showed reduction in Hb, HTC and RBCs values. This results in harmony with Kaoud et al., [71]. Shah, [72] found decline of Hb values of fish exposed to Pb toxicity and suggested these findings could be related to hemolytic crisis caused by HMs mixture. Also, Naz et al., [73] recorded significant reduction of RBCs, Hb, and hematocrit Hct in different fish species exposed to metals including Cd and Pb. Ullah et al., [74] explained the decrease in RBCs, Hb, and HTC in fish exposed to Cd could result from inhibited hemosynthesis or erythropoiesis, or destruction in hematopoietic tissues. Many researches assumed that exposure to Pb induces disruption of hemoglobin synthesis and may lead to anemia [75, 76, 77, 78]. Also, it may accredited to erythropoiesis, hemosynthesis, and osmoregulatory dysfunction and/or to an elevation the rate of erythrocyte damage in hematopoietic organs because of pollution [79]. Increase in WBCs content in HM-mix group agree with studies recorded increase in WBCs of Nile tilapia exposed to Cd stress [21] and Pb- stress [80]. Several studies recorded the increase in WBCs count in different species exposed to metals' combinations [81,72,82, 83]. As the WBCs are attending for defense, alterations of WBCs count documented in metals exposed- fish to specifies immuno-modulation of poisons [84], and/or organs damage [74]. Gaber et al.[85] assumed this is owing to the immune and protective response, lymphopoiesis stimulation and/or releasing of lymphocytes from lymphoid tissue in presence of metals. QS groups showed a significant increase in these parameters compared with HM- mix group. Fish reared in QS₁₅₀ group recorded the lower WBCs count as the control

values. Levavi-sivan *et al.*[65] showed strong hemolytic activities of QS on the human blood. Francis *et al.*[86] informed that there is positive, and others negative QS influences on blood indices. The positive effect might be because of increased oxygen uptake by the fish with a concomitant increase in Hb, RBCs and HTC levels, after the fish were exposed to QS.

Tilapia liver is approximately the best sensitive organ where it appears changes in biochemical features subsequently to the contact with different toxins [87]. It is the target for various water pollutants, including HMs [22]. In the present study, activity of measured enzymes revealed abnormal activities when fish exposed to metals for 40 days (in comparing with the control group). This is concord with many previous studies reported elevation in the fish liver function enzymes (AST, ALT and ALP) exposed to toxicity of metals [88, 21, 89]. Stress might elevate the permeability of animal cell membranes, producing higher enzymes' activities [21] by outflow into the blood stream [90, 91]. Fish of QS₁₅₀ and QS₃₀₀ have significantly enhanced the activities of these enzymes. Fleck *et al.*[29] informed that QS noticeably decreased the ALT activity (57%), AST (66%) and ALP (76%). Fish received 500 mg QS displayed enhancement of values of total protein and globulin. Elkaradawy *et al.*[26] and Elkaradawy *et al.*[67] revealed that QS significantly increased the total protein values in received Nile tilapia. Proteins have important functions as a nitrogenous metabolism and energy source (in stress) and construct the cell structural elements [74]. This might be accompanying the suppressed oxidative metabolism in the fish and hypoxia [36,74]. Lack of produced energy and hypoxia cause respiratory distress because of the buildup of lactic acid. Accordingly, under stress, respiratory pathway would have changed from aerobic to anaerobic. Fish obligates to use proteins as an alternative energy source in case of limited carbohydrates [74]. The improvement in total protein is owing to the augmented secretion of trypsin in the fish received QS. Urea, creatinine and bilirubin are nitrogenous wastes [92]. Increase the levels of urea have been used as nephrotoxicity indicators [22]. Bilirubin is an excretory catabolic product of hemoglobin and its higher levels may cause hypobilirubinea [93]. Fish exposed to pollutants verified higher increase of urea and bilirubin values [22] as a result of generation of various pathological activities in the fish tissues in presence of metals [48]. Different QS supplementations showed decrease levels of these products (specially QS₁₅₀) in comparing with HM-mix group. Abdel-Reheim *et al.*[35] documented rates fed with QS supplementation diet have had a

lower bilirubin value (54%). Fleck *et al.*[29] assumed the reason is the hepatoprotective effect of QS which probably owing to the enhancement of oxidative status and preserving hepatocyte integrity and functioning. The elevation of the lipid energy investments in response to contamination [85]. The excess energy funds (as glucose, triglycerides, and cholesterol) are consumed to mediate the effects of stress. As homeostasis of lipids is regarded one of the main liver functions [85]. The principal function of triglycerides is storing and providing the cellular energy and they are used to estimate growth status and lipid metabolism [94]. Elkaradawy *et al.*[67] showed significant decrease of triglycerides analyzed in Nile tilapia received QS in comparing with the control group. Deng *et al.*[34] showed similar effect on Hybrid groupers fed with steroid saponins supplementations. Abdel-Reheim *et al.*[35] showed decrease in triglycerides of stressed rates to 70% when received QS additives. Abdel-Reheim *et al.*[35] and Elkaradawy *et al.*[67] explained the decrease in triglycerides owing to the reduction in intestinal cholesterol absorption and the saponins' protective activity. In addition, growth in the β -hydroxy β -methylglutaryl- CoA (HMG- CoA) reductase action and low- density lipoprotein receptor quantities in the liver. Unlike, Sørensen *et al.*[95] found that there were no significant changes in plasma triglycerides in Atlantic salmon fed with soya-saponins. HDL values were seriously affected in the presence of metals. This could be accompanying the energy requires in addition the serum carriage of fatty acids through HDLs [96]. Elbialy *et al.*[36] found that *Yucca schidigera* extract added to stressed-fish diet improved the HDL levels. Deng *et al.*[34] explained the shift in HDL values with steroid saponins by way of their ability for improvement the triglycerides breakdown to fatty acids through activating the PPAR signaling path. This stimulates the LDL and HDL receptors families within the cholesterol metabolic path to mediate cholesterol transport. The findings of the present research could indicate that supplementation with 300 and 150 mg QS/kg fish feed may play a significant role in protection of liver. The improved protection of stressed fish liver could have a health, welfare and profitable impact.

Oxidative stress may induces damaging mitochondrial respiration, increasing reactive oxygen species (ROS) generation, promoting lipid peroxidation (LPO is considered one of the major activities stimulated by oxidative stress), and reducing intracellular antioxidants within target cells [97]. The antioxidant enzyme system is a potential biochemical biomarker in environmental biomonitoring remarkably in response to metal

pollution [74]. According to our findings, Cd and Pb decreased the analyzed SOD and GSH. Antioxidants are potential objectives for Pb. Most antioxidants have thiol groups on their active sites and Pb has a high affinity for these groups. So, Pb can fix producing deactivation of the enzyme [98]. The depletion of SOD and GSH and increase of MDA activities is well-matched with previous studies [99, 100, 101]. Our findings also concord with Rajeshkumar et al.[102] who experimented Cd and Pb impacts on antioxidants of common carp for 30 days. Higher MDA levels could be related to the metal-induced ROS generation which would have stimulate LPO production creating loss of cell membrane fluidity in oxidative stress [22]. Wang et al.[88] explained the significant elevation of MDA levels ($p < 0.05$) after 24 h exposure to 10 μM Cd as Cd induced LPO in the hepatocytes. MDA offers a relative measure of prospective oxidative damage caused by toxins [103]. SOD and CAT are crucial enzymes that counteract the harmful impacts of oxygen metabolism [88] and safeguard the organism's body [104, 105]. Operating in a highly coordinated system, these enzymes play a pivotal role in converting reactive oxygen species into non-toxic oxygen within the liver [102, 106]. SOD transforms superoxide anions (O_2^-) to less toxic components, mainly H_2O_2 and O_2 [107]. Next, CAT catalyzes H_2O_2 to water and molecular oxygen [108, 109]. GSH performances as a free radical scavenger, supports the stimulation of other antioxidants and acts as an electron donor in the peroxides reduction mechanism managed by GPx [110]. It is a non-enzymatic antioxidant, works as a reducing agent with xenobiotic conjugation [82], shield against metal cations [111]. Environmental toxicity can affect these enzymes activities causing oxidative stress [21]. Their combined behavior facilitates elimination of the free radicals, and in the standard physiological conditions, a delicate equilibrium is between these components. but, in cases of excessive free radicals' generation, this balance is interrupted, causing oxidative insult [88]. Effects of different concentrations of QS on antioxidants enzymes activities were found as following: $\text{QS}_{150} > \text{QS}_{300} > \text{QS}_{500}$. QS extract has been reported to contain a combination of antioxidants [112]. Elbially et al., [36] informed that yucca extract has saponins which performance as natural antioxidants. This study is in concord with Elkaradawy et al.[26] who found that QS supplementations to Nile tilapia fish diet enhanced SOD and CAT levels. Deng et al.[34] recorded those groupers fed diets comprising 0.1% steroidal saponins showed significant increase in SOD, CAT and GSH levels and decrease in serum MDA levels. Fleck et al.[29] supposed that the ability of QS to scavenge excessive radicals and

consequently defeat oxidative stress is the motive for depletion of liver MDA production. The depletion of hepatic MDA was owing to releasing of ROS (in hypoxia condition), which improved LPO [113].

Cholinesterase is as one of the select neuro-biochemical indices for the estimation of environmental stress [82] by regulating nerve impulses transmission via cholinergic synapses [114, 115]. It is used previously as a biomarker of neurotoxic injures in aquatic pollution studies [116]. Metal ions are studied as neurotoxic compounds with potential inhibition of ChE enzymes activity [117, 92, 115]. Water pollution induces possible toxicity reducing ChE enzyme described as "anticholinesterase agents", which is a key factor on the neurotransmitter acetylcholine [82]. Chandrasekera et al.[118] recorded decrease of ChE percent in brain and muscle by 24-32% and 33-35% respectively of *Oreochromis niloticus* exposed to Cd^{2+} (1 mg/L). Sabullah et al.[119] found that Cd and Pb reduced ChE activity in *Puntius Javanicus* in vitro to 49.14%, 88.68% respectively. Richetti et al.[120] examined significant decrease in acetylcholinesterase of zebrafish has been exposed to lead acetate. Ullah et al.[74] found that *Tor putitora* collected from metals-polluted river sites has a significant reduction in the muscles and liver acetylcholinesterase activity unlike to fish collected from the reference sites. In the present study, Cd and Pb mixture decreased the activity of ChE to about 37.5%. The inhibition might be associated with metals bioaccumulation [74]. Cogun et al.[79] informed that fish serum ChE activity could be a more liver dysfunction specific indicator. He observed a drop in ChE synthesis beside hepatocyte dysfunction because of mercury exposure afterward synthesis restoration with hepatocyte recovery. QS succeeded in restoration of ChE activity and increased its levels to 90% - 91.56% in presence of metals.

Cytochrome CYP, is supported monooxygenase induction that approximately used as a biomarker of exposure to pollution as polyaromatic hydrocarbons, polychlorinated biphenyls [121,116,122] and pesticides [123]. It is a hepatic protein sensitive of natural detoxification processes [124]. CYP P450 has been employed as a susceptible biomarker for monitoring fish exposure to environmental poisons [116]. It is a chief by-product in oxidative stress cases when the free radicals generation during the organism exposed to pollutants [123]. Cyp19 is found to up-regular activate in cases of tissues injuries [125]. GPx is one of the antioxidative component which has a significant role in reduction of the oxidative stress by degenerating the free radicals, producing high antioxidative and immune

responses [126, 127]. Fish of QS₃₀₀ and QS₁₅₀ groups revealed restoration of *CYP* and *GPx* balance of cell proliferation and apoptosis through up-regulation of *GPx* together with down-regulation of *CYP19* to impede tumor initiation and progression.

Nile tilapia gills are the most important portion exposed to metals which in close contact with the water and only a few micrometers separate the blood from the external medium [128]. In the present study, exposure to metals showed necrosis of gill lamellae and this observation agree with previously documents [129,71,73]. In presence of QS, less damage observed on gills. Elkaradawy et al.[26] recorded decrease of gills necrosis, degeneration, and inflammation of Nile tilapia fingerlings. This may because fish of QS has improvement of hematological indices and feeding efficiency. As liver is the detoxification site in the fish body, exposure to Cd and Pb showed hypertrophy and hyperplasia of bill duct cells, eosinophilic debris. Our results agree with many previous studies [130, 102, 12]. Assimilation of QS on fish exposed to metals showed few congested sinusoids and vasculature and a few inflammatory cell infiltrations of hepatocytes. Enhancing of liver enzymes as MDA, SOD and GSH alleviate the liver damage. Deng et al.[34] observed the ability of steroid saponin diets in enhancing the hybrid groupers liver damage. Our result also in concord with Abdel-Reheim et al.[35] and Abdel-Reheim et al.[131] who recorded the same results in rats. Fish spleen recorded high sensitivity in presence of metals [132, 133, 134]. Higher absorbance of QS in intestine may be the cause of the relatively normal appearance of splenic tissue. Previous studies recorded gliosis and meningitis of fish brain exposed to metals [129]. This may because of Pb is known to bioaccumulate in the brain [135]. No available data about the effect of QS on the histology of the fish brain. Enhancement of the cholinesterase activities mirrored in the histopathological healthy brain of the

fish. In the present study, fish received QS showed normal architecture of molecular layer with low degeneration.

Conclusion

Our study work has shown that QS has diminish effect against Cd and Pb negative impacts on fish. QS concentrations 150, 300 and 500 mg/kg fish feed enhanced growth performance, haematological and biochemical indices of fish reared in aquarium containing heavy metals. 150 mg QS/kg fish feed hindered the oxidative stress. It also achieved, especially 500 mg QS/kg, cell balance of proliferation and apoptosis. Therefore, we can recommend QS as a promising feed additive to improve therapeutic substitutes caused by metals stress.

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Data Availability Statement

All relevant data are available from the authors upon request.

Author contributions

All authors contributed equally to this work (conception, acquisition, samples analysis, statistical analysis, data interpretation, manuscript drafting, and manuscript revision).

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Conflict of interest

The authors declare that they have no conflict of interest.

TABLE 1. Primers used for qRT-PCR analysis.

Gene	Primer	Reference
<i>CYP19</i>	F: CGTCATGTTGCTTCTCATCG R: TACCGCAGGCTCTCGTTAAT	Bois et al.[62]
<i>GPx</i>	F: TCGGACATCAGGAGAACTGC R: GCACTGCTCAAAGTTCCAGG	Agus et al.[63]
<i>B-actin</i> *	F: CACACAGTGCCCATCTACGA R: CCACGCTCTGTCAGGATCTTCA	Agus et al.[63]

CYP19: Cytochrome, *GPx*: glutathione peroxidase, *housekeeping gene.

TABLE 2. Growth performance and feed utilization efficiency of Nile tilapia challenged with heavy metals mixture and treatment with *Quillaja Saponaria*.

Parameters	C-G	HM-mix	QS ₅₀₀	QS ₃₀₀	QS ₁₅₀	p-value
IBW (g)	60.05±1.775	62.25±2.865	63.4±2.272	61.03±2.299	62.18±2.868	0.8947
FBW (g)	107.7±3.07 ^a	89.13±3.935 ^b	109.9±2.161 ^a	104.8±3.202 ^a	105.4±2.56 ^a	0.0028
WG (g)	45.51±1.038 ^a	28.29±0.7188 ^b	49.28±0.575 ^a	46.85±0.8725 ^a	43.5±1.328 ^a	0.0001
WG%	75.79±1.728 ^a	45.6±1.162 ^b	77.72±0.9069 ^a	76.77±1.434 ^a	69.96±2.131 ^a	0.0001
FCR	1.584±0.036 ^a	2.107±0.053 ^b	1.544±0.018 ^a	1.564±0.029 ^a	1.717±0.052 ^a	0.0008
SGR %/day	1.46±0.024 ^a	0.9048±0.054 ^b	1.376±0.049 ^a	1.351±0.052 ^a	1.32±0.036 ^a	0.002
Survival rate	100±0.00	95.84±4.165	100±0.00	95.84±4.165	100±0.00	0.7392

IBW: initial body weight, FBW: final body weight, WG %: weight gain percent, WG: weight gain, FCR: Feed conversion ratio, SGR: Specific growth rate. Note: Means within the same row lack common superscripts are significantly different at $p < 0.05$, data expressed as mean $\pm$ SEM.

TABLE 3. Hematological parameters of Nile tilapia challenged with heavy metals mixture and treatment with *Quillaja Saponaria*

Parameters	C-G	HM-mix	QS ₅₀₀	QS ₃₀₀	QS ₁₅₀	p-value
Hb (g/100l)	11.50±0.394 ^a	8.18±1.420 ^b	10.57±0.731 ^a	8.27±0.4041 ^b	10.50±0.625 ^{ab}	0.0216
HCT (%)	28.57±1.733 ^a	18.73±1.977 ^b	26.03±1.658 ^{ab}	18.53±0.504 ^b	25.57±2.404 ^{ab}	0.007
RBCs (x 10 ⁶ /mm ³)	1.77±0.088 ^a	1.167±0.066 ^b	1.7±0.115 ^a	1.2±0.057 ^b	1.7±0.057 ^a	0.0005
MCH (pg)	60.04±3.055	59.33±2.028	63±1.000	65.67±0.333	61±1.528	0.1329
MCHC (%)	40.67±1.202	40.67±1.041	41.00±0.577	43.33±0.882	44.33±0.882	0.0458
WBCs (x10 ³ /μl)	63.13±9.255 ^a	94.57±2.123 ^b	78.03±5.951 ^{ab}	70.87±2.577 ^{ab}	68.50±5.020 ^a	0.0142
Lymphocytes (×10 ³ /μl)	63.00±6.506	77.67±2.848	73.67±4.177	75.67±4.702	70.33±7.881	0.4143
Neutrophils (×10 ³ /μl)	7.67±2.848	8.67±1.202	10.67±0.333	9.67±4.702	9.33±2.848	0.9344
Monocytes (×10 ³ /μl)	14.00±1.528	10.33±1.856	12.67±1.202	8.33±1.333	14.33±2.906	0.1925
Eosinophils (×10 ³ /μl)	4.00±1.155	3.33±1.333	4.67±0.882	2.67±0.333	4.33±1.453	0.7186

WBCs: white blood cell count, RBCs: Red blood cells count, Hb: hemoglobin content, HCT: hematocrit, MCH: mean corpuscular haemoglobin and MCHC: mean corpuscular haemoglobin concentration. Note: Means within the same row lack common superscripts are significantly different at $p < 0.05$, data expressed as mean $\pm$ SEM.

TABLE 4. Serum biochemical- profile of Nile tilapia challenged with heavy metals mixture and treatment with *Quillaja Saponaria*

Parameters	C-G	HVM-mix	QS ₅₀₀	QS ₃₀₀	QS ₁₅₀	p-value
ALT (U/L)	32.33±4.807 ^a	54.33±1.764 ^b	82.33±6.333 ^c	20.67±3.18 ^a	37.33±3.93 ^{ab}	0.0001
AST (U/L)	59±5.292 ^a	260±29.51 ^b	184±14.47 ^c	22.67±3.712 ^a	43±3.786 ^a	0.0001
Albumin (g/dl)	1.267±0.088	1.02±0.057	1.133±0.088	1.133±0.033	1.0±0.057	0.1782
Total protein (g/dl)	3.367±0.145 ^a	2.533±0.202 ^b	3.367±0.145 ^a	3.333±0.12 ^a	2.233±0.218 ^b	0.0015
Globulin (g/dl)	2.10±0.152 ^a	1.433±0.233 ^b	2.233±0.133 ^{ab}	2.2±0.115 ^{ab}	1.233±0.218 ^c	0.0057
Urea (mg/dl)	21±1.155 ^{acd}	26±0.574 ^b	21.33±1.202 ^{ac}	17.33±0.333 ^d	16.33±0.667 ^d	0.0001
Creatinine (mg/dl)	0.2333±0.218 ^a	0.333±0.088 ^a	0.2±0.03 ^a	0.3±0.054 ^a	0.133±0.033 ^b	0.0208
ALP (mg/dl)	118.3±6.566 ^{ac}	157.3±10.27 ^b	134±9.609 ^{ab}	100±7.155 ^c	96.67±3.383 ^c	0.0007
Triglycerides (mg/dl)	241.7±11.14 ^{ac}	229±22.3 ^{ac}	295.3±3.844 ^a	191±3.055 ^{bc}	156.3±15.92 ^b	0.0003
Total lipid (mg/dl)	513.7±14.34 ^a	589.7±18.35 ^b	580.3±20.63 ^b	307±1.732 ^c	260.7±3.48 ^c	0.0001
HDL (mg/dl)	30.67±0.881 ^a	23±1.155 ^b	20.33±0.889 ^b	32±1.155 ^a	34.33±0.881 ^a	0.0001
Glucose (mg/dl)	31.67±1.453 ^a	35±1.155 ^b	30.33±0.881 ^{ab}	28.33±0.882 ^a	29.33±1.856 ^{ab}	0.0353
Cholesterol (mg/dl)	169±21.03	200.3±6.119	196±8.386	196.7±7.265	176.7±7.881	0.2833
Bilirubin (mg/dl)	0.016±0.006 ^a	0.06±0.005 ^b	0.05±0.005 ^b	0.04±0.005 ^{ab}	0.036±0.008 ^{ab}	0.0104

AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline triphosphatase and HDL: High density lipoprotein Note: Means within the same row lack common superscripts are significantly different at $p < 0.05$, data expressed as mean $\pm$ SEM.

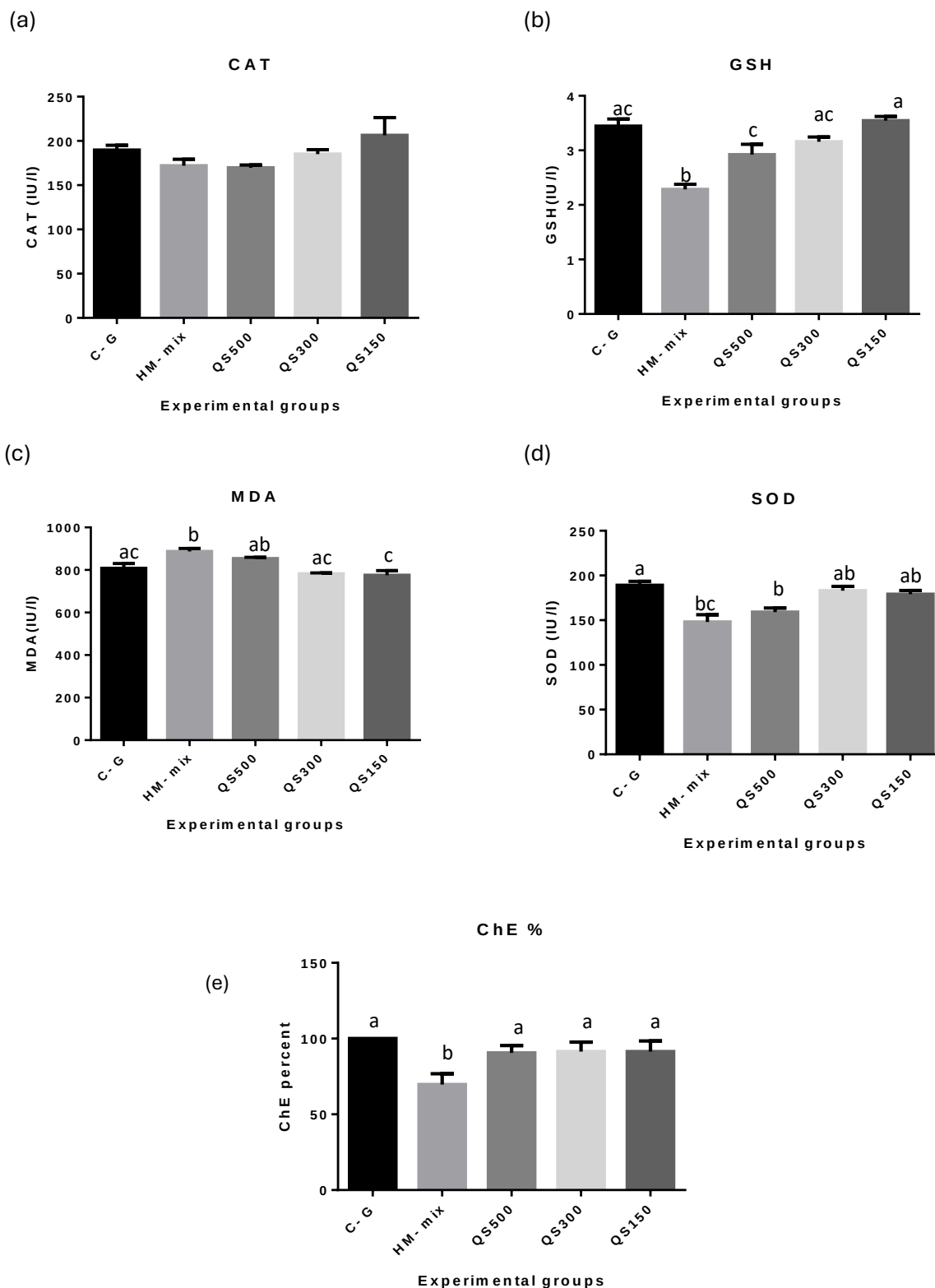


Fig. 1 Oxidative stress assay parameters: (a): CAT (catalase), (b): GSH (glutathione), (c): MDA (malondialdehyde), (d) SOD (superoxide dismutase), (e) ChE % (cholinesterase percent). values are expressed on columns (mean $\pm$ SEM) and different superscripts are significantly different ($p < 0.05$) using one- way ANOVA analysis.

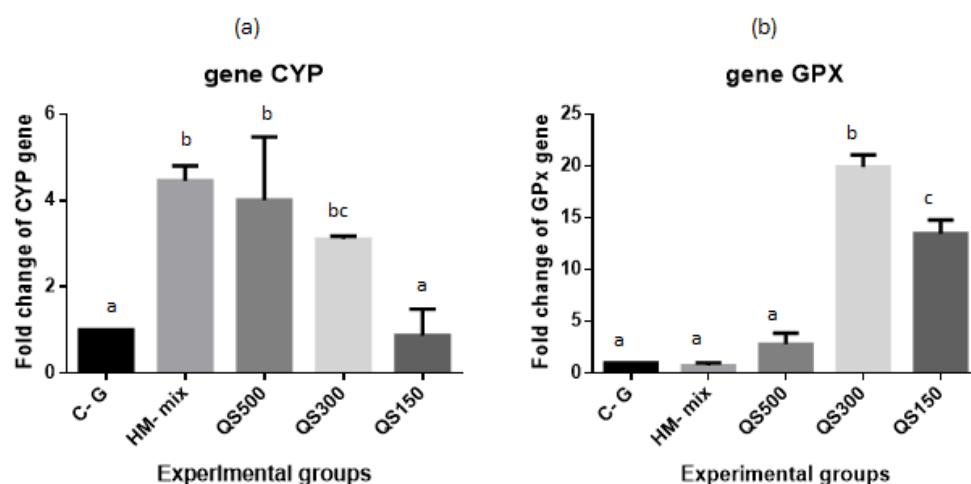


Fig. 2. Relative mRNA expressions of (a) *CYP* (cytochrome) gene and (b) *GPx* (glutathione peroxidase) gene in Nile tilapia liver. Transcriptomic levels are expressed on columns (mean $\pm$ SEM) and different superscripts are significantly different ($p < 0.05$) using one- way ANOVA analysis.

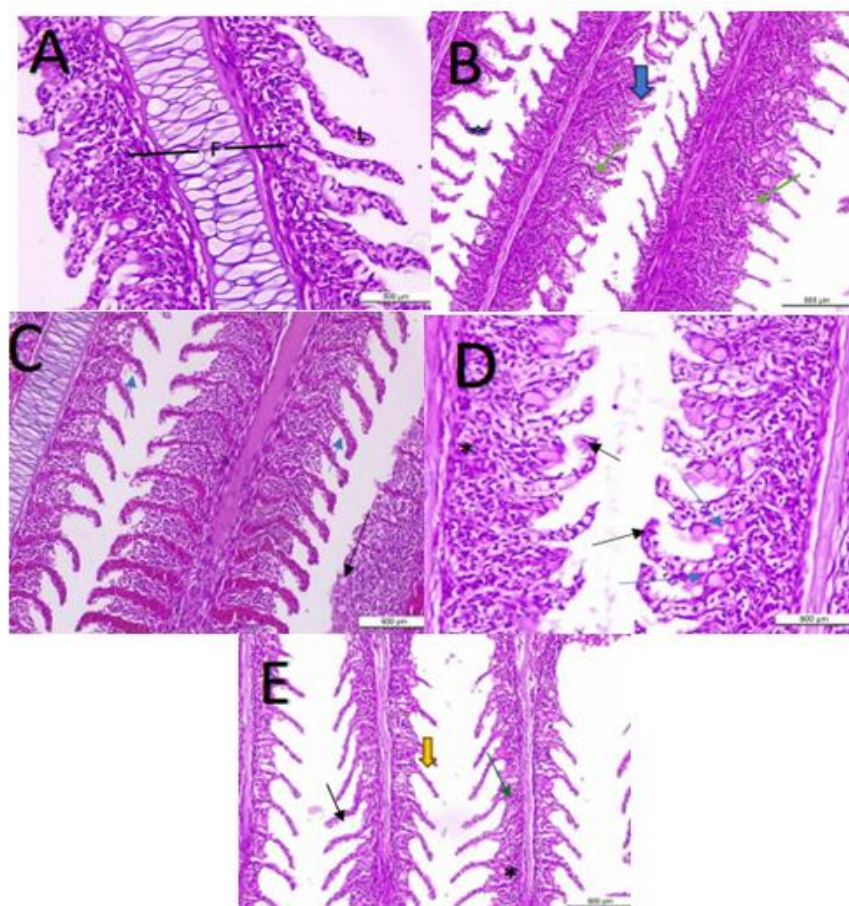


Fig. 3. Photomicrograph of a transverse section of H & E- stained Nile tilapia gill filaments (X 400). (A) Control fish, showing normal appearance of gill filaments (F) and lamellae (L). (B) gills exposed to metals showing hyperplasia leading to shortening secondary lamella with congested blood vessels (blue arrow), hypertrophy of chloride cells and mucus cells (green arrow), curling of secondary lamella (*). (C) Gills treated with 150 mg *Quillaja Saponaria*/ kg fish feed look healthier and there is no adhesion between the filaments (blue arrow) and a few hypertrophies of chloride cells. (D) and (E) Gills treated with 300, and 500 mg *Quillaja Saponaria*/ kg fish feed showed hypertrophies of chloride cells (green arrow), curling of secondary lamella (black arrow), and decreased adhesion of secondary gill lamella (blue arrow) with eosinophilic granular cells (yellow arrow).

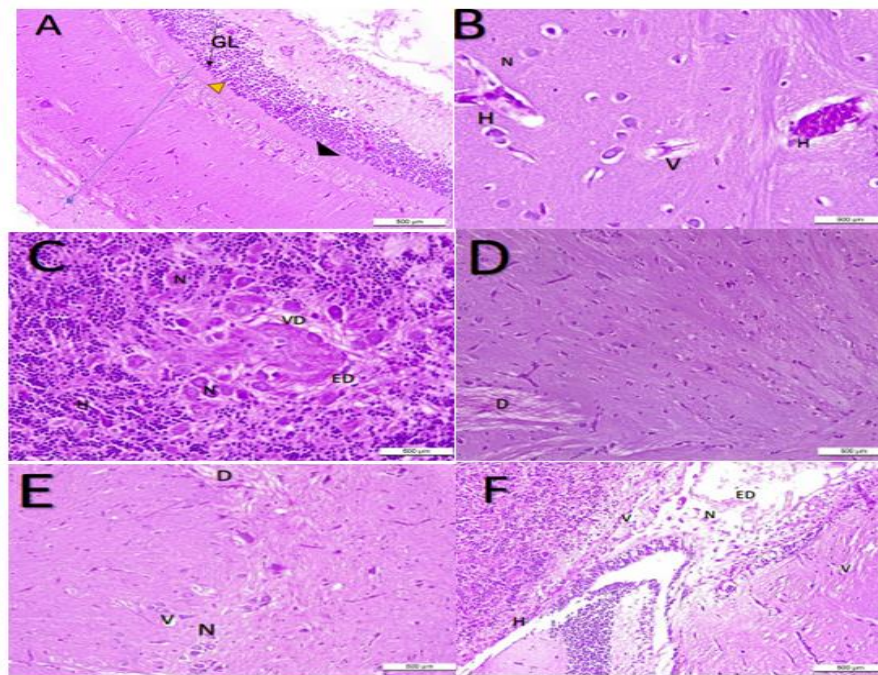


Fig. 4. Photomicrograph of brain section of the Nile tilapia (Stain: H&E; Magnification: $\times 400$). A; control group granular layer (GL), molecular layer (ML), ganglion layer (short arrow), and Purkinje cells (orange arrowheads). (B & C): Fish exposed a mixture of metals showing degenerations (D), vacuolar degeneration (VD), edema (ED), necrosis (N), and hemorrhage (H). fish exposed to 150 μg saponin showed normal architecture of molecular layer with low degeneration. Figure (E) Fish exposed to 300 μg saponin showed vacuolar degeneration (VD), edema (ED), necrosis (N), and hemorrhage (H). Fish treated with saponin 500 μg showed a separation of the granular layer from the molecular layer, focal necrosis, vacuolation in the granular and molecular layers, degeneration of the granular layer, and the emergence of edema in multiple regions of the nervous tissue. There was also bleeding between the components of this tissue.

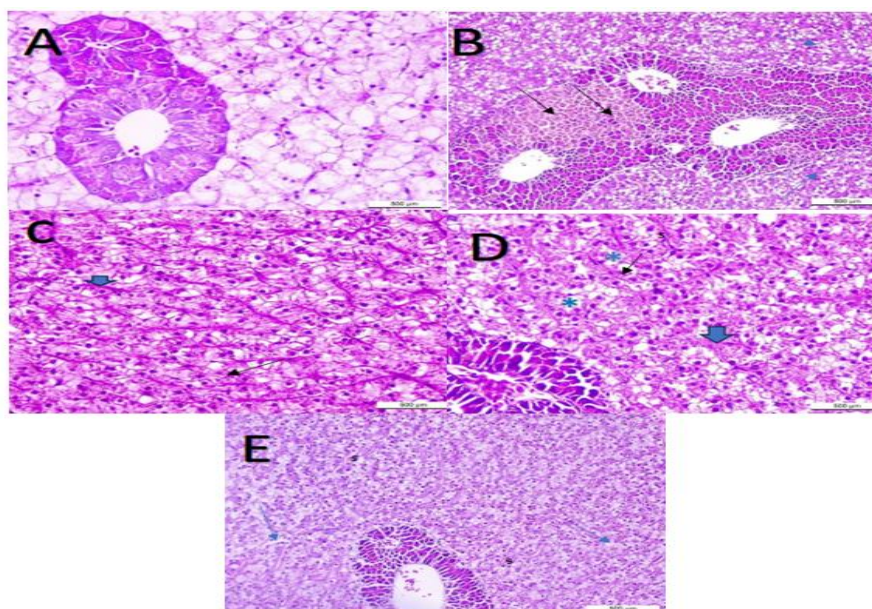


Fig.5. Microscopic examination of H&E-stained Nile tilapia liver sections with representative microphotographs. A: control group; showing normal liver have well-arranged cords of normal hepatocytes, B: liver of fish exposed to metals; showing hypertrophy and hyperplasia of bile duct cells (blue arrow), eosinophilic debris (black arrow). C and D: liver of fish exposed to 500 and 300 mg *Quillaja Saponaria*, respectively; showing Showing a few inflammatory cell infiltrations (blue arrow), vacuolation (*) and few necrosis (black arrow). E: liver of fish exposed to 150 mg *Quillaja Saponaria* showing well-organized architecture of hepatic lobules with few congested sinusoids (s) and vasculature, a few inflammatory cell infiltration, and mild sporadic vacuolar degenerative changes (blue arrow) of hepatocytes

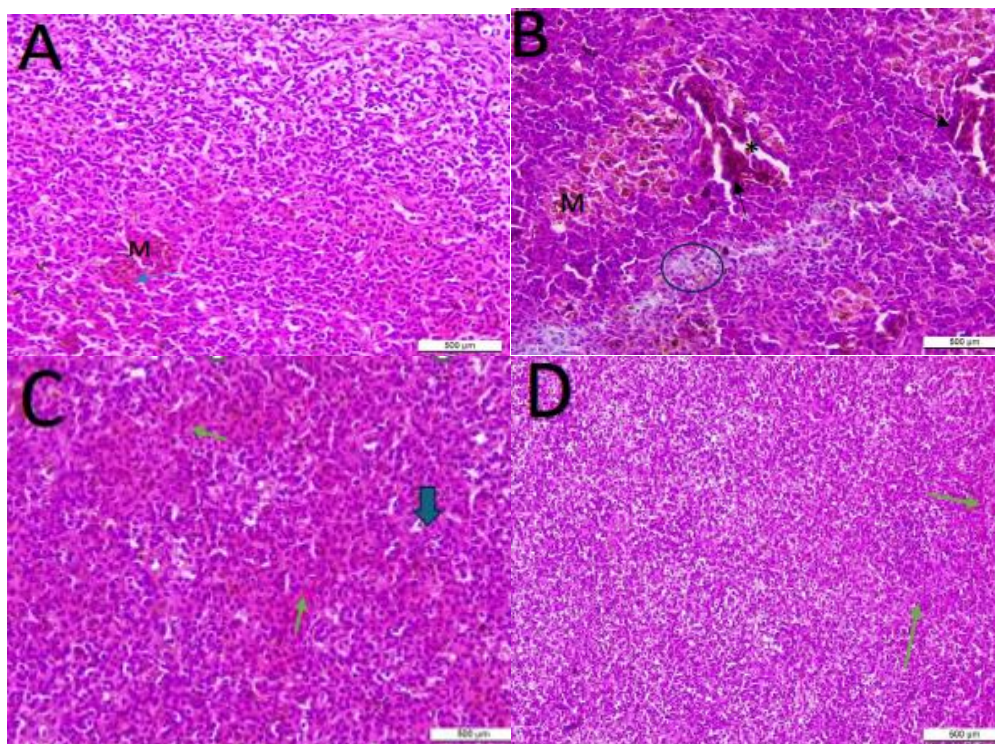


Fig.6. photomicrographs of splenic tissues (H & E stain, scale bar = 500 µm) of Nile tilapia. **A;** The control group; showed normal splenic structures, melanomacrophage aggregation. **B;** fish exposed mixture of metals, splenic tissue showed increasing melanomacrophage aggregation (M), necrosis (*) eosinophilic infiltrate (black arrow) and white pulp (circle). **C and D;** Fish treated with Quillaja Saponaria The splenic tissue has a relatively normal appearance with increased lymphocytic infiltration (green arrow) and hemorrhage (short arrow). There are no significant differences between different concentrations.

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قام *Quillaja saponaria* بتحسين أداء النمو ومكونات الدم ويحمي البلطي النيلي (*Oreochromis niloticus*) من تلف الأنسجة المؤكسدة والالتهابية الناجمة عن الرصاص والكاديوم

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الملخص

واحدة من أهم الوسائل الحديثة في الاستزراع السمكي الاهتمام برعاية الأسماك للحصول على منتج عالي الجودة. هدفت الدراسة الحالية إلى تقدير التأثير المتناقص لنبات *Quillaja saponaria* (QS) على أسماك البلطي النيلي المعرضة للمعادن الثقيلة وتأثيرها على أداء النمو والخصائص الدموية والكيميائية الحيوية والاستجابة التأكسدية وتلف الأنسجة. تم توزيع الأسماك (العدد = ٩٠، متوسط الوزن: ٦١,٧٤ ± ٤,٧٢ جم) بشكل عشوائي في ثلاث مكررات بمعدل ٦ أسماك لكل حوض سمك ٦٠ لتر. تم تعريض الأسماك لخليط الكاديوم (Cd) والرصاص (Pb) بجرعة ١,٣١ ملجم/لتر و ٥٨,٤ ميكروجرام/لتر على التوالي وتم تلقي QS بجرعات ١٥٠، ٣٠٠، ٥٠٠ ملجم/كجم من علف الأسماك. أدى كل من Cd و Pd إلى انخفاض ملحوظ في أداء النمو وتناول العلف كما أدت إلى تدهور مؤشرات الدم والكيمياء الحيوية عن طريق انخفاض نسبة Hb و HTC و RBCs، وزيادة كرات الدم البيضاء، وزيادة مستويات ALT و AST و ALP والبيروكسيد واليورب والدهون الثلاثية والجلوكوز والدهون الكلية وانخفاض البروتين الكلي والجلوبيولين والكوليسترول الجيد. تم تسجيل زيادة ملحوظة في مستويات MDA وانخفاض أنشطة SOD و GSH و ChE في الأسماك المعرضة للمعادن. كشف التحدي مع المعادن عن تأثير منتظم للتعبير الجيني لـ CYP19 في الكبد. عزز *Quillaja saponaria* أداء نمو الأسماك أثناء التعرض للمعادن. استعاد *Quillaja saponaria* مؤشرات الدم والكيمياء الحيوية الطبيعية عند مقارنتها بالمجموعة الضابطة. يعوق استيعاب *Quillaja saponaria* تأثير المعادن على إنزيمات الحالة التأكسدية (انخفاض قيم MDA وزيادة أنشطة SOD و GSH و ChE). حقق *Quillaja saponaria* توازن تكاثر الخلايا وموت الخلايا المبرمج للتعبيرات الجينية CYP19 و GPx. نستنتج من ذلك أن QS قد قلل التأثيرات السلبية للكاديوم والرصاص على الأسماك. عززت تركيزات 150 و 300 و 500 ملجم / كجم من علف الأسماك المحتوى على QS من أداء النمو والمؤشرات البيوكيميائية الدموية للأسماك المعرضة للمعادن الثقيلة. ١٥٠ ملجم QS/كجم علف للأسماك يعوق الإجهاد التأكسدي. كما حقق، وخاصة ٥٠٠ ملجم QS/كجم، توازن الخلايا من التكاثر وموت الخلايا المبرمج.

الكلمات الدالة: سابونين ، البلطي النيلي ، الكاديوم ، الرصاص.