EXTRACT OF GRAPE SEED ENHANCES THE GROWTH PERFORMANCE, HUMORAL AND MUCOSAL IMMUNITY, AND RESISTANCE OF COMMON CARP (*CYPRINUS CARPIO*) AGAINST *AEROMONAS HYDROPHILA*

Zohre Mehrinakhi¹, Ehsan Ahmadifar^{1*}, Najmeh Sheikhzadeh², Mohsen Shahriari Moghadam³,
Mahmoud A.O. Dawood⁴

¹Department of Fisheries, Faculty of Natural Resources, University of Zabol, Zabol, Iran

²Department of Food Hygiene and Aquatic Animals, Faculty of Veterinary Medicine,
University of Tabriz, Tabriz, Iran

³Department of Environmental Sciences, Faculty of Natural Resources, University of Zabol,
Zabol, Iran

⁴Department of Animal Production, Faculty of Agriculture, Kafrelsheikh University,
Kafrelsheikh, Egypt

*Corresponding author: Ehsan.Ahmadifar@uoz.ac.ir

Abstract

This study evaluated the effects of grape seed extract (GSE) on the growth performance, immune response, and disease resistance of common carp (*Cyprinus carpio*). Fish were distributed in four groups and fed diets with varying levels of GSE at 0, 10, 20, and 30 g/kg for 56 days. The final length in fish fed with 20 and 30 g GSE/kg diet significantly increased in comparison with the control group ($P<0.05$). The final weight and weight gain of fish fed with GSE were statistically higher than for the control diet, while the feed conversion ratio was lower in GSE groups than the control. Hematological parameters did not statistically improve with GSE supplemented diets ($P>0.05$). However, fish fed with 30 g GSE/kg diet displayed a significantly higher WBC count than the other group ($P<0.05$). The serum total protein and globulin in fish fed with 20 g GSE/kg diet significantly increased compared to the control ($P<0.05$). A similar pattern was noticed in results for serum and mucus lysozyme activity with a significant increase in fish fed with 20 and 30 g GSE/kg diet compared to the control ($P<0.05$). Total antibody levels in serum and mucus samples were also increased in fish by dietary GSE with maximum levels by 20 and 30 g GSE/kg diet. Besides, mucus protease activity was higher in fish that received 20 and 30 g GSE/kg diet with maximum level shown in fish fed with 30 g GSE/kg diet compared to the control group ($P<0.05$). Meanwhile, all groups showed higher mucus antibacterial activity against *Aeromonas hydrophila* with the highest activity in 30 g GSE/kg diet in comparison with the control group. The cumulative mortality was 36.67% when fish were fed with 30 g GSE/kg diet and challenged with *A. hydrophila*. However, the mortality rate was 40% and 53.33% in fish fed 20 and 30 g GSE/kg diet, respectively. This study suggests that supplementation of GSE could significantly enhance the growth performance, immune responses, and disease resistance against *A. hydrophila* in common carp.

Key words: common carp, grape seed extract, growth, immune response, *Aeromonas hydrophila*

Common carp (*Cyprinus carpio*) contributes to a large proportion of freshwater aquaculture production (Dawood et al., 2020; Jagruthi et al., 2014). Intensified aquaculture environment and high susceptibility to infectious disease make the industry vulnerable to disease outbreaks resulting in significant economic losses (Mehana et al., 2015). For instance, *Aeromonas hydrophila*, the causative agent of motile Aeromonas septicemia in fish, causes skin lesions and septicemia with necrosis in hematopoietic tissues and intestinal mucosa as well as focal necrosis in the heart, pancreas, and gonad tissues (Noga, 2010). The use of chemicals and antibiotics may enhance growth and disease resistance of fish; nevertheless, consequent hazardous impacts on fish and environment are unavoidable (Noga, 2010). Therefore, recent studies have been focused on finding safe and cost-effective alternatives (Al-Deriny et al., 2020; Lazado and Caipang, 2014; Mehana et al., 2015).

Medicinal plants have long been used to improve the performances and health condition of aquatic animals (Dawood et al., 2018). Several studies have considered the utilization of plant extracts, constituting numbers of active substances including flavonoids, phenolics, alkaloids, saponins, terpenoids, tannins, glycosides, steroids, or essential oils as supplements with no side effects (Amin et al., 2019; Moustafa et al., 2019; Srichaiyo et al., 2020; Van Doan et al., 2019 a). Additionally, it has been stated that the immune system and disease resistance of fish can be stimulated and enhanced through a wide range of medicinal plants (Adel et al., 2016 a; Awad and Awaad, 2017; Saeidi Asl et al., 2017). Moreover, plant extracts have been reported as an appetite stimulant and weight-gain enhancers in cultured fish (Adel et al., 2020; Ahmadifar et al., 2019 b; Sarhadi et al., 2020).

Grape seed extract (GSE) is a by-product of the processing of wine and grape (Kao et al., 2010). This additive is the primary source of monomeric phenolic compounds and dimeric, trimeric, and tetrameric procyanidins (Arslan et al., 2018; Lange et al., 2014; Nowshehri et al., 2015; Zhai et al., 2014). These active compounds have anti-mutagenic, anti-viral, anti-aging, anti-diabetic, and anti-inflammatory effects (Gokoglu and Yerlikaya, 2008; Nowshehri et al., 2015). In aquaculture, dietary GSE application was shown to improve the growth performance (Arslan et al., 2018; Lange et al., 2014; Zhai et al., 2014), antioxidant status (Arslan et al., 2018), and disease resistance (Arslan et al., 2018; Kao et al., 2010; Lange et al., 2014) in different fish and shellfish species.

To the best of our knowledge, no reports are available about the use of GSE in common carp diets based on the performance of growth as well as systemic and mucosal immune responses.

Thus, the current study evaluated the effects of GSE on the growth performance and immunity of common carp.

Material and methods

Fish husbandry and ethical statement

The current trial followed a standard operating procedure approved by the animal care and use committee of the Faculty of Natural Resources, University of Zabol, Zabol, Iran.

Diets and experimental design

Common carp (45.67 ± 3.32 g) purchased from a local farm (Zahak, Sistan and Baluchestan, Iran) were transferred to the University of Zabol. After arrival, fish were bathed with sodium chloride (2%) for 10 min and then kept in two 1000 L tanks for 10-day acclimation. Then fish were randomly allocated into fiberglass tanks. The water quality parameters were measured regularly and maintained at the following range: temperature, $25.40 \pm 2.50^\circ\text{C}$; dissolved oxygen, 6.40 ± 0.30 mg/L, and pH 7.5 ± 0.32 , respectively with 50% daily water exchange.

The composition and the preparation of the basal diet were done by following Ahmadifar et al. (2019 a) (Table 1). Four diets were formulated by mixing grape seed extract (GSE) with the basal diet at 0, 10, 20, and 30 g/kg (T0, T1, T2, and T3). The prepared diets were separately kept in plastic bags at 4°C to be used in the experiment. After the adaptation period, fish were accommodated in 12 fiberglass tanks (300 L) (20 fish/tank, triplicates) and fed different diets at the rate of 2–3% of body weight gain by hand three times a day (09:00; 12:00; 15:00) for 56 days.

Table 1. Formulation and proximate composition of the different experimental diets (% dry matter)

Compounds (%)		Chemical composition (%)	
Fish meal	33	Dry matter	89.50
Wheat flour	26	Crude protein	32.40
Soybean meal	12	Crude lipid	8.78
Wheat gluten	6	Ash	5.92
Corn flour	14	Fiber	11.20
Mineral premix ^a	3		
Vitamin premix ^a	3		
Binder ^b	2		
Anti fungal ^c	1		

^aPremix detailed by Hoseinifar et al. (2016).

^bAmet binderTM, Mehr Taban-e-Yazd, Iran.

^cToxiBan antifungal (Vet-A-Mix, Shenan-doah, IA).

Growth performance

In each tank, the initial and final weights of all fish were determined at the end of the trial. The fish survival rate during the 56-day trial was also calculated by counting the live individuals in each tank. Weight gain, thermal growth coefficient (TGC), and feed conversion ratio (FCR) were also expressed as:

Weight gain = W_2 (g) – W_1 (g) / 56 (days),

$TGC = 100 \times W_2$ (g)^{1/3} – W_1 (g)^{1/3} / 56 (days) $\times$ mean water temperature ($^\circ\text{C}$),

$FCR = \text{total feed consumption (g)} / \text{weight gain (g)}$,

Where W_2 and W_1 are the final weight and initial weight, respectively.

Blood and skin mucus sample collection

At the end of the feeding trial, five fish in each tank were sacrificed through bathing in clove oil (50 ml/L) (Hajek et al., 2006). Fish blood sample (2 mL) was gathered from the caudal vein by a non-heparinized syringe. Half of the fish blood was used for hematological examinations after adding heparin as anticoagu-

lants, and remaining blood was used for serum separation. After keeping at room temperature for two hours, fish blood was centrifuged at 1500 g for 15 min. Collected serum from each fish was kept in the Eppendorf tube at -80°C until use for different assays.

Ten fish from each tank were bath anesthetized in clove oil (50 ml/L), then the skin mucus was gathered with gentle scraping (Sheikhzadeh et al., 2012). Mucus from five fish was pooled to get enough samples for all assays. After homogenizing with Tris-buffered saline, mucus samples were centrifuged at 500 g for 30 min at 4°C . The final supernatants were filtered with Whatman no. 1 filter paper. Prepared mucus homogenates were stored at -80°C for specific enzyme activities.

Hematological and biochemical parameters

For counting red blood cell (RBC) and white blood cell (WBC), blood from each fish was diluted with Hayem and Turk solutions for RBC and WBC, respectively. Manual counting was done by hemocytometry using the Neubauer hemocytometer. Hemoglobin concentration (Hb) was determined with the cyanmethemoglobin method using Drabkin's solution. Hematocrit (Ht) was determined with micro hematocrit capillary tubes (Blaxhall and Daisley, 1973). Different parameters, including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) from erythrocytes, were determined according to Blaxhall and Daisley (1973). The WBC differential count was done by preparing the blood smears and staining in Wright-Giemsa.

Serum metabolites namely albumin, cholesterol, triglyceride, as well as metabolic enzyme activities including aspartate aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) were determined using Par Azmoun commercial kits (Tehran, Iran) and a biochemical-type automated analyzer by following the protocol of the manufacturer (Mansour-Ghanaei et al., 2019).

Serum and mucus immunological parameters

Serum and mucus lysozyme activity was measured according to a previous study by Demers and Bayne (1997). Briefly, 25 μL of serum or mucus samples were mixed with a 175 μL *Micrococcus lysodieticus* (Sigma Aldrich) suspension prepared in 0.1 M phosphate citrate buffer (pH 5.8). Turbidity change was calculated every 30 s for 10 min at 450 nm by a microplate reader. For standard, hen egg-white lysozyme (Sigma Aldrich) ranging from 0 to 20 $\mu\text{g/mL}$ in the phosphate citrate buffer was used. The equivalent unit of lysozyme activity for each sample was determined in comparison with the standard and expressed as $\mu\text{g/mL}$ serum or mucus samples.

Serum and mucus total antibody levels were measured according to methods described by Siwicki et al. (1994). Briefly, total protein level was determined before and after precipitation with 12% polyethylene glycol (Sigma Aldrich) using a modified micro-Lowry method (C-690; Sigma Aldrich).

Skin protease activity was measured by the azocasein hydrolysis method (Hosseini et al., 2016). Briefly, skin mucus (100 μL) was mixed with 0.7% azocasein solution (100 μL) and then incubated at 30°C for 20 h with continuous agita-

tion. Trichloroacetic acid (4.5%) was added to stop the reaction. The centrifuging at 15000 g for 5 min was done to obtain the supernatant. Inside the flat-bottomed microplates, the resulting supernatants were mixed with 1 N sodium hydroxide (100 μ L), and optical density was recorded at 450 nm. Protease activity was determined in comparison with the positive control, which was a trypsin solution (5 mg/mL) instead of a mucus sample with 100% protease activity.

Serum antibacterial activity of fish was determined against *A. hydrophila* (IBRC-M 10814) purchased from the Iranian Biological Resource Center. Briefly, *A. hydrophila* was cultured in tryptic soy broth (TSB) and then incubated at 25°C for 24 h and finally adjusted to 1×10^6 CFU/mL. This bacterial suspension was serially diluted (1:10) with phosphate-buffered saline (PBS) five times. Serum bactericidal activity was determined by incubating 2 ml of this diluted *A. hydrophila* suspension with 20 ml of serum in a micro-vial for 1 h at 37°C. For the control group, PBS was replaced with the serum sample. After incubation, the total number of colonies visible on a nutrient agar plate at 37°C for 24 h was considered as the total viable bacteria (Ghiassi et al., 2018).

Challenge test with *A. hydrophila*

A. hydrophila (IBRC-M 10814) cultured in TBS for 24 h at 25°C was adjusted to 1×10^8 CFU mL⁻¹, according to a previous study (Soltanian and Fereidouni, 2016). At the end of the trial, five fish per tank were intraperitoneally injected with 0.1 mL of *A. hydrophila*. For negative control, a group of 15 fish fed with the control diet during the feeding trial was injected with 0.1 mL of PBS. After the challenge, fish were kept under observation for 14 days to record the mortality. Dead fish were examined by culturing on tryptic soy agar to prove the presence of *A. hydrophila* in the fish carcass.

Statistical analysis

The values in this study were indicated as the mean value $\pm$ standard error. After determining the normality and homogeneity of variance, the one-way analysis of variance (ANOVA) was conducted using SPSS ver. 19.0 (IBM Corp. USA). The data values from different groups were compared using the Tukey post hoc test. The $P < 0.05$ was considered as statistically significant level in the analyses.

Results

Growth performance

In this study, initial weight and length showed no differences between all groups. The final length in fish fed with 20 and 30 g GSE/kg diet was significantly increased in comparison with the control group ($P < 0.05$). The final weight, weight gain, and TGC of fish fed with GSE were statistically higher than the control diet, while the FCR was lower than the control ($P < 0.05$). Meanwhile, during this 56-day feeding trial, no mortality occurred in all groups (Table 2).

Table 2. Effect of dietary grape seed extract on growth performances, and survival rate of common carp

	T0	T1	T2	T3
Initial weight (g)	45.66±1.22	45.77±0.80	45.51±1.10	45.75±1.45
Initial length (cm)	13.35±0.40	13.60±0.57	13.48±0.44	13.45±0.42
Final weight (g)	67.40±0.76 a	72.84±1.75 b	74.91±2.57 c	77.23±1.88 c
Final length (cm)	15.96±0.57 a	17.10±0.60 ab	17.35±0.81 b	17.84±0.63 b
Weight gain (g)	21.73±1.97 a	27.05±1.49 b	29.40±3.36 b	31.48±1.00 c
TGC	16.07±0.59 a	18.90±0.95 b	20.60±2.01 bc	21.58±0.36 c
FCR	2.73±0.26 b	2.19±0.12 a	2.02±0.22 a	1.88±0.06 a
Survival rate (%)	100	100	100	100

Values show means ±SE of three replicates. Values in each column with different letters were significantly different ($P<0.05$). T0: 0 g GSE/kg diet; T1: 10 g GSE/kg diet; T2: 20 g GSE/kg diet; T3: 30 g GSE/kg diet.

Blood parameters

Hematological parameters did not statistically differ with GSE supplemented diets ($P>0.05$). However, when fish were fed with 30 g GSE/kg diet, the WBC count significantly increased in comparison with other groups ($P<0.05$) (Table 3). Among the measured serum metabolites, total serum protein, and globulin were significantly increased in fish fed with 20 g GSE/kg diet compared to the control group ($P<0.05$) (Table 4). Conversely, the remaining metabolites and metabolic enzyme activities did not differ in different treated groups in comparison with the control ($P>0.05$).

Table 3. Effect of dietary grape seed extract on hematological parameters of common carp

	T0	T1	T2	T3
RBC $\times 10^6$ (cell/mm ³)	0.90±0.10	0.99±0.14	0.89±0.14	0.93±0.12
WBC $\times 10^3$ (cell/mm ³)	7.51±0.40 a	7.46±0.45 a	7.76±0.32 a	8.56±0.46 b
Hb (g/dL)	3.44±0.34	3.59±0.24	3.05±0.40	3.53±0.11
HCT (%)	24.67±3.06	27.00±1.00	24.00±3.00	28.67±4.16
MCV (fL)	278.74±66.6	275.36±28.95	278.9 $\pm$ 81.1	313.33±80.83
MCH (pg)	38.5 $\pm$ 5.52	36.49±2.76	34.76±5.48	38.33±6.32
MCHC (g/dL)	14.13±2.66	13.28±0.43	12.81±2.12	12.47±1.65
Lymphocyte (%)	95.67±2.31	93.00±4.00	97.67±2.52	99.00±1.00
Neutrophil (%)	4.33±2.31	6.67±4.51	2.33±2.52	0.67±0.58
Monocyte (%)	0.00±0.00	0.33±0.58	0.00±0.00	0.33±0.58

Values show means ±SE of three replicates. Values in each column with different letters were significantly different ($P<0.05$). T0: 0 g GSE/kg diet; T1: 10 g GSE/kg diet; T2: 20 g GSE/kg diet; T3: 30 g GSE/kg diet.

RBC, red blood cell; WBC, white blood cell; Hb, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

Table 4. Effect of dietary grape seed extract on serum biochemical parameters of common carp

	T0	T1	T2	T3
Total protein (g/dL)	3.41±0.34 a	3.50±0.47 a	4.06±0.32 b	3.53±0.51 a
Albumin (g/dL)	1.10±0.30 a	0.93±0.10 a	1.43±0.17 b	1.05±0.19 a
Cholesterol (mg/dL)	258.9±21.62	279.80±13.00	267.30±33.34	240.6±12.39
Triglyceride (mg/dL)	237.78±17.43	231.91±17.00	219.41±15.06	234.54±12.12
AST (IU/dL)	198.26±18.46	159.78±21.86	166.63±28.81	163.71±43.33
ALP (IU/dL)	240.18±25.21	235.08±36.18	247.21±34.57	212.65±23.39
ALT (IU/dL)	11.35±1.11	11.86±2.33	13.15±2.28	14.13±2.69

Values show means ±SE of three replicates. Values in each column with different letters were significantly different ($P<0.05$). T0: 0 g GSE/kg diet; T1: 10 g GSE/kg diet; T2: 20 g GSE/kg diet; T3: 30 g GSE/kg diet.

AST, aspartate aminotransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase.

(A)

(B)

Figure 1. Effect of dietary grape seed extract on (A) serum and (B) mucus lysozyme activity of common carp. Values show means ±SE of three replicates. Values with different letters were significantly different ($P<0.05$). T0: 0 g GSE/kg diet; T1: 10 g GSE/kg diet; T2: 20 g GSE/kg diet; T3: 30 g GSE/kg diet

Immune response

The results of serum and mucus lysozyme activities displayed a significant increase in fish fed with 20 and 30 g GSE/kg diet compared to the control diet ($P<0.05$) (Figures 1 A and 1 B). Total antibody levels in serum and mucus samples were also increased in fish that received different doses of GSE with maximum levels in fish fed 20 and 30 g GSE/kg diet (Figures 2 A and 2 B). Besides, mucus protease activity was higher in fish that received 20 and 30 g GSE/kg diet with maximum level shown in fish fed with 30 g GSE/kg diet compared to the control group ($P<0.05$) (Figure 3). Meanwhile, all groups showed higher mucus antibacterial activity against *A. hydrophila* with the highest activity in 30 g GSE/kg diet in comparison with the control group (Figure 4).

Figure 2. Effect of dietary grape seed extract on (A) serum and (B) mucus total antibody level of common carp. Values show means $\pm$ SE of three replicates. Values with different letters were significantly different ($P<0.05$). T0: 0 g GSE/kg diet; T1: 10 g GSE/kg diet; T2: 20 g GSE/kg diet; T3: 30 g GSE/kg diet

Figure 3. Effect of dietary grape seed extract on mucus protease activity of common carp. Values show means $\pm$ SE of three replicates. Values with different letters were significantly different ($P < 0.05$). T0: 0 g GSE/kg diet; T1: 10 g GSE/kg diet; T2: 20 g GSE/kg diet; T3: 30 g GSE/kg diet

Figure 4. Effect of dietary grape seed extract on serum antibacterial activity against *A. hydrophila* in common carp. Values show means $\pm$ SE of three replicates. Values with different letters were significantly different ($P < 0.05$). T0: 0 g GSE/kg diet; T1: 10 g GSE/kg diet; T2: 20 g GSE/kg diet; T3: 30 g GSE/kg diet

***A. hydrophila* challenge**

The cumulative mortality was 36.67% when fish were fed with 30 g GSE/kg diet. However, the mortality rate was 40% and 53.33% in fish fed 20 and 30 g GSE/kg diet, respectively. The infected fish fed with control diet recorded 63% mortality while the uninfected fish fed the control diet displayed no death (Figure 5).

Figure 5. The cumulative mortality of *A. hydrophila*-infected common carp fed with grape seed extract recorded for 14 days (N=30). T0: 0 g GSE/kg diet; T1: 10 g GSE/kg diet; T2: 20 g GSE/kg diet; T3: 30 g GSE/kg diet

Discussion

Medicinal plants have widely attracted the aquaculture researchers because of their beneficial influence on fish such as improving the innate immune responses, improving growth, and feed digestibility (Adel et al., 2016 b; Mahmoud et al., 2019; Van Doan et al., 2019 b, c). These additives could augment the growth of beneficial microbial colonies in the digestive tract, which leads to enhance feed intake and weight gain of various aquatic species (Safari et al., 2017). Increased resistance against infectious diseases of aquatic species following the administration of different medicinal plants was also shown previously (Van Doan et al., 2019 b, c; Yin et al., 2008).

It has been reported that fish growth performance and immune responses can be induced by several materials such as a plant extract. The grape seed extract contains several bioactive substances such as vitamins C and E, and fatty acids that can stimulate the immune system by the production of bioactive molecules (immunoglobulin, lysozyme, complement) and increasing the activity of phagocytosis (Arslan et al., 2018; Gokoglu and Yerlikaya, 2008; Kao et al., 2010; Lange et al., 2014). Hence, the medicinal utilization of grape seed extract (GSE) is due to the presence of the mentioned bioactive compounds, which can also affect fish growth and immunity.

Weight gain and growth rate are directly correlated with animal capability for digestion and absorption of nutrients (Magouz et al., 2020). Medicinal plants have been used to improve nutrition in human for a long time, and their use has drawn considerable attention in livestock, poultry, and aquatic animals (El-Deep et al., 2019; Saleh et al., 2019; Shekarabi et al., 2019). The current study revealed improved growth performance in fish fed GSE. In parallel, the positive effects of dietary GSE and grape seed oil in tilapia (*Oreochromis niloticus*) and rainbow trout were observed (Arslan et al., 2018; Zhai et al., 2014). The presence of polyphenolic compounds in diet might protect the intestinal mucosa against different pathogens and oxidative damage, thereby leading to better absorption of nutrients (Abu Hafsa et al., 2018). Meanwhile, polyphenols can perform the role of prebiotics by enhancing the composition of the gut microbial community, which results in an impact on host health (Ozdal et al., 2016).

Biochemical and hematological composition of blood can provide valuable information about animal health and is used to monitor metabolic disturbance and disease as a result of biotic and abiotic factors (Dossou et al., 2018; El Basuini et al., 2016). Additionally, these parameters have been assessed as health indicators for the evaluation of immunostimulants' effect on fish (Dawood et al., 2017). The present study demonstrated that dietary GSE could modify the WBC count, which increased with administration of GSE, indicating the immunostimulatory potential of grape seed against infection (Talpur and Ikhwannuddin, 2012).

It has been known for a long time that the number of RBC and WBC has a direct relationship with the immune response in the fish (Ainsworth, 1992). In the current study, we observed that 3% supplementation of GSE in the diet increased WBC significantly, which is in alignment with the results of other studies that evaluated medicinal plants in fish (Van Doan et al., 2019 b; Yin et al., 2008). This might be the result of the increased vitamin C, carotene, calcium, and iron availability in the circulatory system due to improved absorption in the intestine (Arslan et al., 2018; Gokoglu and Yerlikaya, 2008; Kao et al., 2010; Lange et al., 2014).

In parallel, total protein, globulin, and total antibody levels were increased in fish fed GSE. Immunoglobulin, globulin, lysozyme, and alternative complement pathway have bactericidal properties and play a role in the immune defense (Vallejos-Vidal et al., 2016). It has been shown that medicinal plant supplementation can elevate the levels of innate immune response components (Ji et al., 2017; Magnadottir, 2010; Uribe et al., 2011). In the current study, GSE could augment the lysozyme activity in common carp serum. Similarly, feeding tilapia fingerlings with GSE increased the serum lysozyme activity (Zhai et al., 2014). All these results show the potential role of GSE in augmenting the systemic immune responses in common carp. Enhanced blood IgM in the current study is in line with the increased lysozyme activity, suggesting immunomodulatory effects of GSE on carp after *A. hydrophila* challenge.

Fish health relies on two non-specific and specific defense lines (Uribe et al., 2011). The skin mucus and phagocytes are of the first and significant lines of non-specific immunity in fish, which are triggered by various immunostimulants like glucans, growth hormone, vitamin C, etc. (Dawood, 2016; Lazado and Caipang,

2014). Despite the numerous studies on the impact of medicinal plants on fish, there is still a lot to be known, especially on the skin mucus activities (Van Doan et al., 2019 a). Results of the current study showed that lysozyme activity was increased in fish skin mucus following the administration of GSE, which is in parallel with results achieved in fish mucus. The presence of lysozyme enzyme in different organs or tissues in various fish species, including ova, serum, and mucus, shows continuous contact with pathogenic microorganisms (Ji et al., 2017). In this study, higher protease activity in common carp fed with GSE was also shown. This study was in line with previous studies proving the positive effects of different immunostimulants, including Ergosan and Persian hogweed (*Heracleum persicum*) on the protease activity in fish mucosal tissues (Hoseinifar et al., 2016; Sheikhzadeh et al., 2012). Proteases have protective roles against different pathogens by cleaving their proteins direct or enhancing the mucus consistency and sloughing the mucus indirectly. This phenomenon finally results in pathogen removal from the body surface (Sheikhzadeh et al., 2012). Meanwhile, applying the immunomodulatory function by activating other immunological components, such as complement and immunoglobulin, was also shown in mammals (Palaksha et al., 2008). In parallel, in this study, a higher antibody level was also demonstrated in fish treated with GSE.

Although there is a plenty of research on controlling motile *Aeromonas* septicemia disease in aquaculture using natural products (Ardó et al., 2008), the mortalities associated with the disease outbreaks still constitute a significant problem in fish farming (Fernandes et al., 2019). The used GSE in the present study was efficient in the treatment of various human and animal diseases, being rich in flavonoids and polyphenols in addition to tannins, which contribute to enhancing the immune system and antioxidative response (Nowshehri et al., 2015). The cumulative mortality rate is one of the visual parameters indicating the immune effect of an immunostimulant in a challenging experiment. In the current study, the lowest mortality rate against *A. hydrophila* was shown in fish that received a high dose of GSE, followed by medium and low dose groups. This result suggests that GSE can augment different humoral factors in the fish host during pathogen invasion. Furthermore, the skin mucus test also showed strong antibacterial effects against *A. hydrophila* in fish treated with GSE with the highest level in the high dose group. Fish mucosal tissues with immune-related components potentiate the overall physiological response, starting the systemic immune responses upon stimulation (Vallejos-Vidal et al., 2016). Therefore, both mucosal and systemic immune stimulation after feeding GSE might enhance the fish resistance against *A. hydrophila*.

Conclusion

In conclusion, the inclusion of the extract from grape seed improves the growth performance, serum and mucosal immune responses of common carp. Also, GSE resulted in increased resistance against *A. hydrophila*. Therefore, GSE could be used as a promising natural antibacterial additive for protecting the fish from infectious. The optimum dietary GSE is 20 to 30 g/kg diet for improving the performances of common carp.

Acknowledgments

The authors are thankful to the Research Affairs of the University of Zabol for financial support (Grant code: UOZ-GR-9618-104).

Disclosure statement

The authors reported no potential conflict of interest.

References

- Abu Hafsa S., Ibrahim S. (2018). Effect of dietary polyphenol-rich grape seed on growth performance, antioxidant capacity and ileal microflora in broiler chicks. *J. Anim. Physiol. Anim. Nutr.*, 102: 268–275.
- Adel M., Yeganeh S., Dadar M., Sakai M., Dawood M.A.O. (2016 a). Effects of dietary *Spirulina platensis* on growth performance, humoral and mucosal immune responses and disease resistance in juvenile great sturgeon (*Huso huso* Linnaeus, 1754). *Fish Shellfish Immunol.* 56: 436–444.
- Adel M., Yeganeh S., Dadar M., Sakai M., Dawood M.A. (2016 b). Effects of dietary *Spirulina platensis* on growth performance, humoral and mucosal immune responses and disease resistance in juvenile great sturgeon (*Huso huso* Linnaeus, 1754). *Fish Shellfish Immunol.*, 56: 436–444.
- Adel M., Dawood M.A.O., Shafiei S., Sakhaie F., Shekarabi S.P.H. (2020). Dietary *Polygonum minus* extract ameliorated the growth performance, humoral immune parameters, immune-related gene expression and resistance against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 519: 734738.
- Ahmadifar E., Sheikhzadeh N., Roshanaei K., Dargahi N., Faggio C. (2019 a). Can dietary ginger (*Zingiber officinale*) alter biochemical and immunological parameters and gene expression related to growth, immunity and antioxidant system in zebrafish (*Danio rerio*)? *Aquaculture*, 507: 341–348.
- Ahmadifar E., Dawood M.A.O., Moghadam M.S., Sheikhzadeh N., Hoseinifar S.H., Musthafa M.S. (2019 b). Modulation of immune parameters and antioxidant defense in zebrafish (*Danio rerio*) using dietary apple cider vinegar. *Aquaculture*, <https://doi.org/10.1016/j.aquaculture.2019.734412>
- Ainsworth A.J. (1992). Fish granulocytes: morphology, distribution, and function. *Ann. Rev. Fish Dis.*, 2: 123–148.
- Al-Deriny S.H., Dawood M.A.O., Elbialy Z.I., El-Tras W.F., Mohamed R.A. (2020). Selenium nanoparticles and *Spirulina* alleviate growth performance, hemato-biochemical, immune-related genes, and heat shock protein in Nile tilapia (*Oreochromis niloticus*). *Biol. Trace Elem. Res.*, 10.1007/s12011-020-02096-w.
- Amin A., El Asely A., Abd El-Naby A.S., Samir F., El-Ashram A., Sudhakaran R., Dawood M.A.O. (2019). Growth performance, intestinal histomorphology and growth-related gene expression in response to dietary *Ziziphus mauritiana* in Nile tilapia (*Oreochromis niloticus*). *Aquaculture*, 512: 734301.
- Ardó L., Yin G., Xu P., Váradi L., Szigeti G., Jeney Z., Jeney G. (2008). 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: 26–33.
- Arslan G., Sönmez A.Y., Yank T. (2018). Effects of grape *Vitis vinifera* seed oil supplementation on growth, survival, fatty acid profiles, antioxidant contents and blood parameters in rainbow trout *Oncorhynchus mykiss*. *Aquaculture Res.*, 49: 2256–2266.
- Awad E., Awad A. (2017). Role of medicinal plants on growth performance and immune status in fish. *Fish Shellfish Immunol.*, 67: 40–54.
- Blaxhall P., Daisley K. (1973). Routine haematological methods for use with fish blood. *J. Fish Biol.*, 5: 771–781.

- Dawood M.A., Koshio S., Ishikawa M., El-Sabagh M., Yokoyama S., Wang W.L., Yukun Z., Olivier A. (2017). Physiological response, blood chemistry profile and mucus secretion of red sea bream (*Pagrus major*) fed diets supplemented with *Lactobacillus rhamnosus* under low salinity stress. *Fish Physiol. Biochem.*, 43: 179–192.
- Dawood M.A.O. (2016). Effect of various feed additives on the performance of aquatic animals. Kagoshima University.
- Dawood M.A.O., Koshio S., Esteban M.Á. (2018). Beneficial roles of feed additives as immunostimulants in aquaculture: a review. *Rev. Aquacult.*, 10: 950–974.
- Dawood M.A.O., Eweedah N.M., Moustafa E.M., El-Sharawy M.E., Soliman A.A., Amer A.A., Atia M.H. (2020). Copper nanoparticles mitigate the growth, immunity, and oxidation resistance in common carp (*Cyprinus carpio*). *Biol. Trace Elem. Res.*, 10.1007/s12011-020-02068-0.
- Demers N.E., Bayne C.J. (1997). The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. *Develop. Comp. Immunol.*, 21: 363–373.
- Dossou S., Koshio S., Ishikawa M., Yokoyama S., Dawood M.A.O., El Basuni M.F., Olivier A., Zaineldin A.I. (2018). Growth performance, blood health, antioxidant status and immune response in red sea bream (*Pagrus major*) fed *Aspergillus oryzae* fermented rapeseed meal (RM-Koji). *Fish Shellfish Immunol.*, 75: 253–262.
- El Basuni M.F., El-Hais A.M., Dawood M.A.O., Abou-Zeid A.E.-S., El-Damrawy S.Z., Khalafalla M.M.E.L.S., Koshio S., Ishikawa M., Dossou S. (2016). Effect of different levels of dietary copper nanoparticles and copper sulfate on growth performance, blood biochemical profiles, antioxidant status and immune response of red sea bream (*Pagrus major*). *Aquaculture*, 455: 32–40.
- El-Deep M.H., Dawood M.A.O., Assar M.H., Ijiri D., Ohtsuka A. (2019). Dietary *Moringa oleifera* improves growth performance, oxidative status, and immune related gene expression in broilers under normal and high temperature conditions. *J. Therm. Biol.*, 82: 157–163.
- Fernandes D.C., Eto S.F., Funnicielli M.I., Fernandes C.C., Charlie-Silva I., Bello M.A., Pizauro J.M. (2019). Immunoglobulin Y in the diagnosis of *Aeromonas hydrophila* infection in Nile tilapia (*Oreochromis niloticus*). *Aquaculture*, 500: 576–585.
- Ghiassi M., Binaii M., Naghavi A., Rostami H.K., Nori H., Amerizadeh A. (2018). Inclusion of *Pediococcus acidilactici* as probiotic candidate in diets for beluga (*Huso huso*) modifies biochemical parameters and improves immune functions. *Fish Physiol. Biochem.*, 44: 1099–1107.
- Gokoglu N., Yerlikaya P. (2008). Inhibition effects of grape seed extracts on melanosis formation in shrimp (*Parapenaeus longirostris*). *Int. J. Food Sci. Technol.*, 43: 1004–1008.
- Hajek G., Klyszejko B., Dziaman R. (2006). The anaesthetic effects of clove oil on common carp, *Cyprinus carpio* L. *Acta Ichthyol. Piscatoria*, 2: 93–97.
- Hoseinifar S.H., Zoheiri F., Lazado C.C. (2016). Dietary phytoimmunostimulant Persian hogweed (*Heracleum persicum*) has more remarkable impacts on skin mucus than on serum in common carp (*Cyprinus carpio*). *Fish Shellfish Immunol.*, 59: 77–82.
- Jagruthi C., Yogeshwari G., Anbazahan S.M., Mari L.S.S., Arockiaraj J., Mariappan P., Sudhakar G.R.L., Balasundaram C., Harikrishnan R. (2014). Effect of dietary astaxanthin against *Aeromonas hydrophila* infection in common carp, *Cyprinus carpio*. *Fish Shellfish Immunol.*, 41: 674–680.
- Ji L., Sun G., Li J., Wang Y., Du Y., Li X., Liu Y. (2017). Effect of dietary β -glucan on growth, survival and regulation of immune processes in rainbow trout (*Oncorhynchus mykiss*) infected by *Aeromonas salmonicida*. *Fish Shellfish Immunol.*, 64: 56–67.
- Kao T.-T., Tu H.-C., Chang W.-N., Chen B.-H., Shi Y.-Y., Chang T.-C., Fu T.-F. (2010). Grape seed extract inhibits the growth and pathogenicity of *Staphylococcus aureus* by interfering with dihydrofolate reductase activity and folate-mediated one-carbon metabolism. *Int. J. Food Microbiol.*, 141: 17–27.
- Lange B., Currie K.-L., Howarth G.S., Stone D.A. (2014). Grape seed extract and dried macroalgae, *Ulva lactuca* Linnaeus, improve survival of greenlip abalone, *Haliotis laevis* Donovan, at high water temperature. *Aquaculture*, 433: 348–360.
- Lazado C.C., Caipang C.M.A. (2014). Mucosal immunity and probiotics in fish. *Fish Shellfish Immunol.*, 39: 78–89.

- Magnadottir B. (2010). Immunological control of fish diseases. *Marine Biotechnol.*, 12: 361–379.
- Magouz F.I., Dawood M.A.O., Salem M.F.I., Mohamed A.A.I. (2020). The effects of fish feed supplemented with Azolla meal on the growth performance, digestive enzyme activity, and health condition of genetically-improved farmed tilapia (*Oreochromis niloticus*). *Ann. Anim. Sci.*, 20: 1029–1045.
- Mahmoud H., Dawood M.A., Assar M.H., Ijiri D., Ohtsuka A. (2019). Dietary *Moringa oleifera* improves growth performance, oxidative status, and immune related gene expression in broilers under normal and high temperature conditions. *J. Therm. Biol.*, 82: 157–163.
- Mansour-Ghanaei R., Mansour-Ghanaei F., Naghipour M., Joukar F. (2019). Biochemical markers and lipid profile in nonalcoholic fatty liver disease patients in the PERSIAN Guilan cohort study (PGCS), Iran. *J. Fam. Med. Prim. Care.*, 8: 923–928.
- Mehana E., Rahmani A., Aly S. (2015). Immunostimulants and fish culture: an overview. *Ann. Res. Rev. Biol.*, 5: 477–489.
- Moustafa E.M., Dawood M.A.O., Assar D.H., Omar A.A., Elbially Z.I., Farrag F.A., Shukry M., Zayed M.M. (2019). Modulatory effects of fenugreek seeds powder on the histopathology, oxidative status, and immune related gene expression in Nile tilapia (*Oreochromis niloticus*) infected with *Aeromonas hydrophila*. *Aquaculture*, 734589.
- Noga E.J. (2010). Fish disease: diagnosis and treatment. John Wiley & Sons.
- Nowshetri J.A., Bhat Z.A., Shah M.Y. (2015). Blessings in disguise: Bio-functional benefits of grape seed extracts. *Food Res. Int.*, 77: 333–348.
- Ozdal T., Sela D.A., Xiao J., Boyacioglu D., Chen F., Capanoglu E. (2016). The reciprocal interactions between polyphenols and gut microbiota and effects on bioaccessibility. *Nutrients*, 8: 78.
- Palaksha K., Shin G.-W., Kim Y.-R., Jung T.-S. (2008). Evaluation of non-specific immune components from the skin mucus of olive flounder (*Paralichthys olivaceus*). *Fish Shellfish Immunol.*, 24: 479–488.
- Saeidi Asl M.R., Adel M., Caipang C.M.A., Dawood M.A.O. (2017). Immunological responses and disease resistance of rainbow trout (*Oncorhynchus mykiss*) juveniles following dietary administration of stinging nettle (*Urtica dioica*). *Fish Shellfish Immunol.*, 71: 230–238.
- Safari R., Hoseinifar S.H., Van Doan H., Dadar M. (2017). The effects of dietary Myrtle (*Myrtus communis*) on skin mucus immune parameters and mRNA levels of growth, antioxidant and immune related genes in zebrafish (*Danio rerio*). *Fish Shellfish Immunol.*, 66: 264–269.
- Saleh A.A., Kirrella A.A., Dawood M.A.O., Ebeid T.A. (2019). Effect of dietary inclusion of cumin seed oil on the performance, egg quality, immune response and ovarian development in laying hens under high ambient temperature. *J. Anim. Physiol. Anim. Nutr.*, 103: 1810–1817.
- Sarhadi I., Alizadeh E., Ahmadi Far E., Adineh H., Dawood M.A.O. (2020). Skin mucosal, serum immunity and antioxidant capacity of common carp (*Cyprinus carpio*) fed artemisia (*Artemisia annua*). *Ann. Anim. Sci.*, 20: 1011–1027.
- Sheikhzadeh N., Pashaki A.K., Nofouzi K., Heidarieh M., Tayefi-Nasrabadi H. (2012). Effects of dietary Ergosan on cutaneous mucosal immune response in rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.*, 32: 407–410.
- Shekarabi S.P.H., Omid A.H., Dawood M.A.O., Adel M., Avazeh A., Heidari F. (2019). Effect of black mulberry (*Morus nigra*) powder on growth performance, biochemical parameters, blood carotenoid concentration, and fillet color of rainbow trout. *Ann. Anim. Sci.*, 20: 125–136.
- Siwicki A.K., Anderson D.P., Rumsey G.L. (1994). Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. *Vet. Immunol. Immunopathol.*, 41: 125–139.
- Soltanian S., Fereidouni M.S. (2016). Effect of Henna (*Lawsonia inermis*) extract on the immunity and survival of common carp, *Cyprinus carpio* infected with *Aeromonas hydrophila*. *Int. Aquatic Res.*, 8: 247–261.
- Srichaiyo N., Tongsiri S., Hoseinifar S.H., Dawood M.A.O., Jaturasitha S., Esteban M.Á., Ringø E., Van Doan H. (2020). The effects gotu kola (*Centella asiatica*) powder on growth performance, skin mucus, and serum immunity of Nile tilapia (*Oreochromis niloticus*) fingerlings. *Aquacult. Rep.*, 16: 100239.

- Talpur A.D., Ikhwanuddin M. (2012). Dietary effects of garlic (*Allium sativum*) on haemato-immunological parameters, survival, growth, and disease resistance against *Vibrio harveyi* infection in Asian sea bass, *Lates calcarifer* (Bloch). *Aquaculture*, 364: 6–12.
- Uribe C., Folch H., Enríquez R., Moran G. (2011). Innate and adaptive immunity in teleost fish: a review. *Vet. Med.*, 56: 486–503.
- Vallejos-Vidal E., Reyes-López F., Teles M., MacKenzie S. (2016). The response of fish to immunostimulant diets. *Fish Shellfish Immunol.*, 56: 34–69.
- Van Doan H., Hoseinifar S.H., Chitmanat C., Jaturasitha S., Paolucci M., Ashouri G., Dawood M.A.O., Esteban M.Á. (2019 a). The effects of Thai ginseng, *Boesenbergia rotunda* powder on mucosal and serum immunity, disease resistance, and growth performance of Nile tilapia (*Oreochromis niloticus*) fingerlings. *Aquaculture*, 734388.
- Van Doan H., Hoseinifar S.H., Sringarm K., Jaturasitha S., Khamlor T., Dawood M.A., Esteban M.Á., Soltani M., Musthafa M.S. (2019 b). Effects of elephant's foot (*Elephantopus scaber*) extract on growth performance, immune response, and disease resistance of Nile tilapia (*Oreochromis niloticus*) fingerlings. *Fish Shellfish Immunol.*, 93: 328–335.
- Van Doan H., Hoseinifar S.H., Sringarm K., Jaturasitha S., Yuangsoi B., Dawood M.A., Esteban M.Á., Ringø E., Faggio C. (2019 c). Effects of Assam tea extract on growth, skin mucus, serum immunity and disease resistance of Nile tilapia (*Oreochromis niloticus*) against *Streptococcus agalactiae*. *Fish Shellfish Immunol.*, 93: 428–435.
- Yin G., Ardo L., Jeney Z., Xu P., Jeney G. (2008). Chinese herbs (*Lonicera japonica* and *Ganoderma lucidum*) enhance non-specific immune response of tilapia, *Oreochromis niloticus*, and protection against *Aeromonas hydrophila*. *Diseases in Asian Aquaculture VI. Fish Health Section*, Asian Fisheries Society, Manila, Philippines, 269–282.
- Zhai S.-W., Lu J.-J., Chen X.-H. (2014). Effects of dietary grape seed proanthocyanidins on growth performance, some serum biochemical parameters and body composition of tilapia (*Oreochromis niloticus*) fingerlings. *It. J. Anim. Sci.*, 13: 3357.

Received: 27 XII 2019

Accepted: 5 V 2020