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Research Article

Effect of dietary supplementation with olive leaf extract on growth performance, hematological parameters and resistance against *Yersinia ruckeri* in rainbow trout, *Oncorhynchus mykiss*

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Abstract: This study investigated effects of dietary olive leaf extract (OLE) on growth performance, hematological parameters and resistance against *Yersinia ruckeri* in rainbow trout. Four groups were designed including a control diet and OLE added in fish feed at 0.5, 1.0, and 1.5 ml/100 g feed, which were administered for 30 days. The weight gain (WG), specific and absolute growth rate (SGR and AGR), feed conversion ratio (FCR) and feed efficiency ratio (FER) of rainbow trout fed with OLE were found to be significantly ($p < 0.05$) differed compared with control. Fish fed 1.0 ml/100g feed OLE supplemented diet significantly improved ($p < 0.05$) WG, AGR and SGR. The lowest FCR was observed with 1.0 ml OLE/100 g diet. An enhancing effect of OLE on hematological parameters occurred, as shown by the significant increase in red blood cell (RBC), white blood cell (WBC) and haematocrit (Ht) parameters in fish fed with 1.0% OLE. Haemoglobin (Hb) level of fish fed the 1.5% added OLE diet was significantly lower than those fed the 0.5 or 1.0% added OLE diet and control groups. Mortality at 14-day post-challenge with *Y. ruckeri* significantly decreased at all the dietary OLE groups. Dietary OLE at the level of 1.0 and 1.5 ml/100 g feed showed significantly ($P < 0.05$) higher relative percentage survival (RPS) (73.91 and 78.26%, respectively) against *Y. ruckeri* infection. The present results suggest that diets supplemented with OLE feed level positively enhance the growth parameters and affords protection from *Y. ruckeri*.

Keywords: Feed additive, Growth, Olive leaf extract, Rainbow trout, *Yersinia ruckeri*

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1. INTRODUCTION

Antibiotics, drugs, and chemicals have been used for treating fish disease caused by environmental stress and other factors for years (Sakai, 1999). To control bacterial fish pathogens, feeding infected fish with antibiotic-medicated feed is a general practice. But, this practice is expensive and usually infective as sick fish tend to remain of feed. Also, pathogenic bacteria can develop antibiotic resistance. Therefore, the need to look for alternative techniques with eco-friendly disease prevention has been taken into account. Recently, plants and their derivates are commonly used against fish diseases in aquaculture (Olusola et al., 2013).

The olive tree (*Olea europea*) is widely cultivated in Mediterranean countries. This tree shows strong resistance against microorganism invasion and insect attacks (Kubo et al., 1995). The extracts of olive leaves contain the compounds oleuropein and oleuropeoside, which are polyphenols, or antioxidants that can protect against cell damage from harmful free radicals. Olive leaves include oleuropeoside compounds such as oleuropein and verbascoside and various flavonoid compounds (Govaris et al., 2010). Oleuropein and oleuropeoside dilate blood vessels, they are antimicrobial, and they support the immune system (Juven et al., 1972; Bisignano et al., 1999; Khayyal et al., 2002; Liu et al., 2017). Studies with other animals have demonstrated an enhancement of immune function with the administration of olive oil, olive leaf extract (OLE), oleuropein and other derivates from *Olea europea*. Phenolic compounds derived from olive oil decreased inflammatory mediator production by human whole blood cultures, which may contribute to its anti-atherogenic properties (Miles et al., 2005). Oleuropein, a immunomodulator derived from the olive tree, was found to prolong survival against *Pseudomonas aeruginosa* infection (Giamarellos-Bourboulis et al., 2006). A commercial plant extract derived from olive tree leaf and its derivate, oleuropein, successfully controlled fish viruses namely salmonid rhabdovirus, viral haemorrhagic septicaemia virus (Micol et al., 2005). Extract preparation of many medicinal plants for which an ability to increase immune system and protective effect against diseases has been demonstrated include OLE (Pereira et al., 2007); cinnamon extract (Shan et al., 2011); *Echinacea angustifolia* extract (Morazzoni et al., 2005); garlic extract (Kyo et al., 2001; Dash et al., 2014); *Euphorbia hitra* extract (Pratheepa & Sukumaran, 2014); *Polygonum minus* leaf extract (Veerasamy et al., 2014) and *Muscari cumosum* extract (Baba et al., 2014) in several fish species and other animals.

The present study was conducted to determine the effects of OLE on the growth parameters, hematological parameters and protection against *Yersinia ruckeri* in rainbow trout. By using these kinds of substrate, the loss of valuable fish species caused by pathogens in fish culture may be prevented and there may also be an economic benefit for fish farming.

2. MATERIAL AND METHOD

2.1. Fish and Experimental Design

Rainbow trout for the experiment (approximate weight: 20 g) were obtained from Inonu University, College of Surgu, Aquaculture Station in Malatya (Turkey). The fish were kept in a 200 L fiberglass tank. Prior to each experiment, the fish were transferred tank containing aerated well water and acclimatised for a minimum of 14 days. Fish were fed ad libitum with a commercial (Ecobio, Turkey) feed throughout the experiments. To verify the pathogenic bacteria-free status of the rainbow trout, samples were obtained for bacterial culture from the kidney. The samples were incubated at 22^{o}C for 24 hours. Pathogenic agents were not isolated from selected rainbow trout.

Fish were stocked in 4 groups (50 fish/per group) in the fiberglass tanks supplied with fresh water. Light/dark cycle was 12L:12D. Water quality parameters were monitored daily for each tank and pH, temperature, and dissolved oxygen were maintained at 7.7-8.25; 12-13°C and 7.5-8.3 mg/L, respectively.

After 2 weeks of acclimation to the condition, to study the hemato-immune mechanisms, fish were allocated into 4 tanks (50 fish/tank) and fed diets containing three doses of olive leaf (0.5, 1.0, and 2.0 ml/100 g feed) extract. Also, the control group only was fed with commercial feed. Fish were fed to apparent satiation twice daily (between 08:00-09:00 and 16:00-17:00) for 30 days.

2.2. Growth Performance Parameters and Organ-Somatic Indices

Fish and compound feed were weighed at 30 days was calculated: condition factor (*K*), average daily gain (ADG) and specific growth ratio (SGR), feed efficiency ratio (FER), feed conversion ratio (FCR) and Organ-somatic parameters (El-Asely et al., 2014). Mortality was recorded daily and with obtained data was calculated the percentage of viability.

The total fish length (L) was measured to the nearest 0.1 cm and body weight (W) was weighted to using a portable digital balance to the nearest 0.01 g.

Condition (*K*) factor: $K=100 \times W \times L^3$

Average daily gain (ADG) = [Average final weight (g) - average initial weight (g)]/feeding period (days).

Feed conversion ratio (FCR) = F/(Wf - Wi); where F is the weight of feed offered to fish, Wf is the final weight of fish and Wi is the weight of fish at stocking.

Feed efficiency ratio (FER) =Weight gain (g)/dry feed fed (g).

Specific growth rate (SGR) (% g/day) =100 (ln final body weight (g)) - (ln initial body weight (g))/feeding period (day).

The hepato-somatic index and Viscero-somatic index index was measured according to the following formulas:

Hepato-somatic index (HSI) = (weight of liver (g)/total body weight (g)) x100.

Viscero-somatic index (VSI) =(weight of intestine (g)/total body weight (g)) x100.

2.3. Hematological Parameters

The erytrocyte count was performed with the technique described by Wintrobe (1934) in hemacytometer using a Natt-Herrick solution (1952). Hemoglobin (Hb) contents were determined spectrophotometrically at 540 nm using the cyanomethemoglobin method (Drabkin, 1946). The hematocrit (Ht) was determined by the volume occupied by erytrocytes in heparinized microhematoctit tubes, and the hematimetric indicates how secondary indices, MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), and MCHC (mean corpuscular hemoglobin concentration), were determinedusind Standard formulas described by Wintrobe (1934). White blood cells (WBCs) count were realized according to Tavares- Dias & Moraes (2006).

2.4. Bacterial Challenge

Yersinia ruckeri strain was originally isolated from rainbow trout farmed in Turkey with clinical signs of Enteric Red Mouth. The strain was stored in tryptic soy broth (TSB) supplemented with 15% (v/v) sterile glycerol at –80°C. The isolate was presumptively identified using the biochemical method of Austin & Austin, (1999) and definitively identified using the polymerase chain reaction method of Seker et al., (2012). For each experiment, the bacteria were inoculated on tryptic soy agar (TSA), and 1 to 3 colonies were transferred into TSB and incubated for 24 h at 22°C. The cells were harvested and washed twice with sterile phosphate-buffered saline (PBS, pH 7.2) by centrifugation at 2500 g for 10 min. After the final centrifugation, the cells were re-suspended in sterile PBS and immediately used in the experiments. The number of bacteria (expressed as colony forming units, CFU) in the suspension was determined using 10-fold serial dilutions and plate count technique on TSA.

At 4 weeks of first feeding, experimental and control groups were challenged with bacteria at cell concentration (1.5 x 10^8 CFU/ml). Fish were injected by intraperitoneal injection (IP) (100 µl/fish). Control fish were IP injected with sterile TSB at the same volume. They monitored daily for clinical signs and mortality for 14 days. Dead fish were removed and bacterial samples were obtained aseptically from the viscera organs of morbid and dead fish to confirm the presence of *Y. ruckeri*. The efficacy of the experimental diets was calculated as cumulative mortality and relative percentage survival (RPS) (Amend, 1981).

RPS: 1-[(per cent mortality in treated group)/(per cent mortality in control group)] x 100

2.5. Statistical Analysis

All experiments were conducted mean values and standard deviations of the data of immune parameters were calculated from the experimental data obtained. The mean significance of the immune parameter for experimental groups was analyzed using analysis of variance. The significance level was 0.05.

3. RESULTS

3.1. Growth Performances and Organo-Somatic Indexes

Mean final weight gain (WG), feed intake (FI), and feed conversion ratio (FCR) are presented in Table 1. WG did not significantly differ in fish fed with the control diet and diet containing 0.5 and 2.0 ml/100 g diet OLE. However, WG was significantly increased for diets containing 1.0 ml/100 g diet OLE (Table 2). A significant reduction in FCR was detected in fish-fed diets containing 1.0 ml/100 g diet OLE. No significant differences were observed in FCR of 0.5 and 2.0 ml/100 g diet OLE.

Table 1. Response of rainbow trout to the olive leaf extract diet after 30 days of feeding

	Diets containing OLE (ml/100g diet)			
	0	**0.5**	**1**	**2**
Initial weight (g/fish)	19.05±4.01	19.65±3.88	19.80±2.13	20.01±1.80
Final weight (g/fish)	31.05±6.47	33.76±7.39^{a,d}	36.35±8.38^{a,b,d}	31.98±7.80
Initial length (cm)	12.81±1.15	12.39±1.12	12.49±1.41	12.83±1,08
Final length (cm)	14.23±9.83	14.04±11.21	14.16±12.34	14.27±10.22
Condition Factor (CF)	1.11±0.13	1.24±0.14^{a,d}	1.32±0.18^{a,d}	1.09±0.14
Feed Efficiency Ratio (FER)	1.63^{b}	1.37^{d}	1.57^{b,d}	1.14
Absolute Growth Ratio (AGR)	0.40	0.47	0.55^{a,b,d}	0.40
Feed Intake (FI)	10.32	10.28	10.55	10.52
Weight gain (WG)	12.00^{d}	14.11^{a}	16.55^{a,b,d}	11.97
Feed Conversion Ratio (FCR)	0.86	0.73^{a,d}	0.68^{a,b,d}	0.88
Specific growth rate (SGR)	1.628^{a}	1.803^{a,d}	2.025^{a,b,d}	1.563

a: control, b: 0.5 OLE, c: 1.0 OLE, d: 2.0 OLE

^{abcd}: lowercase letters represent groups and indicate differences between groups ($p < 0.05$).

The viscerosomatic index (VSI) of the fish was not significantly different from the value for the fish fed 0.5 ml/100 g diet OLE ($p > 0.05$) but was significantly different from the values of fish fed all the other dietary treatments ($p < 0.05$) (Table 2). VSI of fish feed supplemented with 1.0 ml/100 g diet OLE was decreased. But, VSI of fish feed supplemented with 2.0 ml/100 g diet OLE was increased. The hepatosomatic index of fish fed 0.5 and 1.0 ml/100 g diet was significantly lower than that of fish fed the control diet (Table 2).

Table 2. Body indices of rainbow trout fed the test diets

	Diets containing OLE (ml/100 g diet)			
	0 (Control)	**0.5**	**1**	**1.5**
Viscero-Somatic Index	14.01±2.98^{c}	14.56±3.05	13.13±3.61	15.39±3.35^{a,b,c}
Hepato-Somatic Index	1.41±0.38^{b,c}	1.32±0.31	1.27±0.40	1.45±0.32^{a,b,c}

a: control, b: 0.5 OLE, c: 1.0 OLE, d: 2.0 OLE
^{abcd}: lowercase letters represent groups and indicate differences between groups ($p < 0.05$).

3.2. Hematological Parameters

The hematological parameters of rainbow trout fed diets containing different levels of olive leaf extract following the 30 days feding period are shown in Table 3. An enhancing effect of OLE on hematological parameters occurred in this study, as shown by the significant increase in RBC, WBC and Ht parameters in fish fed with 1.0% OLE. Exceptionally, the level of RBC and WBC in the end of 30 days was higher than in the control group and other experimental groups ($p < 0.05$). Hematocrit value were not affected by dietary OLE levels, except that was significantly increased in fish fed the 1.0% OLE group compared with the groups fed the control or other added OLE diets. Hb level of fish fed the 1.5% added OLE diet was significantly lower than those fed the 0.5 or 1.0% added OLE diet and control groups. treatment.

Table 3. Hematological parameters of rainbow trout fed dietary olive leaf extract for 30 days

	RBC (x10^{6}/ml)	**WBC (x10^{3}/ml)**	**Hb (%)**	**Ht (%)**	**MCV (µm^{3})**	**MCH (pg)**	**MCHC (gd/L)**
Control	1.53±0.14	11.19±1.33	7.42±0.29	24.93±1.08	164.90±16.40^{c,d}	50.94±3.64 ^{c,d}	30.77±1.60
0.5%	1.60±0.17	12.32±1.61	7.56±0.71	25.50±1.76	160.47±9.13^{c,d}	49.25±2.87 ^{c,d}	29.82±1.93
1.0%	1.82±0.12^{a,b,d}	14.41±1.08^{a,b}	7.30±0.83	26.10±2.10	147.40±11.08	43.11±4.82	28.88±2.09
1.5%	1.62±0.43	13.80±1.21^{a}	6.98±0.69	24.17±1.17	151.39±9.83	44.09±2.71	30.69±1.05

$^{a, b, c, d}$ Hematological parameter values in rows with different letters significantly differ ($p < 0.05$).
RBC: Red blood cell, WBC: White blood cell, Hb: Haemoglobin, Ht: Haematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration

3.3. Bacterial Challenge

Typical external symptoms of diseases were not obvious following experiments, and no internal lessons were noted in any of the fish. Mortality, control, and OLE treatment groups fish began 5 days after the challenge, and almost all mortalities in groups occurred during the 14d post-challenge. The control group experienced 92% mortalities while the treatment groups experienced 32%, 24%, and 20% mortalities, respectively. Relative percent survival (RPS) and days of survival between control and treatment groups during the challenge period were different. The percent survival in the control group was 8% on 14 days, while the relative percent survival for the vaccinated was 65.22%, 73.91%, and 78.26%, respectively (Figure 1).

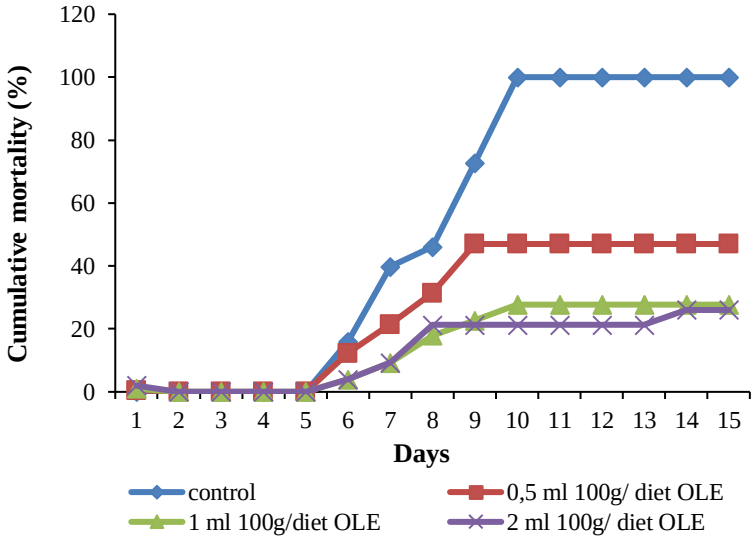

Figure 1. Percentage cumulative mortality of fish infected with *Y. ruckeri*

4. DISCUSSION AND CONCLUSION

Considering the increasing human population around the world and the importance of nutrition, the importance of aquaculture is increasing day by day to meet the food needs of future generations. Aquaculture is known as the fastest-growing agricultural activity contributing to the global demand for food. The estimated value of aquaculture production, which reached a record amount of approximately 87.5 million tons worldwide in 2020, was determined as 281.5 billion US dollars (FAO, 2022). With the increasing development of aquaculture, both bacterial and viral diseases have become a concern in the aquatic environment (Lieke et al., 2020). Many stress factors, such as the physicochemical structure and microbial content of water in breeding areas, malnutrition, and high stock density, can induce mass deaths due to infectious diseases (Kumari, 2020). For this reason, in rainbow trout farming, the emergence of many bacterial and viral diseases and the fight against related epidemics are in question (Radosavljević et al., 2022). Various antibiotics, chemotherapeutics, and vaccines control or prevent outbreaks of viral, bacterial, parasitic, and fungal diseases in fish (Mehana et al., 2015). It is known that antibiotics and chemical products have undesirable effects on fish, fish meat, the environment, and human health. Because of this, fish breeders need to find methods to ensure healthy fish growth, due to the constant threat of epidemics and economic losses in fish farms. An effective way to achieve these goals is to use feed supplements that will increase the growth performance of the fish and strengthen the immune system (Paray et al., 2020). For this reason, there are many studies on feed supplements in the literature on fish (Acar et al., 2018; Parrino et al., 2020; Yılmaz et al., 2020; Salem et al., 2022; Yousefi et al., 2022).

Hematological characteristics is important parameters for monitor of physio-pathological changes in fish (Satheeshkumar et al., 2011). In the present study, OLE were showed statistically significant effect on RBC. Similarly, Nahak & Sahu (2014a, b, c) reported a depletion of total content of RBC in the blood of *Clarias batrachus* when fed to *Ocimum basilicum*, *Ocimum gratissimum* and *Ocimum sanctum*. Adel et al. (2015) recorded a increase in the RBC levels in Caspian white fish (*Rutilus frisii kutum*) when fed to *Mentha piperita*. In the present study, WBC were also increased in a dose dependent manner in fish fed supplemented diets being the highest increments recorded in fish fed on diet supplemented with 1.0% of OLE. The total white blood cells (WBCs) count plays a major role in the defense system of fish. In the present investigation the significant increase in leucocyte count cell during Ibuprofen treatment indicated the presence of tissue damage such as necrosis in fish. Adel et. al. (2015) evaluated the effect of supplemented diets *Mentha piperita* extract on haematological and immunological parameters in Caspian white fish (*Rutilus frisii kutum*). They reported that WBC counts were higher in experimental fish fed *M. piperita* compared with control. Bahrami Babaheydari et. al (2015) investigated the effect of *Stachys lavandulifolia* extract on common carp. Hemoglobin, hematocrit, mean erythrocytes of hemoglobin, mean erythrocyte volume, mean hemoglobin erythrocyte concentration, white blood cell and red blood cells were evaluated. The highest values were observed in WBC of the group fed with *S. lavandulifolia*. Nahak & Sahu (2014c) also reported that there was an increase in the WBC count after feeding the *Clarias batrachus* with *Ocimum sanctum*. Our results for this parameter showed similarity to the results in previous studies.

This study investigated the effects of dietary OLE on growth performance and resistance to *Yersinia ruckeri* in rainbow trout. As a result of the study, it was determined that the growth parameters of fish fed with OLE-supplemented feed increased and protected *Y. ruckeri*. Similar results were obtained in many studies using feed supplements in various fish. Adel et. al. (2015) investigated the effect of *Mentha piperita* diet on growth, feed conversion ratio in Caspian white fish (*Rutilus frisii kutum*) in a 8 weeks feeding trail. The *M. piperita* extract improved the weight gain; and specific growth rate. Bahrami Babaheydari et. al. (2015) evaluated the effect of supplemented diets *Stachys lavandulifolia* extract on growth in Common carp, *Cyprinus carpio*. The weight gain was significantly higher in the experimental groups than in the control group. This study indicated that the growth performance can be improved by dietary *S. lavandulifolia*. There are great importance of food conversion ratio for economic success in aquaculture (Akbulut et al., 2002). Amirkhani & Firouzbakhsh (2015) incorporated to *Ocimum basilicum* in the experimental diet of common carp. They reported that the fish fed the herbal diets had higher SGR and better FCR than the fish fed the control diet. Maslinic acid, common compound in the leaves of *O. europaea*, was found to act as a growth factor when added to rainbow trout trout diets (Fernández-Navarro et al., 2008). Hidalgo et al. (2006) did not observe significant differences in growth of dentex (*Dentex dentex*). However, Fernández-Navarro et al. (2008) reported rainbow trout fed diets containing maslinic acid for 225 days showed good whole-body growth rate compared with fish fed the control diet without maslinic acid supplementation.

Parrino et al. (2020) applied hot pepper (Capsicum sp.) oil as a feed additive to rainbow trout and as a result of the study, they stated that application at a dose of 4 mg/kg showed the highest growth percentage and the lowest feed conversion rate. Acar et al. (2018) conducted a study on the effects of pomegranate seed oil supplementation on growth performance and disease resistance against *Y. ruckeri* in rainbow trout. As a result of the study, they stated that there was an increase in weight gain, growth, and feed conversion in fish fed with diets supplemented with pomegranate seed oil. They also concluded that pomegranate seed oil reduced the mortality of rainbow trout infected with *Y. ruckeri* and produced significant differences in growth performance between treated groups.

Yousefi et al. (2022) examined the effects of thyme essential oil and prebiotic supplementation on growth performance in rainbow trout (*Oncorhynchus mykiss*). The study concluded that all experimental groups had similar final weight, weight gain, specific growth rate, and survival rate, which were significantly higher than the control. Yousefi et al. (2021) fed rainbow trout with diets supplemented with 0%, 0.5%, 1.5%, 2.5%, and 4% mistletoe (*Viscum album*) extract for eight weeks. As a result, the highest final weight, weight gain (%), and lowest FCR were observed in 1.5% and 2.5% applications. Salem et al. (2022) examined the effects of white mustard (*Sinapis alba*) oil dietary supplementation on growth performance in juvenile rainbow trout. Fish for 9 weeks; They fed diets containing 0%, 0.5%, 1%, and 1.5% white mustard oil. They also conducted a study on the effects of white mustard oil on disease resistance against *Aeromonas hydrophila* and *Y. ruckeri*. As a result, they found that final weight, weight gain, and specific growth rate increased significantly in all experimental groups compared to the control group ($p<0.05$). However, as a result of the control test against *A. hydrophila*, no significant difference was observed between the groups; However, they stated that they observed an increased survival rate against *Y. ruckeri* in the experimental groups compared to the control group ($p<0.05$). In another study, Yılmaz et al. (2020) stated that L-alliin and oleuropein added to the feed did not change the survival rate against the *Aeromonas salmonicida* pathogen in rainbow trout, however, the addition of 10 mg/kg L-alliin to the trout feed increased the growth performance of the fish.

There are also results in the literature that are not similar to the results obtained in this study. For example, Paray et al. (2020) found in their study that oak leaf extract had no growth-promoting effect on common carp (*Cyprinus carpio*) and stated that this extract may not stimulate digestive enzymes or intestinal absorption. Moreover, in other studies, the negative effects on the growth performance of hybrid grouper (*Epinephelus lanceolatus*♂ × *Epinephelus fuscoguttatus*♀) and Nile tilapia (*Oreochromis niloticus*) fish fed with feed supplemented with *Ginkgo biloba* and *Moringa oleifera* leaf extract were detected (Dongmeza et al., 2006; Tan et al., 2018). As in these studies, it has been reported that some leaf extracts may have negative effects on fish growth performance.

Our study demonstrated that feeding enriched diets of OLE could significantly enhance growth activity. This preliminary study suggests that supplementation of OLE at levels up to 1.0 ml/100 g to a commercial rainbow trout diet affected growth, feed intake, utilization efficiency, and survival of rainbow trout reared under laboratory conditions. Moreover, the results of the present study showed that the administration of OLE caused a significant ($P<0.05$) increase in the mobilization of immune responses of treated rainbow trout.

Authors' Contributions

Üİ: Manuscript design, Field sampling, Laboratory experiments, Draft checking, Writing, Reading, Editing, Statistical analyses.
MK: Manuscript design, Field sampling, Laboratory experiments, Draft checking, Writing, Reading, Editing, Statistical analyses.
MÖ: Manuscript design, Field sampling, Laboratory experiments, Draft checking, Writing, Reading, Editing.
MaK: Manuscript design, Laboratory experiments, Draft checking, Writing, Reading, Editing.
All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Statement on the Welfare of Animals

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Data Availability Statements

The authors confirm that the data supporting the findings of this study are available within the article.

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