Fish and Shellfish Immunology 89 (2019) 672–676

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Dietary supplementation of olive leaf extract increases haematological, serum biochemical parameters and immune related genes expression level in common carp (*Cyprinus carpio*) juveniles

Fahriye Zemheri-Navruz[a], Ümit Acar[b,*], Sevdan Yılmaz[c]

[a] Bartın University, Faculty of Science, Department of Molecular Biology and Genetics, Bartın, Turkey
[b] Department of Forestry, Bayramiç Vocational School, Çanakkale Onsekiz Mart University, Çanakkale, Turkey
[c] Department of Aquaculture, Faculty of Marine Science and Technology, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

ARTICLE INFO

Keywords:
Common carp
Gene expression
Olive leaf extract
Blood parameter

ABSTRACT

The present study investigated the effects of dietary olive leaf (*Olea europea* L.) extract (OLE) on some blood parameters and immune (*TNF-α*, *IL-1ß* and *IL-8*) related genes in different tissues (head kidney, liver and spleen) and resistance of common carp, *Cyprinus carpio* to *Edwardsiella tarda*. Five diets were prepared for fed fish (mean body weight 15.90 ± 0.93 g) with different rates of OLE (0.0%, 0.1%, 0.25%, 0.50% and 1.0%). A control diet was prepared non-supplemented with OLE. Increased haematocrit ratio, serum myeloperoxidase activity, immune response gene levels (*IL-1β* in head kidney tissue and *TNF-α* in spleen tissue) and survival rate against *E. tarda* particularly in the 0.1% OLE treatment group. In conclusion, results of the present study show that feeding common carp with a diet containing 1 g/kg OLE over a period of 60 days might be adequate to improve fish immune parameters, and survival rate against *E. tarda*. Therefore, OLE can be used as a dietary additive to prevent *E. tarda* in common carp.

1. Introduction

Aquaculture continues to grow faster than other major food production sectors in the World. Especially World production of farmed food fish relies increasingly on inland aquaculture. Global aquaculture production of common carp was 4.5 million tons in 2016 [1]. The intensive rearing of species created some stressful conditions due to high stocking density. As a result of stress which lead to various diseases [2]. Therefore various infectious may cause huge economic loses in aquaculture. The use of antibiotics in aquaculture has debated for long time due to its negative impacts on environment, development of drug resistance and residues in fish [2]. Therefore to provide the sustainability of aquaculture and preventing of diseases its necessary to find eco-friendly drug for use in aquaculture.

Recently herbs extracts or their essential oils offer an alternative to antibiotics because of their immunomodulatory effects in aquaculture [3]. Immunostimulant effects of some herbal origin feed additives have ability to increase disease resistance by enhancing immunity of fish [4–6]. Olive leaf is the most important source of oleuropein known in nature. It is well known that the metabolic effects of polyphenolic substances such as oleuropein, hydroxytyrosol and verbascositis, which form the main bioactive components of Olive Leaf Extract (OLE), are multifaceted. Several researchers revealed the fact that oleuropein has many pharmacological qualities including antioxidant, antimicrobial, anti-inflammatory, antiaterogenic and antiviral activities [7–9]. In addition, it is stated that oleuropein has a strong antioxidant effect with its quality of bonding endogenous peptides in addition to its anti-inflammatory, anti-atherosclerotic and anticarcinogenic properties [10].

Edwardsiella tarda, a gram negative bacterium which is associated with stressful condition caused septicemia in fish [11]. Experimental or natural infectious by *E. tarda* have also been reported in carp [12,13]. Earlier researches have shown that significant correlation exist between herbal feed additives and disease resistance in fish which affect the immune response [14–16].

The present study was aimed to investigate the changes in some hematological and serum biochemical parameters in common carp (*C. carpio*) fed with olive leaf extract supplemented diets. Also, expression pattern of some immune related genes such as *IL-8*, *IL-1ß*, *TNF-α*, in the head kidney, liver and spleen tissue.

* Corresponding author.
E-mail address: umitacar@comu.edu.tr (Ü. Acar).

https://doi.org/10.1016/j.fsi.2019.04.037
Received 28 January 2019; Received in revised form 8 April 2019; Accepted 11 April 2019
Available online 13 April 2019

Table 1
Ingredients (%), composition of the experimental diets containing graded replacement levels of OLE for common carp as (% of dietary protein).

Raw materials	OLE_0	$OLE_{0.1}$	$OLE_{0.25}$	$OLE_{0.50}$	OLE_1
Fish meal[1]	23.00	23.00	23.00	23.00	23.00
Soybean meal[2]	37.00	37.00	37.00	37.00	37.00
Wheat meal[2]	12.00	12.00	12.00	12.00	12.00
Fish oil[3]	5.00	5.00	5.00	5.00	5.00
Vitamin-mineral mix[4]	4.00	4.00	4.00	4.00	4.00
Corn starch[2]	19.00	18.90	18.75	18.50	18.00
Olive leaf extract	0.00	0.10	0.25	0.50	1.00
Crude protein	35.27	35.63	35.78	35.73	35.78
Crude lipid	7.62	7.58	7.71	7.58	7.71
Crude ash	5.88	5.96	5.68	5.96	5.68

[1] Anchovy fish meal, Koptur Balıkçılık Ltd. Şti, Trabzon.
[2] Soybean meal, Agromarin Yem San. ve Tic. A.Ş.
[3] Anchovy fish oil, Koptur Balıkçılık Ltd. Şti, Trabzon.
[4] Vitamin-mineral mix: Vitamin A, 18000 IU kg^{-1}; Vitamin D_3, 2500 IU kg^{-1}; Vitamin E, 250 mg kg^{-1}; Vitamin K_3, 12 mg kg^{-1}; Vitamin B_1, 25 mg kg^{-1}; Vitamin B_2, 50 mg kg^{-1}; Vitamin B_3, 270 mg kg^{-1}; Vitamin B_6, 20 mg kg^{-1}; Vitamin B_{12}, 0,06 mg kg^{-1}; Vitamin C, 200 mg kg^{-1}; Folic asit, 10 mg kg^{-1}; Calcium d–pantotenat, 50 mg kg^{-1}; Biotin, 1 mg kg^{-1}; İnositol, 120 mg kg^{-1}; Fe, 75,3 mg; Cu, 12,2 mg; Mn, 206 mg; Zn, 85 mg; I, 3 mg; Se, 0,35 mg; Co, 1 mg.

2. Material and methods

2.1. Fish and experimantal conditions

Three hundred common carp obtained from Mediterranean Fisheries Research, Production and Training Institute in Antalya, Turkey were used in the experimental study. Fish were acclimatized for fourteen days in to the 1000 L tank fed with commercial carp diet. During the acclimatized and experiment period fish were kept at the ambient photoperiod (12L:12D), temperature was 25 ± 1 °C, dissolved oxygen 5.96 ± 0.75 mg/L, and pH 7.48 ± 0.56. The feed supplement used was dried leaves of olive (*O. europea*) were collected from their natural habitat from Balıkesir Edremit region in Turkey. Olive leaves were dried in shade and crushed. After crushing, 200 g of powdered leaves were extracted by stirring 1000 ml of 70% aqueous ethanol at a temperature of 38 °C incubated for 24 h in a water bath and filtered using a Whatman filter (0.45 µm). The remaining aqueous solutions were lyophilized and kept at 4 °C in dark bottles [17]. Experimental diets were prepared by adding 0.1%, 0.25%, 0.50% and 1% olive leaves extract (OLE). A control diet was prepared non-supplemented with OLE (Table 1). A laboratory food mixer was used to mix the dry ingredients for the diet preparation. The mixtures were primed with tap water to yield an applicable pulp. 1-mm pellets were fabricated using the wet ingredients, which were later dried at 40 °C in a drying cabinet, and stored at −20 °C until feeding. In this experiment 300 individuals (15.90 ± 0.93 g) were randomly distributed in to five groups (15 aquarium) and coded OLE_0, $OLE_{0.1}$, $OLE_{0.25}$ $OLE_{0.50}$ and OLE_1. The each experimental group was assembled in triplicate. The fish were fed two times each days at 9:00 and 16:00 for 60 days.

2.2. Blood collection and analyzing

After feeding with the supplemented diet of OLE, 1 ml of blood was withdrawn from caudal vein with a syringe after anesthetizing with clove oil (25 mg/L) five fish from each aquarium [18]. The extracted blood was then centrifuged at 4000 rpm for 10 min to separate the serum for biochemical analyses. Biochemical indices, including glucose (GLU), cholesterol (CHOL), serum total protein (TP), in serum were analyzed using bioanalytical test kits (Bioanalytic Diagnostic Industry, Co) and measured by a Shimadzu spectrophotometer (PG Instruments, UK). One mL blood samples were allocated for the haematological assays and the rest were added to heparin containing tubes for the other biochemical analyses. Red blood cells, haematocrit and haemoglobin concentration were determined by using the method of Blaxhall & Daisley (1973). RBC was counted with a Thoma hemocytometer with the usage of Dacie's diluting fluid. The haematocrit was determined by using a capillary hematocrit tube. The haemoglobin concentration was determined with spectrophotometry (540 nm) by using the cyanomethahemoglobin method.

2.3. Challenge test

E. tarda SY-ED14 was provided by the Fish Health Research Laboratory, Department of Aquaculture, Çanakkale Onsekiz Mart University. Challenge experiment were performed according to the methods previously described in our laboratory [5]. Recorded mortality data was used for calculating Relative Percentage Survival (RPS) following Amend (1981) [19].

RPS = 1 − [(Mortality (%) in treated group) / (Mortality (%) in control group)] × 100.

2.4. Total RNA isolation

For immune-related gene expression study, head kidney, spleen and liver tissue from **six** fish each aquarium were dissected and stored in RNAlater solution (Thermo Scientific, USA) at - 20 °C -until extraction of total RNA. RNA isolation was performed by using GeneJet RNA purification kit (Thermo Scientific, USA) from liver, head kidney and spleen tissue samples. Purity and quantity of isolated RNAs was measured with Multiskan™ FC Microplate Photometer (Thermo Scientific, USA).

2.5. Primer design and cDNA synthesis

DNase-I (Thermo Scientific, USA) was used to eliminate DNA contamination from RNA samples and cDNA was synthesized by means of RevertAid H Minus Single Strand cDNA Synthesis Kit (Thermo Scientific, USA). National Center of Biotechnology (NCBI) website was used for mRNA sequences of *β-actin, TNF-α*, *IL-1ß* and *IL-8* genes and primers were designed using FastPCR 6.0 program [20]. Primer sequences, - product size (bp) and gene bank numbers are given in Table 2.

2.6. Real-time PCR analysis

Real-time PCR (Bio RAD, ABD) was used to determine differences in gene expression levels of experimental groups. PCR analysis was performed in PCR mix containing Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA), cDNA, forward and revers primer and nuclease free water.

Table 2
Primer sequences used in this study.

Gene		Oligonucleotide sequence	Product size (bp)	Gene Bank No.
β-Aktin	F	CTGGTATCGTGATGGACTCT	204	M24113
	R	CAGAGCTTCTCCTTGATGTC		
TNF-α	F	GTGTCTACAGAAACCCTGGA	109	AJ311800
	R	AGTAAATGCCGTCAGTAGGA		
IL-1ß	F	TTACAGTAAGACCAGCCTGA	89	AJ245635
	R	AGGCTCGTCACTTAGTTTGT		
IL-8	F	GTCTTAGAGGACTGGGTGTA	120	AB470924.1
	R	ACAGTGTGAGCTTGGAGGGA		

2.7. Identification of gene expression levels

Analysis of real-time PCR results was carried out by CFX Manager 3.1 software. Relative changes in mRNA expression levels of target genes were calculated using $2^{-\Delta\Delta Ct}$ method based on cycle thresholds (Ct) of amplification curves obtained following amplification process comprising denaturation, annealing of primer and chain extension steps [21].

2.8. Statistical analysis

Statistical significance was tested by one-way analysis of variance (ANOVA) and differences between group means were evaluated using Tukey multiple range test. In all analyses, the significance level was set at 0.05.

3. Results

3.1. Hematological and serum biochemical parameters

The results of hematology and serum biochemistry values obtained between the groups at the end of feeding experiment are given in Table 3. When the results were examined, it was determined that there was no statistically significant difference between the number of erythrocyte counts and hemoglobin levels in all experimental groups ($p > 0.05$). However, the amounts of hematocrit in the $OLE_{0.1}$ $OLE_{0.25}$ groups were significantly higher compared to the other experimental groups ($p < 0.05$). Serum biochemistry parameters of GLU, TPROT and CHOL values were found to be adversely affected by the increase of OLE in the ration. The lowest serum MPO value was obtained in the group fed with OLE_1 diet.

3.2. Expression of immune related genes

Expression profiles of immune related genes (*TNF-α*, *IL-1β* and *IL-8*) were examined in head kidney, spleen and liver (Fig. 1) at the end of the experiment. Transcription levels of *TNF-α*, *IL-1β* and *IL-8* were slightly up-regulated in all three tissue in fish fed the OLE supplemented diets (Fig. 1). However, significant differences obtained the expression of *IL-1β* in fish head kidney and *TNF-α* in spleen tissue fish fed with $OLE_{0.1}$ diet.

3.3. Challenge test

After 60 days of feeding, fishes were challenged with *E. tarda* and cumulative mortality was recorded for 6 days. Dietary supplementation of OLE at level of 0.1% enhanced common carp resistance againist to *E. tarda* infection (Table 4). The highest post-challenge survival (70%) was obtained in $OLE_{0.1}$ group whereas the lowest post-challenge survival rate (36.7%) was observed for fish fed with OLE_1 diet.

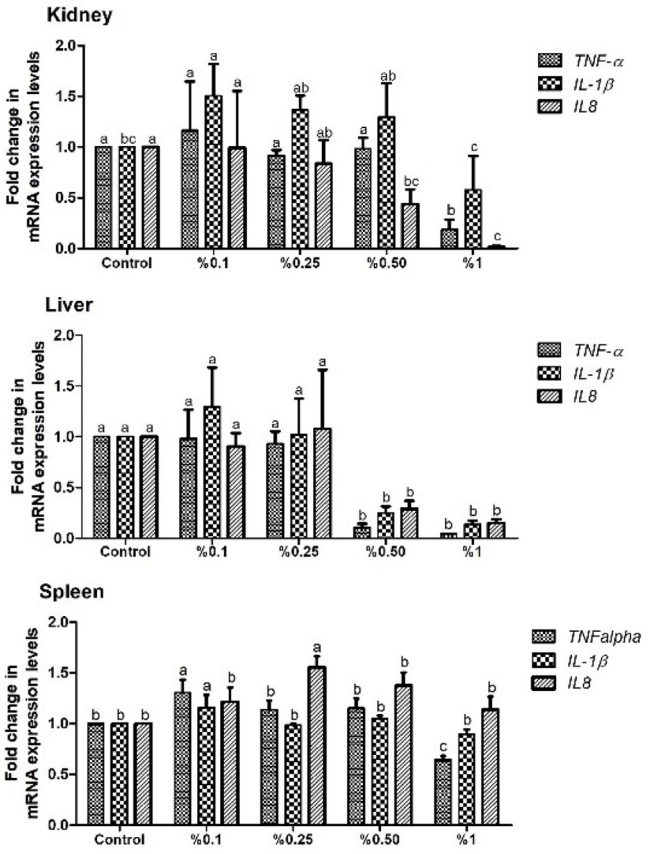

Fig. 1. Effect of OLE diet on *TNF-α*, *IL-1β* and *IL-8* mRNA expression levels of head kidney, liver and spleen tissues. Values were reported as means ± SD of 6 replicates. Results were expressed statistically significant at $p < 0.05$. Different letters (a, b, and c) show statistically significant differences between all groups.

Table 4
The effect of experimental groups on survival rates of fish against E. tarda pathogen.

Experimental Groups	Survival rate (%)	Mortality (%)	RPS
OLE_0 (Control)	53.3	46.7	
$OLE_{0.10}$	70	30	43.75
$OLE_{0.25}$	56.7	43.3	18.75
$OLE_{0.50}$	56.7	43.3	18.75
OLE_1	36.7	63.3	−18.75

4. Discussion

Antibiotics, which are used as resistance against to bacterial diseases in aquaculture and used as growth promoted agents, are not undesirable due to the antibiotic resistance of the fish and the health of

Table 3
Hematological and serum biochemical parameters of common carp fed with experimental diets for 60 days.

	OLE_0	$OLE_{0.1}$	$OLE_{0.25}$	$OLE_{0.50}$	OLE_1
Erythrocytes count (10^6 mm^3)	2.07 ± 0.14	2.20 ± 0.09	2.27 ± 0.06	2.24 ± 0.19	2.14 ± 0.15
Hemoglobin (g × dL^{-1})	9.38 ± 0.46	9.88 ± 0.33	10.12 ± 0.67	10.16 ± 0.43	9.73 ± 0.45
Hematocrit (%)	23.43 ± 1.62^{b}	26.12 ± 0.85^{a}	26.32 ± 0.55^{a}	24.63 ± 1.83^{ab}	23.03 ± 1.13^{b}
GLU (mg dL^{-1})	102.41 ± 10.44^{b}	96.35 ± 9.63^{b}	109.14 ± 9.19^{b}	132.54 ± 13.18^{a}	149.58 ± 21.11^{a}
TPROT (g dL^{-1})	6.71 ± 0.54^{b}	7.02 ± 0.58^{b}	8.53 ± 0.71^{a}	7.31 ± 0.96^{b}	5.45 ± 0.15^{c}
CHOL (mg dL^{-1})	163.91 ± 36.89^{b}	163.57 ± 27.93^{b}	222.59 ± 40.38^{ab}	197.44 ± 39.73^{ab}	224.73 ± 29.73^{a}
MPO (U L^{-1})	141.05 ± 3.49^{bc}	149.40 ± 4.60^{a}	143.25 ± 4.54^{ab}	136.04 ± 4.86^{cd}	133.46 ± 2.32^{d}

Data are represented as mean ± SE (n = 15). Different letters represent the significant differences at $p < 0.05$. GLU.glucose; CHOL.cholesterol; TPROT.total protein; MPO, myeloperoxidase.

the consumers [22]. Therefore, traditional medicinal herb and their extracts widely used because of various pharmacological properties and immunomodulatory effects in fish diets [2,23]. In the present study, we determined the effects of OLE on some hematological, serum biochemical parameters, resistance to *E. tarda* and immune related genes expression level in different tissue of common carp.

Haematological and serum biochemical parameters are widely used in order to determine the effects of feed additives on fish health [24,25]. In our study the Hct levels significantly increased in the group fed with 0.1% and 0.5%. Similar results were also obtained in Asian sea bass, *Lates calcarifer* fed with garlic supplemented feeds [26]. Increased levels of Hct in OLE supplemented groups show the improved health status of fish.

The hypoglycaemic and hypolipidemic effects of OLE was reported in rat [27] and mice [28] respectively. However, in present study, serum glucose levels increased in fish fed with diets containing 0.5% and 1% OLE. Moreover, cholesterol level was also increased in the 1% OLE group. This suggests that addition of high doses of OLE in common carp diets negatively affect fish health. In support of serum glucose and cholesterol results, immune related variables (total protein and myeloperoxidase activity) decreased significantly in fish fed with high doses of OLE in this study. This results is in agreement with a previous study in fish treated with different herbs oil [29].

Apart from the benefit of the improvement the serum biochemical variables of common carp, we also determine that OLE can effectively up-regulate the expression of immune related genes such as *TNF-α*, *IL-1β* and *IL-8*. For example, *TNF-α*, *IL-1β*, *IL-8* were up-regulated in spleen, head kidney and liver. Due to complexity of understanding the immune response evaluating from different aspects is important. For this reason studying the immunostimulant effects of herb extract by mRNA expression of genes is also valuable tool [30]. *IL-1β* is recognized as an indicator of innate immunity and body response to various microbial agents and toxins [31]. It can stimulate immune response by procuding blood monocyte and tissue macrophage [32]. In the present study, the effects of OLE on *IL-1β* gene expression was dose specific. In spleen, head kidney and liver tissue expression level of *IL-1β* was down regulated in fish fed more than 2.5 g/kg OLE in diets. Similar results reported by Nootash et al. (2013) for rainbow trout (*Oncorhynchus mykiss*) when the fish fed 20 mg kg^{-1} green tea extract [30]. According to the limited study about the effects of OLE on fish immune response, Baba et al. (2018) reported, *IL-1β* gene expression up-regulated in liver tissue of rainbow trout (*O. mykiss*) fed with 1 g/kg OLE in diets [33]. *TNF-α* is cell-inflammatory cytokines involved in cell proliferation, apoptosis, leukocyte migration, phagocytic activity and other pro-inflammatory cytokine [34]. In the present study, OLE increased significantly *TNF-α* gene expression levels in all three immune related organ especially at 1 g/kg and 2 g/kg compared to the other experimental groups. *TNF-α* gene expression level was also up-regulated in rainbow trout (*O. mykiss*) liver, spleen and head kidney that fed appropriate dose of green tea extract [30]. In common carp gill and spleen *TNF-α* gene expression levels were up-regulated fish fed with high dose of Astragalus polysaccharides [34]. *IL-8* is produced by a number of cell types, such as macrophages/monocytes, epithelial cells, neutrophils, fibroblasts, and endothelial cells, which exhibit a strong inflammatory response to neutrophils and other leukocytes to the site of infection, or which is a chemokine induced by cytokines such as *IL-1* and *TNF-α* [35]. In the present study *IL-8* gene expression levels in spleen, head kidney and liver up-regulated fish fed with 1 g/kg and 2 g/kg OLE supplemented diets. Similarly, in rainbow trout fed with green tea extract and OLE supplemented diets *IL-8* gene expression levels up-regulated in tissue specific and dose-dependent manner [30,33]. It is not surprising that the immune response in fish is positively affected by feed additives such as green tea [30], fenugreek [36], spirulina [37] and alginic acid [38]. Specifically, OLE has strong antioxidant properties due to the content of oleurpein [39,40]. Therefore, antioxidant compounds can protect cells against the damaging effects of reactive oxygen species, such as superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite [30]. The main point of using the herbal extracts in fish diets should be considered appropriate doses are preferred.

In our study, increased haematocrit ratio, serum myeloperoxidase activity, immune response gene levels (*IL-1β* and *TNF-α*) and survival rate against *E. tarda* particularly in the OLE0.1 treatment group showed the immunomodulatory effects of OLE in fish. Similar with our study, Baba et al. (2018) reported that a significant increase of survival rate against *Yersinia ruckeri* was recorded for rainbow trout fed with 1 g/kg OLE in diet [33].

In conclusion, results of the present study show that feeding common carp with a diet containing 1 g/kg OLE over a period of 60 days might be adequate to improve fish immune parameters, and survival rate against *E. tarda*. Therefore, OLE can be used as a dietary additive for prevent to *E. tarda* in common carp. This study is a single experimental model, it's necessary in the future use a different concentration of OLE in different fish species and with different feed concentration. The response to feed integration should be change in relation to different dose and other blood parameters should be investigate.

Acknowledgement

Authors thank to Dr. Yonca Surgun-Acar for valuable help to writing the manuscript. This work was supported by Bartın University Scientific Research Projects Coordination Unit. Project Number: 2017-FEN-A-015. Also, this study was conducted with the approval of the local ethics committee for animal experiments, Çanakkale Onsekiz Mart University, Çanakkale, Turkey (Approval Number:2017/04-01).

References

[1] FAO, The state of world fisheries and aquaculture 2018, Food and Agriculture Organization of the United Nations, Rome, FAO, 2018, p. 224.
[2] J.L. Wang, X. Meng, R. Lub, C. Wu, Y.T. Luo, X. Yan, X.J. Li, X.H. Kong, G.X. Nie, Effects of Rehmannia glutinosa on growth performance, immunological parameters and disease resistance to Aeromonas hydrophila in common carp (*Cyprinus carpio L.*), Aquaculture 435 (2015) 293–300.
[3] J. Galina, G. Yin, L. Ardo, Z. Jeney, The use of immunostimulating herbs in fish. An overview of research, Fish Physiol. Biochem. 35 (4) (2009) 669–676.
[4] Ü. Acar, O. Kesbiç, S. Yilmaz, N. Gültepe, A. Türker, Evaluation of the effects of essential oil extracted from sweet orange peel (*Citrus sinensis*) on growth rate of tilapia (*Oreochromis mossambicus*) and possible disease resistance against *Streptococcus iniae*, Aquaculture 437 (2015) 282–286.
[5] E. Baba, Ü. Acar, C. Öntaş, O.S. Kesbiç, S. Yılmaz, Evaluation of Citrus limon peels essential oil on growth performance, immune response of Mozambique tilapia Oreochromis mossambicus challenged with *Edwardsiella tarda*, Aquaculture 465 (2016) 13–18.
[6] Ü. Acar, Effect of St. John's worth oil (*Hypericum perforatum*) on growth performance and some blood parameters of fry carp (*Cyprinus carpio*), Alınteri Zirai Bilimler Dergisi 33 (1) (2018) 21–27.
[7] F. Visioli, S. Bellosta, C. Galli, Oleuropein, the bitter principles of olives, enhances nitric oxide production by mouse macrophages, Life Sci. 62 (6) (1998) 541–546.
[8] R.W. Owen, A. Giacosa, W.E. Hull, R. Haubner, G. Würtele, B. Spiegelhalder, H. Bartsch, Olive oil consumption and health: the possible role of antioxidants, Lancet Oncol. 1 (2) (2000) 107–112.
[9] F. Visioli, A. Poli, C. Galli, Antioxidant and other biological activities of phenols from olives and olive oil, Med. Res. Rev. 22 (1) (2002) 65–75.
[10] E. Gikas, F.N. Bazoti, A. Tsarbopoulos, Conformation of Oleuropein, the major bioactive compound of Olea europea, J. Mol. Struct.: THEOCHEM 821 (1–3) (2007) 125–132.
[11] B.R. Mohanty, P.K. Sahoo, Edwardsiellosis in fish: a brief review, J. Biosci. 32 (3) (2007) 1331–1344.
[12] P.K. Sahoo, S.C. Mukherjee, The effect of dietary immunomodulation upon *Edwardsiella tarda* vaccination in healthy and immunocompromised Indian major carp (*Labeo rohita*), Fish Shellfish Immunol. 12 (1) (2002) 1–16.
[13] P. Swain, S.K. Nayak, Comparative sensitivity of different serological tests for seromonitoring and surveillance of Edwardsiella tarda infection of Indian major carps, Fish Shellfish Immunol. 15 (4) (2003) 333–340.
[14] M.R.I. Sarder, K.D. Thompson, D.J. Penman, B.J. McAndrew, Immune responses of Nile tilapia (*Oreochromis niloticus L.*) clones: I. Non-specific responses, Dev. Comp. Immunol. 25 (1) (2001) 37–46.
[15] B.R. Mohanty, P.K. Sahoo, K.D. Mahapatra, J.N. Saha, Innate immune responses in families of Indian major carp, Labeo rohita, differing in their resistance to *Edwardsiella tarda* infection, Curr. Sci. (2007) 1270–1274.
[16] B.R. Mohanty, P.K. Sahoo, Immune responses and expression profiles of some immune-related genes in Indian major carp, Labeo rohita to Edwardsiella tarda

infection, Fish Shellfish Immunol. 28 (4) (2010) 613–621.
[17] T.H. Lee, F. Qiu, G.R. Waller, C.H. Chou, Three new flavonol galloylglycosides from leaves of Acacia confusa, J. Nat. Prod. 63 (2000) 710–712.
[18] M. Iversen, B. Finstad, R.S. McKinley, R.A. Eliassen, The efficiency of metomidate clove oil aqui-S™ and Benzoak® as anaesthetics in atlantic salmon (*Salmo salar L.*) smolts and their potential stress-reducing capacity, Aquaculture 221 (1–4) (2003) 549–566.
[19] D.F. Amend, Potency testing of fish vaccines, Fish biologics: serodiagnostics and vaccines (1981) 447–454.
[20] R. Kalendar, D. Lee, A.H. Schulman, Fast PCR Software for PCR primer and probe design and repeat search, Genes, Genomes Genomics 3 (1) (2009) 1–14.
[21] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Asids Research 29 (9) (2001).
[22] S. Yılmaz, S. Ergün, Trans-cinnamic acid application for rainbow trout (Oncorhynchus mykiss): I. Effects on haematological, serum biochemical, non-specific immune and head kidney gene expression responses, Fish Shellfish Immunol. 78 (2018) 140–157.
[23] P. Rattanachaikunsopon, P. Phumkhachorn, Protective effect of clove oil-supplemented fish diets on experimental Lactococcus garvieae infection in tilapia, Biosci. Biotechnol. Biochem. 73 (2009) 2085–2089.
[24] V. Parrino, T. Cappello, G. Costa, C. Cannavà, M. Sanfilippo, F. Fazio, S. Fasulo, Comparative study of haematology of two teleost fish (Mugil Cephalus and Carassius Auratus) from different environments and feeding habits, the European Zoological Journal 85 (1) (2018) 194–200.
[25] F. Fazio, Fish hematology analysis as an important tool of aquaculture: a review. 579 Aquaculture, 500 (2019) 237–242.
[26] A.D. Talpur, M. Ikhwanuddin, 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 (2012) 6–12.
[27] J. Wainstein, T. Ganz, M. Boaz, Y. Bar Dayan, E. Dolev, Z. Kerem, Z. Madar, Olive leaf extract as a hypoglycemic agent in both human diabetic subjects and in rats, J. Med. Food 15 (7) (2012) 605–610.
[28] O. Said, B. Saad, S. Fulder, R. Amin, E. Kassis, K. Khalil, Hypolipidemic activity of extracts from Eriobotrya japonica and Olea europaea, traditionally used in the Greco-Arab medicine in maintaining healthy fat levels in the blood, Open Compl. Med. J. 1 (1) (2009).
[29] S. Yilmaz, S. Ergun, Effects of garlic and ginger oils on hematological and biochemical variables of sea bass Dicentrarchus labrax, J. Aquat. Anim. Health 24 (4) (2012) 219–224, https://doi.org/10.1080/08997659.2012.711266.
[30] S. Nootash, N. Sheikhzadeh, B. Baradaran, A.K. Oushani, M.R.M. Moghadam, K. Nofouzi, S. Shabanzadeh, Green tea (*Camellia sinensis*) administration induces expression of immune relevant genes and biochemical parameters in rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 35 (6) (2013) 1916–1923.
[31] C.J. Secombes, The nonspecific immune system: cellular defenses, The fish immune system: organism, pathogen and environment 15 (1996) 63–103.
[32] Y. Corripio-Miyar, S. Bird, K. Tsamopoulos, C.J. Secombes, Cloning and expression analysis of two pro-inflammatory cytokines, *IL-1β* and *IL-8*, in haddock (*Melanogrammus aeglefinus*), Mol. Immunol. 44 (6) (2007) 1361–1373.
[33] E. Baba, Ü. Acar, S. Yılmaz, F. Zemheri, S. Ergün, Dietary olive leaf (*Olea europea L.*) extract alters some immune gene expression levels and disease resistance to *Yersinia ruckeri* infection in rainbow trout *Oncorhynchus mykiss*, Fish Shellfish Immunol. 79 (2018) 28–33.
[34] C. Yuan, X. Pan, Y. Gong, A. Xia, G. Wu, J. Tang, X. Han, Effects of Astragalus polysaccharides (APS) on the expression of immune response genes in head kidney, gill and spleen of the common carp, *Cyprinus carpio L*, Int. Immunopharmacol. 8 (1) (2008) 51–58.
[35] N. Jimenez, J. Coll, F.J. Salguero, C. Tafalla, Co-injection of interleukin 8 with the glycoprotein gene from viral haemorrhagic septicemia virus (VHSV) modulates the cytokine response in rainbow trout (*Oncorhynchus mykiss*), Vaccine 24 (27–28) (2006) 5615–5626.
[36] E. Awad, R. Cerezuel, M.A. Esteban, Effects of fenugreek (*Trigonella foenum graecum*) on gilthead seabream (*Sparus aurata L.*) immune status and growth performance, Fish Shellfish Immunol. 45 (2) (2015) 454–464.
[37] H. Watanuki, K. Ota, A.C.M. Tassakka, T. Kato, S. Masahiro, Immunostimulant effects of dietary Spirulina platensis on carp, *Cyprinus carpio*, Aquaculture 258 (2006) 157–163.
[38] G. Gioacchini, F. Lombard, M.A. Avella, I. Olivotto, O. Carnevali, Welfare improvement using alginic acid in rainbow trout (*Oncorhynchus mykiss*) juveniles, Chem. Ecol. 26 (2010) 111–121.
[39] O.H. Lee, B.Y. Lee, Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract, Bioresour. Technol. 101 (10) (2010) 3751–3754.
[40] J.E. Hayes, P. Allen, N. Brunton, M.N. O'grady, J.P. Kerry, Phenolic composition and in vitro antioxidant capacity of four commercial phytochemical products: olive leaf extract (*Olea europaea L.*), lutein, sesamol and ellagic acid, Food Chem. 126 (3) (2011) 948–955.