



OPEN Silymarin enhances the response to oxytetracycline treatment in *Oreochromis niloticus* experimentally infected with *Aeromonas hydrophila*

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Many governments have approved the use of oxytetracycline as an antibiotic additive to food fish, with oxytetracycline now routinely used in many nations. However, oxytetracycline is known to have immunosuppression impacts. We, therefore, evaluated the immunological, antioxidative, and histopathological status of Nile tilapia fed a diet containing silymarin (100 mg/kg fish feed) for 0, 2, 4, 6, and 8 weeks. The protective effects of silymarin against *Aeromonas hydrophila* (*A. hydrophila*) infection and oxytetracycline treatment were evaluated. Blood parameters (erythrocyte count, white blood cell count, hemoglobin, and packed cell volume) improved over time in fish fed on dietary silymarin. Serum levels of alanine aminotransferase (ALT) were lower in fish fed on dietary silymarin, whereas serum levels of aspartate transferase (AST) and alkaline phosphatase (ALK) were unchanged. Dietary silymarin affected serum lipid profiles as decreases in serum triglyceride and low-density lipoprotein cholesterol levels and a trend toward lower cholesterol levels, whereas serum high-density lipoprotein cholesterol levels were increased compared to fish fed on the control diet. Dietary silymarin resulted in an increase of serum total protein levels and globulin fractions. Significant and progressive increases in catalase and glutathione peroxidase levels were observed after six weeks of feeding on a dietary silymarin before decreasing to control levels at the end of the experimental period. Fish fed on dietary silymarin, interleukin-1 and fish tumor necrosis factor- α were upregulated in hepatic tissues; however, interleukin-10 levels decreased to comparable levels to controls after eight weeks. Fish infected with *A. hydrophila* displayed septicemia (opaque eye, hemorrhagic ulcers, dentated fins, hepatomegaly, and splenomegaly). Reduced mortality was observed in Nile tilapia infected with *A. hydrophila* and fed a diet containing silymarin, indicating that silymarin improves fish responses to oxytetracycline with a 37% reduction in mortality.

Silymarin is a flavonolignan derived from the seed of milk thistle (*Silybum marianum* L.) that contains silybinin A and B, isosilybinin A and B, and silydianin^{1,2}. Given its phytotherapeutic characteristics, silymarin has been widely employed as a fish feed additive for the treatment and prevention of diseases in recent years^{3,4}. Dietary silymarin reportedly improves the growth performance of grass carp (*Ctenopharyngodon idellus*)⁵ and increases the activity of superoxide dismutase (SOD), an antioxidant enzyme, in the liver of turbot (*Scophthalmus maximus* L.)⁶.

The liver is the primary organ responsible for both endogenous and external chemical metabolism and one of the first organs to be affected by toxic exposures⁷. Silymarin, a hepatoprotective antioxidant with anti-lipid and anti-inflammatory properties, has been shown to have hepatoprotective effects in common carp (*Cyprinus*

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carpio)⁸. The steroid structure of silymarin may alter the hepatocyte cell membrane by blocking the entry of xenobiotics and trapping free radicals, thereby increasing intracellular concentrations of glutathione and inhibiting lipid peroxidation^{9,10}. Previous studies have examined the effects of silymarin on juvenile grass carp at doses of 0, 100, and 200 mg/kg⁵. Although compounds derived from plants may be used for chemotherapy, compounds used as supplements in fish diets must undergo physiological and biological examinations before their routine use.

The rapid rise of the global aquaculture sector over the last seven decades has generated a multibillion-dollar business (aquatic animal protein and global food security)¹¹. The intensification of aquaculture to meet the growing demand for seafood has led to an increase in the prevalence of infectious diseases among fish stocks, with increasing stock densities leading to increased transmission of pathogens. Further, decreased water quality has been shown to induce stress factors that impact disease persistence within fish. The annual cost of infectious disease epidemics among fish stocks has been reported as 6 billion USD¹².

Only three antibiotics used in fish are Food and Drug Administration approved and commercially available in the United States: oxytetracycline (OTC, Terramycin for Fish, Phibro Animal Health, Inc., Fairfield, NJ); florfenicol (Aquaflor, Intervet/Schering-Plough, Animal Health Corp. Summit, NJ); and sulfadimethoxine and ormetoprim (Romet-30, Pharmaq AS, Oslo, Norway)¹³. Tetracyclines have broad-spectrum bacteriostatic action which prevents the growth of both Gram-negative and Gram-positive bacteria by inhibiting bacterial protein synthesis. Tetracyclines have been widely employed in aquaculture due to excellent penetration into bodily fluids and tissues, and low cost^{14,15}. As tetracyclines are inexpensive, simple to administer, and cause less stress to fish, the oral route is frequently used for the administration of tetracyclines to aquatic animals, with in-feed oxytetracycline widely used in the treatment of Nile tilapia (*Oreochromis spp*)^{16–18}. Many studies have reported that oxytetracycline has histopathological, immunosuppressive, and genotoxic characteristics in farmed fish species, including rainbow trout^{19,20}. Accordingly, dietary supplementation is required to limit oxytetracycline withdrawal and increase fish responses to oxytetracycline treatment.

Materials and methods

Experimental design

In the present study, two hundred Nile tilapia (*O. niloticus*) with an average weight of 50 ± 3 g were collected from local freshwater fish farms (Tolompat7 village in Kafrelsheikh governorate Egypt) and transferred to the wet laboratory at Animal Health Research Institute at Kafrelsheikh. Fish were maintained in a water tank for acclimation for two weeks. Fish were distributed into fifteen glass aquaria (aquarium size: $50 \times 50 \times 40$ cm) after sedation with tricaine methanesulfonate. Antiseptic therapy was administered before transfer into new aquaria^{21,22}. Silymarin was used as a feed additive at a dose of 0.1% (1 g per kg of dry ration)²³ for 2, 4, 6, and 8 weeks, and were abbreviated Sil-0, Sil-2, Sil-4, Sil-6, and Sil-8, respectively. Fish were divided into two groups. The control group was maintained in ten aquaria (10 fish per aquarium). The second group (100 fish) was maintained in ten glass aquaria. Blood and tissues samples were collected at 0, 2, 4, 6, and 8 weeks. Fish were challenged with *Aeromonas hydrophila* (*A. hydrophila*) via intraperitoneal injection at a dose of 2.4×10^5 CFU/mL at 0, 2, 4, 6, and 8 weeks and then treated with dietary oxytetracycline (83 mg/kg b w) for 10 days²⁴.

Commercial fish food (in the form of pellets) was soaked in water and blended into a paste, to enhance the consistency and viscoelastic properties of the food, gelatin (Nutri-B-Gel) produced by Canal Aqua Cure (Port-Said, Egypt) was mixed with the formed paste to a final level of 5% w/w. The diet pastes were sited into syringes that yielded a 2 mm-thick diet. The extruded diets were left to dry before cutting into similar size pellets. Fish feed was provided twice a day at 08.00 am and 02.00 pm. The amount of feed was changed weekly to provide a weight of feed corresponding to 3% body weight²⁵.

Silymarin (capsules, 140 mg) was purchased from a local market. Silymarin capsules were manufactured by Safe for pharmaceutical products, new Borg El-Arab, Egypt, packed by South Egypt Drug Industries Company SEDICO (Batch No.: 0920468/2020). Oxytetracycline (powder, 20%) was purchased from a local market produced by the Arab Company for Gelatin and Pharmaceutical Products (Arab Caps) under registration no. (M.O.H. Reg. No.: 838/2004).

Serological analyses of experimental fish

Blood samples were collected after the anaesthetization of fish with tricaine methanesulfonate (MS222; Sigma, St. Louis, MO, USA). Blood samples were drawn from the caudal vein of experimental Nile tilapia using a syringe coated with heparin (100 IU/ml) and without anticoagulant to obtain whole blood and serum, respectively. Red blood cell (RBC) and white blood cell (WBC) counts were obtained using a hemocytometer²⁶. Packed cell volume (PCV) was determined using the microhematocrit technique. Serum hemoglobin (Hb) levels were calculated using the cyanmethemoglobin method²⁷. All serum samples were stored at -80 °C till analyses were performed.

Liver enzymes and lipid profile in fish serum

Hepatic enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) levels were measured colorimetrically²⁸ using kit reagents supplied by Diamond Diagnostic Co. (Holliston, USA). Lipid profiles were colorimetrically measured in experimental fish serum including triglycerides (TG)²⁹, cholesterol (CH)³⁰, high-density lipoprotein cholesterol (HDL)³¹, and low-density lipoprotein cholesterol (LDL)³².

Serum protein levels in Nile tilapia

Serum concentrations of total protein (TP)³³ and albumin (ALB)³⁴ were measured by colorimetric methods. Globulin (GLO) concentrations were calculated by subtracting the ALB concentration from the concentration of TP. The electrophoretic pattern of serum α , β , and γ GLOs was assessed using polyacrylamide gel columns³⁵. Gels were imaged and analyzed according to previously described methods³⁶.

Hepatic antioxidant enzyme levels in Nile tilapia

The levels of glutathione peroxidase (GPx, EC 1.11.1.9) and catalase (CAT; EC 1.11.1.6) in hepatic tissue of experimental Nile tilapia were determined^{37,38}.

Serum cytokine levels in experimental fish

Serum levels of interleukin-1 β (IL-1 β), interleukin-10 (IL-10), and fish tumor necrosis factor- α (TNF- α) were measured using solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) test kits (My BioSource Co., San Diego, California, USA). ELISA was performed according to the manufacturer's protocols.

Infective signs, mortality rate, and relative level of protection

Experimental fish were challenged with virulent *A. hydrophila* (AHRAS2) at 0, 2, 4, 6, and 8 weeks. At each time point, fish were divided into two groups, one group was treated with oxytetracycline for ten days while the other group received no antimicrobial treatment. Each fish was intraperitoneally administered 0.2 mL of a bacterial suspension containing 2.4×10^5 CFU/mL in phosphate buffer saline using the McFarland scale. *A. hydrophila* (AHRAS2)³⁹ and deposited in GenBank with the accession number MW092007. *A. hydrophila* expresses cytotoxic enterotoxin genes (*act* and *alt*). Mortality rate (MR) and relative level of protection (RLP) were determined in all groups. MR (%) was estimated according to the following formula.

$$\text{MR (\%)} = (\text{No. of deaths in a specific period} \div \text{total population during that period}) \times 100$$

Meanwhile, RLP was calculated in challenged fish⁴⁰ as follows:

$$\text{RLP (\%)} = (1 - (\% \text{ mortality in the treated group} \div \% \text{ mortality in the control group})) \times 100$$

Histopathology

From experimental fish, liver samples were collected and fixed in 10% buffered formalin to assess the impact of oxytetracycline and the ameliorative role of silymarin. After alcohol dehydration and xylol clearing, specimens were embedded in paraffin, then cut into sections and stained with haematoxylin–eosin for microscopic examination⁴¹.

Statistical analyses

The impact of silymarin on the health of Nile tilapia was evaluated by determining the mean and standard error of the collected data. Groups were compared using ANOVA and Duncan's Multiple Range⁴² using SPSS software⁴³. *P* values less than 0.05 were considered statistically significant.

Biosafety

Dead fish were burned in fixed incinerators at the laboratory. The present study followed the biosafety measures concerning pathogen safety described by the Pathogen Regulation Directorate (Infectious substances—*A. hydrophila* datasheet)⁴⁴.

Ethical approval

The above described methodology was approved by the Ethics Committee at the Animal Health Research Institute and European Union directive 2010/63UE, and all methods were carried out in accordance with relevant guidelines and regulations. This study is reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>). This paper does not contain any studies with human participants by any of the authors. No specific permissions were required for access to the artificial pond in wet laboratory Animal Health Research Institute, Kafrelsheikh, Egypt. The field studies did not involve endangered or protected species.

Results

Serological parameters

Nile tilapia fed a dietary silymarin had improvements in serological indices at four weeks (RBC, 6.45×10^6 ; WBC, 49.27×10^3 ; Hb, 14.13 g/dl; PCV, 45.57%; Table 1) compared to the control. No significant differences

Items	RBC ($\times 10^6$)	WBCs ($\times 10^3$)	Hb (g/dl)	PCV (%)	SR (%)
Sil-0	2.31 \pm 0.1 ^C	35.7 \pm 2.45 ^C	9.46 \pm 0.4 ^C	30.49 \pm 1.28 ^C	27/30 (90)
Sil-2	3.13 \pm 0.12 ^{AB}	42.67 \pm 1.23 ^B	12.85 \pm 0.5 ^{AB}	41.37 \pm 1.59 ^{AB}	95/100 (95)
Sil-4	3.45 \pm 0.18 ^A	49.27 \pm 2.66 ^A	14.13 \pm 0.74 ^A	45.57 \pm 2.38 ^A	80/85 (94.11)
Sil-6	2.87 \pm 0.12 ^B	38.3 \pm 1.96 ^{BC}	11.75 \pm 0.5 ^B	37.8 \pm 1.6 ^B	67/70 (95.71)
Sil-8	2.75 \pm 0.08 ^B	41.1 \pm 0.67 ^{BC}	11.28 \pm 0.31 ^B	36.35 \pm 0.99 ^B	55/57 (96.5)

Table 1. Serological parameters and survival rate. Superscript letter within the same column indicate significant differences between values at $P \leq 0.05$. Sil-0, control group; Sil-2, group fed silymarin-containing diet for 2 weeks; Sil-4, 4 weeks; Sil-6, 6 weeks; Sil-8, 8 weeks; RBC, red blood cell; WBC, white blood cell; Hb, hemoglobin; PCV, packed cell volume.

were observed between time points or groups. The survival rate (SR; %) of experimental Nile tilapia was higher in silymarin-fed fish at two weeks (95%) compared to baseline (90%) and remained higher than baseline until the end of the 8-week experimental period.

Serum enzymes and lipid profiles

Serum ALT levels were significantly decreased in Nile tilapia fed on a silymarin-containing diet for four weeks compared to baseline (4 weeks, 20 IU/l vs. baseline, 25.07 IU/l). No significant differences in serum ALT levels were observed between other time points. No significant differences in serum levels of AST or ALK were observed between time points (Fig. 1).

No significant differences in serum lipid profiles in Nile tilapia including TG, CH, HDL, and LDL levels were observed between timepoints. CH levels were significantly lower than baseline after tilapias were fed a silymarin-containing diet for 6 weeks. Serum HDL levels were significantly increased at six weeks compared to baseline (6 weeks, 70.17 mg/dl vs. baseline, 56.4 mg/dl; Fig. 2).

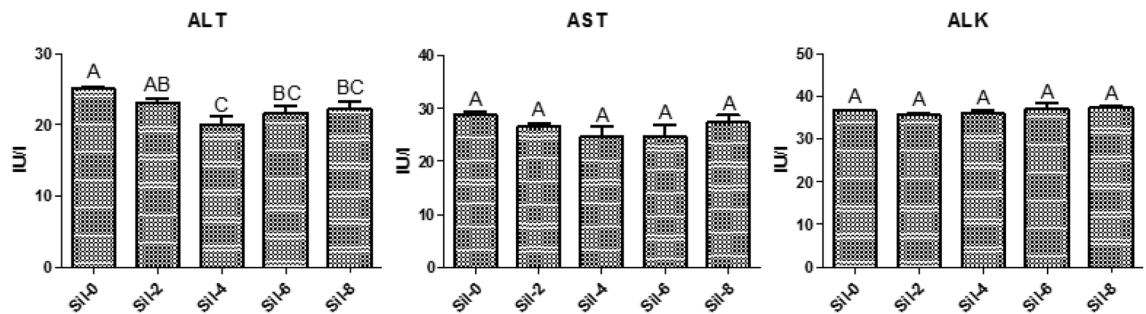


Figure 1. Serum liver enzyme levels and lipid profiles. Different letters indicate significant differences between values at $P \leq 0.05$. Sil-0, control group; Sil-2, group fed silymarin-containing diet for 2 weeks; Sil-4, 4 weeks; Sil-6, 6 weeks; Sil-8, 8 weeks; ALT, alanine aminotransferase; AST, Aspartate aminotransferase; ALK, alkaline phosphatase.

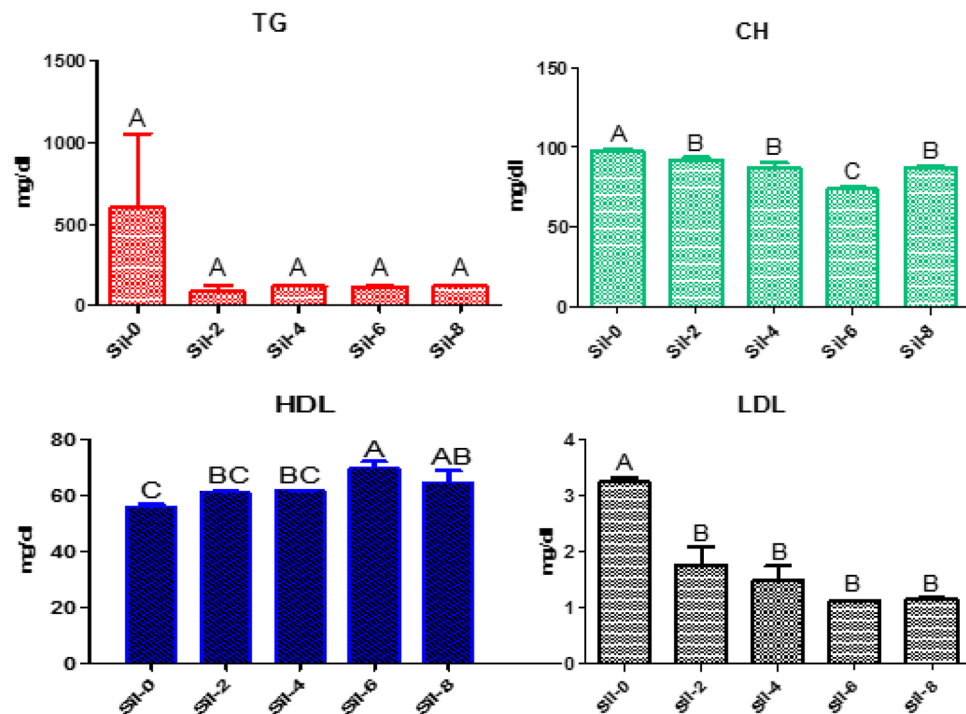


Figure 2. Serum lipid profiles. Different letters indicate significant differences between values at $P \leq 0.05$. Sil-0, control group; Sil-2, group fed silymarin-containing diet for 2 weeks; Sil-4, 4 weeks; Sil-6, 6 weeks; Sil-8, 8 weeks; TG, triglycerides; CH, cholesterol; HDL, high-density lipoprotein (HDL) cholesterol; and LDL, low-density lipoprotein cholesterol.

Serum protein levels and globulin electrophoresis

Serum TP levels were significantly increased from baseline to 5.74 g/dl in Nile tilapia fed a silymarin-containing diet for 6 weeks. Significant increases in serum ALB levels at four weeks (2.95 g/dl); however, no significant differences in serum ALB levels were observed between 8 weeks (2.73 g/dl) and baseline (2.76 g/dl).

Serum GLO levels were increased in fish fed a silymarin-containing diet compared to baseline values. Alpha GLO levels (1.03 g/dl) and beta GLO levels (0.81 g/dl) were significantly increased at 6 weeks compared to baseline (0.64 g/d and 0.46 g/dl, respectively). Gamma GLO levels were significantly increased at four weeks compared to baseline (4 weeks, 0.4 g/dl vs. baseline, 0.62 g/dl; Table 2).

Antioxidant responses

In Nile tilapia fed a silymarin-containing diet, hepatic levels of GPx and CAT reached a maximum level at four weeks (GPx, 5.3 U/mg; CAT, 2.99 U/mg) compared to baseline (GPx, 2.29 U/mg; CAT, 0.61 U/mg) and then decreased at eight weeks (GPx, 2.33 U/mg; CAT, 1.12 U/mg), as shown in Table 3.

Serum cytokines levels

Cytokines (IL-1 β , TNF- α , and IL-10) levels were altered in liver tissues of Nile tilapia fed a silymarin-containing diet. Levels of proinflammatory cytokines were significantly increased at two weeks (IL-1 β , 4.87 pg/mg; TNF- α , 3.47 pg/mg) compared to baseline (IL-1 β , 2.48 pg/mg; TNF- α , 1.52 pg/mg) and then decreased thereafter. IL-10 levels in hepatic tissues were significantly increased at four weeks (4.43 pg/mg) compared to baseline (1.2 pg/mg) and then decreased at eight weeks (1.4 pg/mg; Fig. 3).

Items	TP (g/dl)	ALB (g/dl)	GLO (g/dl)	GLO- α (g/dl)	GLO- β (g/dl)	GLO- γ (g/dl)
Sil-0	4.56 \pm 0.05 ^D	2.76 \pm 0.03 ^B	1.8 \pm 0.02 ^C	0.64 \pm 0.03 ^D	0.46 \pm 0.03 ^C	0.4 \pm 0.02 ^C
Sil-2	5.17 \pm 0.02 ^C	2.71 \pm 0.02 ^B	2.46 \pm 0.01 ^B	0.83 \pm 0.01 ^C	0.71 \pm 0.01 ^B	0.6 \pm 0.02 ^{AB}
Sil-4	5.47 \pm 0.05 ^B	2.95 \pm 0.03 ^A	2.52 \pm 0.04 ^B	0.88 \pm 0.003 ^{BC}	0.72 \pm 0.01 ^B	0.62 \pm 0.03 ^A
Sil-6	5.74 \pm 0.05 ^A	2.97 \pm 0.08 ^A	2.76 \pm 0.04 ^A	1.03 \pm 0.04 ^A	0.81 \pm 0.01 ^A	0.63 \pm 0.07 ^A
Sil-8	5.21 \pm 0.05 ^C	2.73 \pm 0.06 ^B	2.47 \pm 0.02 ^B	0.94 \pm 0.04 ^B	0.74 \pm 0.02 ^B	0.49 \pm 0.01 ^{BC}

Table 2. Serum protein analyses. Superscript letters within the same column indicate significant differences between values at $P \leq 0.05$. Sil-0, control group; Sil-2, group fed silymarin-containing diet for 2 weeks; Sil-4, 4 weeks; Sil-6, 6 weeks; Sil-8, 8 weeks; TP, total protein; ALB, albumin; GLO, globulin.

Items	GPx (U/mg prot)	CAT (U/mg prot)
Sil-0	2.29 \pm 0.1 ^C	0.61 \pm 0.07 ^D
Sil-2	3.41 \pm 0.08 ^B	1.32 \pm 0.07 ^C
Sil-4	5.3 \pm 0.58 ^A	2.99 \pm 0.06 ^A
Sil-6	3.09 \pm 0.06 ^{BC}	1.84 \pm 0.06 ^B
Sil-8	2.33 \pm 0.18 ^C	1.12 \pm 0.13 ^C

Table 3. Glutathione and catalase levels in hepatic tissues. Superscript letters within the same column indicate significant differences between values at $P \leq 0.05$. Sil-0, control group; Sil-2, group fed silymarin-containing diet for 2 weeks; Sil-4, 4 weeks; Sil-6, 6 weeks; Sil-8, 8 weeks; GPx, glutathione peroxidase; CAT, catalase.

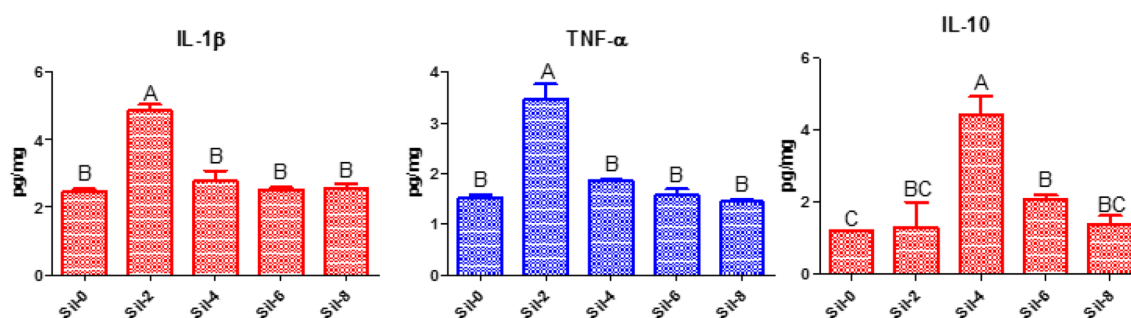


Figure 3. Cytokines levels in the liver tissues of the experimental fish. Different letters indicate significant differences between values at $P \leq 0.05$. Sil-0, control group; Sil-2, group fed silymarin-containing diet for 2 weeks; Sil-4, 4 weeks; Sil-6, 6 weeks; Sil-8, 8 weeks; IL, interleukin; TNE, tumor necrosis factor.

Infection of experimental fish with *A. hydrophila*

Infective signs and post-mortem examinations, Nile tilapia infected with *A. hydrophila* developed multiple hemorrhagic petechia, hemorrhagic ulcers, and pop eye (Figs. 4 and 5). Post-mortem examinations revealed signs of septicemia (Figs. 6 and 7) as splenomegaly and yellow- or brown-colored hepatic tissue. Some fish had empty intestines (Fig. 6), while others had full intestines (Fig. 7).

Among *O. niloticus* infected with *A. hydrophila*, fish fed a silymarin-containing diet had lower mortality than controls, with silymarin supplementation providing a protection level of 25% at six weeks. Oxytetracycline treatment decreased the MR to 70% compared to 80% in controls. In addition, treatment with oxytetracycline resulted in a relative protection level (RPL) of 37.5% after six weeks of silymarin supplementation as shown in Table 4.

Histopathological examination of experimental fish with *A. hydrophila*

After two weeks of silymarin supplementation, there were no changes in the hepatic tissue of Nile tilapia criteria (Fig. 8A), but after four weeks of supplementation, the hepatic tissue revealed scanty hyaline bodies and focal zones of hepatocyte degeneration (Fig. 8B). After six and eight weeks of silymarin supplementation, no hyaline bodies or degenerated areas were observed (Fig. 8C,D).

A. hydrophila demonstrated septicemic infection, intra-vascular thrombus development, and haemolysis after two weeks of silymarin supplementation followed by challenge (Fig. 9A). Hyaline bodies were shown, and pancreatic cells showed significant vacuolation and even necrosis (Fig. 9B), in addition to necrotic foci (Fig. 9C). At challenge post four weeks of silymarin supplementation, intra-vascular hemolysis could be detected with a high incidence of hyaline bodies' formation (Fig. 9D). Supplementation with silymarin for six weeks followed by challenge, no intra-vascular hemolysis nor thrombus could be detected (Fig. 10A), meanwhile multiple areas of hepatocytes lysis was detected (Fig. 10B). Challenge after silymarin supplementation for 8 weeks revealed a



Figure 4. *Oreochromis niloticus* with hemorrhagic ulcers (arrow A) and popeye (arrow B).



Figure 5. *Oreochromis niloticus* with mild popeye (arrow A) and multiple hemorrhagic petechia (arrow B).

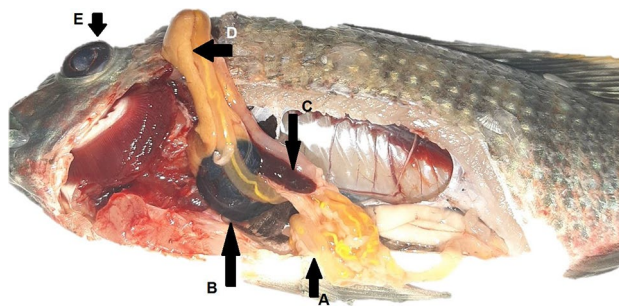


Figure 6. *Oreochromis niloticus* with an empty intestine (arrow A), distended gall bladder (arrow B), splenomegaly (arrow C), yellow-colored liver (arrow D), and mild popeye (arrow E).

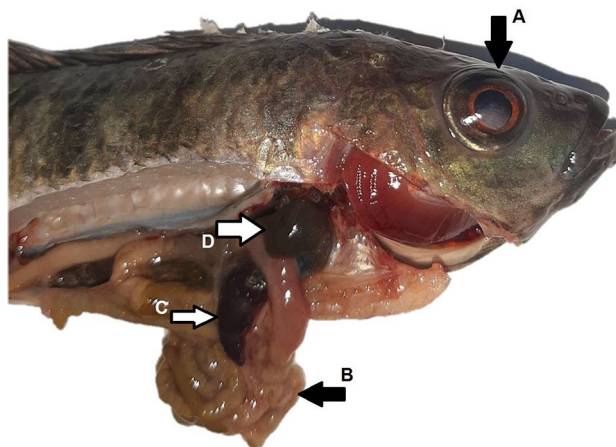


Figure 7. *Oreochromis niloticus* with popeye (arrow A), a full intestine (arrow B), splenomegaly (arrow C), and brown-colored liver (arrow D).

Items	Fish no	Silymarin		OTC	
		MR (%)	RPL (%)	MR (%)	RPL (%)
Sil-0	10	80	–	70	12.5
Sil-2	10	80	0	60	25
Sil-4	10	70	12.5	60	25
Sil-6	10	60	25	50	37.5
Sil-8	10	60	25	50	37.5

Table 4. Mortality rate (MR) and relative protection level (RPL). No., number; MR, mortality rate; RPL, relative protection level; OTC, oxytetracycline.

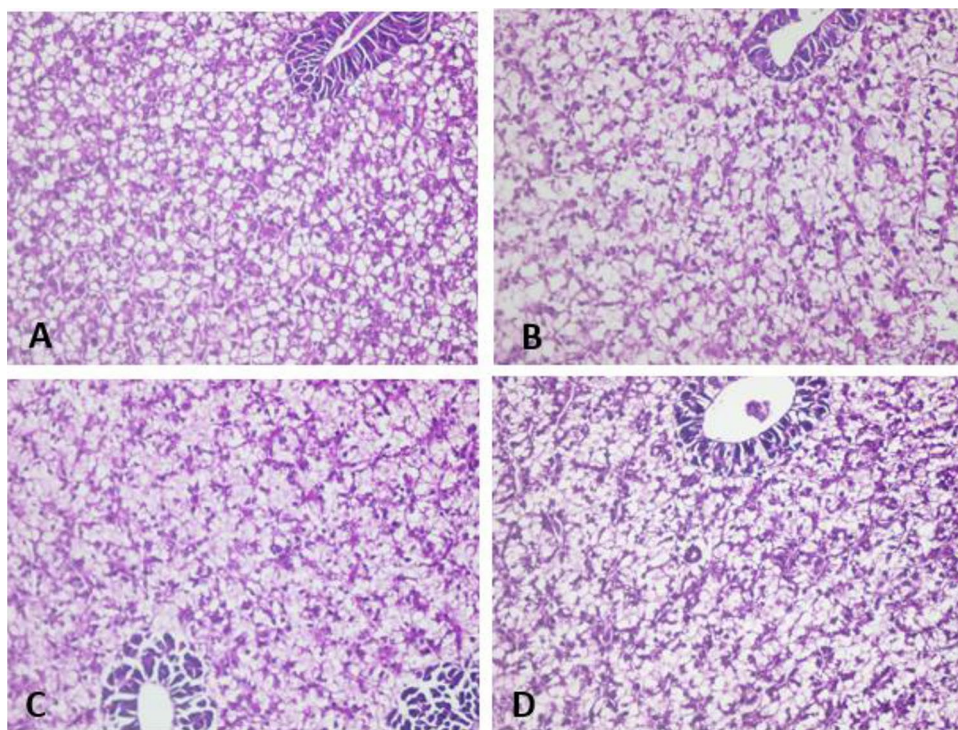


Figure 8. Liver of Sil-2 showed normal *Tilapia* hepatic tissue criteria (A), liver of Sil-2 showed significantly scanty hyaline bodies detection and focal areas of hepatocytes degeneration (B). liver of Sil-6 (C) and Sil-8 (D) neither hyaline bodies nor focal necrotic areas could be detected. Hand E $\times 400$.

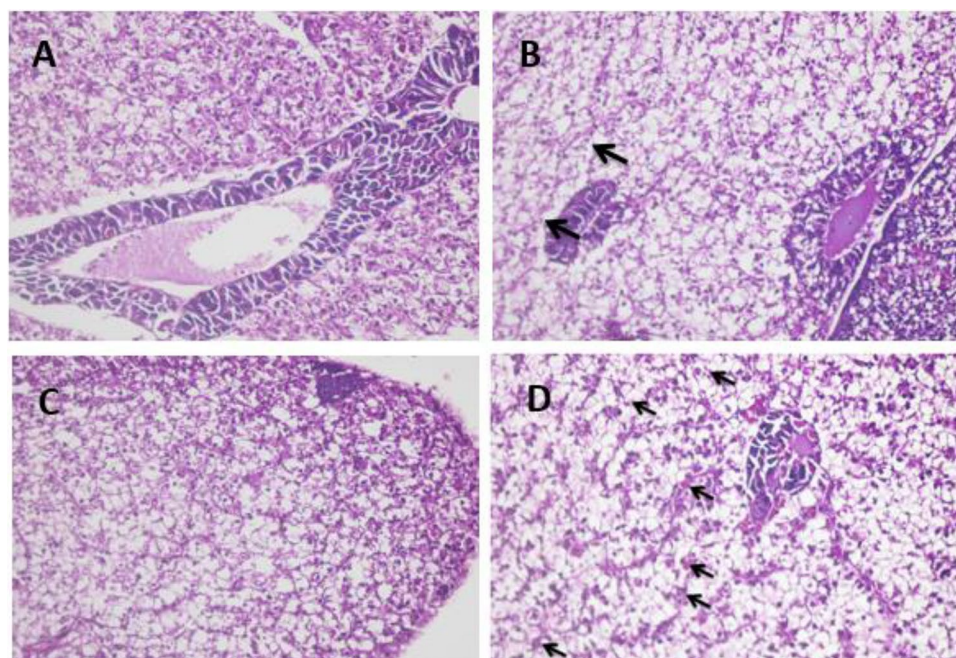


Figure 9. Liver of Sil-2/Challenge showed intra-vascular thrombus formation (A) and hemolysis with scant number hyaline bodies (arrows), there is marked vacuolation and necrosis of pancreatic cells (B), in addition to foci of necrosis and mono-nuclear cells infiltration (C). Liver of Sil-4/Challenge (D) showed intra-vascular haemolysis with high incidence of hyaline bodies' formation. Hand E \times 400.

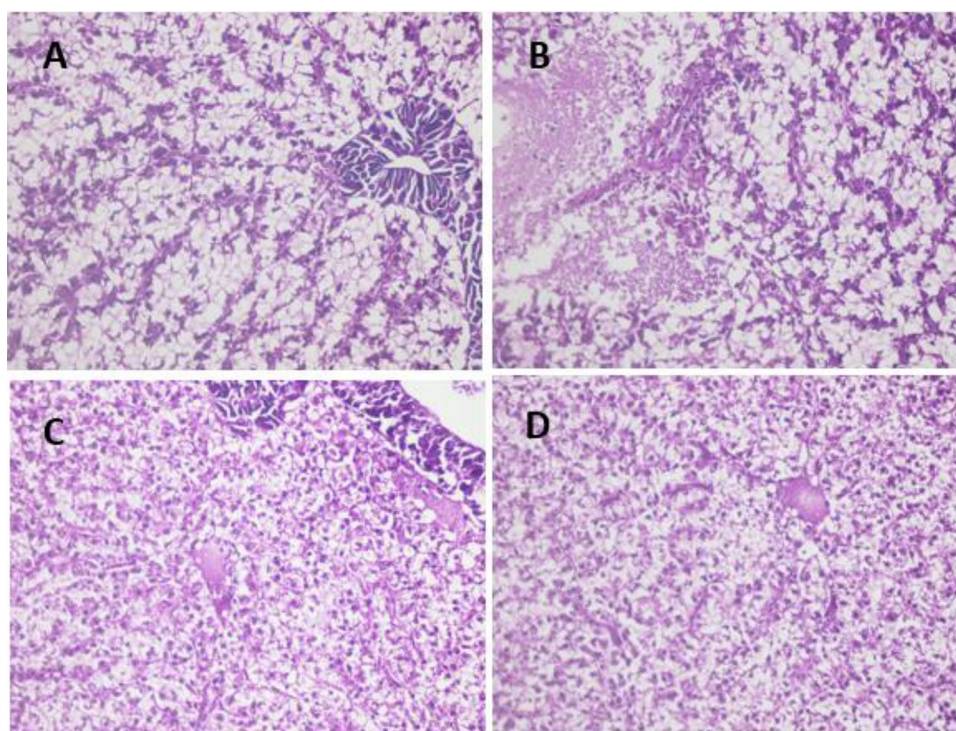


Figure 10. Liver of Sil- 6/Challenge no intra-vascular hemolysis nor thrombus could be detected (A), meanwhile multiple areas of hepatocytes lysis was detected (B). Liver of Sil-8/Challenge high incidence of lytic areas (C, D). H and E \times 400.

high incidence of lysis area occurrences, meanwhile, no intra-vascular hemolysis nor thrombus could be detected (Fig. 10C,D). The protection estimation of the silymarin supplementation showed no effect at two and four weeks. At periods of six and eight weeks, the intra-vascular effect of *A. hydrophila* infection (thrombus and hemolysis) was inhibited, meanwhile the cellular degenerative effect could not be improved.

Discussion

Compared to the control group, Nile tilapia fed a silymarin-containing diet had a higher survival rate after two weeks (95%) until the end of the experimental period (eight weeks). These results demonstrate fish fed a diet supplemented with different doses of silymarin had significantly greater survival compared to fed the control fish⁴⁵.

In the present study, Nile tilapia fed a silymarin-containing diet had improvements in blood indices after four weeks (RBC, 6.45×10^3 ; WBC, 49.27×10^3 ; Hb, 14.13 g/dl; PCV, 45.57%) compared to baseline. Values were comparable to the control group at other time points. Similar to our findings, Nile tilapia fed a diet supplemented with silymarin for 45 days have been reported to have higher RBC compared to fish fed a control diet. An increase of WBC count was observed in *Oncorhynchus mykiss* on day 15 of feeding with a dietary silymarin 0.1 g/kg, with higher RBCs on days 15 and 30 in fish fed a dietary silymarin 0.8 or 0.1 g/kg compared to the controls⁴⁶. Conversely, blood indices including WBC, Hb, and PCV were similar between fish fed on dietary silymarin and control diets²³. These differences between the results of the present study and previous studies may be attributable to the longer duration of feeding with silymarin-supplemented diets as blood indices were comparable to baseline levels after 6 weeks of feeding with a silymarin-containing diet in the present study.

The liver is the primary organ responsible for the metabolism (biotransformation and detoxification) of toxicants including microbial toxins, viruses, heavy metals, aquatic pollutants, pesticides, and chemotherapeutic drugs which may cause organ damage^{47,48}. After four weeks of silymarin supplementation, serum ALT levels were significantly decreased compared to fish fed a control diet, while serum AST and ALK were comparable between fish fed a silymarin-containing diet and the control diet throughout the experimental period. Further, serum TG and CH levels were unaffected by silymarin-containing diets; however, serum HDL levels were significantly increased in fish fed a silymarin-containing diet compared to fish fed the control diet. A previous study reported decreased serum AST and ALT levels in Nile tilapia fed a diet with higher *S. marianum* content, with serum AST and ALT levels lowest in Nile tilapia fed a diet supplemented with 10 g/kg fish feed⁴⁵. These decreases in serum AST and ALT levels may be attributable to the strong antioxidant activity of silymarin increasing intracellular levels of glutathione, thereby increasing the elimination of free radicals and inhibition of lipid peroxidation. These effects may protect the cell membranes and ultimately prevent the release of liver enzymes into the circulating plasma. Further, the hepatoprotective properties of silymarin may prevent the transport of xenobiotics across the cell membrane^{9,49,50}.

In general, fish with higher serum TP, ALB, and globulin levels are considered to have more robust innate immune responses⁵¹. After four weeks of silymarin supplementation. Serum proteins were significantly increased compared to baseline values. Further, serum gamma GLO levels were increased in fish fed a silymarin-containing diet compared to fish fed the control diet. Similarly, significant increases in total plasma protein levels have been reported in rainbow trout fed on diets containing silymarin at a dose of 100 and 800 mg/kg compared to the control group⁵². Serum ALB, GLO, and TP levels in Nile tilapia were affected by diets containing *S. marianum* seeds, with TP and GLO content markedly higher in fish fed either diets containing *S. marianum* seeds at 7.5 or 10 g/kg diet compared to other diets. In addition, serum ALB levels were significantly higher in fish fed on diets supplemented with *S. marianum* seeds⁴⁵. On the contrary, no differences in serum TP levels, immunoglobulin levels, agglutinating titers, or antimicrobial activity were observed between Nile tilapia fed a silymarin-supplemented diet and tilapia fed a control diet²³.

The use of natural herbs or their extracts as feed additives is beneficial for the growth of fish due to their antioxidant, growth-promoting, appetite-stimulating, and immunostimulant properties. This impact is attributed to the presence of bioactive components including flavonoids, phenolics, essential oils, and pigments^{7,53,54}. Superoxide dismutase (SOD) and catalase (CAT) are important biomarkers of the antioxidant capacity of cells and are frequently utilized as non-specific immunological indices in fish⁵⁵. In this work, hepatic levels of GPx and CAT, antioxidant enzymes, increased after four weeks of feeding with a dietary silymarin and then returned to baseline levels. A previous study reported the activities of CAT, GST, and SOD were comparable between Nile tilapia fed on a silymarin-containing diet for 6 weeks compared to fish fed a control diet²³. Silymarin supplementation in the form of *S. marianum* seeds improved antioxidant activity in hepatic tissues and had a direct impact on immune cells⁵⁶. Also, Nile tilapia fed a diet containing *S. marianum* at doses of 7.5 or 10 g/kg had higher SOD activity compared to fish fed other diets, with a trend toward increase of CAT activity in fish provided a diet containing *S. marianum* seeds⁴⁵. These results may be explained by the findings of previous studies^{9,47,57} demonstrating silymarin has cytoprotective properties due to antioxidant and free radical scavenging effects, these authors posited that stress causes the formation of reactive oxygen species (ROS) in organ tissues leading to lipid peroxidation of cell membranes and modification of cell transporters, glycoproteins, estrogenic receptors, and nuclear receptors, as silymarin supplementation is thought to protect against these deleterious impacts of ROS.

Levels of IL-1 β , TNF- α , and IL-10 were increased in the hepatic tissues of Nile tilapia fed on dietary silymarin in the present study; however, levels had returned to baseline levels at eight weeks. Dietary silymarin was reported to increase the expression of TNF- α , nitric oxide synthase (iNOS), and interleukins (IL-1 β and IL-6) in a dose-dependent manner in rats, further, silymarin at doses up to 10 mg/kg could suppress WBC function, with higher doses between 50 and 250 mg/kg found to stimulate inflammatory processes¹⁰.

Nile tilapia infected with *A. hydrophila* and fed a silymarin-containing diet had lower mortality compared to the control fish, providing a protection level of 25%. Accordingly, it was reported that 28% of Nile tilapia fed a silymarin-supplemented diet survived streptococcosis while all fish fed a control diet died by 192 h²³. Similarly,

Rutin, a dietary flavonoid, had hepatoprotective effects in silver catfish treated with oxytetracycline, with reduced oxidative stress and apoptosis⁵⁸. Nile tilapia fed on diets containing *Moringa oleifera*, a flavonoid, were protected against oxidative stress in hepatic tissues induced by oxytetracycline treatment¹⁸.

Oxytetracycline treatment decreased mortality in the present study. In addition, treatment with oxytetracycline after six weeks of feeding with a silymarin supplementation diet provided an RPL of 37.5% compared with 12.5% in fish fed a control diet. Oxytetracycline is an approved therapeutic agent for aquaculture use^{59–61} and oral administration is the most common route of antibiotic administration in aquaculture raising the risk of environmental pollution⁶².

This study has demonstrated that the provision of a silymarin-supplemented diet could reduce the increased antioxidant and immune activities caused by oxytetracycline withdrawal. Accordingly, oral oxytetracycline administration at a dose of at 80 mg/kg biomass/day for 10 consecutive days reduced the antioxidant capacity of liver and muscle tissues and reduced the expression of TNF α and IL-1 in hepatic tissues, indicating that oxytetracycline compromises the innate immunity of *O. niloticus*⁶³. These authors posited that the cessation of oxytetracycline treatment partially restored the expression of IL-1 β . Likewise, low doses of doxycycline have been shown to down-regulate proinflammatory cytokines, including TNF- α and IL-1 β , in multiple models⁶⁴. Further, a trend toward a decrease in renal IL-1 β expression has been reported in gilthead seabream (*Sparus aurata*) after administration of oxytetracycline at 4 or 8 g/kg feed for 21 days⁶⁵.

In our study, silymarin supplementation could maintain healthy hepatic tissue in Nile tilapia. While some authors detected some drawbacks effect of silymarin supplementation in the form of hepatic vacuolization and necrosis^{66,67} on the other side recorded the improvement of hepatic steatosis by silymarin supplementation. In this study, *A. hydrophila* infection resulted in hepatic degenerative changes, which were previously recorded in the form of necrosis⁶⁸, in association with mononuclear cell infiltrations^{69,70}. While some authors⁷¹ detected the haemosiderin pigment granules in the infected fish tissues, our study recorded the earlier haemolytic changes in the form of intra-vascular blood haemolysis. These changes were attributed to the bacterial toxins and extra-cellular products such as hemolysin of *Aeromonas*^{72,73}. In the present study, silymarin supplementation could ameliorate the intra-vascular effect of *A. hydrophila* infection however, the cellular degenerative effect could not be improved. For induction of an effective role, silymarin should be administrated in early life stages⁷⁴. In accordance, silymarin with its flavonolignans, flavonoids (taxifolin, quercetin), and polyphenolic molecules⁴⁷ has a hepatoprotective effect mediated by its free radical scavenger effect with modulation of enzymes involved in cellular damage and cirrhosis⁷⁴. Generally, the hepatoprotective effect of silymarin could be summarized; as an anti-inflammatory, antioxidant, anti-proliferative, anti-lipidemic, anti-fibrotic, nuclear expression regulation, mitochondrial membrane stabilization, preserving hepatic mitochondrial bioenergetics, and decreased elevation of AST and ALT in serum^{75,76}.

Conclusion

Silymarin supplementation decreases serum levels of liver enzymes, low-density lipoprotein cholesterol, and cholesterol and significantly increases hepatic levels of glutathione peroxidase and catalase in Nile tilapia. Further, serum TP levels and circulating levels of IL-1 β and fish TNF- α were increased, while serum levels of IL-10 were decreased, in tilapia fed a diet containing silymarin. Finally, oxytetracycline treatment provided a RPL of 37.5% against *A. hydrophila* in Nile tilapia fed a silymarin-supplemented diet.

Data availability

Data are available on request from the corresponding author.

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References

- Wellington, K. & Jarvis, B. Silymarin: A review of its clinical properties in the management of hepatic disorders. *BioDrugs* **15**(7), 465–489. <https://doi.org/10.2165/00063030-200115070-00005> (2001).
- Jacobs, B. P. *et al.* Milk thistle for the treatment of liver disease: A systematic review and meta-analysis. *Am. J. Med.* **113**(6), 506–515. [https://doi.org/10.1016/S0002-9343\(02\)01244-5](https://doi.org/10.1016/S0002-9343(02)01244-5) (2002).
- Karkanis, A., Bilalis, D. & Efthimiadou, A. Cultivation of milk thistle (*Silybum marianum* L. Gaertn.), a medicinal weed. *Ind. Crops Prod.* **34**(1), 825–830. <https://doi.org/10.1016/j.indcrop.2011.03.027> (2011).
- Colturato, C. P. *et al.* Metabolic effects of silybinin in the rat liver. *Chem. Biol. Interact.* **195**(2), 119–132. <https://doi.org/10.1016/j.cbi.2011.11.006> (2012).
- Xiao, P. *et al.* Dietary silymarin supplementation promotes growth performance and improves lipid metabolism and health status in grass carp (*Ctenopharyngodon idellus*) fed diets with elevated lipid levels. *Fish. Physiol. Biochem.* **43**(1), 245–263. <https://doi.org/10.1007/s10695-016-0283-6> (2017).
- Wang, J. *et al.* Effects of silymarin on growth performance, antioxidant capacity and immune response in turbot (*Scophthalmus maximus* L.). *J. World. Aquac. Soc.* **50**(6), 1168–1181. <https://doi.org/10.1111/jwas.12614> (2019).
- Cao, L. *et al.* Hepatoprotective and antioxidant effects of dietary Angelica sinensis extract against carbon tetrachloride-induced hepatic injury in Jian Carp (*Cyprinus carpio* var Jian). *Aquac. Res.* **47**(6), 1852–1863. <https://doi.org/10.1111/are.12643> (2016).
- Jia, R. *et al.* The protective effect of silymarin on the carbon tetrachloride (CCl₄)-induced liver injury in common carp (*Cyprinus carpio*). *In Vitro Cell. Dev. Biol.* **49**(3), 155–161. <https://doi.org/10.1007/s11626-013-9587-3> (2013).
- Karimi, G., Vahabzadeh, M., Lari, P., Rashedinia, M. & Moshiri, M. “Silymarin”, a promising pharmacological agent for treatment of diseases. *Iran. J. Basic Med. Sci.* **14**(4), 308–317 (2011).
- Saller, R., Melzer, J., Reichling, J., Brignoli, R. & Meier, R. An updated systematic review of the pharmacology of silymarin. *J. Complement. Med. Res.* **14**(2), 70–80. <https://doi.org/10.1159/000100581> (2007).
- Metian, M., Troell, M., Christensen, V., Steenbeek, J. & Pouil, S. Mapping diversity of species in global aquaculture. *Rev. Aquac.* **12**(2), 1090–1100. <https://doi.org/10.1111/raq.12374> (2020).

12. Brummett, R. E., Alvial, A., Kibenge, F., Forster J., Burgos, J. M., Ibarra, R., & Nikuli, H. L. *Reducing disease risk in aquaculture* Agriculture and environmental services discussion paper No. 9. World Bank report number 88257-GLB, Washington, DC, (2014).
13. Stoffregen, D. A., Bowser, P. R. & Babish, J. G. Antibacterial chemotherapeutics for finfish aquaculture: A synopsis of laboratory and field efficacy and safety studies. *J. Aquat. Anim. Health*. **8**(3), 181–207. [https://doi.org/10.1577/1548-8667\(1996\)008%3c0181:ACFFAA%3e2.3.CO;2](https://doi.org/10.1577/1548-8667(1996)008%3c0181:ACFFAA%3e2.3.CO;2) (1996).
14. Riviere, J. E. & Spoo, J. W. Tetracycline antibiotics. In *Veterinary Pharmacology and Therapeutics* (eds Booth, N. H. & McDonald, L. E.) 784–796 (Iowa State University Press, 1995).
15. Mog, M. *et al.* Problems of antibiotic resistance associated with oxytetracycline use in aquaculture: A review. *J. Entomol. Zool. Stud* **8**, 1075–1082 (2020).
16. Sidhu, P. K. *et al.* Comparative pharmacokinetics of oxytetracycline in tilapia (*Oreochromis spp.*) maintained at three different salinities. *Aquaculture* **495**, 675–681 (2018).
17. Lulijwa, R., Rupia, E. J. & Alfaro, A. C. Antibiotic use in aquaculture, policies and regulation, health and environmental risks: A review of the top 15 major producers. *Rev. Aquac.* **12**(2), 640–663. <https://doi.org/10.1111/raq.12344> (2020).
18. Sherif, A. H. *et al.* Moringa oleifera mitigates oxytetracycline stress in *Oreochromis niloticus*. *Aquac. Res.* **53**(5), 1790–1799. <https://doi.org/10.1111/are.15707> (2022).
19. Lunden, T., Miettinen, S., Lönnström, L. G., Lilius, E. M. & Bylund, G. Influence of oxytetracycline and oxolinic acid on the immune response of rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* **8**(3), 217–230. <https://doi.org/10.1006/fsim.1998.0142> (1998).
20. Rodrigues, S., Antunes, S. C., Nunes, B. & Correia, A. T. Histopathological effects in gills and liver of *Sparus aurata* following acute and chronic exposures to erythromycin and oxytetracycline. *Environ. Sci. Pollut. Res.* **26**(15), 15481–15495. <https://doi.org/10.1007/s11356-019-04954-0> (2019).
21. Sherif, A. H., Eldessouki, E. A., Sabry, N. M. & Ali, N. G. The protective role of iodine and MS-222 against stress response and bacterial infections during Nile tilapia (*Oreochromis niloticus*) transportation. *Aquac. Int.* <https://doi.org/10.1007/s10499-022-00984-7> (2023).
22. Eldessouki, E. A., Salama, S. S. A., Mohamed, R. & Sherif, A. H. Using nutraceutical to alleviate transportation stress in the Nile tilapia. *Egypt J. Aquat. Biol. Fish.* **27**(1), 413–429. <https://doi.org/10.21608/ejabf.2023.287741> (2023).
23. Owatari, M. S. *et al.* Silymarin as hepatic protector and immunomodulator in Nile tilapia during *Streptococcus agalactiae* infection. *Fish shellfish Immunol.* **82**, 565–572. <https://doi.org/10.1016/j.fsi.2018.08.061> (2018).
24. Noga, E. J. *Fish Diseases Diagnosis and Treatment* 2nd edn. (Wiley-Blackwell, 2010).
25. Sherif, A. H., Gouda, M. Y., Naena, N. A. & Ali, A. H. Alternate weekly exchanges of feeding regime affect the diversity of intestinal microbiota and immune status of Nile tilapia *Oreochromis niloticus*. *Aquac. Res.* **51**(10), 4327–4339. <https://doi.org/10.1111/are.14778> (2020).
26. Stoskopf, M. K. Bacterial diseases of fresh water tropical fishes. In *Fish Medicine* (ed. Stoskopf, M. K.) 559–562 (Saunders Company, 1993).
27. Drabkin, D. W. Spectrophotometric studies. XIV. The crystallographic and optical properties of man Hbs in comparison with those of other species. *J. Biol. Chem.* **164**, 702–723 (1964).
28. Reitman, S. & Frankel, S. Determination of AST and ALT in serum. *Am. J. Clin. Pathol.* **28**, 56–68 (1957).
29. Wahlefeld, A. W. Enzymatic determination of triglycerides. In *Methods of Enzymatic Analysis* (ed. Bergmeyer, H. U.) 1931–1835 (Academic Press, 1974).
30. Allain, C. C., Poon, L. S. & Chan, C. S. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20**, 470–475 (1974).
31. Albers, N., Henderson, V. & Warnick, G. Enzymatic determination of high density lipoprotein cholesterol. *Sel. Methods Clin. Chem.* **10**, 91–99 (1983).
32. Friedewald, W. T., Leve, R. I. & Fredrickson, D. S. Estimation of the concentration of low-density lipoprotein. *Clin. Chem.* **18**, 499–502 (1972).
33. Weichsellbaum, T. E. An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. *Am. J. Clin. Pathol.* **16**(3), 40–49. https://doi.org/10.1093/ajcp/16.3_ts.40 (1946).
34. Doumas, B. T., Waston, W. A. & Biggs, H. G. Albumin standards and the measurements of serum albumin with bro-mocresol green. *Clin. Chim. Acta.* **31**, 87–96. [https://doi.org/10.1016/0009-8981\(71\)90365-2](https://doi.org/10.1016/0009-8981(71)90365-2) (1971).
35. Maurer, R. *Disc Electrophoresis and Related Technique of Polyacrylamide Gel Electrophoresis* (Water de Gruyter Berlin, 1971).
36. Glick, B. Serum protein electrophoresis pattern in acrylamide gel. Pattern from normal and bursa less birds. *Poultry Sci.* **47**(3), 807–814. <https://doi.org/10.3382/ps.0470807> (1968).
37. Mohandas, J., Marshall, J. J., Duggin, G. G., Horvath, J. S. & Tiller, D. Differential distribution of glutathione and glutathione related enzymes in rabbit kidney: Possible implications in analgesic neuropathy. *Biochem. Pharmacol.* **33**(11), 1801–1807 (1984).
38. Lartillot, S., Kedziora, P. & Athias, A. Purification and characterization of a new fungal catalase. *Prep. Biochem.* **18**(3), 241–246. <https://doi.org/10.1080/00327488808062526> (1988).
39. Sherif, A. H. & AbuLeila, R. H. Prevalence of some pathogenic bacteria in caged- Nile Tilapia (*Oreochromis Niloticus*) and their possible treatment. *Jordan J. Biol. Sci.* **15**(2), 239–247. <https://doi.org/10.54319/jjbs/150211> (2022).
40. Ruangroupan, L., Kitao, T. & Yoshida, T. (Protective efficacy of *Aeromonas hydrophila* vaccines in Nile tilapia. *Vet. Immunol. Immunopathol.* **12**(1–4), 345–350. [https://doi.org/10.1016/0165-2427\(86\)90139-X](https://doi.org/10.1016/0165-2427(86)90139-X) (1986).
41. Bancroft, D. J., Cook, C. H., Stirling, R. W. & Turer, D. R. *Manual of Histological Techniques and their Diagnostic Applications* (Churchill Livingstone, 1999).
42. Duncan. Multiple range and multiple “F” test. *Biometrics* **11**, 10, (1955)
43. SPSS. "Statistical and package for social science, SPSS for windows release14.0.0, 19 June, 2004." Standard Version, Copyright SPSS Inc. pp. 1989–2004, (2004).
44. Public Health Agency of Canada. The Honourable Leona Aglukkaq, P.C., M.P. Minister of Health, (2010).
45. Hassaan, M. S. *et al.* Effect of Silybum marianum seeds as a feed additive on growth performance, serum biochemical indices, antioxidant status, and gene expression of Nile tilapia, *Oreochromis niloticus* (L.) fingerlings. *Aquaculture* **509**, 178–187. <https://doi.org/10.1016/j.aquaculture.2019.05.006> (2019).
46. Ahmadi, K., Banaee, M., Vosoghei, A. R., Mirvaghefi, A. R. & Ataieimehr, B. Evaluation of the immunomodulatory effects of silymarin extract (Silybum marianum) on some immune parameters of rainbow trout, *Oncorhynchus mykiss* (Actinopterygii: Salmoniformes: Salmonidae). *Acta Ichthyologica Et Piscatoria* **42**(2), 13–120 (2012).
47. Surai, P. F. Silymarin as a natural antioxidant: An overview of the current evidence and perspectives. *Antioxidants* **4**(1), 204–247. <https://doi.org/10.3390/antiox4010204> (2015).
48. Ha, K. T. *et al.* Protective effect of Lycium chinense fruit on carbon tetrachloride-induced hepatotoxicity. *J. Ethnopharmacol.* **96**(3), 529–535 (2005).
49. Kvasnička, F., Biba, B., Ševčík, R., Voldřich, M. & Kratka, J. Analysis of the active components of silymarin. *J. Chromatogr. A.* **990**, 239–245 (2003).
50. Akrami, R., Gharaei, A., Mansour, M. R. & Galeshi, A. Effects of dietary onion (*Allium cepa*) powder on growth, innate immune response and hemato-biochemical parameters of beluga (*Huso huso* Linnaeus, 1754) juvenile. *Fish Shellfish Immunol.* **45**(2), 828–834. <https://doi.org/10.1016/j.fsi.2015.06.005> (2015).

51. Bernet, D., Schmidt, H., Wahli, T. & Burkhardt-Holm, P. Effluent from a sewage treatment works causes changes in serum chemistry of brown trout (*Salmo trutta* L.). *Ecotoxicol. Environ. Safety* **48**(2), 140–147. <https://doi.org/10.1006/eesa.2000.2012> (2001).
52. Banaee, M., Sureda, A., Mirvaghefi, A. R. & Rafei, G. R. Effects of long-term silymarin oral supplementation on the blood biochemical profile of rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol. Biochem.* **37**(4), 885–896. <https://doi.org/10.1007/s10695-011-9486-z> (2011).
53. Hassaan, M. S. & Soltan, M. A. Evaluation of essential oil of fennel and garlic separately or combined with *Bacillus licheniformis* on the growth, feeding behaviour, hemato-biochemical indices of *Oreochromis niloticus* (L.) fry. *J. Aquac. Res. Dev.* **7**, 422–429 (2016).
54. Sherif, A. H. *et al.* Lactobacillus plantarum ameliorates the immunological impacts of titanium dioxide nanoparticles (rutile) in *Oreochromis niloticus*. *Aquac. Res.* **53**, 3736–3747. <https://doi.org/10.1111/are.15877> (2022).
55. Shiau, S. Y., Gabaudan, J. & Lin, Y. H. Dietary nucleotide supplementation enhances immune responses and survival to *Streptococcus iniae* in hybrid tilapia fed diet containing low fish meal. *Aquac. Rep.* **2**, 77–81. <https://doi.org/10.1016/j.aqrep.2015.08.002> (2015).
56. Chand, N., Muhammad, D., Durrani, F. R., Qureshi, M. S. & Ullah, S. S. Protective effects of milk thistle (*Silybum marianum*) against aflatoxin B1 in broiler chicks. *Asian-Australas. J. Anim. Sci.* **24**(7), 1011–1018 (2011).
57. Ardó, L. *et al.* Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron enhance the non-specific immune response of Nile tilapia (*Oreochromis niloticus*) and resistance against *Aeromonas hydrophila*. *Aquaculture* **275**(1–4), 26–33. <https://doi.org/10.1016/j.aquaculture.2007.12.022> (2008).
58. Londero, E. P. *et al.* Rutin-added diet protects silver catfish liver against oxytetracycline-induced oxidative stress and apoptosis. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **239**, 108848 (2021).
59. USFWS. Approved Drugs for Use in Aquaculture, second ed. U.S. Fish and Wildlife Service's Aquatic Animal Drug Approval Partnership Program, American Fisheries Society's Fish Culture and Fish Health Sections, Association of Fish and Wildlife Agencies, and Fisheries and Water Resources Policy Committee's Drug Approval Working Group, (2015).
60. USFDA. Aquaculture. Silver Spring: U.S. Food and Drug Administration (Accessed 22 October 2017). <https://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/Aquaculture/efault.htm>, (2017).
61. Chen, J. *et al.* Antibiotics and food safety in aquaculture. *J. Agric. Food Chem.* **68**(43), 11908–11919 (2020).
62. Leal, J. F., Santos, E. B. & Esteves, V. I. Oxytetracycline in intensive aquaculture: water quality during and after its administration, environmental fate, toxicity and bacterial resistance. *Rev. Aquac.* **11**(4), 1176–1194. <https://doi.org/10.1111/raq.12286> (2019).
63. Abraham, T. J. *et al.* Dietary therapeutic dose of oxytetracycline negatively influences the antioxidant capacity and immune-related genes expression in Nile tilapia *Oreochromis niloticus* (L.). *Environ. Toxicol. Pharmacol.* **87**, 103685. <https://doi.org/10.1016/j.etap.2021.103685> (2021).
64. Bahrami, F. L., Morris, D. & Pourgholami, H. Tetracyclines: Drugs with huge therapeutic potential. *Mini-Rev. Med. Chem.* **12**(1), 44–52. <https://doi.org/10.2174/138955712798868977> (2012).
65. Guardiola, F. A., Cerezuela, R., Meseguer, J. & Esteban, M. A. Modulation of the immune parameters and expression of genes of gilthead seabream (*Sparus aurata* L.) by dietary administration of oxytetracycline. *Aquaculture* **334**, 51–57. <https://doi.org/10.1016/j.aquaculture.2012.01.003> (2012).
66. Abdel-Kader, M. S. *et al.* Evaluation of the hepatoprotective effect of combination between hinokiflavone and Glycyrrhizin against CCl4 induced toxicity in rats. *Saudi Pharm. J.* **26**(4), 496–503 (2018).
67. Mengesha, T., Gnanasekaran, N. & Mehare, T. Hepatoprotective effect of silymarin on fructose induced nonalcoholic fatty liver disease in male albino wistar rats. *BMC Complement. Med. Ther.* **21**(1), 1–13 (2021).
68. Abdelhamed, H., Ibrahim, I., Baumgartner, W., Lawrence, M. L. & Karsi, A. Characterization of histopathological and ultrastructural changes in channel catfish experimentally infected with virulent *Aeromonas hydrophila*. *Front. Microbiol.* **8**, 1519 (2017).
69. Islam, M. T., Mostafa, K. & Rashid, M. M. Histopathological studies of experimentally infected shing, *Heteropneustes fossilis* with *Aeromonas hydrophila* Bacteria. *Progress. Agric.* **19**(1), 89–96 (2008).
70. Julinta, R. B. *et al.* Histopathology and wound healing in oxytetracycline treated *Oreochromis niloticus* (L.) against *Aeromonas hydrophila* intramuscular challenge. *J. Aquac. Res. Dev.* **8**, 488 (2017).
71. Roy, A., Singha, J. & Abraham, T. J. Histopathology of *Aeromonas caviae* infection in challenged Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758). *Int. J. Aquac.* **8**(20), 151–155 (2018).
72. Afifi, S. H., Al-Thobiati, S. & Hazaa, M. S. Bacteriological and histopathological studies on *Aeromonas hydrophila* infection of Nile tilapia (*Oreochromis niloticus*) from fish farms in Saudi Arabia. *Assiut Vet. Med. J.* **42**(84), 195–205 (2000).
73. Yardimci, B. & Aydin, Y. Pathological findings of experimental *Aeromonas hydrophila* infection in Nile tilapia (*Oreochromis niloticus*). *Ankara Üniversitesi Veteriner Fakültesi Dergisi* **58**(1), 47–54 (2011).
74. Gillissen, A. & Schmidt, H. H. J. Silymarin as supportive treatment in liver diseases: A narrative review. *Adv. Ther.* **37**(4), 1279–1301 (2020).
75. Trappoliere, M. *et al.* Silybin, a component of silymarin, exerts anti-inflammatory and anti-fibrogenic effects on human hepatic stellate cells. *J. Hepatol.* **50**(6), 1102–1111 (2009).
76. Kwon, D., Jun, D. & Kim, Y. A novel mechanism involved in the enhancement of glutathione synthesis in liver by silymarin and its pharmacological significance. *Planta Med.* **80**(16), SL5 (2014).

Author contributions

All authors equally contributed to this work. All authors analysed and interpreted the data regarding gene expression and enzymes. All authors performed the experimental study and were major contributors to writing the manuscript. All authors read, reviewed and approved the final manuscript.

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Competing interests

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Additional information

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