Ziziphus mauritiana supplementation of Nile tilapia (*Oreochromis niloticus*) diet for improvement of immune response to *Aeromonas hydrophila* infection

Amel El Asely · Aziza Amin · Asmaa S. Abd El-Naby ·
Fatma Samir · Ahmed El-Ashram ·
Mahmoud A. O. Dawood

Abstract This study evaluated the effectiveness of dietary *Ziziphus mauritiana* leaf powder (ZLP) to control *Aeromonas hydrophila* infection in Nile tilapia and reduce damage to vital immune organs. Four experimental groups were fed a diet supplemented with ZLP at concentrations of 0, 5, 10, and 20 g/kg (*w/w*) for 6 weeks. At the end of the feeding trial, all groups were intraperitoneally injected with pathogenic *A. hydrophila*. It was found that *Z. mauritiana* significantly ($P < 0.05$) upregulated (lysozyme, interleukin 1 beta) and superoxide dismutase gene expressions as well as improved the activity of serum lysozyme and liver antioxidant enzymes. The fish that were fed a ZLP-supplemented diet also exhibited significantly higher survival rates after *A. hydrophila* challenge than those that were fed a ZLP-free diet ($P < 0.05$). Supplementation of 10 g/kg ZLP most effectively reduced the histopathological alterations caused by *A. hydrophila* challenge in the liver, spleen, kidney, and muscle of the fish. In conclusion, ZLP can be effective in controlling *A. hydrophila* infection in Nile tilapia (particularly at a concentration of 10 g/kg) through enhancement of its immune and antioxidant status.

Keywords *Ziziphus mauritiana* · Nile tilapia · Histopathology · Immune gene expression · Pathogenic challenge · Oxidative stress

A. El Asely
Department of Aquatic Animals Diseases and Management, Faculty of Veterinary Medicine, Benha University, Benha 13736, Egypt

A. Amin
Department of Pathology, Faculty of Veterinary Medicine, Benha University, Benha 13736, Egypt

A. S. Abd El-Naby · F. Samir
Fish Nutrition Department, Central Laboratory for Aquaculture Research, Abassa, Abu Hammad, Sharkia, Tell El Kebir, Egypt

A. El-Ashram
Private Consultant, Cairo, Egypt

M. A. O. Dawood (✉)
Animal Production Department, Faculty of Agriculture, Kafrelsheikh University, Kafrelsheikh 33516, Egypt
e-mail: Mahmouddawood55@gmail.com

Introduction

Aquaculture is an important sector that serves as a valuable and essential protein source for human consumption (Murray and Munro 2018). However, the intensification and extension of this industry have resulted in susceptibility to disease outbreaks (Dawood et al. 2018a). Antimicrobial substances were extensively used in aquaculture as prophylactic measures and metaphylactic treatments (Smith 2012). However, controlling the outbreak of diseases in aquaculture using antimicrobial substances has led to the emergence of drug-resistant pathogens. Recent microbiological and clinical evidence has revealed that antimicrobial resistance genes and bacteria can be transferred from livestock and aquatic animals to humans (Dawood et al. 2018b). Consequently, there is ongoing research on the

effectiveness of alternatives such as antibiotics and other chemotherapeutics. The use of medicinal plants is one of the most promising means for the prevention and/or treatment of such diseases in aquaculture (Awad et al. 2015; Van Hai 2015). Medicinal plants are proven to be cost-effective, biodegradable, and safe options for the control of such diseases and widely used in the aquaculture industry (Abd El-Gawad et al. 2020; Sarhadi et al. 2020). Additionally, they provide longer protection periods than synthetic drugs, which have shorter recovery rates (Yostawonkul et al. 2019). It is well-documented that many types of medicinal plants have antioxidant properties that can delay or stop oxidative damage and thereby play an important role in disease prevention (Awad et al. 2019).

The leaves of *Ziziphus mauritiana* (common names: Ber, Chinese apple, and Indian jujube) have long been considered to have health-promoting effects (Abdallah et al. 2016; Abdulameer et al. 2017). Its leaves contain many bioactive compounds such as polyphenols, nitrogenous compounds, vitamins, inorganic elements, carbohydrates, lipids, proteins, free sugars, and mucilage (Dkhil et al. 2018). Previous studies have demonstrated the beneficial impacts of *Ziziphus*-supplemented diets for the treatment of digestive disorders, fever, diabetes, diarrhea, insomnia, and bronchitis in both humans and animals (AL-Marzooq 2014; Preeti and Shalini 2014). In the aquaculture sector, *Z. mauritiana* leave powder (ZLP) has effectively increased the growth of Nile tilapia fingerlings (Amin et al. 2019). However, limited information is available on the immune stimulatory effects of *Z. mauritiana*. To the best of the authors' knowledge, only one relevant study has reported about the positive effects of *Ziziphus* on common carp (*Cyprinus carpio*) immunity (Hoseinifar et al. 2018). Generally, its immune-modulatory perspective remains unclear.

Nile tilapia remains one of the most commonly cultured fish species worldwide due to their easy breeding, tolerance to varied environments and diseases, fast growth, and high market demand (Dawood et al. 2019a, b). However, tilapia farms face challenges due to the infections of *Streptococcus* spp., *Vibrio* spp., *Aeromonas hydrophila*, and *Flavobacterium* spp. *A. hydrophila*, which threaten the health of fish by causing Motile *Aeromonas* Septicemia, a serious threat to cultured freshwater fishes including tilapia (Cipriano et al. 1984). *A. hydrophila* has developed into the most damaging impediment to the expansion of the tilapia industry worldwide.

Accordingly, the present study was conducted to assess the effectiveness of diet supplementation with different concentrations of *Z. mauritiana* leaf powder in reducing the deleterious effects of *A. hydrophila* on vital immune organs, as well as protective and immune-modulatory effects in Nile tilapia.

Materials and methods

Ethical statement

The experimental design was approved by the Central Laboratory for Aquaculture Research (CLAR), Abassa, Egypt (Approval no. 43429), and all procedures followed the guidelines for the care and use of fish.

Fish and experimental diet

A total of 260 healthy Nile tilapia (*Oreochromis niloticus*) with a mean weight of 9.63 ± 0.05 g were obtained from the Central Laboratory for Aquaculture Research (CLAR) hatchery, Abassa, Abu-Hammad, Sharkia, Egypt. The fish were acclimated to the lab conditions in 1000-L fiberglass tanks for 14 days. Next, 240 fish were randomly distributed in 12 glass aquaria ($75 \times 40 \times 50$ cm^3) filled with dechlorinated water (120 L/aquarium) (4 groups, triplicates, 20 fish/aquarium). The remaining 20 fish were stocked in a separate aquarium and fed the control diet (negative control group) for the pathogenic challenge experiment.

The experimental diet (30% crude protein and 8.5% lipid) (Table 1) was prepared to add *Z. mauritiana*. The plant was collected from El Arish, North Sinai, Egypt, and identified by plant taxonomists of the Faculty of Environmental Agricultural Sciences, Suez University. Finely ground ZLP was added to the experimental diet at concentrations of 0 (control), 5, 10, and 20 g/kg diet (G1, G2, G3, and G4, respectively). The experimental diet was prepared following the methods adopted at the Fish Nutrition Laboratory, CLAR. The nutritional value of the experimental diet was analyzed following standard methods (AOAC 1990).

Fish were fed for 6 weeks at a rate of 5% of their body weight per day. Water temperature was adjusted to 28 °C ± 2 °C using heaters. Oxygen and total ammonia levels were measured daily and maintained at acceptable levels, i.e., 6.25 ± 0.53 and 0.02 mg/l, respectively (pH ~ 7.5). The

Table 1 Ingredients and chemical analysis of the experimental diets (on dry matter basis) containing different concentrations of *Ziziphus mauritiana*

Ingredients	*Zizyphus mauritiana* levels (g/kg diet)			
	0.0	5	10	20
Fish meal (Cp 72%)	104.0	104.0	104.0	104.0
Soybean meal (Cp 44%)	429.8	429.8	429.8	429.8
Ground corn	203.2	203.2	203.2	203.2
Wheat bran	154.9	154.9	154.9	154.9
Cod fish oil	23.1	23.1	23.1	23.1
Corn oil	15.0	15.0	15.0	15.0
Vitamins premix[a]	10.0	10.0	10.0	10.0
Minerals premix[b]	20.0	20.0	20.0	20.0
Starch	40.0	35.0	30.0	20.0
Zizyphus mauritiana dry leaves powder	0.0	5.0	10.0	20.0
Total	1000	1000	1000	1000
Chemical analysis (g/kg)				
Dry matter	910.1	912.9	915.9	917.2
Crude protein	301.4	302.1	303.5	302.8
Ether extract	85.5	84.9	84.8	8 3.9
Total ash	86.5	88.6	86.6	87.6
Crude fiber	51.0	50.0	50.0	49.0
Nitrogen free extract	475.6	474.4	475.1	476.7
Gross energy (kcal/100 g)	4441.6	4454.9	4464.8	4458.9

[a] Vitamin premix (per kg of premix): thiamine, 2.5 g; riboflavin, 2.5 g; pyridoxine, 2.0 g; inositol, 100.0 g; biotin, 0.3 g; pantothenic acid, 100.0 g; folic acid, 0.75 g; para-aminobenzoic acid, 2.5 g; choline, 200.0 g; nicotinic acid, 10.0 g; cyanocobalamine, 0.005 g; a-tocopherol acetate, 20.1 g; menadione, 2.0 g; retinol palmitate, 100,000 IU; cholecalciferol, 500,000 IU

[b] Mineral premix (g/kg of premix): $CaHPO_4 \cdot 2H_2O$, 727.2; $MgCO_4 \cdot 7H_2O$, 127.5; KCl 50.0; NaCl, 60.0; $FeC_6H_5O_7 \cdot 3H_2O$, 25.0; $ZnCO_3$, 5.5; $MnCl_2 \cdot 4H_2O$, 2.5; $Cu(OAc)2 \cdot H_2O$, 0.785; $CoCl_3 \cdot 6H_2O$, 0.477; $CaIO_3 \cdot 6H_2O$, 0.295; $CrCl_3 \cdot 6H_2O$, 0.128; $AlCl_3 \cdot 6H_2O$, 0.54; Na_2SeO_3, 0.03

fish were exposed to 12-h light/dark cycles using artificial lighting. Daily partial water exchange was performed to remove fecal matter and excess feed. Special care was taken during this exchange to avoid stressful consequences on the fish.

Pathogenic challenge

A. hydrophila (verified virulent strain kindly provided by the Department of Aquatic Animals Diseases and Management, Faculty of Veterinary Medicine, Benha University) was grown in tryptic soy broth at 28 °C for 18 h. Bacterial cells were collected by centrifugation at 2550*g* for 20 min at 4 °C and harvested in sterile physiological saline. Bacterial concentration was adjusted to 9×10^8 cells/ml following the method reported by El Asely et al. (2014). Ten fish from each replicate (30

fish/group) were transferred to adjusted aquaria and maintained at 26 °C with 6 mg/l dissolved oxygen. The day before infection, fish were intraperitoneally (IP) injected with 0.2 ml of the bacterial suspension. The negative control group was IP injected with 0.2 ml of 0.9% saline. Fish were observed daily for any clinical signs or mortalities, and the number of dead fish was recorded for 10 successive days. Daily relative percentage of survival was calculated using the Kaplan–Meier equation.
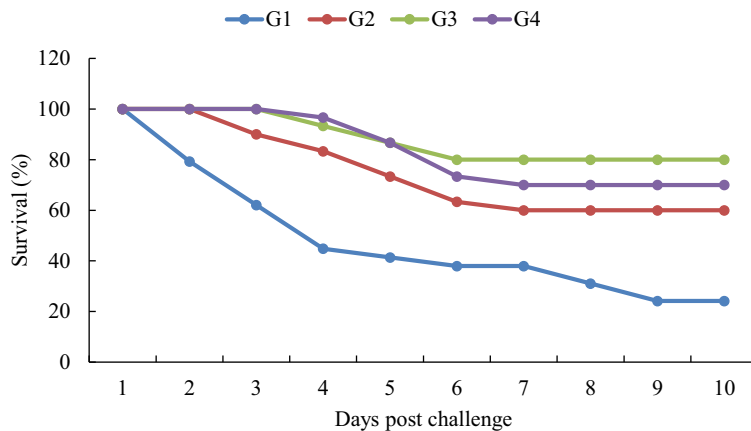
Sampling

Fish were sedated with 150 mg/l MS222 (Argent Laboratories, Redmond, Washington) following the procedure reported by Barreto et al. (2007), and whole-blood samples were collected from the

Gene	Primer	Sequence (5′-3′)	Annealing Temp.
β-actin	Forward	TGGCATCACACCTT CTATAACGA	60 °C
	Reverse	TGGCAGGAGTGTTG AAGGTCT	
EF-1α	Forward	CCTTCAACGCTCAG GTCATC	62 °C
	Reverse	TGTGGGCAGTGTGG CAATC	
Lysozyme	Forward	ACACCATGGCCAAA AACACC	62 °C
	Reverse	AAAACGCACTGCAG TCCATG	
SOD	Forward	GACGTGACAACACA GGTTGC	63 °C
	Reverse	TACAGCCACCGTAA CAGCAG	
IL1β	Forward	TGCACTGTCACTGA CAGCCAA	65 °C
	Reverse	ATGTTCAGGTGCAC TATGCGG	

caudal vessels ($n = 5$ fish/aquarium) using a syringe without anticoagulant for serum separation. Clotted blood was centrifuged (3000 rpm, 15 min, 4 °C) and stored at − 20 °C for measurement of serum lysozyme activity. After bleeding, liver and spleen tissues were immediately collected, stored in RNAlater (Bioshop, Germany), and preserved at − 80 °C until RNA extraction and gene expression analysis. From the liver, another 50 mg of samples was excised ($n = 5$ fish/aquarium) and kept in cold phosphate-buffered saline to measure alkaline phosphatase (ALP) and antioxidant enzyme activity and stored at − 20 °C until determination.

Immune gene expression

Liver and spleen tissues (50–100 mg) were used for RNA isolation using Trizol Reagent (Invitrogen, USA) following the manufacturer's instructions. The integrity and quantity of total RNA were determined by passing samples through formamide gel and using a spectrophotometer, respectively. The primer sequences of lysozyme, superoxide dismutase (SOD), and interleukin 1β (IL-1β) genes were specifically designed using Primer3 (Table 2). cDNA synthesis was conducted using a first-strand cDNA synthesis kit (Roche Diagnostic GmbH, Germany) in a thermal cycler (Prime Pro48, Techne, UK) under the following parameters: reverse transcription (RT) at 45 °C for 45 min and denaturation at 94 °C for 10 min. Each round of quantitative RT polymerase chain reaction (qRT-PCR) was performed in duplicate. Two housekeeping genes, β-actin and elongation factor 1 alpha, obtained using the primer designed by Gröner et al. (2015). Relative mRNA expressions were calculated using the $2^{-\Delta\Delta Ct}$ method to determine fold difference.

Serum lysozyme activity

Serum lysozyme activity was determined following the protocol reported by Milla et al. (2010) using *Micrococcus lysodeikticus* (Sigma Chemical Co, USA). Absorbance was determined using a spectrophotometer at a wavelength of 450 nm after 0.5 and 4.5 min.

Fig. 1 The survival rate of Nile tilapia after *Aeromonas hydrophila* infection, fish were fed with the control 0 g (G1), 5 g (G2), 10 g (G3), and 20 g (G4) of ZLP per kg diet for 6 weeks
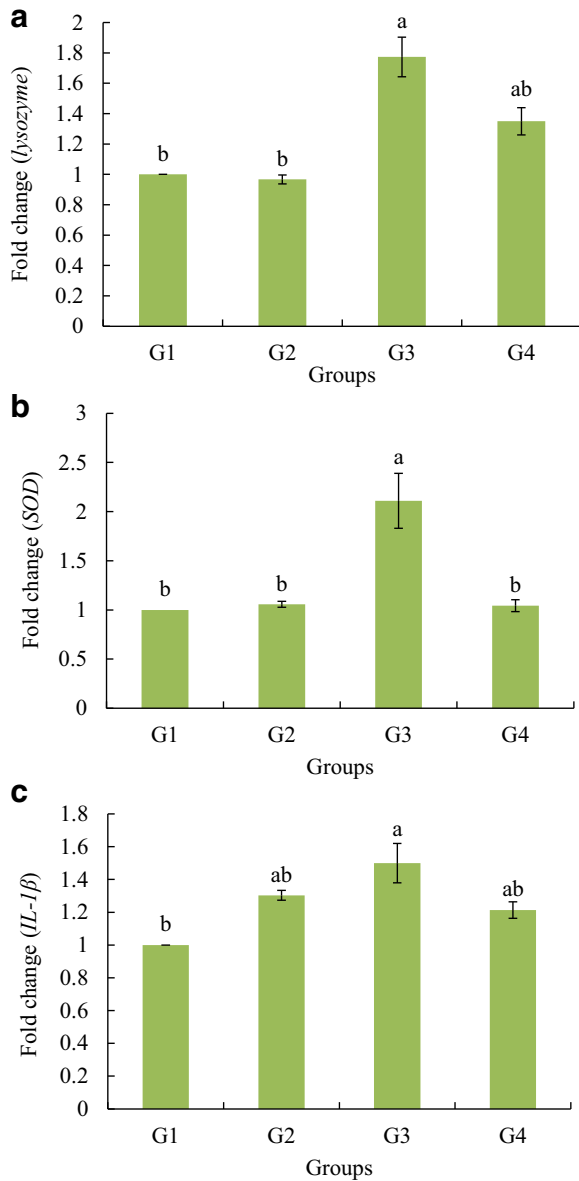
Fig. 2 Immune-related genes **a** *lysozyme*, **b** *IL-1β*, and **c** antioxidant related gene *SOD* expression of Nile tilapia fed with control 0 g (G1), 5 g (G2), 10 g (G3), and 20 g (G4) of ZLP per kg diet for 6 weeks and challenged with *A. hydrophila*. Data are expressed as mean ± SE, bars with different letters indicate significance at $P < 0.05$

ALP and antioxidant enzyme activity

Liver tissue homogenates were centrifuged at 4000 rpm for 15 min at 4 °C. Supernatants were used for the determination of ALP activity using commercial kits (Abcam, UK) at a wavelength of 405 nm. Total SOD and reduced glutathione (GSH) levels were determined using commercially available kits (Biodiagnostic Lab, Egypt) at wavelengths of 560 and 405 nm, respectively, according to the manufacturer's instructions.

Histopathological analysis

Liver, kidney, spleen, and muscle samples from all sacrificed fish in all groups (challenged and unchallenged fish) were excised and fixed in 10% neutral buffered formalin. All histopathological procedures were performed following the methods described by Bancroft et al. (1996). Hematoxylin–eosin-stained sections were examined under a light microscope for the evaluation of pathological changes. Severity of microscopic lesions was classified according to the classification reported by Bernet et al. (1999): degree 1, mild pathological changes such as circulatory disturbance, congestion, hemorrhage, and edema)with mild degenerative changes); degree 2, moderate pathological alterations including degenerative changes, in association with cell deposits with mild inflammatory cell infiltration; and degree 3, severe alterations such as necrotic areas with leukocyte aggregation.

Statistical analysis

All data were subjected to one-way ANOVA. Difference in means was tested at a 5% probability level using Tukey's post hoc test. All statistical analyses were conducted using SPSS V.16 (SPSS, Richmond, USA).

Results

Experimental infection

No mortalities were recorded in the negative control group or unchallenged ZLP-fed groups (data not shown). After IP injection of *A. hydrophila*, fish mortality began at post-infection 2 days in G1 and at post-infection 3 days in G2–G4 (Fig. 1). The cumulative survival rate at post-infection 10 days was the lowest in G1 (24.14%), whereas it was the highest (80%) in G3.

Fig. 3 Serum lysozyme activity of Nile tilapia fed with control 0 g (G1), 5 g (G2), 10 g (G3), and 20 g (G4) of ZLP per kg diet for 6 weeks and challenged with *A. hydrophila*. Data are expressed as mean ± SE, bars with different letters indicate significance at $P < 0.05$
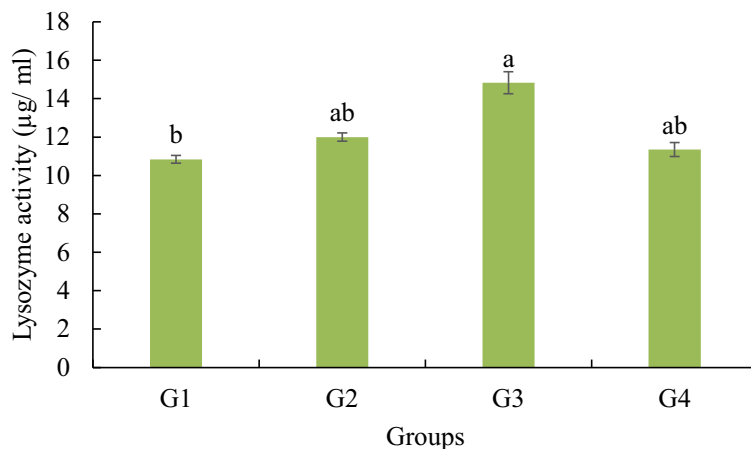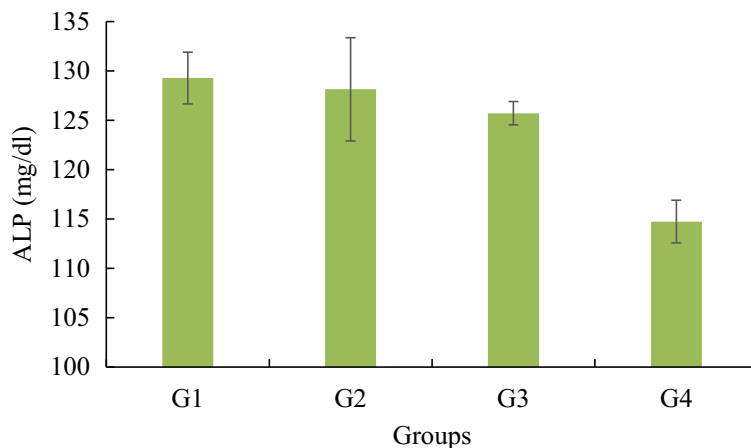
Immune gene and *SOD* expressions

Compared with G1, G3 showed significantly higher upregulation ($P < 0.05$) of lysozyme gene expression in the liver, followed by G4 (Fig. 2a). Similarly, *Z. mauritiana* supplementation significantly ($P < 0.05$) increased *IL-1β* expression in fish, with the highest values observed in G3 (Fig. 2b). In the same respect, *SOD* expression was significantly upregulated ($P < 0.05$) in G3, followed by in G2 and G4 compared with in G1 (Fig. 2c).

Serum lysozyme activity

Feeding of Nile tilapia fingerlings with ZLP-supplemented diet significantly ($P < 0.05$) increased serum lysozyme activity in all groups, with higher activity in G3 than in G1 (Fig. 3).

ALP and antioxidant activity

No significant effects of dietary ZLP supplementation ($P > 0.05$) were observed on ALP activity in the liver in G2–G4 compared with in G1 (Fig. 4).

As shown in Fig. 5a, GSH level significantly increased ($P < 0.05$) in G3 compared with that in G1. There was a significant increase ($P < 0.05$) in SOD activity in G2–G4 compared with that in G1 (Fig. 5b).

Histopathological findings

Histopathological examination of the liver, kidney, spleen, and muscle indicated normal morphological characteristics in all groups (data not shown). The main characteristic lesions in the liver samples obtained from G1 revealed dilatation with congestion of hepatic sinusoids and hepatic portal
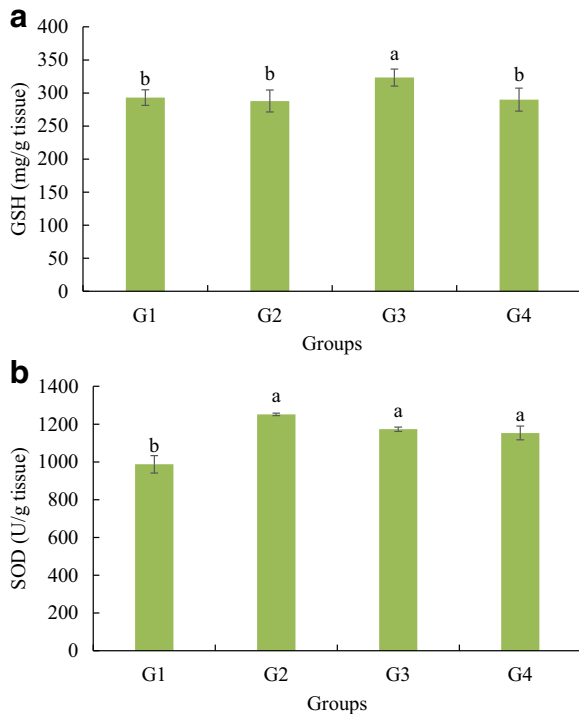

Fig. 4 Alkaline phosphatase activity (ALP) of Nile tilapia fed with control 0 g (G1), 5 g (G2), 10 g (G3), and 20 g (G4) of ZLP per kg diet for 6 weeks and challenged with *A. hydrophila*. Data are expressed as mean ± SE, bars with different letters indicate significance at $P < 0.05$

Fig. 5 **a** Glutathione (GSH) and **b** total superoxide dismutase (SOD) of Nile tilapia fed with control 0 g (G1), 5 g (G2), 10 g (G3), and 20 g (G4) of ZLP per kg diet for 6 weeks and challenged with *A. hydrophila*. Data are expressed as mean ± SE, bars with different letters indicate significance at $P < 0.05$

vessels. Perivascular infiltration of mononuclear leukocytes, mainly lymphocytes and macrophages, and diffuse hemorrhage admixed with mononuclear inflammatory cells were observed in the hepatic parenchyma. Further, scattered degenerative changes were seen in hepatocytes as extensive hydropic degeneration as well as hepatic steatosis characterized by clear cytoplasmic vacuolation of hepatocytes with pushing of their nucleus to the periphery (Fig. 6a). Additionally, focal areas of coagulative necrotic hepatic cells with pyknotic nuclei associated with the loss of structural integrity were seen. Distributed areas of lytic necrosis characterized by replacement of hepatocytes by homogenous eosinophilic substances infiltrated by inflammatory cells (Fig. 6b) and eosinophilic necrotic debris admixed with mononuclear inflammatory cells were also observed (Fig. 6c). Moreover, extensive necrosis of pancreatic acinar cells with peri-pancreatic hemorrhage that replaced the hepatic parenchyma was detected (Fig. 6d). Vacuolation

of most pancreatic acinar cells with peri-pancreatic infiltration of mononuclear leukocytes was also observed. Generally, the mean lesion score was degree 3 in the challenged fish.

Severity of the mentioned histopathological changes that were recorded for liver samples in G1 was alleviated to varying degrees in ZLP-fed groups. Mild improvement in altered liver tissues was demonstrated in G2, with lesion score varying between degree 3 in two fish and degree 2 in the remaining fish. The main pathological changes detected in this group were marked dilatation with congestion of hepatic sinusoids and hepatic portal vessels, degenerative changes such as mild cytoplasmic vacuolation of hepatocytes, vacuolation and necrosis of pancreatic acinar cells with deposition of hemosiderin pigment in some pancreatic acinar cells (Fig. 6e), as well as small focal areas of coagulative necrosis in the hepatic parenchyma characterized by hypereosinophilic cytoplasm with pyknotic nucleus (Fig. 6f).

In contrast, marked improvement in the hepatocellular architecture with more regular and less altered hepatocytes was noticed in liver tissues obtained from G3 compared with in G1. The mean lesion score of this group was degree 1, with the most pronounced pathological alterations being only mild congestion of hepatic sinusoids and hepatic portal vessels with mild degenerative changes in hepatocellular architecture in some examined fish (Fig. 6g).

The lesion score of G4 was degree 2 based on congestion of hepatic sinusoids and hepatic portal vessels, degenerative changes in the wall of hepatic blood vessels with mild perivascular infiltration of mononuclear leukocytes including lymphocytes and macrophages, as well as mononuclear leukocyte infiltration (Fig. 6h) with degeneration of pancreatic acinar cells.

Microscopic examination of renal samples obtained from G1 fish challenged with *A. hydrophila* showed extensive congestion of renal blood vessels and perivascular edema admixed with erythrocytes and few leukocytes (Fig. 7a); this was associated with clear interstitial hemorrhage with degeneration of renal tubules (Fig. 7b). Additionally, hemosiderin deposits were observed in the lumen of degenerated renal tubules. Further, extensive cytoplasmic vacuolation and necrotic pyknosis of the nuclei of the renal tubular epithelial cells as
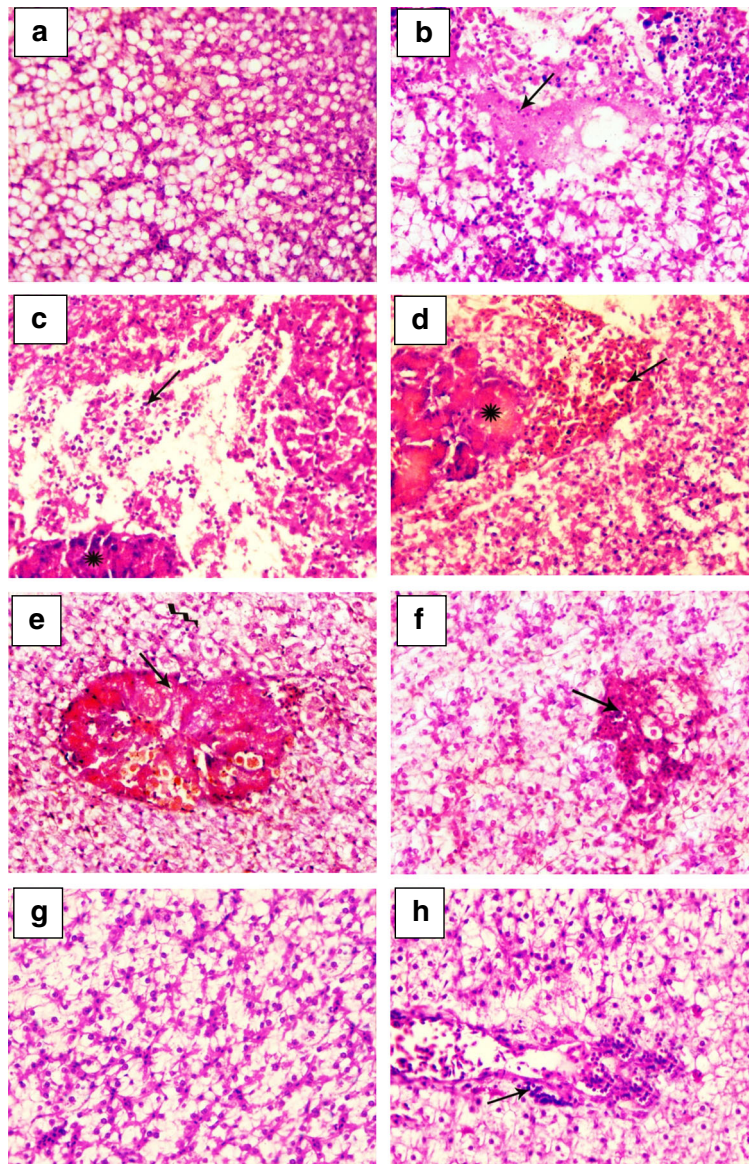
Fig. 6 H&E stained sections of liver (**a**–**d**) of Nile tilapia infected with *A. hydrophila*, **e**–**g** fish infected with *A. hydrophila* and treated with different concentrations of ZLP, G2 (**e**, **f**), G3 (**g**), and G4 (**h**). **a** Clear vacuolation of most hepatocytes with squeezing of the nucleus to the periphery (arrow, × 200), **b** scattered areas of lytic necrosis characterized by disappearance of hepatocytes and replaced by homogenous eosinophilic substances infiltrated by inflammatory cells (arrow, × 200), **c** diffuse areas of lytic necrosis characterized by disappearance of hepatocytes and replaced by eosinophilic necrotic debris admixed with mononuclear inflammatory cells (arrow) with necrosis of pancreatic acinar cells

as well as necrosis of pancreatic acinar cells (asterisk, × 200), **d** extensive necrosis of pancreatic acinar cell (asterisk) with peri-pancreatic hemorrhage that replace to the hepatic parenchyma (arrow, × 200), **e** vacuolation and necrosis of pancreatic acinar cells with deposition of hemosiderin pigment in some pancreatic acini (arrow), notice also degenerative changes in hepatocytes (zigzag arrow, × 200), **f** focal area of coagulative necrosis in hepatic parenchyma (arrow, × 200), **g** mild degenerative changes in hepatocytes (× 200), **h** degenerative changes in the wall of hepatic blood vessels with few perivascular mononuclear leukocytic cellular infiltrations (arrow, × 200)

well as shrinkage and hyalinization of the glomerular tuft were seen (Fig. 7c). Additionally, eosinophilic hyaline casts were detected in the lumen of

some renal tubules. Multifocally, small areas of lytic necrosis that were characterized by replacement of some renal tubules by mononuclear

Fig. 7 Light micrograph of kidney (**a**–**d**) of Nile tilapia infected with *A. hydrophila*, **e**–**g** fish infected with *A. hydrophila* and treated with different concentratins of ZLP, G2 (**e**), G3 (**f**), and G4 (**g**), showing **a**, marked congestion of renal blood vessels and perivascular edema admixed with erythrocytes and few leukocytes (arrow, × 200). **b** Clear interstitial hemorrhage (H) with disconnection of renal tubules (×200). **c** Severe degenerative (arrow) and necrotic (zigzag arrow) changes of the lining epithelium of renal tubules with necrosis (N) and hyalinization (H) of the glomerular tuft (× 200). **d** Small areas of lytic necrosis of the renal tissues with infiltration of mononuclear cells (arrow, × 200). **e** Vacuolar degeneration of the renal tubular epithelium with necrosis and shrinkage of the glomerular tuft (× 200). **f** Mild vacuolar degeneration in lining epithelium of renal (× 200). **g** Vacuolation (arrow) of the lining epithelium of renal tubules with eosinophilic hyaline casts in their lumen (zigzag arrow, × 200) (H&E stain)
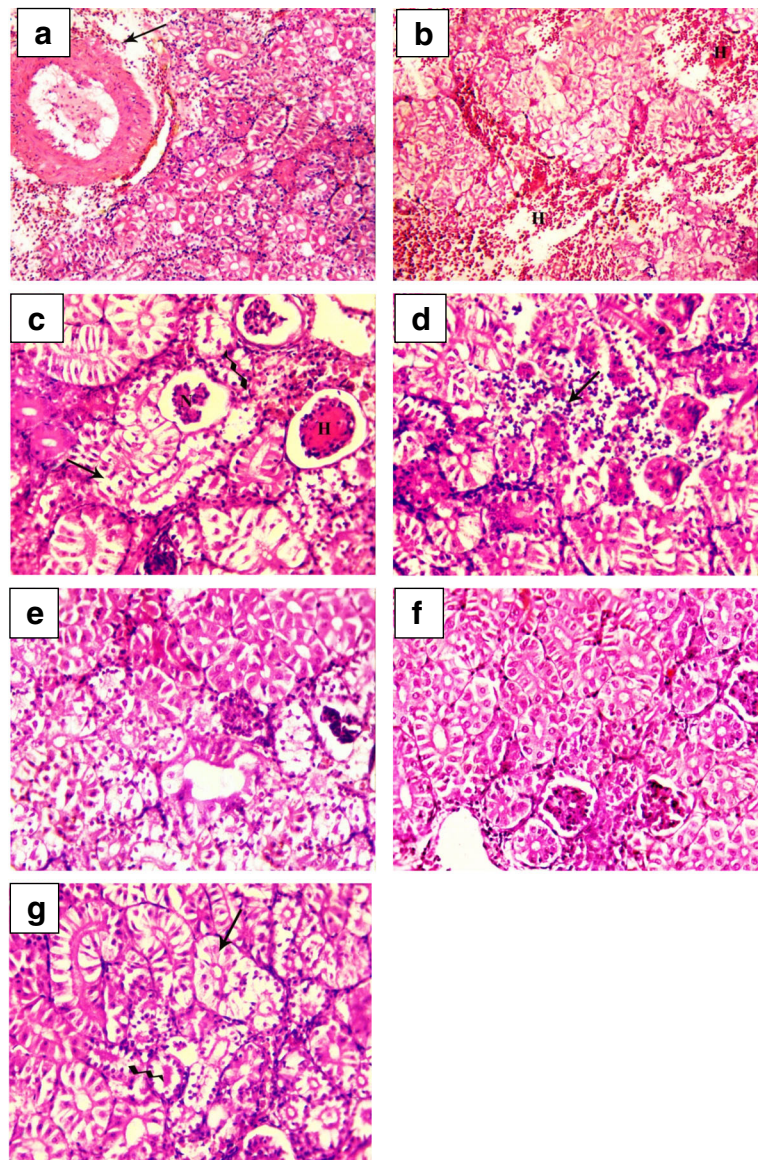
leukocytes were also noticed (Fig. 7d). Collectively, a lesion score of degree 3 was observed in most challenged G1 fish.

The severity of the pathological alterations induced by *A. hydrophila* reduced to a certain extent in ZLP-fed fish. However, most lesions detected in G2 were of degrees 2 and 3 based on congestion of renal blood vessels, mild perivascular hemorrhage, vacuolar degeneration, necrosis of renal tubular epithelial cells, as well as necrosis and shrinkage of the glomerular tuft (Fig. 7e) as the most common pathological changes observed during microscopic examination.

Overall, renal samples obtained from G3 displayed distinct histological improvement compared with those obtained from G1. Mild congestion of renal blood vessels and mild vacuolar degeneration of renal tubular epithelial cells (degree 1) were observed (Fig. 7f). Vacuolation of renal tubular epithelial cells with eosinophilic hyaline casts in their lumen (degree 2) was recorded in G4 (Fig. 7g).

Spleen samples of *A. hydrophila*-challenged fish showed severe pathological changes of degree 3 based on marked congestion of splenic blood vessels with diffuse splenic hemorrhage (Fig. 8a) and reduced

lymphocyte population, resulting in severe lymphoid depletion with eosinophilic mesh free of lymphocytes (Fig. 8b). Moreover, the accumulation of hemosiderin with reduced number of melanomacrophage centers (MMCs) was demonstrated (Fig. 8c), where the density of pigmented granules and cell density in MMCs reduced. Generally, mild improvement in the splenic architecture was demonstrated (degree 2) because congestion of splenic blood vessels, mild hemorrhage in the red pulp (Fig. 8d) with mild lymphoid depletion, and mild reduction in the number of MMCs in some examined fish were observed. Interestingly, most detected lesions in the splenic tissues of G3 were of degree 0 or 1 because considerable increase was observed in the lymphocyte population with well-developed MMCs but, to a certain extent, mild reduction was observed in some examined fish (Fig. 8e). Meanwhile, there was an increase in the lymphocyte population and mild hemorrhage in the red pulp with mild activation of MMCs in challenged G4 fish (Fig. 8f).

Extensive intermuscular edema admixed with mononuclear leukocyte infiltration, mainly of lymphocytes and macrophages, was the most common pathological alterations in the muscular tissues obtained from G1 (Fig. 9a). Hyalinization of muscle fiber was observed based on muscle sarcoplasm appearing more eosinophilic with pyknotic nuclei (Fig. 9a). Additionally, extensive myomalacia with complete vacuolation of muscle sarcoplasm was detected (Fig. 9b).

The most common pathological alterations in muscle samples obtained from G2 were mild intermuscular hemorrhage and degenerative changes in muscle sarcoplasm (Fig. 9c). Its muscle fiber appeared similar to that in the negative control group, except mild degenerative changes in muscle sarcoplasm (Fig. 9d). However, small focal areas of intermuscular edema admixed with leukocyte infiltration, mainly of lymphocytes and macrophages, were observed in muscle samples obtained from G4 (Fig. 9e).

Fig. 8 H&E-stained sections of spleen (**a**–**c**) of Nile tilapia infected with *A. hydrophila*. **d**–**f** Fish infected with *A. hydrophila* and treated with different concentrations of ZLP, G2 (**d**), G3 (**e**), and G4 (**f**), showing **a**, diffuse splenic hemorrhage (× 200). **b** Severe lymphoid depletion (arrow, × 200). **c** Accumulation of hemosiderin with inhibition of melanomacrophage center (MMC, arrow, × 200). **d** Mild hemorrhage in the red pulp (arrow, × 200). **e** Extensive improvement in the lymphocytic population with well-developed melanomacrophage center (arrow, × 200). **f** Hemorrhage in red pulp with mild activation in melanomacrophage center (× 200)
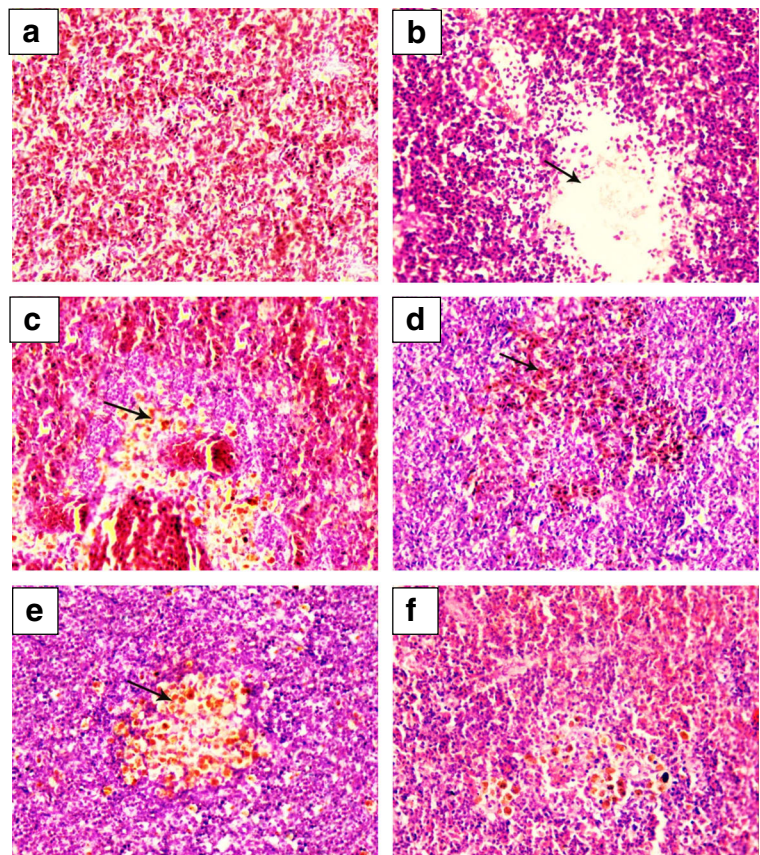
Fig. 9 H&E-stained sections (**a**, **b**) of muscular tissue of Nile tilapia infected with *A. hydrophila*. **c**–**e** Fish infected with *A. hydrophila* and treated with different concentrations of ZLP G2 (**c**), G3 (**d**), and G4 (**e**), showing **a** inter-muscular edema admixed with mononuclear leukocytic cellular infiltrations (arrow) with hyalinization of muscle fiber (asterisk, × 200). **b** Extensive myomalaciain which entire vacuolation of sarcoplasm (asterisk, × 200). **c** Mild intermuscular hemorrhage (arrow, × 200). **d** Muscle fiber appeared nearly similar to negative control group (× 200). **e** Small focal areas of intermuscular edema admixed with leukocytic cellular infiltration (× 200)
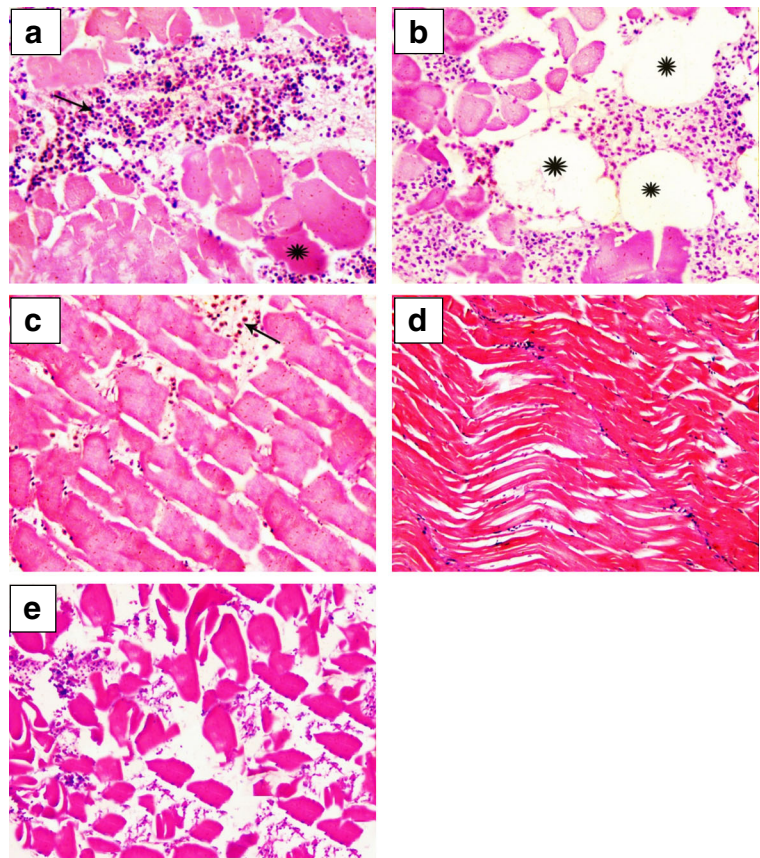
Discussion

The use of herbal medicine for improving the immune status of fish takes a great interest from researchers to prove its effectiveness in different fish species (Awad and Awaad 2017). However, limited information is available about the role of herbs in alleviating the damaging effect of pathogenic bacterial infection at the gene and cellular levels.

Although there is adequate research on the use of natural products for controlling Motile *Aeromonas* Septicemia in Nile tilapia (Ardo' et al. 2008; El Asely et al. 2012; El Asely et al. 2014; Negm et al. 2016), the mortality rate associated with disease outbreaks remain a major problem in tilapia farming (Fernandes et al. 2019).

The present study demonstrated that G2–G4 exhibited tolerance to *A. hydrophila* challenge, which resulted in significantly reduced mortality compared with G1. Jiang et al. (2016) reported that *A. hydrophila* pathogenesis is highly

correlated with oxidative stress. Based on this concept, the protective effect of *Ziziphus* may be associated with its richness in flavonoids and polyphenol, in addition to the high concentrations of ascorbic acid that neutralizes excessive free radicals and contributes to enhanced immune system (Abdallah et al. 2016; Elaloui et al. 2016). It is worth noting that *Z. mauritiana* was effective in the treatment of various human and animal diseases (Abdulameer et al. 2017).

In the present study, ZLP supplementation of Nile tilapia juvenile diet significantly upregulated lysozyme gene expression in G3, followed by in G4. Similarly, dietary ginger significantly increased lysozyme gene expression in zebrafish (Ahmadifar et al. 2019), and methanolic extract of marine macroalga significantly increased lysozyme gene expression in the spleen of Nile tilapia at post-infection 24 h (Yengkhom et al. 2019).

A significant increase was observed in *IL-1β* expression in ZLP-fed groups. Contrary to the present results, *Z.*

spina-christi L. leaf extract significantly decreased *IL-1β* expression in the renal tissues of mice with induced sepsis (Dkhil et al. 2018). This may be due to the difference in plant species and animal subjects used in the present study. In the present study, ZLP supplementation significantly upregulated *SOD* expression. Similar results were reported by Hassaan et al. (2019) when they studied the effects of *Silybum marianum* seeds as feed additives on tilapia. In another study, 20 mg/kg dietary anthocyanin significantly increased *SOD* expression (Yilmaz 2019).

In the present study, serum lysozyme activity significantly increased in G2–G4 compared with in G1. Lie et al. (1989) linked the increase in lysozyme activity in fish spleen and kidney to the abundance of immune cells such as monocytes, macrophages, and polymorphonuclear granulocytes, which are the main sources of such proteolytic enzymes. Engstad et al. (1992) reported that an increase in lysozyme population in the blood of stimulated fish was associated with proliferating phagocytes or increased lysozyme population produced from lysosomes, rendering lysozyme activity to be one of the best markers for evaluating the bactericidal effect of feed additives. The increase in lysozyme activity observed in the present study could be due to flavonoids, which stimulate leucocytes and phagocytosis (Awad et al. 2015). Similar results were recorded in tilapia that were fed *Withania sominefera* (Zahran et al. 2018). The elevation in ALP level serves an alarm for the occurrence of cell damage (Kumar and Sharma 2012). In the present study, no changes were observed in ALP level between the treatment and control groups, confirming the safety of *Z. mauritiana* treatments.

SOD is an enzyme that acts as the first line of defense against reactive oxygen species (ROS) damage by converting ROS to less harmful H_2O_2 (Wang et al. 2018). In this context, the application of medicinal herbs can potentially increase endogenous antioxidants in addition to the natural antioxidant activity (Rajasekaran et al. 2005). Significant upregulation of SOD levels in liver homogenates was recorded in ZLP-fed groups. Similarly, higher SOD levels were reported in genetically improved farmed tilapia that were fed *Aloe vera* for 8 weeks (Gabriel et al. 2015) and in the serum of *O. mossambicus* that were fed *Psidium guajava* leaf extract (Gobi et al. 2016).

GSH is one of the antioxidants that exert cellular defense against oxidative stress by scavenging H_2O_2, free radicals, and other peroxides (Srikanth et al. 2013). A significant increase was observed in GSH level in G3 compared with in the other groups.

Histopathological examination is widely performed to study the pathological alterations caused by chemicals or biological infectious agents (Camargo and Martinez 2007). In addition, the inclusion of immune cells within the head kidney, spleen, and liver is involved in fish immunity (Zapata et al. 2006). In the present study, the addition of *Z. mauritania* to the diet of Nile tilapia reduced the pathological changes associated with *A. hydrophila* infection in all examined organs, with an alleviating effect observed in G3. In the same context, Ostaszewska et al. (2008) reported structural improvement in hepatocytes in silver bream (*Vimba vimba*) fed natural feed additives. In line with the present findings, Owatari et al. (2018) proved that sylimarin significantly reduced the pathological alterations of the liver and spleen of Nile tilapia challenged with *Streptococcus agalactiae*.

The damaging effects of bacterial infection on host tissues are, in part, associated with induction of oxidative stress (Tkachenko et al. 2014). The protective mode of action of most flavonoid-containing plants originates from the ability of flavonoids to quench ROS and consequently relieve harmful effects (Brunetti et al. 2013).

MMCs are pigmented macrophage aggregations in the kidney and spleen that are associated with immunity through phagocytosis of foreign materials (Steinel and Bolnick 2017). Similar to the present findings, an increase in the number of MMCs in Nile tilapia that were fed an *Echinacea purpurea*-supplemented diet (El Asely et al. 2012) and in farmed sea bass that were fed polyphenol-enriched diet has been reported (Magrone et al. 2016).

The immune response of fish muscles to different stimuli, including infections, was studied by Chatterjee et al. (2016) and Valenzuela et al. (2017). Remarkably, the muscles of ZLP-fed groups in the present study exhibited mild degenerative changes in the muscle sarcoplasm.

Conclusion

ZLP supplementation of tilapia feed, particularly at a concentration of 10 g/kg, reduced mortalities associated with *A. hydrophila* infection. The damaging effects of oxidative stress were reduced through the elevation of antioxidant enzyme activity associated with the infection, reducing histopathological alterations and

improving fish immune status. Thus, ZLP supplementation of tilapia diet is recommended as an effective treatment for the avoidance of harmful impacts of *A. hydrophila* infection.

Compliance with ethical standards

Ethical statement The experimental design was approved by the Central Laboratory for Aquaculture Research (CLAR), Abassa, Egypt (Approval no. 43429), and all procedures followed the guidelines for the care and use of fish.

Conflict of interest The authors declare that they have no conflict of interest.

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