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Modulatory effects of laurel-leaf cistus (*Cistus laurifolius*) ethanolic extract on innate immune responses and disease resistance in common carp (*Cyprinus carpio*)

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ABSTRACT

Medicinal herbs are used for growth promotion, disease control and other health benefits in aquaculture industry. Here, we examined the effect of dietary laurel-leaf cistus (*Cistus laurifolius*) ethanolic extract on growth performance, digestive enzyme activity, haematological profile and nonspecific immune responses in common carp (*Cyprinus carpio*). In addition, resistance against *Aeromonas hydrophila* infection was examined. Common carp was fed diets containing 0 (Control), 0.1 (CL0.1), 0.5 (CL0.5) and 1 (CL1) g kg^{-1} laurel-leaf cistus extract for 45 days. After 30 days, superoxide anion production (SAP) increased in CL0.1 and CL0.5 fish groups and at the end of the study all experimental fish groups had higher SAP compared to that of the control ($P < 0.05$). Lysozyme activity (LA) was elevated in CL0.5 and CL1 treated groups on 30th day ($P < 0.05$), and this increase was only observed in C0.1 fish group at the end of study compared to control ($P < 0.05$). Myeloperoxidase activity was significantly increased in CL0.5 and CL1 fish groups at the end of study. IL-1βgene expression was significantly increased in treated fish in a dose-depended manner. Similar results were observed for transcription of IL-6 and IL-8 ($P < 0.05$). Anti-inflammatory cytokines, IL-10 and TGF-β were highly up-regulated in the intestine and head kidney of CL treated fish groups compared to control ($P < 0.05$). At the end of experiment, significantly higher final body weight, weight gain, and specific growth rate were obtained in CL0.1 treated fish group compared to control. However, growth was negatively affected in CL1 fish group ($P < 0.05$). CL1 fish group had also a significantly higher FCR. Amylase activity was significantly increased in all experimental fish groups compared to control ($P < 0.05$). Trypsin activity was decreased in CL0.1 and CL1 fish groups ($P < 0.05$). WBC and RBC were significantly increased ($P < 0.05$) in CL0.5 and CL1 fish groups, whereas haemoglobin, haematocrit, mean cell, mean cell haemoglobin contents were no significantly changed among control and treatment groups. Result of challenge test with *A. hydrophila* exhibited that survival rate in all treatment groups was significantly higher than that of control. These findings demonstrated that laurel-leaf cistus at 0.1 g kg^{-1} can be a suitable candidate for growth promotion, immune system induction and infection control in fish.

1. Introduction

Fish are one of the prime protein sources in nutrient requirement of human. Therefore, since last two decades, intensive and semi-intensive aquaculture systems have developed rapidly to meet the protein requirement in human diet [1]. Animal welfare is an important factor to maintain efficient and sustainable production process in intensive aquaculture. The health of fish directly depends on water quality, handling, stocking densities, diseases and nutrition management [2]. Cutting edge technologies allow to increase fish production by maintaining high stocking density [3,4]. However, those new technologies could result in high mortality by increased disease incidences in aquaculture [5].

The use of antibiotics and chemotherapeutics is one of the common methods to prevent and control of microbial infections [6,7]. However, therapeutic agents can give rise to toxic residual effects to fish and

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human health [8,9]. Moreover, they affect water quality and cause environmental pollution [10]. In recent years, several alternative methods have been tried to minimize residual risks and devise environment-friendly treatments. In this regard, medicinal plants have emerged considerably well with their antimicrobial activity [11,12] and reproduction promoting [13] properties. Medicinal plants have also become an effective candidate for control and prevention of fish diseases [14–18].

Many pathogenic bacteria belonging to the genus *Aeromonas* are primary aetiologies for diseases of freshwater fish species, such as common carp, tilapia, catfish, other tropical and ornamental fishes [5, 19–21]. *Aeromonas hydrophila* is a gram-negative, anaerobic, rod-shaped bacterium [22,23], distributed worldwide and one of the major disease causing agents in common carp (*Cyprinus carpio*).

Laurel-leaf cistus (*Cistus laurifolius*) belonging to the family Cistaceae, is native to Southern Europe and North Africa regions [24]. It has been used to treat rheumatic disease, hypoglycaemia disease, urinary inflammations, common cold in Turkish traditional medicine [24,25]. Laurel-leaf cistus contains rich antioxidant components, such as flavonoids, coumarins and terpenes [24,26]. Several studies have reported that laurel-leaf cistus has antioxidant, anti-inflammatory, antidiabetic, antinociceptive, analgesic, hepatoprotective and antiulcerogenic properties [24,25]. However, effects of laurel-leaf cistus on immune response and disease resistance have not been evaluated in fish till date.

In this study, we have examined the efficacy of laurel-leaf cistus supplemented diets on growth performance, digestive enzymes activity, haematological profile and innate immune responses in common carp. In addition, the potential of using laurel-leaf cistus incorporated diet as a therapeutic agent against *A. hydrophila* infection in common carp was investigated.

2. Material and methods

2.1. Procurement of experimental fish and acclimatization

Common carp fingerlings (2.95 ± 0.01 g) were obtained from a local fish farm in Mediterranean Fisheries Research, Production and Training Institute in Turkey. Fish were transported to the indoor wet laboratory of the Faculty of Fisheries, Kastamonu University, Turkey. A total of 480 fish were acclimatized in water with 7.2–8.1 mg/L dissolved oxygen, pH 7.7–8.5 and temperature at 20–24 °C for 2 weeks. Photoperiod was maintained at 12 h light: 12 h dark cycle. Water parameters were monitored daily. Fish were fed with a commercial feed twice a day at a rate of 2% body weight until commencement of the experiment.

2.2. Diet preparation and experimental design

Leaves of *C. laurifolius* were obtained from Kastamonu province (41° 25′50 N, 33° 45′19 E) in Turkey. Leaves were washed with sterilized water and dried at room temperature. The 50 g of leaves was homogenized in 1 L of 96% ethanol (Sigma-Aldrich, St. Louis, MO, USA) using a laboratory blender at room temperature and kept for 3 days. The supernatant was filtered through a 150 μm filter paper and then evaporated in a rotary evaporator at 55–65 °C to remove the ethanol. The final product was dissolved in distilled water and stored in plastic tubes at 4 °C [27]. The ethanolic extract of *C. laurifolius* (CL) was diluted with 50 mL of distilled water and then sprayed on the fish diet [28] at concentrations of 0.1, 0.5 and 1 g kg^{-1} and designated as CL01, CL0.5 and CL1, respectively. The basal diet without containing CL served as the control diet. The CL diets were stored in plastic zip packs at −20 °C until use.

Fish were randomly selected and divided into four experimental groups consisting of 3 CL supplemented diets, CL0.1, CL0.5 and CL1 and a control diet. Forty fish were placed in each glass aquarium (100 L) provided with aeration and biological filtration. Water parameters (temperature, oxygen and pH) were monitored daily. Water parameters and photoperiod were kept similar to those described above. The fish were fed twice a day *ad libitum* for 45 days.

2.3. Growth performances

After the 45-day feeding trial, growth performance parameters and survival rate were determined as follows:

Weight gain (WG %) = [(final body weight–initial body weight)/ initial body weight/] × 100;

Specific growth rate (SGR, % g day^{-1}) = [(Ln final weight) – Ln (initial weight)/days of the experiment] × 100;

Feed conversion ratio (FCR) = wet weight gain (g)/dry feed consumed (g);

Fish survival rate (%) = (Number of fish remaining at the end of the experiment/initial number of fish) × 100.

2.4. Determination of digestive enzyme activities

Anterior parts of intestine (0.1 g) were measured in microbalance and homogenized in 1 mL cold double distilled water using Potter Elvehjem homogenizer. After that, samples were centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatant was gently collected with a plastic pipette and stored at −80 °C until analysis. Amylase activity was determined by using 2% starch (Sigma-Aldrich, St. Louis, MO, USA) as a substrate [29]. Lipase activity was determined by hydrolysis of 4-nitrophenyl myristate (Sigma-Aldrich, St. Louis, MO, USA) [30]. Trypsin activity was measured by using benzoyl-DL-arginine-p-nitroanilide (Sigma-Aldrich, St. Louis, MO, USA) [31].

2.5. Haematological analysis

At the end of experiment, fish were anesthetized using MS-222 (100 mg/L) for blood sampling. The blood samples (10–50 μL) were collected from caudal vein puncture with heparinized syringes and transferred to EDTA rinsed tubes to analyse haematological parameter. White blood cell (WBC × 10^3 mm^{-3}), red blood cell (RBC × 10^6 mm^{-3}) counts, haemoglobin (Hb, g dL^{-1}) and haematocrit (Hct, %) were measured using BC 3000 Heamatometer. Blood indices, such as mean cell (MCV, fL), mean cell Hb (MCH, pg) and mean cell Hb concentration (MCHC, %) were calculated according to Lewis, Bain [32].

2.6. Analysis of non-specific immune parameters

2.6.1. Isolation of head kidney (HK) cells

Individual fish was scooped out of holding tank and anesthetized with 2-phenoxyethanol (0.05%, Sigma–Aldrich, St. Louis, MO, USA) in a bucket containing aerated water before being sacrificed for tissue collection. Carp HK cells were isolated following the method described elsewhere [33]. Finally, the number of prepared cells was adjusted to 1 × 10^7 cells mL^{-1}.

2.6.2. Humoral immune parameters

In this study, superoxide anion production in HK cells was determined by reduction of nitroblue tetrazolium (NBT) (Sigma–Aldrich, St. Louis, MO, USA) assay on the 15th, 30th and 45th day, as per the previously described method (Biswas, Korenaga [34]. Lysozyme activity (LYS) was performed according to Bilen, Biswas [35]. The isolated HK cells were inoculated with *Micrococcus lysodeikticus* on 15th, 30th and 45th day. After 1 h of incubation, optical density (OD) of the cell supernatant was measured at 520 nm in a microplate reader. Total myeloperoxidase activity in serum was determined as described by Sahoo, Kumari [36] with a slight modification. Hundred μL of serum was diluted with 100 μL of Hank's Balanced Salt Solution without Ca^{2+} or Mg^{2+}. Hundred μL of 0.1 mg mL^{-1} 3, 3′, 5, 5′ - tetramethylbenzidine dihydrochloride and 0.006% fresh hydrogen peroxide were mixed with the diluted serum. The reaction was followed kinetically by measuring the increase in absorbance.

2.7. Cytokine gene expression

Head kidney and intestine samples (10–20 mg) from each selected fish were collected and the tissues were stored in RNALater solution. Total RNA isolation was performed using BIOLINE kit (ISOLATE II RNA Mini Kit) and cDNA was synthesized via reverse transcription from 1 μg mRNA using a BIOLINE kit (SensiFAST™ cDNA Synthesis Kit) as described previously (Bilen and Elbeshti, 2019). qRT-PCR analysis was completed using Bio-Rad and Wizpure qPCR Master SYBR Kit (Wizbio solutions, ABD). Gene-specific primer sequences with references are listed in Table 1.

Table 1. Cytokine gene specific primers used for qRT-PCR in carp.

2.8. Challenge test

A. hydrophila with a density of 1×10^8 CFUs (previously determined LD_{50} dose) mixed in 100 μL PBS was injected intraperitoneally to all experimental fish (31 numbers in each) at the end of dietary administration. Survival rate of each experimental group was examined and recorded for 8 days. Bacterium isolated from the infected fish was confirmed as *A. hydrophila* using conventional methods.

2.9. Statistical analyses

Result data were analysed using SPSS software version 23.0 (IBM Corp., Armonk, NY, USA). Difference among parameters between treatments of the experiment was determined by one-way ANOVA. Multiple comparisons were made using Tukey's multiple range test. Level of significance was set at <0.05. The survival in fish groups after challenged with *A. hydrophila* were analysed by Kaplan-Meier survival test. Differences among groups were determined using Log-Rank Test.

3. Results

3.1. Growth performances

Growth performances of fish fed different diets are shown in Table 2. Final weight of fish in CL0.1 group was significantly higher than that of all other groups ($P < 0.05$). WG and SGR also significantly increased in CL0.1 diet fed group compared to control. CL1 diet fed fish group had a significantly higher FCR. However, decreased FCR was recorded in CL0.1 fish group compared to that of all other experimental diet per fish groups (Table 2).

3.2. Digestive enzymes activities

Amylase activity significantly increased ($P < 0.05$) in all CL diet fed groups compared to control (Table 3). No significant changes in lipase activity were observed in all experimental fish groups. However, in CL0.1 and CL1 diet fed fish groups, significantly decreased trypsin activity was noticed compared to that of CL0.5 diet fed and control groups (Table 3).

Table 1
Cytokine gene specific primers used for qRT-PCR in common carp (*C. carpio*).

Gene	Primer Sequences	References
β-Actin	Forward: AGACATCAGGGTGTCATGGTTGGT Reverse: CTCAAACATGATCTGTGTCAT	[37]
IL-1β	Forward: ACCAGCTGGATTTGTCAGAAG Reverse: ACATACTGAATTGAACTTTG	[37]
TNF-α	Forward: GGTGATGGTGTCGAGGAGGAA Reverse: TGGAAAGACACCTGGCTGTA	[37]
IL-6	Forward: CCGCACATGAAGACAGTGAT Reverse: GGGTATATTTGGCTGCAGGA	[38]
IL-8	Forward: TGGAGCTCTTCCCTCCAAG Reverse: AGGGTGCAGTAGGGTCCAG	[38]
IL-10	Forward: TGATGATTTGGAACCATTATTGAA Reverse: CACCTTTTTCCTTCATCTTTTCAT	[39]
TGF-β	Forward: TACAATACTTTCACGCTTTCCC Reverse: GGCTCAACACCTCCTTCAC	[37]

F: Forward primer; R: Reverse primer.

Table 2
Growth performance of common carp (*C. carpio*) fed diets containing different levels of methanolic extract of *Cistus laurifolius*.

	Control	CL0.1	CL0.5	CL1
Initial weight (g)	2.94 ± 0.03	2.97 ± 0.01	2.93 ± 0.01	2.96 ± 0.01
Final weight (g)	5.25 ± 0.15^b	6.31 ± 0.34^a	5.34 ± 0.06^b	4.69 ± 0.2^c
WG (%)	73.9 ± 4.36^b	107.29 ± 10.1^a	82.21 ± 3.61^b	58.53 ± 2.17^c
SGR (% g day^{-1})	1.28 ± 0.05^b	1.66 ± 0.01^a	1.31 ± 0.02^b	0.95 ± 0.1^c
FCR	1.41 ± 0.05^b	1.32 ± 0.01^c	1.40 ± 0.02^b	1.61 ± 0.1^a

CL0.1, CL0.5 and CL1, diets containing extract of *Cistus laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively; WG is weight gain, SGR is specific growth rate, and FCR is feed conversion ratio; Values are expressed as mean ± SE. Different superscript letters in a row indicate significant differences between groups ($P < 0.05$).

Table 3
The digestive enzyme activities in common carp (*C. carpio*) fed diets containing different levels of methanolic extract of *Cistus laurifolius*.

	Amylase (U/mg protein)	Lipase (U/mg protein)	Trypsin (U/mg protein)
Control	0.14 ± 0.08^d	0.01 ± 0.007	0.11 ± 0.01^a
CL0.1	1.29 ± 0.84^c	0.02 ± 0.009	0.07 ± 0.002^b
CL0.5	3.39 ± 0.89^a	0.01 ± 0.004	0.12 ± 0.006^a
CL1	2.43 ± 0.94^b	0.01 ± 0.006	0.05 ± 0.01^b

CL0.1, CL0.5 and CL1, diets containing extract of *Cistus laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively; Values are expressed as mean ± SE. Different superscript letters in a column indicate significant differences between groups ($P < 0.05$).

3.3. Haematological analysis

At the end of study, significantly elevated WBC was observed in CL1 and CL0.5 fish groups compared to control ($P < 0.05$). Similarly, increased RBC was determined in CL1 and CL0.5 fish groups. HGB was not affected by laurel extract treatment. However, HCT was decreased only in CL0.5 fish group compared to that of other treated and control groups ($P < 0.05$). HGB, MCV, MCH and MCHC did not have any significant variation between control and CL diet fed fish groups (Table 4).

3.4. Non-specific immune parameters

Result of superoxide anion production (SAP) increased in CL0.1 and CL0.5 diet fed groups compared to the control on 30th day of the study (Fig. 1). At the end of experiment, SAP level significantly increased in all treated fish groups compared to that of control ($P < 0.05$).

LA was found higher ($P < 0.05$) in CL0.1 and CL0.5 diet fed groups on 15th day of the study. However, this increase was similar to that of the control. However, control fish group also had similar value compared to CL0.5 group. On 30th day, similar result was observed to that of 15th day. At the end of study, no differences were observed among experimental fish groups compared to the control ($P > 0.05$) (Fig. 2.)

At the first and second sampling time of the study (on 15th and 30th day), MPO activity did not vary among treated and control fish groups. However, on 45th day of the study, MPO activity was significantly elevated in CL0.5 and CL1 fish groups ($P < 0.05$) (Fig. 3).

Table 4
Haematological indices of common carp (*C. carpio*) fed diets containing different levels of methanolic extract of *Cistus laurifolius*.

	WBC	RBC	HGB	HCT	MCV	MCH	MCHC
Control	1.1 ± 0.47^{c}	0.56 ± 0.02^{c}	5.53 ± 0.27	21.90 ± 0.68^{a}	138.03 ± 5.52	35.10 ± 1.56	250.67 ± 6.01
CL0.1	1.17 ± 0.72^{c}	0.29 ± 0.07^{d}	5.37 ± 0.38	15.63 ± 0.29^{b}	138.70 ± 0.84	35.97 ± 0.47	245.33 ± 4.01
CL0.5	2.57 ± 0.27^{b}	1.20 ± 0.16^{b}	6.17 ± 0.38	23.50 ± 0.42^{a}	140.90 ± 4.77	36.97 ± 0.97	253.33 ± 3.41
CL1	5.33 ± 0.72^{a}	1.36 ± 0.21^{a}	6.23 ± 0.45	22.30 ± 1.24^{a}	147.03 ± 5.63	33.07 ± 3.61	245.33 ± 3.57

CL0.1, CL0.5 and CL1, diets containing extract of *Cistus laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively . WBC: white blood cell (WBC: 10^3 mm^{-3}), RBC: red blood cells (RBC: 10^6 mm^{-3}), HGB: Haemoglobin (g dl^{-1}), CT: Haematocrit (%), MCV: mean corpuscular volume (fl) MCH: mean cell haemoglobin (pg) MCHC: mean cell haemoglobin concentration (g dl^{-1}). Values are expressed as mean ± SE. Different superscript letters in a column indicate significant differences between groups ($P < 0.05$).

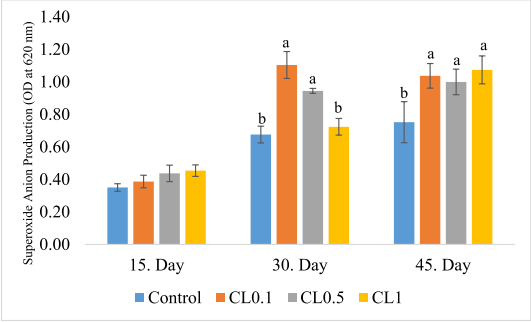

Fig. 1. Effect of dietary methanolic extract of *Cistus laurifolius* on superoxide anion production of head kidney leucocytes of common carp (*Cyprinus carpio*). CL0.1, CL0.5 and CL1, diets containing extract of *C. laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively; Values are expressed as mean ± SE. Different letters on bars indicate significant differences between groups ($P < 0.05$).

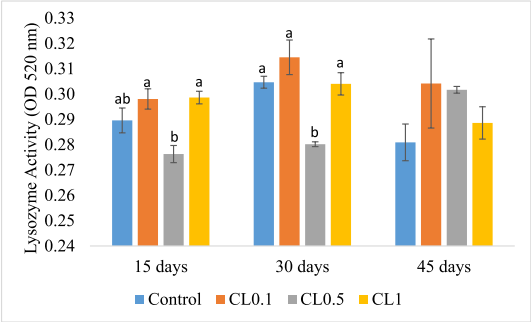

Fig. 2. Effect of dietary methanolic extract of *Cistus laurifolius* on lysozyme activity of head kidney of common carp (*Cyprinus carpio*). CL0.1, CL0.5 and CL1, diets containing extract of *C. laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively; Values are expressed as mean ± SE. Different letters on bars indicate significant differences between groups ($P < 0.05$).

3.5. Gene expression

Results of cytokine gene expressions were presented in Figs. 4–9. IL-1β gene expression was significantly elevated in the kidney and intestine of treated fish groups on all the sampling days of study ($P < 0.05$)

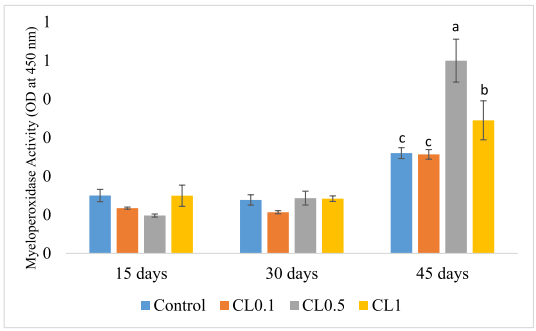

Fig. 3. Effect of dietary methanolic extract of *Cistus laurifolius* on myeloperoxidase activity of head kidney of common carp (*Cyprinus carpio*). CL0.1, CL0.5 and CL1, diets containing extract of *C. laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively; Values are expressed as mean ± SE. Different letters on bars indicate significant differences between groups ($P < 0.05$).

(Fig. 4).

IL-6 gene expression was up-regulated in the kidney of CL treated groups compared to control ($P < 0.05$) on all the sampling times. In the intestine, elevated expression of IL-6 gene was observed only at the end of study (Fig. 5).

The results on effect of dietary administration of CL on IL-8 gene expression are presented in Fig. 6. Expression of IL-8 gene was significantly enhanced in tissues of all experimental fish groups at all the sampling times.

TNF-α expression in the kidney of CL fed fish groups was up-regulated at all the sampling times compared to that of the control ($P < 0.05$). However, in the intestine, TNF-α gene expression was not affected in treated and control groups at any sampling time ($P > 0.05$) (Fig. 7).

IL-10 gene expression was also elevated in both kidney and intestine tissues (Fig. 8). This elevation was found at very high level in the intestine on 45th day of the study (up to 32 folds). On 15th and 30th day of the study, IL-10 gene expression level was raised more than 10 folds in the intestine of treated fish groups and this elevation reached up to 37 folds in the CL1 fish group at the end of study.

Results of TGF-β gene expression were illustrated in Fig. 9. The result exhibited up-regulated expression in the kidney of the treated fish at all the sampling times. Similar to kidney results, intestine also had elevated expression of TGF-β gene. This elevation reached to 31 folds on 15th day and to 33 folds on 45th day of the study.

3.6. Survival of fish during challenge test

Survival rate was significantly higher ($P < 0.05$) in all CL treated fish groups compared to the control (Fig. 10). The highest survival rate was observed in CL0.1 fish group ($P < 0.05$).

4. Discussion

Many medicinal plants and their bioactive compounds have been used in animal and human health for several years due to their beneficial properties, such as antipathogenic, anticancer, anti-inflammatory, immunostimulant activities, growth promotion and others [15,40–43]. Until now, to our knowledge, there has been no information regarding the effects of dietary laurel-leaf cistus on fish health. Hence, this study was designed to examine some growth and clinical parameters (growth performance, digestive enzymes activity, haematological profile, immunological responses and resistance to *A. hydrophila*) of common carp after oral administration of laurel-leaf cistus supplemented diets.

Growth performance of fish is one of the key success factors to efficient production in aquaculture [44]. Our results demonstrated that feeding laurel-leaf cistus increased the growth in common carp. Several

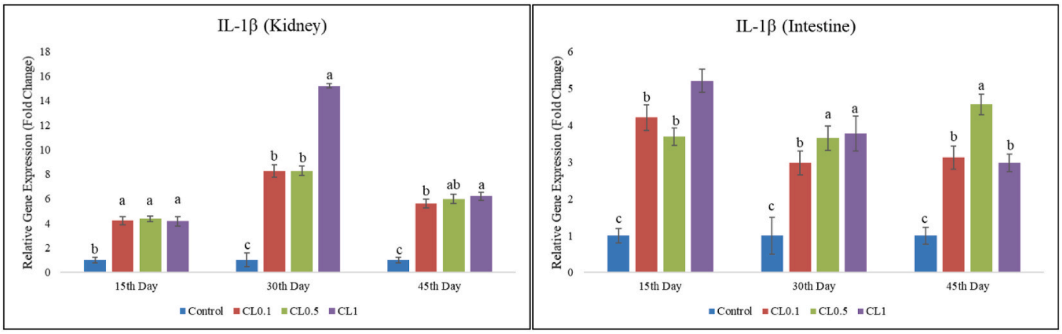

Fig. 4. The effect of laurel-leaf cistus extract on IL-1β gene expression in the kidney and intestine of common carp (n = 3). Letters on the bars denote a significant difference among the treatments on a particular sampling day ($P < 0.05$). CL0.1, CL0.5 and CL1, diets containing extract of *Cistus laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively.

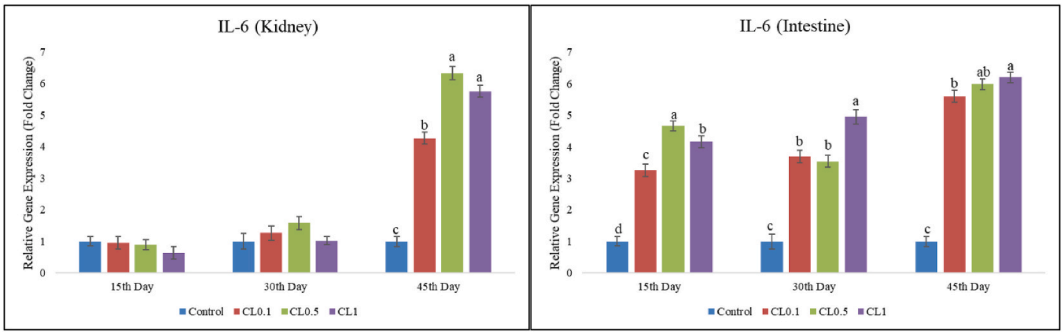

Fig. 5. The effect of laurel-leaf cistus extract on IL-6 gene expression in the kidney and intestine of common carp (n = 3). Letters on the bars denote a significant difference among the treatments on a particular sampling day ($P < 0.05$). CL0.1, CL0.5 and CL1, diets containing extract of *Cistus laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively.

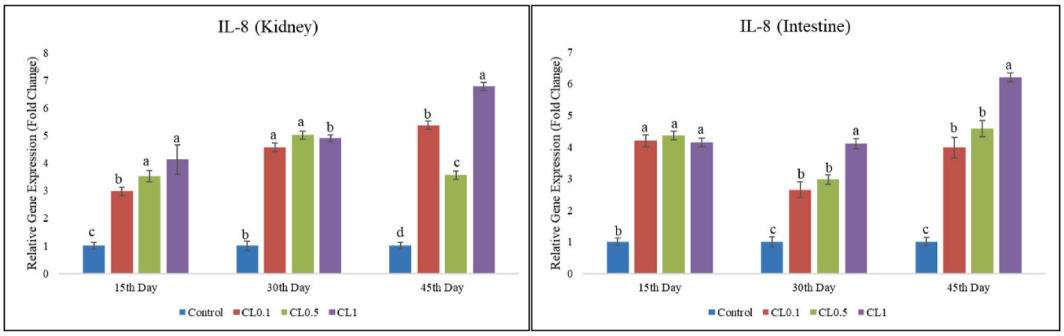

Fig. 6. The effect of laurel-leaf cistus extract on IL-8 gene expression in the kidney and intestine of common carp (n = 3). Letters on the bars denote a significant difference among the treatments on a particular sampling day ($P < 0.05$). CL0.1, CL0.5 and CL1, diets containing extract of *Cistus laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively.

researchers reported that some medicinal plants stimulate growth of fish. For example, common mallow (*Malva sylvestris*) [45] and *Tilia tomentosa* [46] supplemented feeds increased the common carp growth. Mohamed, Amhamed [47] observed an increased growth in common carp (*C. carpio*) fed with *Apium graveolens* aquaeous methanolic extract. Bilen, Altief [48] also found growth promoting effect of lemon balm in rainbow trout. Xie, Liu [49] reported that dietary anthraquinone from rhubarb (*Rheum officinale*) has growth promoting effect in common carp. Dietary rehmannia (*Rehmannia glutinosa*) was reported to exert positive effect on growth in common carp (*Cyprinus carpio*) [44]. These studies reported similar growth promoting effects with our candidate herb in common carp. On the contrary, Salem, Salem [50] ascertained no effects on growth performance in rainbow trout fed with dandelion (*Taraxacum officinalis*) and lichen (*Usnea barbata*) extracts. Also, Bilen and Bilen [51] found no differences on growth performance in rainbow trout after fed with *Cotinus coggygria* and *Laurus nobilis*.

Digestive enzyme (amylase, lipase, trypsin and others) activities correlate directly with growth parameters (SGR, FCR and WG) in fish and play an important role in the hydrolysis of protein, lipids and carbohydrates [52]. The higher digestive enzyme activity results in obtaining better growth in fish. In our study, amylase activity increased significantly in CL0.5 and CL1 diet fed fish. Glycolytic chains, glycogen and starch can trigger amylase activity in fish. The laurel-leaf cistus contains rich organic and inorganic compounds, such as coumarins, flavonoids, terpenoids and sugars [24,26,53]. Therefore, components of laurel-leaf cistus might have affected amylase enzyme activity which facilitated better utilization of diet in fish. Similar to our study, Amhamed, Mohamed [54] reported an increased amylase activity in

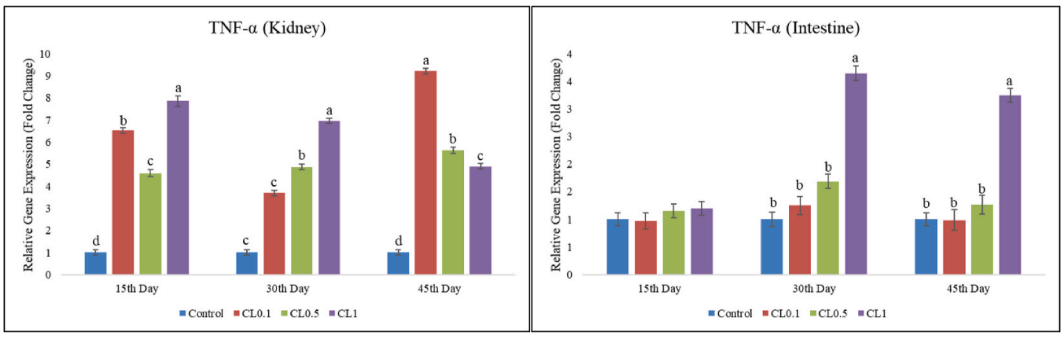

Fig. 7. The effect of laurel-leaf cistus extract on TNF-α gene expression in the kidney and intestine of common carp (n = 3). Letters on the bars denote a significant difference among the treatments on a particular sampling day ($P < 0.05$). CL0.1, CL0.5 and CL1, diets containing extract of *Cistus laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively.

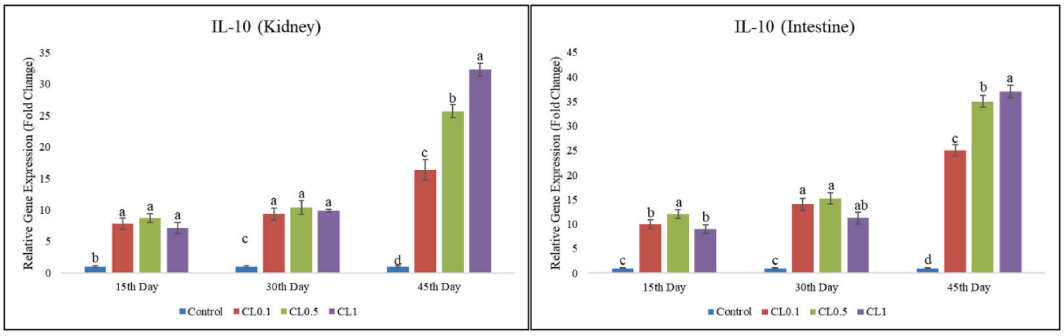

Fig. 8. The effect of laurel-leaf cistus extract on IL-10 gene expression in the kidney and intestine of common carp (n = 3). Letters on the bars denote a significant difference among the treatments on a particular sampling day ($P < 0.05$). CL0.1, CL0.5 and CL1, diets containing extract of *Cistus laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively.

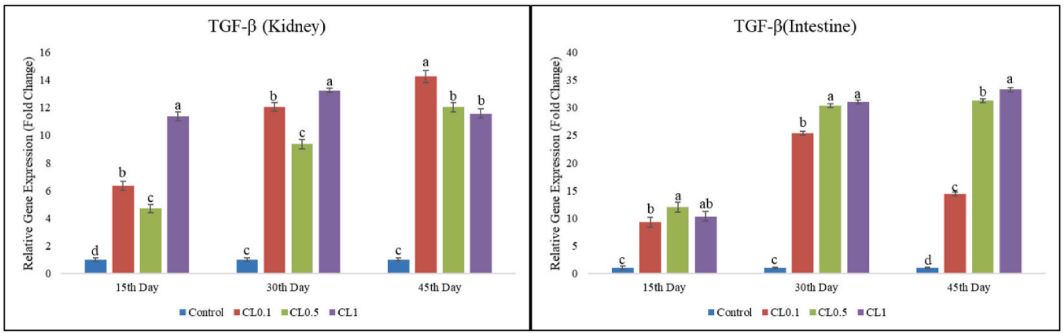

Fig. 9. The effect of laurel-leaf cistus extract on TGF-β gene expression in the kidney and intestine of common carp (n = 3). Letters on the bars denote a significant difference among the treatments on a particular sampling day ($P < 0.05$). CL0.1, CL0.5 and CL1, diets containing extract of *Cistus laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively.

carp treated with *Chenopodium album* extract.

Haematological parameters, such as counts of white and red blood cell are important health indicators in immune system [23]. WBC is indicative of many beneficial features, such as phagocytosis, antibody production and release of immune-related molecules for treating infection [23]. Our haematological analysis exhibited that WBC and RBC increased in CL0.5 and CL1 diet fed fish. Similar results of increased WBC and RBC levels were observed in beluga (*Huso huso*) fed diet containing rose hip and safflower [55].

Oxidative radicals are important antimicrobial effectors [56]. According to our results, SRP increased in CL0.1 and CL0.5 fish groups on 30th day, and in all treatment fish groups at the end of the study. Many biological activities and antibacterial effects have been reported from the use of plant products which contain flavonoids and terpenes [57]. Robak and Gryglewski [58] reported that flavonoids can affect oxidative radical production. Similar to our result, increased ORP was also reported in goldfish (*Carassius auratus*) fed with nettle extract (*Urtica dioica*) [59], and in common carp (*C. carpio*) fed with common mallow extract (*Malva sylvestris*) [45] and rehmannia (*R. glutinosa*) diet [44].

The LA plays a key role in nonspecific immune defence against pathogens, such as bacteria, viruses, fungus and parasites [60,61]. Enhanced LA can control and prevent infection, and keep the mortality rates low in aquaculture (Magnadottir, 2006). LA increased at doses CL0.1 and CL0.5 diets after 30 days in the present study. Further, we observed LA activity was increased in CL1 diet fed fish after 45 days. Previous studies have reported that common mallow (*Malva sylvestris*) and false daisy (*Eclipta alba*) supplemented diets increased LA activity in fishes [62,63]. Contrarily, Mohamed, Amhamed [47] observed no effect

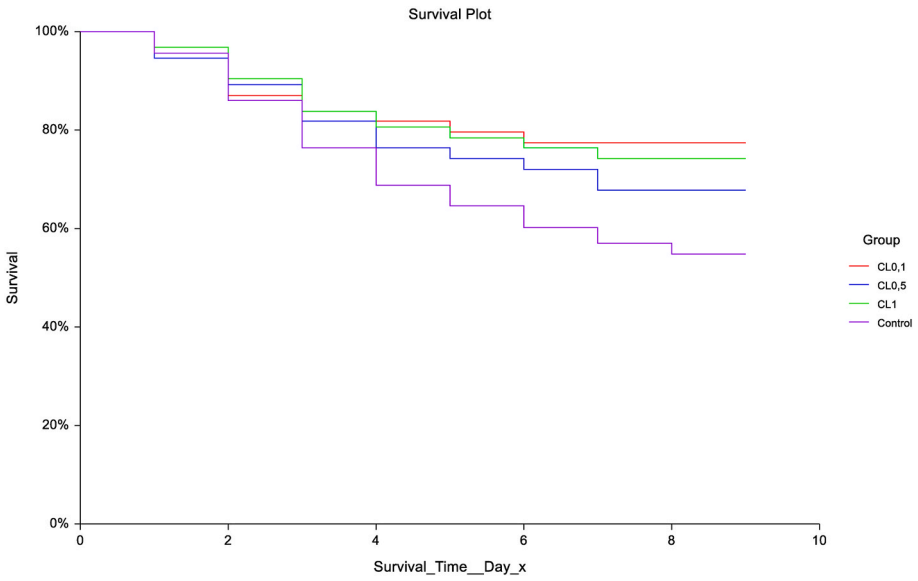

Fig. 10. Cumulative survival (Kaplan-Meier) of common carp fed with extract of *Cistus laurifolius* after challenged with *Aeromonas hydrophila*. CL0.1, CL0.5 and CL1, diets containing extract of *C. laurifolius* at 0.1, 0.5 and 1 g kg^{-1} diet, respectively.

on LA in common carp fed diet containing celery (*Apium graveolens*) aqueous methanolic extract. In our study, all these results (haematological parameters, ORP and LA activity) indicated that CL supplemented diets can support nonspecific immune system in fish.

In the present study, expression of all pro-inflammatory cytokine genes was elevated in the head kidney cells of common carp. Head kidney is the main immune-competent organ and performs many different immune activities. Moreover, early and quick immune responses are generally provided by the head kidney. Previously, up-regulated IL-1β, TNF-α and IL-8 gene expressions were reported by Hoseinifar, Zou [37] in common carp fed with a diet supplemented with loquat (*Eriobotrya japonica*) leaf extract and by Terzi, Kucukkosker [64] in rainbow trout fed with a diet supplemented with *Prunus domestica* extract. Significant up-regulation of IL-1β and TNF-α gene expressions was observed in the yeast extract-treated common carp as well [34]. Up-regulated IL-1β and TNF-α genes were also reported in common carp fed with Spirulina [65]. IL-6 is produced and released by different organs in fish. In the present study, an enhanced IL-6 gene expression was observed in the intestine of treated fish at all sampling times. However, this enhancement was observed in the kidney only at the end of the study. This may indicate that IL-6 could be highly expressed by the gut associated immune cells. Elevated pro-inflammatory cytokines could also be resulted from increased phagocytic activity. All these elevated inflammatory cytokine gene expressions could also support high survival of common carp against *A. hydrophila*.

Expression of anti-inflammatory cytokine genes, such as IL-10 and TGF-β was also investigated in the present study. The results demonstrated a very high elevation of both IL-10 and TGF-β genes initially in the intestine and then in the kidney. TGF-β, an anti-inflammatory cytokine suppresses or regulates inflammation [66]. Our results suggest that CL also have a high anti-inflammatory characteristic. In ethanol extracted CL, three flavonoids; 3-O-methylquercetin, 3,7-O-dimethylquercetin and 3,7-O-dimethylkaempferol were previously isolated as the main compounds [67]. All those compounds also possess anti-inflammatory properties. Our results, especially IL-10 and TGF-β gene expressions were supported by this previous study. Moreover, it is evident that CL ethanolic extract contains those three compounds which are anti-inflammatory in nature.

After challenge test, all CL diet fed fish groups had higher survival rates than the control against *A. hydrophila* infection. This result indicated two different that oral administration of CL can control and prevent disease. Previous studies reported that flavonoids and terpenes are antibacterial agents against various bacterial infections [68,69]. The rainbow trout fed with the extract of oyster mushroom exhibited higher survival rate during the *A. hydrophila* infection [27]. Similar to the laurel-leaf cistus, oyster mushroom contains flavonoids and terpenes [70]. Hence, it is necessary to identify the functional compound profile of those herbs and fungus. In addition, further investigation on the pharmacokinetics of the main active compounds, such as flavonoids or terpenes of laurel-leaf cistus is necessary to confirm the antibacterial effect against *A. hydrophila in vitro*.

In conclusion, our results demonstrated that laurel-leaf cistus is a potential candidate medicinal herb as a growth promoter and antibacterial agent against *A. hydrophila*, as well as it supports nonspecific immune system of fish for aquaculture industry. Laurel-leaf cistus at 0.1 g kg^{-1} can be suitable for application with feed to fish. However, before field application of laurel-leaf cistus diet to common carp, toxicity and pharmacokinetic studies are needed for fish health and drug safety.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authorship contribution statement

Conceptualization and study design were performed by Soner Bilen. Extract preparation, fish culture and sampling were conducted by Gamaia Ali Mohamed Ali, Iman Daw Amhamed, Ahmed Alhadi Almabrok. Blood and plasma analysis were conducted by Gamaia Ali Mohamed Ali, Iman Daw Amhamed, Ahmed Alhadi Almabrok. Data analysis and manuscript drafting were conducted by Soner Bilen.

Declaration of competing interest

None.

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