

Full length article

Dietary *Syzygium cumini* leaf extract influences growth performance, immunological responses and gene expression in pathogen-challenged *Cyprinus carpio*

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ABSTRACT

This study evaluated the effects of jamun leaf extract (JLE) as a feed supplement on growth performance, haemato-immunological, oxidative stress-related parameters, and cytokine gene expression in *Cyprinus carpio* challenged with *Aeromonas hydrophila*. Diets containing four different JLE concentrations, that is, 0 (basal diet), 5 (JLE5), 10 (JLE10), and 15 g kg⁻¹ (JLE15), were fed to carp (6.17 ± 0.43 g) for eight weeks. Growth performance was significantly higher in JLE10. Haemato-immunological and antioxidant parameters were determined in fish at 48 h post-challenge with *A. hydrophila*. The cumulative survival was highest in JLE10 (69.69%) 14 days post-challenge. Serum protein (2.18 ± 0.06 g dL⁻¹), lysozyme (32.38 ± 1.2 U mL⁻¹), alternative complement pathway (70.43 ± 1.61 U mL⁻¹), phagocytic activity (21.18 ± 0.48%), respiratory burst activity (0.289 ± 0.09 OD_{630nm}), and immunoglobulin levels (6.67 ± 0.36 U mg mL⁻¹) were significantly higher in JLE10 than in the control. Serum alanine aminotransferase (44.06 ± 1.62 Unit mL⁻¹), aspartate aminotransferase (31.58 ± 1.82 Unit mL⁻¹), and malondialdehyde (2.57 ± 0.26 nmol mL⁻¹) levels were lower in JLE10 than in the control ($p < 0.05$), whereas myeloperoxidase activity was significantly higher in JLE5 and JLE10 than in the control. Superoxide dismutase levels in the serum were higher ($p < 0.05$) in JLE5 and JLE10 than in the other groups. Gene expression analysis revealed that the mRNA expression of pro-inflammatory cytokines *TNF-α* and *IL-1β* was upregulated ($p < 0.05$) in the liver, head-kidney, and intestine of challenged carp in JLE10. The signalling molecule *NF-κB p65* was upregulated in lymphoid organs in JLE10 but not in the liver. The anti-inflammatory cytokine *IL-10* was significantly downregulated in challenged carp in JLE10 compared with that in the control. Quadratic regression analysis showed that optimal dietary JLE was estimated to be 9.03–10.15 g kg⁻¹ to maximize the growth performance. Results of the present study revealed that dietary JLE at 10 g kg⁻¹ can significantly improve the immunity and disease resistance of *C. carpio*. Thus, JLE is a promising food additive for carp aquaculture.

1. Introduction

Aquaculture is one of the fastest-growing food-producing sectors and is an ideal source of high-quality protein and micronutrients in humans [1]. Global fisheries and aquaculture production is up from 148.1

million metric tons (mmt) in 2010 to 184.6 mmt in 2022 [2]. Yet there is a need to increase aquaculture production to meet the protein needs of humans. However, intensive aquaculture often generates stressors. When fish are subjected to severe stress conditions, an acute drop in oxygen causes irregular oxidative reactions that lead to excessive

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amounts of reactive oxygen species (ROS) being produced [3]. Any change in the equilibrium between antioxidants and ROS, in support of ROS, is considered oxidative stress. Oxidative stress weakens the immune system of fish and renders them susceptible to various bacterial, parasitic, fungal, and viral pathogens [4]. The use of antibiotics and chemotherapeutic drugs in aquaculture leads to drug-resistant pathogens, immune suppression in fish, environmental pollution, and the accumulation of chemical residues in fish, which can potentially be hazardous to humans [5]. The need to replace synthetic drugs with eco-friendly options is becoming increasingly important. Currently, research approaches are being extended to vaccination, probiotics, herbal plants, and immunostimulants. However, the success rate of vaccines varies because they are species-specific, and developing vaccines against intracellular pathogens is challenging. Natural immunostimulants are attractive alternatives for sustainable aquaculture because they are biodegradable, eco-friendly, often easily available, and have little or no side effects on humans [6].

A wide range of investigations has revealed that medicinal plants could be potential substitutes for antibiotics in fish farming [7]. Plant extracts show various activities such as growth promotion, appetite enhancement, stress management, immunostimulation, anti-pathogen activity, and maturation of culture species owing to the presence of active compounds, such as alkaloids, tannins, terpenoids, glycosides, saponins, phenolics, flavonoids, and essential oils [7–9]. They also facilitate growth, reduce stress, and have antimicrobial properties [10]. Whole plants, extracted plant compounds, or plant parts (leaves, seeds, or roots) have been used as feed supplements in aquaculture. It has been demonstrated that various plant substances, including *Astragalus polysaccharides* [1], grape pomace flour [10], azadirachtin [11], dehydrated lemon peels [12], and extracts of *Pandanus tectorius* [13], ginger [14], date palm fruit [15], *Origanum majorana* [16], guava leaf [GLE; 17], *Rehmannia glutinosa* (or root powder) [18], *Artemisia absinthium* [19], and dandelion [20] have been found to improve immune responses and disease resistance in fish. However, the effects of many herbal plants and their compounds on fish yet to be investigated in detail.

Syzygium cumini (java plum or jamun tree) is a very important medicinal plant that has long been used in traditional medicine. It is widely available in Bangladesh, India, Indonesia, Nepal, the Philippines, and Malaysia [21]. Jamun leaves are available throughout the year and exhibit antioxidant [22], antibacterial [22], and anti-inflammatory [23] activities. Jamun leaves possess various bioactive substances, such as anthocyanins, alkaloids, flavonoids, myricetin, quercetin, tannins, kaempferol, and ellagic acid [24]. The methanolic extract of *S. cumini* leaves showed satisfactory 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic and superoxide radical scavenging activities *in vitro* [25]. Furthermore, Prabu et al. [26] demonstrated that jamun leaf powder could mitigate oxidative stress and improve innate immune responses in snubnose pompano (*Trachinotus blochii*) infected with *Vibrio parahaemolyticus*.

Cyprinus carpio is a highly valued aquaculture species worldwide, but outbreaks of *Aeromonas hydrophila*, a gram-negative pathogenic bacterium associated with various fish diseases [16], in carp ponds have been reported [27]. This study was conducted to evaluate the effect of jamun leaf extract (JLE) supplementation on growth, haemato-immunological and antioxidant parameters, disease resistance, and cytokine gene expression in *C. carpio*. To our knowledge, this is the first study to investigate the effect of JLE on antioxidant activity, immune parameters, and cytokine gene expression in *A. hydrophila*-affected *C. carpio*.

2. Materials and methods

2.1. Preparation of jamun leaf extract

Fresh *Syzygium cumini* leaves were collected from Thanjavur, TamilNadu, India. Leaves were washed in running tap water, and shade dried for few days to reduce the moisture content to approximately 8% on a

dry basis. Dried leaves were brought to the laboratory, pulverised, milled to a fine powder, and dissolved in methanol. The flasks were wrapped in aluminium foil and kept in a thermostatic shaking water bath. The mixture was filtered using Whitman no. 1 filter paper, and the filtrate was further concentrated in a rotary evaporator (BUCHI Rotavapor). The solvent-free extract was dried in a lyophiliser and stored at 4 °C until use.

2.2. Diet preparation

A basal diet (crude protein: 33.17%; crude lipid: 8.94; ash: 9.63%) was prepared, the composition of which is shown in Table 1. JLE was mixed with the basal diet at various concentrations (g kg⁻¹) to prepare the experimental diets: 0 (control), 05 (JLE5), 10 (JLE10), and 15 (JLE15). The ingredients were mixed thoroughly, pelleted (2 mm diameter), and air-dried in an oven (60 °C) until the moisture level was <10%. Prepared feeds were packed, labelled, and stored at -4 °C until use.

2.3. Fish and experimental design

Healthy *C. carpio* (mean average weight: 6.17 ± 0.43 g) were acclimatised to laboratory conditions for 2 weeks at 23 ± 2 °C and fed the basal diet. Approximately 20% of the tank water was renewed daily, and the water was completely replaced weekly. Basic physicochemical water parameters were monitored weekly: Oxygen and ammonia concentrations were 6.2–7.1 mg L⁻¹ and 0.03–0.07 mg L⁻¹, respectively, and pH ranged from 7.0 to 7.8 [13].

A total of 252 fish were randomly assigned to four experimental groups. In each independent group, 63 fish were distributed into three aerated 300 L tanks (21 fish per tank). Fish were fed corresponding experimental diets twice daily for 8 weeks. This was adjusted fortnightly by batch weighing after 24 h of starvation. The amount of feed consumed was determined by the daily recovery of excess feed (in any). All experiments complied with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.4. Growth performances

At the end of 8 weeks, fish were weighed to evaluate growth

Table 1
Composition of Basal diet formulation and composition.

Ingredients	Concentration (g kg ⁻¹ dry matter)			
	Basal diet	JLE (5.0)	JLE (10)	JLE (15)
Soybean meal	300	300	300	300
Wheat flour	350	345	340	335
Groundnut meal	160	160	160	160
Fish meal	100	100	100	100
Vegetable oil	40	40	40	40
Fish oil (Menhaden)	30	30	30	30
Mineral and Vitamin mix ^a	19	19	19	19
Vitamin C ^b	01	01	01	01
Jamun leaf extract	0	05	10	15
Proximate analysis (g Kg ⁻¹)				
Crude protein	331.7	336.8	335.9	334.4
Crude lipid	89.4	89.1	88.9	88.7
Ash	96.3	96.7	96.4	96.6

^a Every 250 g of mineral-vitamin mixture (Supplivite-M, Sarabhai Zydus Animal Health Ltd., India) provides vitamin A, 500000 IU; vitamin D3, 100000 IU; vitamin B2, 0.2 g; vitamin E, 75 units; vitamin K, 0.1 g; calcium pantothenate, 0.25 g; nicotinamide, 0.1 g; vitamin B12, 0.6 mg; choline chloride, 15 g; calcium, 75 g; manganese, 2.75 g; iodine, 0.1 g; iron, 0.75 g; zinc, 1.5 g; copper, 0.2 g; and cobalt, 0.045 g.

^b Merck, USA.

parameters:

$$\text{Weight gain (g)} = W_t - W_0$$

$$\text{Weight gain rate (WGR; \%)} = [(W_t - W_0) / W_0] \times 100$$

$$\text{Specific growth rate (SGR; \% day}^{-1}\text{)} = [(\ln W_t - \ln W_0) / t] \times 100$$

$$\text{Feed conversion ratio (FCR)} = [\text{total dry feed intake (g)} / \text{wet weight gain (g)}]$$

$$\text{Survival rate (\%)} = 100 \times (\text{final number of fish} / \text{initial number of fish})$$

In the above equations, W_t and W_0 are the final and initial weights (g) of the fish, respectively, and 't' is the duration of feeding (in days).

2.5. Challenge study

At the end of the feeding trial, 14 fish from each of the three tanks (42 fish per group) were selected for the challenge test. All fish were intraperitoneally (i.p.) injected with 100 μ L of phosphate-buffered saline (PBS) containing 1×10^7 live *Aeromonas hydrophila* cells [14]. Another group of 10 fish (fed the basal diet) was i.p. injected with 100 μ L of PBS as a control. The fish were fed a basal diet and observed for two weeks. The cause of death and pathological changes were confirmed by re-isolation of the pathogen from dead or moribund fish. To investigate survival over time, a Kaplan–Meier (K–M) survival analysis was performed separately for each treatment (IBM SPSS Statistics, v26; IBM Corp). The log-rank test was used for pairwise comparisons to identify the significant differences among JLE-supplement groups.

2.6. Sample collection

After 48 h of pathogen challenge, fish from each tank ($n = 3$) were anaesthetised with MS222 (100 mg L^{-1}) (Merck, USA). Blood samples were collected from the caudal vein and transferred into EDTA-coated heparinised tubes and non-heparinised tubes. Heparinised tubes were agitated vigorously to prevent blood clotting and stored at 4 °C. Non-heparinised tubes were allowed to clot at 32 ± 2 °C, centrifuged (4000 g, 5 min, 4 °C) to collect serum, and stored at -20 °C until use.

2.7. Haemato-immunological parameters

Total serum protein and albumin levels were measured using commercial assay kits (Merck, USA) according to the manufacturer's instructions. The globulin value was calculated by subtracting the albumin values from the total serum protein. The A/G ratio was calculated by dividing the albumin and globulin values.

Serum lysozyme activity (LA) was measured using a turbidimetric method based on the lysis of the lysozyme-sensitive gram-positive bacterium *Micrococcus lysodeikticus* (Merck, USA) [28]. The alternative complement pathway (ACP) in the serum was measured according to the procedure described by Yano [29].

Serum immunoglobulin (Ig) levels were determined using the method described by Hoseinifar et al. [15]. Immunoglobulin molecules were precipitated using 12% polyethylene glycol (Merck, USA). The difference in protein content before and after Ig precipitation was considered the Ig content.

The respiratory burst activity (RBA) of phagocytes was measured using the Nitroblue tetrazolium test (NBT; Merck, USA), following the method described by Geng et al. [30]. Colour development was measured at 630 nm using a spectrophotometer, and KOH/DMSO was used as a blank. The phagocytic activity (PA; %) was assessed according to the microscopic blood count method described by Siwicki et al. [31].

2.8. Oxidative stress-related parameters

Malondialdehyde (MDA) content was measured using barbituric acid reaction chromometry [32] and expressed as nmol mL^{-1} . The total

myeloperoxidase (MPO) content in the blood serum was measured using a commercial kit (Abcam, USA).

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined using commercial assay kits (Sigma-Aldrich, USA). ALT and AST activities were expressed as $U\ mL^{-1}$.

Superoxide dismutase (SOD) and catalase (CAT) activities were determined using an enzymatic assay and a commercial kit (Randox, Crumlin, UK).

2.9. Gene expression study

After blood collection, liver, head-kidney, and intestine tissues were aseptically collected from the fish. Samples were collected in 1.5 mL tubes containing TRIZOL reagent (Invitrogen, USA) and stored at -80 °C. Total cellular RNA was extracted from the tissues following the standard protocol. The purity and concentration of the isolated RNA were assessed using a spectrophotometer (Bio-Rad, CA, USA) and agarose gel electrophoresis. The extracted RNA was treated with DNase (Takara, Shiga, Japan), and re-suspended in DEPC pyrocarbonate-treated water. A Nanodrop spectrophotometer (Thermo Scientific) was used to quantify RNA samples, and they were assessed for purity based on the A_{260nm}/A_{280nm} ratio. Complementary DNA (cDNA) was synthesised using a cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). Prepared cDNA was stored at < 20 °C until further analysis. Real-time polymerase chain reaction analyses (qPCR) of interleukin (*IL*)-1 β , tumour necrosis factor (*TNF*)- α , *IL*-10, nuclear factor kappa B 65 (*NF- κ B* p65), heat-shock protein 70 (*HSP*70), and β -actin (as a reference housekeeping gene) were performed using a CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA, USA) following standard protocols. The primers and thermocycling conditions used for the qPCR are listed in Table S1. The expression of all genes was determined in triplicate. After amplification, a melting curve analysis was performed to verify the accuracy of each amplicon. Gene expression results were analysed using the $2^{-\Delta\Delta Ct}$ method after verifying that the primers had an efficiency of approximately 100% [33]. The expression levels in all treatment groups were compared to those in the control group. Fold changes = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [Ct(\text{treatment group}) - Ct(\text{treatment } \beta\text{-actin})] - [Ct(\text{control group}) - Ct(\text{control } \beta\text{-actin})]$. Polymerase chain reaction products were validated for their correct size and single band amplification using 2% agarose gel electrophoresis with 8 μ L of the sample.

2.10. Statistical analysis

The data were analysed using one-way analyses of variance (ANOVA), and multiple comparisons were performed with Tukey's test using OriginPro software (version 8; OriginLab Corporation, Northampton, MA, USA). Results are expressed as the mean $\pm$ standard error of the mean (SE) and were considered statistically significant at $p < 0.05$. To determine the optimal JLE dose, regression analysis was performed. Best-fitted curve between linear and quadratic equations based on the R-square values was selected [34]. Finally, the classical optimization technique (solving first-order derivative as zero) was used to determine the optimal supplementary dose that maximizes FBW, WGR, and SGR, and minimize FCR value.

3. Results

3.1. Growth parameters

The growth parameters of fish at the end of the feeding trial are shown in Table 2. Supplementing feed with JLE significantly increased the FWG and WGR in the experimental groups. The highest FWG and WGR were recorded in JLE10. The SGR was significantly higher only in JLE10, compared to the control. Similarly, the best (i.e. lowest) FCR was recorded in JLE10 ($p < 0.05$). The highest FCR was recorded in the control group, followed by JLE15. No mortality was recorded during the

Table 2

Growth performance after 8 weeks of feeding trial with dietary JLE in *Cyprinus carpio*. Different superscript letters in the same row are significantly different ($p < 0.05$). Values are presented as mean $\pm$ SEM ($n = 3$).

Parameters	Control	JLE5	JLE10	JLE15
Initial weight (g)	5.93 $\pm$ 0.8	6.22 $\pm$ 0.23	6.14 $\pm$ 0.24	6.31 $\pm$ 0.24
FBW (g)	38.02 $\pm$ 1.04 ^a	43.75 $\pm$ 1.53 ^b	49.16 $\pm$ 0.58 ^c	44.97 $\pm$ 0.95 ^{bc}
WGR (%)	525.11 $\pm$ 14.87 ^a	603.59 $\pm$ 8.02 ^b	699.65 $\pm$ 12.7 ^c	613.65 $\pm$ 12.89 ^b
SGR (% day ⁻¹)	3.32 $\pm$ 0.09 ^a	3.47 $\pm$ 0.02 ^{ab}	3.71 $\pm$ 0.03 ^b	3.5 $\pm$ 0.03 ^{a,b}
FCR	1.62 $\pm$ 0.06 ^a	1.32 $\pm$ 0.05 ^b	1.26 $\pm$ 0.02 ^b	1.41 $\pm$ 0.05 ^{ba}
Survival rate (%)	100 ^a	100 ^a	100 ^a	100 ^a

Note: FBW = final body weight; WGR = weight gain rate; SGR = specific growth rate; FCR = feed conversion ratio. values are presented as mean $\pm$ SEM ($n = 3$).

eight-week feeding trial.

The quadratic curves showing relationship of growth parameters (FBW, SGR, WGR, and FCR) with JLE level are shown in Fig. 1. Those parameters were best fitted with quadratic trends (R-square was high) (Fig. 1). The classical optimization demonstrated that the optimal value of JLE inclusion level as 1.015%, 0.968%, 0.962%, and the 0.903% for maximum FBW, WGR, and SGR and minimum FCR, respectively.

3.2. Challenge study

The effect of dietary administration of JLE on the resistance of fish against *A. hydrophila* was demonstrated using a Kaplan-Meier survival curve and log rank test (Fig. 2; Table s2). The highest survival rate was recorded in JLE10 (69.69%), followed by JLE5 (60.6%) and JLE15 (45.45%), while the control group had the highest mortality ((81.82%). Mortality started in the control group 24 h post-challenge with the pathogen. In JLE10, the first mortality was recorded on day four post-challenge.

The Kaplan-Meier analysis and log rank test revealed significantly different time-to-event estimates for JLE treatments. Mean survival rate detected significant differences among different treatments, and mean survival rate is maximum in JLE10.

3.3. Haemato-immunological parameters

Feed supplementation with JLE at 10 g kg⁻¹ significantly increased the carps' serum protein level (2.11 ± 0.06 g dL⁻¹) relative to the control and JLE15 and albumin levels (0.87 ± 0.07 g dL⁻¹) relative to the control (Table 3). Globulin levels did not differ significantly at supplementation rates of up to 10 g kg⁻¹. However, carp in the JLE15 group had significantly lower globulin rates than all the other groups. The A/G ratio was the highest in JLE10 ($p < 0.05$).

Dietary JLE had a profound effect on serum lysozyme activity (Table 4). The highest lysozyme activity was recorded in the JLE10 group (32.38 ± 1.21 U mL⁻¹, $p < 0.05$). Serum ACP activity was significantly higher in the 5–10 g kg⁻¹ JLE groups than in the control and JLE15. The highest ACP was recorded in JLE10 (70.43 ± 1.61 U mL⁻¹). The JLE supplementation at 10 g kg⁻¹ significantly increased the serum Ig level relative to the control.

The respiratory burst and phagocytic activity were significantly higher in JLE10 and JLE15 than in the control; the highest activity level was found in JLE10. The lysozyme, ACP, PA, IgM, and respiratory burst activities were all highest in JLE10 and decreased when the supplementation rate exceeded 10 g kg⁻¹ (Table 4).

3.4. Serum oxidative-stress-related parameters

Supplementation with JLE at an appropriate level reduced oxidative stress in the fish (Fig. 3). JLE supplementation at 10 g kg⁻¹ (JLE10) significantly reduced serum MDA level (2.57 nmol mL⁻¹) relative to the control. MPO levels were significantly higher in JLE5 and JLE10 than in the control and JLE15. Higher levels of JLE (15 g kg⁻¹) had no significant effect on serum MDA or MPO levels.

Serum AST and ALT levels declined with increasing supplementation

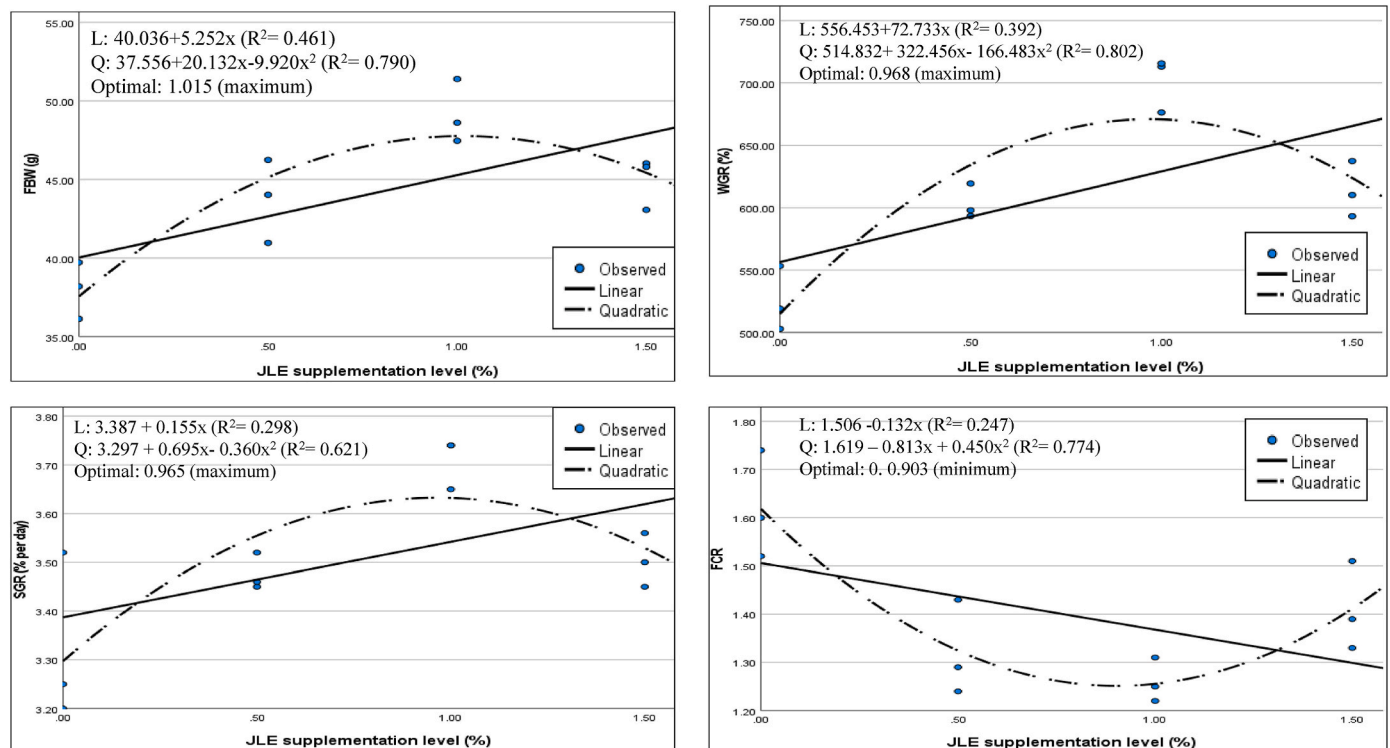


Fig. 1. The relationship between FBW (g), WGR (%), (SGR, % day⁻¹), and FCR against dietary JLE based on regression analysis.

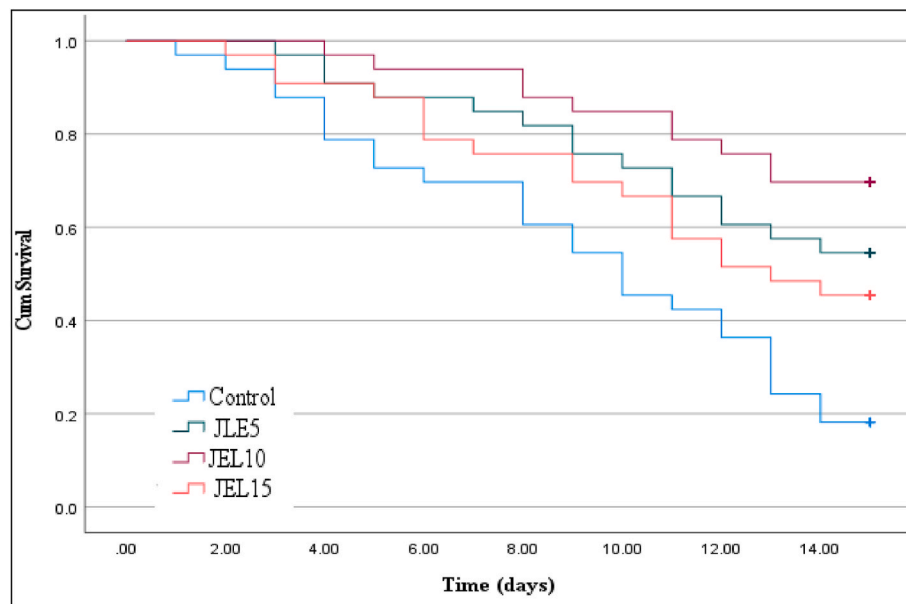


Fig. 2. Kaplan-Meier survival curve [(cumulative survival (%) two weeks)] of fish challenged with *Aeromonas hydrophila*. Log Rank (Mantel-Cox): $\chi^2 = 20.477$, $df = 3$, $p < 0.01$).

Table 3

Serum total protein, albumin, and globulin in fish fed with JLE supplemented diets for 8 weeks.

Fish group	Total protein (g dL ⁻¹)	Albumin (g dL ⁻¹)	Globulin (g dL ⁻¹)	A/G ratio
Control	1.86 ± 0.04 ^{ac}	0.59 ± 0.06 ^a	1.27 ± 0.06 ^a	0.46 ± 0.02 ^{ac}
JLE5	2.03 ± 0.1 ^{ab}	0.68 ± 0.03 ^{ac}	1.29 ± 0.02 ^a	0.52 ± 0.06 ^{ac}
JLE10	2.18 ± 0.06 ^b	0.87 ± 0.07 ^{bc}	1.24 ± 0.04 ^a	0.70 ± 0.01 ^b
JLE15	1.96 ± 0.09 ^c	0.84 ± 0.04 ^c	0.95 ± 0.03 ^b	0.46 ± 0.04 ^c

Different superscript letters in the same column are significantly different ($p < 0.05$); values are presented as mean ± SEM ($n = 3$).

Table 4

Serum immune activities in *C. carpio* fed with various graded level of JLE supplemented diets.

Fish group	Lysozyme (U mL ⁻¹)	ACP (U mL ⁻¹)	Phagocytic activity IgM (PA %)	RBT (Unit-mg mL ⁻¹)
Control	23.61 ± 1.1 ^a	62.08 ± 1.47 ^a	16.23 ± 0.39 ^a	5.1 ± 0.27 ^a
JLE5	27.02 ± 0.63 ^b	67.11 ± 0.86 ^{bc}	17.36 ± 0.61 ^{ab}	5.82 ± 0.43 ^{ab}
JLE10	32.38 ± 1.21 ^c	70.43 ± 1.61 ^b	21.18 ± 0.48 ^b	6.67 ± 0.36 ^b
JLE15	27.64 ± 0.53 ^b	65.82 ± 1.33 ^{ac}	19.44 ± 0.33 ^b	5.63 ± 0.23 ^b

Values in the same column with different superscripts letters are significantly different ($P < 0.05$); values are presented as mean ± SEM ($n = 3$).

rate up to JLE10. The lowest AST (31.58 U mL⁻¹) and ALT (44.07 U mL⁻¹) values were recorded in JLE10.

SOD activity was significantly higher in JLE5 and JLE10 than in the other groups, and CAT activity was highest in JLE10 (24.29 ± 0.98 U mL⁻¹; $p < 0.05$). The maximum SOD activity was recorded in JLE10 (18.76 ± 1.42 U mL⁻¹).

3.5. Gene expression

The expression of *IL-1β* in the liver, head-kidney, and intestine of *A. hydrophila*-challenged fish fed a JLE-supplemented diet was significantly modulated (Fig. 4). In the liver, *IL-1β* expression was significantly higher in the JLE groups than in the control, with the highest expression observed in JLE10 (2.1-fold higher than that in the control). In the head-kidney, *IL-1β* expression was significantly higher in JLE10 than in the control and JLE5, whereas in the intestine, expression was higher ($p < 0.05$) in JLE5 and JLE10 than the control. Upregulation of *IL-1β* expression was highest in JLE10 and declined at the higher supplementation rate (i.e., JLE15).

The expression of *TNF-α* in all three tissues was the highest ($p < 0.05$) in JLE10 (Fig. 4). In the liver, expression levels in JLE10 were significantly higher than in all other groups, whereas in the kidney *TNF-α* expression was significantly higher in JLE5 and JLE10 than in the control. In the intestine, all three treatment groups had significantly higher *TNF-α* expression levels than the control. In general, *TNF-α* expression was lowest among the treatment groups at the highest level of JLE supplementation.

The expression of the anti-inflammatory cytokine *IL-10* was down-regulated in the JLE groups (Fig. 4). *IL-10* expression was significantly downregulated in the liver and intestinal tissues of JLE5 and JLE10. However, JLE supplementation at 15 g kg⁻¹ had no significant effect on *IL-10* expression in liver and intestinal tissues. In the head-kidneys, *IL-10* expression was significantly lower in JLE10 and JLE15 than in the control and JLE5.

Supplementation with JLE had no significant effect on the expression of *NF-κB p65* in liver tissue (Fig. 4). However, in the head-kidney and intestine, the highest ($p < 0.05$) *NF-κB p65* expression was recorded in JLE10. The expression of *HSP70* was not significantly affected by JLE supplementation in the kidney and intestinal tissues of the challenged fish. However, liver tissue from JLE15 had significantly higher *HSP70* expression levels than the control.

4. Discussion

Faster growth, good health, and resistance to diseases are key parameters for successful and sustainable aquaculture practices. Eco-friendly alternatives to chemical or synthetic agents are becoming

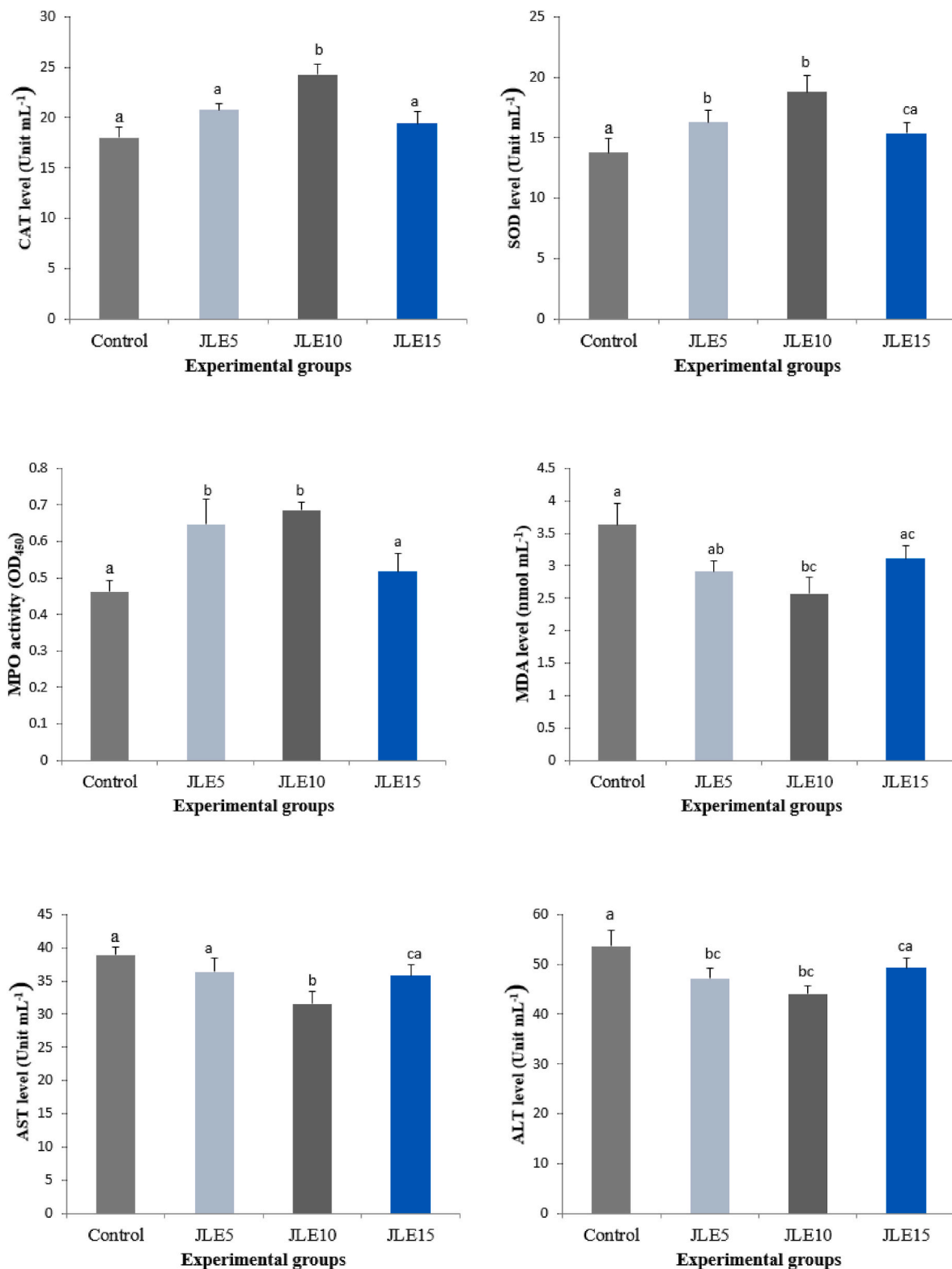


Fig. 3. Changes in serum oxidative stress-related parameters in fish fed with various graded level of JLE supplemented diets. Column with different superscripts letters are significantly different ($P < 0.05$); values are presented as mean $\pm$ SEM ($n = 3$).

increasingly important for aquaculture sustainability [8], and research on the use of phytobiotics in fish cultures is increasing. Several plant extracts, including those from marjoram [16], oak leaf [35], lavender leaf [36], *P. tectorius* [13], GLE [20] in *C. carpio*, and Tulsi [37], have been reported to boost immune responses in cultured fish species. The immune functions and resistance of tilapia against *A. hydrophila* have

been boosted by JLE [38]. However, studies pertaining to the impact of dietary JLE on immune function and disease resistance in pathogen-challenged carp are unavailable. This study aimed to determine the impact of dietary JLE on immune function in *C. carpio*.

Growth enhancement is a trait of particular interest in fish farming as it is directly linked to the productivity and profitability of aquaculture

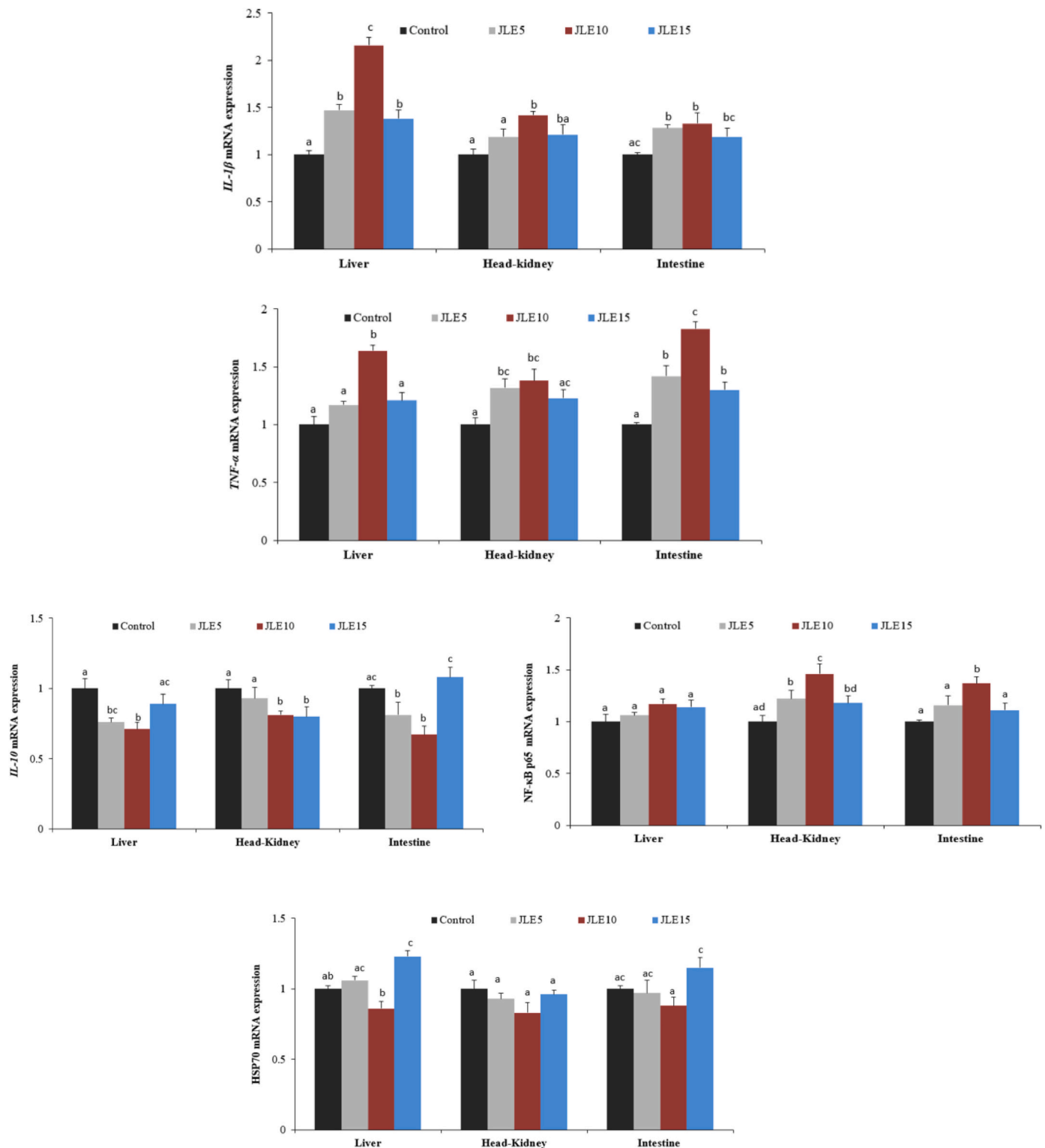


Fig. 4. Expression of cytokine-related genes in liver, kidney, and intestine of fish fed different concentrations of JLE supplemented diets and challenged with *A. hydrophila*. Different superscript letters on the top of error bars differ significantly. Data are shown as mean $\pm$ SEM ($n = 3$ ($P < 0.05$)).

practices [18]. In this study, dietary JLE (particularly at 10 g kg⁻¹) increased the growth performance of carp. Similarly, dietary JLE at 10 g kg⁻¹ increased the growth performance of genetically improved farmed tilapia (GIFT) [38]. Similarly, dietary *Rehmania glutinosa* [18], marjoram [16], *P. tectorius* [13], and palm fruit extracts [15] significantly increased the growth performance of *C. carpio*. Dietary jamun leaf powder had no significant impact on the WGR or SGR of snubnose

pompano [26]. The primary purpose of feed supplementation is to achieve faster growth and lower FCR. The low FCR value and high FBW in the group supplemented at 10 g kg⁻¹ indicates that this treatment achieved that primary purpose. The higher supplementation rate did not result in better growth performance which is consistent with the results of carp fed on herbal-supplemented diets [13,39,40]. Excess JLE may lead to higher energy consumption for metabolism, resulting in

unfavourable effects in fish. Furthermore, the presence of anti-nutritional factors, such as tannins and polyphenols, have been reported in JLE [38]. Consuming excessive amount of these anti-nutrients factors may interfere with digestion and absorption of biomolecules (e.g: proteins, and fats); thus affecting the fish health [41].

Serum total protein is an indicator of the broader health, stress, and nutritional condition of fish. Protein components, such as albumin, lysozyme, immunoglobulins, complement proteins, lectins, and transferrin are included in the protein spectrum [42]. In this study, total serum protein and albumin levels were significantly higher in JLE10, indicating an improved nutritional and health status of the fish. Dietary jamun leaves at 1% for 45 days increased the serum protein levels in snubnose pompano [26]. Similarly, dietary ginger extract [14] and pomegranate peel extract (PPE) [40] elevated serum protein levels in carp. Furthermore, the lowered total protein in JLE15 might be related to immunosuppression in fish. Lysozyme is a protective protein found in the blood, mucus, plasma, and other body fluids of fish. It cleaves the β -1,4 glycosidic bond between N-acetyl glucosamine and N-acetyl muramic acid in the peptidoglycan layer, which catalyses the hydrolysis of most bacterial cell walls [43]. On the other hand, ACP is a vital serum factor that stimulates the cellular defence mechanisms by phagocytosis via the opsonin process [10]. In this study, 10 g kg⁻¹ JLE increased the lysozyme and ACP levels. Similarly, significantly higher lysozyme and complement activities were recorded in *C. carpio* fed a diet supplemented with 200 mg kg⁻¹ GLE [17], 5 g kg⁻¹ PPE [40], or dietary oak leaf extract at 1–2 g kg⁻¹ [35], and grass carp fed a diet supplemented with 438.20 mg kg⁻¹ curcumin [43]. The enhanced lysozyme activity in JLE10 could be attributed to the presence of phenolic compounds in JLE [45], which function as potent antimicrobial agents by mediating the sensitisation of membrane phospholipids in pathogenic microorganisms [46]. Higher complement activity in JLE10 enhances inflammatory and phagocytic activity (PA) via enhanced opsonisation and chemotaxis [10].

Immunoglobulin plays a key role in systemic immune responses. It participates in the opsonisation of pathogens by facilitating phagocytosis [47]. Dietary administration of plant compounds, such as ginger extract at 0.5% [14], *P. tectorius* extract at 2% [13], or PPE at 0.5% [40] increased the serum IgM levels in carp. Myeloperoxidase is a vital enzyme expressed mainly in neutrophils and produces hypochlorous acid from oxidative radicals (H₂O₂) [48]. Dietary inclusion of *P. tectorius* extract or dried lemon peels increases serum MPO levels in fish [12,13], which were in agreement with the results of the present study. The enhanced lysozyme and MPO activities increase the leucocyte count, which may, in turn, enhance PA [12].

Phagocytosis is a vital cellular component of the innate immune system in fishes [42]. Phagocytes produce respiratory bursts that attack invasive pathogens during the process of phagocytosis. Excessive accumulation of reactive oxygen intermediates is extremely toxic to the cells [48]. In this experiment, significant increases in PA and RBT were observed in JLE10. Similarly, dietary azadirachtin at 4 g kg⁻¹ for 28 days significantly increased PA and RBT in goldfish [11]. Dietary PPE at 0.5% [40] and grape pomace flour at 200 mg kg⁻¹ [10] increased the RBT in pathogen-challenged *L. rohita*. Dietary *R. glutinosa* root powder at 4% for 80 days [18] or *P. tectorius* extract at 20 g kg⁻¹ for eight weeks [13] increased the serum PA level in *C. carpio*. In this study, the maximum LA, ACP, PA, RBT, and IgM activities were found in JLE10 but not in JLE15. This suggests that long-term feeding with high levels of immunostimulants may lead to immune suppression in fish, which is consistent with earlier studies [10,13,14,16,40,49].

Antioxidant systems are vital for fish health, and plant compounds are useful additives to augment the system owing to the presence of natural antioxidants. Antioxidant enzymes (e.g: SOD, CAT, GPx) protect cells from oxidative stress and attendant damage by scavenging hydrogen peroxide (H₂O₂), lipid peroxides, and superoxide (O₂⁻) radicals under stress [50]. In this study, 10 g kg⁻¹ of JLE significantly decreased MDA content and increased the activities of SOD and CAT in

the serum. As the first line of defence against oxidative stress, SOD, CAT, and glutathione peroxidase convert superoxide radicals into H₂O₂ and then into water and molecular oxygen [51]. Malondialdehyde content can be used to measure the degree of lipid peroxidation [52] but a high MDA content can negatively affect cell morphology and function [50]. Dietary supplementation with an appropriate level of marjoram [19], *Artemisia absinthium* extract [19], GLE [17], and *P. tectorius* extract [13] decreased the MDA level in fish while increasing the antioxidant activities. The low MDA level in JLE10 indicates that the endogenous antioxidant defence system was enhanced in the fish. The higher antioxidant activities of JLE10 may be associated with the presence of bioactive compounds in JLE.

ALT and AST are important indicators of the physiological state of the liver in fish. Generally, ALT and AST are released into the blood when the liver is impaired [53]. A significantly lower serum ALT and AST levels were recorded in JLE10. Similarly, dietary administration of jamun leaf powder reduced AST and ALT levels in snubnose pompano under pre- and post-challenge conditions [26]. Dietary supplementation with emodin [49], GLE [17], and *Artemisia absinthium* extract [19] also decreased the serum ALT and AST levels in carp. Furthermore, ALT and AST levels are inversely correlated with serum protein levels, indicating that AST and ALT levels may play a role in protein metabolism [54].

In fish, the inflammatory response is a key element against various challenges and is mediated primarily by cytokines, including pro- and anti-inflammatory cytokines [55]. *IL-1 β* is a pro-inflammatory cytokine that plays a role in phagocyte function, eicosanoid production, and lymphocyte stimulation and proliferation by major histocompatibility complex (MHC) presented antigens [56]. The main immunocompetent organs in fish, such as the liver, head-kidney, and intestine, demonstrated a strong elevation in *IL-1 β* mRNA expression in JLE10. Therefore, JLE activated immune cells and triggered *IL-1 β* expression. Similarly, dietary banana peel flour at 5% increased the expression of *IL-1 β* in the hepatopancreas and head-kidney of *L. rohita* [39]. Dietary supplementation with PPE at 0.5% for 60 days significantly increased the expression of *IL-1 β* in *L. rohita* challenged with *A. hydrophila* [40]. Tumour necrosis factor- α is vital for diverse cellular processes, including cell proliferation and differentiation [57]. Its expression was significantly upregulated in the tissues tested in JLE10. Similarly, upregulation of *IL-1 β* and *TNF- α* was recorded in the head-kidney, intestine, and hepatopancreas of *L. rohita* fed a diet supplemented with 0.5% guava leaf flour [58]. Grass carp fed a diet supplemented with *Ficus carica* polysaccharide exhibited strong upregulation of *IL-1 β* and *TNF- α* [59]. The pattern of variability in expression levels between the treatment groups was the same for *IL-1 β* and *TNF- α* in all the tested organs.

Monocytes, B cells, T cells, natural killer cells, macrophages, and dendritic cells produce IL-10, which inhibits chemokine receptors and the effects of pro-inflammatory cytokines [60]. We observed a significantly lower *IL-10* expression in the tested tissues of JLE10 than that of the control. Similarly, Dietary supplementation with banana peel flour [39] or PPE [40] significantly downregulated the expression of *IL-10* in various tissues of *L. rohita*. The inverse pattern of anti-inflammatory cytokines (*IL-10*) and pro-inflammatory cytokines (*IL-1 β* , *TNF- α*) in fish after dietary administration of plant immunostimulants has been reported in numerous studies [13,14,40,44,49]. Nuclear factor kappa B plays a critical role in the regulation of cell survival genes and induces the expression of inflammatory enzymes and cytokines such as iNOS, COX-2, *TNF- α* , *IL-1 β* , and *IL-6* [61]. NF- κ B p65 is associated with the classical NF- κ B pathway. In this study, JLE supplementation (10 g kg⁻¹) significantly upregulated the expression of NF- κ B p65. The upregulated expression of NF- κ B p65 can upregulate the mRNA levels of pro-inflammatory cytokines [62], which is in agreement with the results of this study. Thus, the presence of bioactive compounds in JLE may regulate cytokine gene expression. However, the exact mechanism of inflammatory cytokine regulation requires further investigation.

Heat-shock proteins are stress-resistant proteins and can be used as biomarkers for different types of environmental stress, such as hypoxia,

microbial invasion, and starvation in fish [63]. In the present study, 10 g kg⁻¹ JLE had no significant effect on *HSP70* expression under post-challenge conditions. This revealed that dietary JLE can modulate stress conditions in fish.

Bacterial challenge studies are key to evaluating the effectiveness of immunostimulants in terms of protection against pathogen attack [20]. Several plant compounds have been reported to protect fish against pathogen invasion [14,16–20,30,37,49,58]. Dietary administration of PPE at 5 g kg⁻¹ for 60 days [40] and *P. tectorius* extract at 20 g kg⁻¹ for eight weeks [13] improved the protection of *L. rohita* and *C. carpio*, respectively, against *A. hydrophila*. In this study, JLE at 10 g kg⁻¹ provided higher protection to fish against pathogen attack, and the relative per cent survival (RPS) for JLE10 was 62.95%. According to Amend [64], a RPS of more than 50% indicates a positive impact of administered bioactive compounds or vaccines. The enhanced immune parameters (LA, ACP, IgM, RBT, MPO, and PA), antioxidant enzymes (SOD and CAT), and declines in certain biochemical parameters (MDA, AST, and ALT) in the fish fed diets supplemented with 10 g kg⁻¹ JLE may be associated with the improved resistance of fish against *A. hydrophila*, resulting in higher post-challenge survival rates. The negative impact of higher doses of JLE (15 g kg⁻¹) could be related to the immunosuppressive effect of JLE in fish at higher doses.

5. Conclusions

The results of this study revealed that dietary administration of JLE at 10 g kg⁻¹ could increase the antioxidant status, serum immune parameters, and modulate the expression of cytokine genes in carp. The supplementation of JLE (10 g kg⁻¹) for eight weeks strongly increased the resistance of fish against *A. hydrophila* challenge. Based on regression analysis, the recommended JLE inclusion level was 9.03–10.15 g kg⁻¹ in the carp diet for maximum growth performance. Moreover, JLE may modulate inflammatory and stress responses in fish. The optimum dose of JLE could be an economically viable alternative to chemical or synthetic agents in carp production because of its potential. However, excessive dietary JLE may negatively affect fish health. Further investigations are warranted to characterise JLE as a feed additive while addressing its specific immunological mechanisms.

CRediT authorship contribution statement

Sib Sankar Giri: Conceptualization, design, experiment, Writing – original draft. **Sang Guen Kim:** manuscript revision, Data curation. **Won Joon Jung:** Formal analysis, Validation. **Sung Bin Lee:** Manuscript formatting; revision. **Young Min Lee:** Formal analysis, manuscript preparation. **Su Jin Jo:** Manuscript preparation, Validation. **Mae Hyun Hwang:** Manuscript revision, proof reading. **Jae Hong Park:** Data curation, proof reading. **Ji Hyung Kim:** Manuscript, Writing – review & editing. **Subrata Saha:** Formal analysis. **Venkatachalam Sukumaran:** Methodology, Supervision. **Se Chang Park:** Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

Authors do not have any competing interest to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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