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Growth performance, haematological changes, immune response, antioxidant activity and disease resistance in rainbow trout (*Oncorhynchus mykiss*) fed diet supplemented with ellagic acid

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ABSTRACT

The present study was performed to investigate the effects of various levels of dietary ellagic acid (EA) on growth performance, haematological values, immune response, protection against *Yersinia ruckeri* infection, and oxidant/antioxidant status in rainbow trout, *Oncorhynchus mykiss*. Fish were fed with the control diet and three different experimental diets containing three graded levels of EA (50, 100 and 200 mg kg^{-1} diet) for 8 weeks. At the end of the experiment, the growth performance [weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR)], haematological values [the red blood cell (RBC) count, haemoglobin (Hb) concentration, haematocrit (Ht) level and erythrocyte indices: mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC)], immune response [white blood cell (WBC) count, oxidative radical production (nitroblue tetrazolium (NBT) assay), phagocytic activity (PA) and phagocytic index (PI), total protein (TP) and immunoglobulin M (IgM) levels, serum bactericidal activity (BA), lysozyme (LYZ) and myeloperoxidase (MPO) activities] and oxidant/antioxidant status [tissue malondialdehyde (MDA) levels and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities] were analysed. In addition, fish were challenged by *Y. ruckeri* and survival rate was recorded for 14 days. In the groups fed with EA the growth parameters such as WG, SGR, and FCR did not vary significantly. The RBC count, Hb concentration, and Ht level increased in the groups fed with EA when compared with the control group. However, there were no significant differences in the MCV, MCH and MCHC values among the groups. The results demonstrated enhancement in all the immunological parameters in the groups fed with EA compared to the control group. The results obtained from challenge with *Y. ruckeri* revealed reduction in the mortalities in the groups fed with EA. The dietary EA stimulated the SOD, CAT and GSH-Px activities in liver, head kidney and spleen as compared to the control group; however, a reverse trend was observed in the MDA levels of tissues. The present study suggest that EA can effectively enhance the haematological values, immune response, antioxidant capacity, and disease resistance in rainbow trout.

1. Introduction

Aquaculture is a key sector of the global food system and has made a significant contribution for human consumption [1]. Aquaculture products represent a very valuable source of animal protein and essential nutrients micronutrients for balanced nutrition and good health. However, the intensive and semi-intensive practices of aquaculture production caused an increase of disease outbreaks resulting in partial or total loss of fish production. Furthermore, factors such as overcrowding, periodic handling, high or sudden changes in temperature, poor water quality and poor nutrition are responsible for creating a state of stress leading to immunosuppression in fish, and thus boosting the susceptibility to infectious diseases [2].

In order to avoid economic losses related to infectious diseases, several veterinary drugs and antimicrobials have been widely used in aquaculture industries. The use of antibiotics and other chemotherapeutics for controlling diseases have been widely criticized due to it is expensive and spreading of drug-resistant pathogens, immunosuppression, oxidative stress, nephrotoxicity, growth retardation, environmental pollution and accumulation of chemicals in aquatic animal tissues, which can be possibly dangerous to public health [3,4]. On the other hand, commercial vaccines are expensive for fish producers and may not be available for all species and against emerging diseases [5]. Alternatively, natural immunostimulants such as probiotics, prebiotics, synbiotics, complex carbohydrates, nutritional factors, herbs, hormones and cytokines are generally being suggested to use in aqua feeds to

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effectively promote growth, immune response and control various diseases in aquatic animals [4].

Ellagic acid (EA) is a natural polyphenolic compound present in fruits and berries such as pomegranates, strawberries, raspberries, grapes blackberries, walnuts and many other plant-based foods [6]. Important biological activities such as antioxidant, chemopreventive, neuroprotective, antimicrobial, estrogenic/antiestrogenic, antiinflammatory, anticarcinogenic, antidepressant, antidiabetic, antifibrosis, and antiviral activities have been ascribed to EA [6–8]. Despite the unique biological properties of EA, the information on the use of EA is limited in the aquaculture and few studies have been reported in the fish. For example, Mişe Yonar et al. [9] have documented that EA can be used in the aquaculture to improve the haematological values, immune responses, antioxidant capacity in rainbow trout (*Oncorhynchus mykiss*). Also, Ural et al. [10] have reported that EA may alleviate malathion-induced oxidative stress in carp.

The aim of the present investigation was to evaluate the effects of dietary EA on the growth performance, haematological values, cellular and humoral immunity, oxidant/antioxidant capacity and disease resistance against Y. ruckeri infection in rainbow trout, *O. mykiss*.

2. Materials and methods

2.1. Ethics statement

This study was carried out in accordance with the principles adopted by the Animal Experimentation Ethics Committee of Firat University (FUAEEC, Elazig, Turkey) and approved by the Committee on Ethics in the Use of Animals (Protocol number: 2014/14, 136).

2.2. Chemicals

EA (Synonym: 4,4′,5,5′,6,6′-Hexahydroxydiphenic acid 2,6,2′,6′-dilactone; CAS No: 476-66-4; Empirical formula: $C_{14}H_6O_8$) and all the other chemicals were supplied by Sigma-Aldrich Chemical Co. and Merck. All reagents were of analytical grade.

2.3. Diet preparation

EA is hardly dissolved under natural condition. Therefore, it was dissolved in alkaline solution (0.01 M NaOH; approximately pH 12). The pH of the final solution after the addition of EA was approximately 7.6 [11]. This final solution (pH ≈ 7.6) was added to diets.

A normal pellet diet (Ecobio Inc., trout grower feed; including 45% crude protein, 20% crude fat, 11% ash, 3% crude fibre, 8.5% moisture, 12.5% nitrogen free extract, and 5124 kcal/kg gross energy) was crushed, mixed with the final solution containing the adequate amount of EA and made again into pellets, thus obtaining diets supplemented with 50 mg (EA-50), 100 mg (EA-100) or 200 mg (EA-200) EA kg^{-1} diet. The diets were reformed into pellets, spread to dry and stored at +4 °C for the feeding experiment. Control group (C) received the normal pellet diet which not contained EA. The doses of EA used in this study were selected on the basis of the previous studies [9,12].

2.4. Fish and experiment design

O. mykiss juveniles of average weight 33.74 ± 2.43 g were procured from a trout farm (Keban, Elazig, Turkey) and transported to laboratory. Health statuses of fish were examined instantaneously upon arrival. Fish were quarantine bathed in 250 ppm formalin for 30 min [13]. Fish were acclimatised for 4 weeks. All fish were fed with the control diet until satiation twice a day in two equal parts at 9.00 a.m. and 4.00 p.m. Water quality was monitored daily throughout the experiment. Water exchange was done daily at a rate of 50% and 100% of the water was exchanged once a week. Temperature was maintained at 15 ± 1 °C, dissolved oxygen concentration 7.8 mg/L and pH 7.6 ± 0.2.

The juveniles divided randomly into 4 groups (C, EA-50, EA-100 and EA-200) with 75 fish in each group, maintained in 12 tanks (540 L capacity) each containing 25 fish (4 experimental groups x 3 replicates, totally 300 fish). Control (C) was fed with the normal pellet diet without EA. The remaining groups were fed with 50 mg (EA-50), 100 (EA-100), 200 (EA-200) EA for 8 weeks. All experimental groups were fed until satiation two times a day at 9.00 a.m. and 4.00 p.m.

2.5. Sample collection

At the end of the feeding trial, all fish in each tank were weighed and counted to calculate growth performance including weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) and survival. Five fish from each tank (fifteen fish per dietary treatment) were randomly captured, anaesthetized in benzocaine solution (25 mg/L water), and blood samples were taken from the caudal vein with plastic syringes. A part of the blood samples was transferred into tubes containing K_3EDTA for the haematological [red blood cell (RBC) count, haemoglobin (Hb) concentration, haematocrit (Ht) level and erythrocyte indices: mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC)] and immunological [white blood cell (WBC) counts, oxidative radical production (nitroblue tetrazolium (NBT) assay), phagocytic activity (PA) and phagocytic index (PI)] analysis. The remaining of the blood was transferred into serum tubes. The serum tubes were allowed to clot at room temperature for 30 min and the serum samples were separated by centrifugation for 10 min at 5000 × *g* and stored at −20 °C for analysis of the other immune parameters including total protein (TP) and immunoglobulin M (IgM) levels, serum bactericidal activity (BA), lysozyme (LYZ) and myeloperoxidase (MPO) activities.

After the blood samples were collected, liver, head kidney and spleen were carefully removed, washed with physiological saline (0.9% NaCl) and stored at −40 °C until the biochemical assays, which were performed within 1 month after extraction. The tissue was homogenised in a Teflon-glass homogeniser in buffer containing 1.15% KCl at a 1:10 (w/v) ratio to the whole homogenate. The homogenate was centrifuged at 18,000 g at 4 °C for 30 min to determine malondialdehyde (MDA) level and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities [14].

2.6. Growth study

Calculations for the growth performance (the WG, SGR and FCR) were performed using following equations:

$WG\ (g) = Wt – W_0$;

$SGR\ (\%) = 100 \times (\ln Wt - \ln W_0)/t$;

$FCR = FI/(Wt - W_0)$.

(Wt and W_0: Final and initial weight of fish, respectively; t: experimental days; FI: Feed intake).

2.7. Haematological analysis

The RBC count was performed using a haemocytometer and the solution developed by Natt and Herrick [15]. The Hb concentration was determined based on the absorbance of Drabkin's reagent at 540 nm [16]. The Ht level was determined through a microhematocrit centrifugation technique. The erythrocyte indices (MCV, MCH, and MCHC) were calculated through standard formulas using the Ht, RBC, and Hb data [17,18].

2.8. Immunological analysis

2.8.1. WBC count

The WBC count was simultaneously determined with the RBC count Natt and Herrick, [15].

2.8.2. Oxidative radical production of neutrophil (NBT assay)

The NBT assay was used to analyse radical oxygen production by phagocytes. Briefly, 0.1 ml of blood was placed into a microtitre plate well, and an equal amount of 0.2% NBT solution was added. The mixture was incubated at room temperature for 30 min, and 0.05 ml of the NBT-blood cell suspension was removed and added to a glass tube containing 1.0 ml of N,N dimethyl formamide. After centrifugation, the sample absorbance was read at 620 nm in a 1.0 ml cuvette [19].

2.8.3. Phagocytic activity (PA) and phagocytic index (PI)

The PA and PI were measured according to the method described by Siwicki and Anderson [20]. The heparinized blood was immediately used for the phagocytic assay. Briefly, 1×10^7 cells *Yersinia ruckeri* in 0.1 ml of phosphate buffer saline (PBS) were added to 0.1 ml of blood samples in a microplate and incubated for 30 min after through mixing in the well. After incubation, the plate was mixed gently, and 0.05 ml of this suspension was smeared on the glass slide. After air-drying, the smears were fixed in ethanol and stained with Giemsa solution for 10 min. The phagocytic cells and phagocytosed bacteria were counted. PA and PI were determined by enumerating 100 phagocytes per slide under a microscope. The mean of the slides was calculated as below;

PA: (Number of phagocytic cells with engulfed bacteria/number of phagocytes) × 100

PI: (Number of engulfed bacteria/phagocytic cells).

2.8.4. Total protein (TP) level

The TP level was estimated following the Biuret method with slight modification. Serum (0.1 mL) and 0.9 mL of distilled water were pipetted into a spectrophotometer tube and mixed thoroughly by inversion. The Biuret reagent (4.0 mL) was added, and the mixture was incubated for 30 min at room temperature in the dark. The sample absorbance was read at 540 nm with a spectrophotometer [3,19,21].

2.8.5. Total immunoglobulin (IgM) level

The IgM level was determined following the method of Siwicki et al. [19]. The assay was based on the measurement of total protein contents in plasma using a micro protein determination method (Biuret method) prior to and after precipitating down the IgM molecules employing a 12% (w/v) solution of polyethyleneglycol (Sigma). The difference in the protein contents was considered as the IgM content.

2.8.6. Serum bactericidal activity (BA)

The BA was done following the procedure of Kajita et al. [22], with some modifications. An equal volume (100 mL) of serum and bacterial suspension were mixed and incubated for 1 h at 25 °C. Blank control was also prepared by replacing serum with sterile PBS. The mixture was then diluted with sterile PBS at a ratio 1:10. The serum-bacterial mixture (100 mL) was plated onto TSA and plates were incubated for 24 h. Bacterial colonies grown in the nutrient agar plates were counted to determine the number of viable bacteria.

2.8.7. Serum lysozyme (LYZ) activity

The LYZ activity was studied following the protocol provided by Ellis [23]. Concisely, 200 µl of *Micrococcus lysodeikticus* (ATCC 4698) (2 mg mL^{-1}) suspension in 0.05 M sodium phosphate buffer, pH 6.2 was taken, to which 50 µl of serum sample was added to a microplate. The reaction was done at 25 °C and the absorbance was read at 450 nm after 0.5 and 10 min. A unit of lysozyme activity was defined as the enzyme amount reducing the absorbance by 0.001 min^{-1} mL^{-1} serum.

2.8.8. Serum myeloperoxidase (MPO) activity

The MPO activity was determined following Quade and Roth [24] with slight modification of Sahoo et al. [25]. Briefly, 20 µl of serum was diluted with Hanks Balanced Salt Solution (HBSS, Sigma) without Ca^{2+} and Mg^{2+} in 96-well plates. Then, 35 µl of 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB, 20 mM) (Sigma) and H_2O_2 (5 mM) were added. The colour change reaction was stopped after 2 min by adding 35 µl of 4 M sulphuric acid. Finally, optical density was read at 450 nm in a microplate reader.

2.9. Challenge test with Y. ruckeri

Y. ruckeri (NCTC 10,476) was used for the challenge test. *Y. ruckeri* was grown in tryptic soy broth for 24 h at 22 °C. Fresh culture was suspended in sterile PBS. The cell suspension was adjusted to OD 0.5 at 600 nm (10^5 cfu/mL) [26].

At the end of the feeding trial, the remaining fish from each tank were experimentally exposed to live *Y. ruckeri*. Thirty fish (ten fish/tank) were intraperitoneally injected with 0.1 mL PBS containing 1×10^5 cfu/mL live *Y. ruckeri*. The challenged fish were kept under observation for 14 days of post-infection and all fish groups were fed only the control diet. The cause of death was confirmed by re-isolating the organism from the liver, kidney and spleen of dead fish using conventional methods.

Relative percentage survival (RPS) was quantified using the following formula [27]:

RPS (%) = (Number of surviving fish after challenge/Number of fish injected with *Y. ruckeri*) × 100.

2.10. Oxidative stress and antioxidant capacity

2.10.1. The tissue MDA level

The tissue MDA level, which is an index of oxidative stress in tissues, was measured with the thiobarbituric acid (TBA) reaction [28]. The TBA reactive substance was quantified by comparing the absorption of the sample to a standard curve of malondialdehyde equivalents that was generated by the acid-catalysed hydrolysis of 1,1,3,3-tetramethoxypropane.

2.10.2. The tissue SOD activity

The tissue SOD activity was determined according to the method described by Sun et al. [29]. Briefly, the method is based on the SOD in the sample preventing the reduction of nitroblue tetrazolium (NBT). To analyse the SOD activity, 600 µl of the SOD reaction mixture containing 0.1 mM of xanthine, 0.1 mM of (Ethylenedinitrilo)tetraacetic acid (EDTA), 50 mg of bovine serum albumin and 25 µmol of NBT per litre was added to 125 µl of the supernatant or 125 µl of an SOD standard solution; 25 µl of 9.9 nM xanthine oxidase solution was added to each tube at 30 s intervals. The tube was incubated for 20 min at 25 °C, and the reaction was terminated by the addition of 0.5 ml of 0.8 mM copper (II) chloride ($CuCl_2$) solution every 30 s. The amount of formazan was determined by measuring the absorbance at 560 nm with a spectrophotometer.

2.10.3. The tissue CAT activity

The tissue CAT activity was determined according to the method previously published by Aebi [30]. Briefly, the assay principle is based on determining the rate constant of hydrogen peroxide (H_2O_2) decomposition by the CAT enzyme. To determine the CAT activity, 2 ml of the sample was added to 1 ml of 40 mM H_2O_2 in phosphate buffer [50 mM, pH 7.0; prepared by mixing 0.681 g potassium dihydrogen phosphate (KH_2PO_4) in 100 ml and 1.335 g disodium hydrogen

phosphate dehydrate ($Na_2HPO_4{\cdot}2H_2O$) in 150 ml]. The decrease in H_2O_2 was assessed by measuring the absorbance at 240 nm for 3 min with a spectrophotometer.

2.10.4. The tissue GSH-Px activity

The tissue GSH-Px activity was determined using the method of Beutler [31], which records at 340 nm, the disappearance of β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.2 mM NADPH, 1 EU/ml GSH-Px, 1 mM GSH, and 0.25 mM H_2O_2. An enzyme source (0.1 ml) was added to 0.8 ml of this mixture, and this mixture was incubated at 25 °C for 5 min before the initiation of the reaction, which was induced by the addition of 0.1 ml peroxide solution. The absorbance at 340 nm was recorded for 5 min. The activity was then calculated from the slope of the lines as micromoles of NADPH oxidized per minute.

2.10.5. The tissue protein levels

The protein levels in the tissues were determined by the method described by Lowry et al. [32].

2.11. Statistical analysis

Data obtained from the control and experimental groups were expressed as the mean ± standard error. The statistical significance of differences between the control and experimental groups was analysed by analysis of variance (one-way ANOVA) and Duncan's post-hoc test using the SPSS 21 computer program (SPSS). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of EA on growth parameters

The growth performance of control and experimental groups are presented in Table 1. All experimental diets were well accepted by fish in the experimental groups. Additives of EA to the diets led to no statistically significant differences in the WG, SGR and FCR values ($p > 0.05$). No mortality or any signs of disease were observed in all treatment groups. Fish survival was 100% in all treatments ($p > 0.05$).

3.2. Effects of EA on haematological values

The results of different dosages of EA on the haematological values are shown in Table 2. The RBC count, Hb concentration and Ht level of EA-50, EA-100 and EA-200 groups were found to be statistically higher than the control group ($p < 0.05$). Additionally, the haematological values of EA-100 and EA-200 groups were significantly higher than the EA-50 group ($p < 0.05$). No significant difference in the erythrocyte indices (MCV, MCH and MCHC) was observed among the experimental groups ($p > 0.05$).

Table 1
Growth performance of the control group and experimental groups fed with different levels of EA for 8 weeks.

Parameters	Groups			
	C	EA-50	EA-100	EA-200
W_0	33.78 ± 2.15[a]	33.92 ± 2.24[a]	33.49 ± 3.08[a]	33.83 ± 2.30[a]
Wt	65.27 ± 1.79[a]	66.74 ± 1.60[a]	66.88 ± 1.83[a]	66.13 ± 1.99[a]
WG	31.49 ± 1.81[a]	32.82 ± 1.95[a]	31.39 ± 1.58[a]	33.30 ± 1.62[a]
SGR	1.18 ± 0.02[a]	1.20 ± 0.02[a]	1.21 ± 0.02[a]	1.19 ± 0.02[a]
FCR	1.05 ± 0.02[a]	1.04 ± 0.02[a]	1.04 ± 0.02[a]	1.05 ± 0.02[a]
Survival	100	100	100	100

W_0: Initial weight of fish (g), Wt: Final weight of fish (g), WG: Weight gain (g), SGR: Specific growth rate (%), FCR: Feed conversion ratio.
Survival is presented as %.
The values (n = 75) are expressed as mean ± standard error. The mean values in same line with different superscripts are significantly different ($p < 0.05$).

3.3. Effects of EA on immune response

The results of different dosages of EA on the immunological parameters are presented in Table 3. The results indicated that the immunological values of EA-50, EA-100 and EA-200 groups were significantly ($p < 0.05$) higher than the control group. Additionally, the immunological values of EA-100 and EA-200 groups were significantly higher than the EA-50 group ($p < 0.05$).

3.4. Challenge test with Y. ruckeri

The RPS rates of experimental groups are given in Table 3. The RPS rate was significantly affected by different dosages of EA. In the EA-50, EA-100 and EA-200 groups, the RPS rate was significantly higher ($p < 0.05$) than in the control group. Among the groups fed diets containing EA, the RPS rates of EA-100 and EA-200 groups were significantly higher than the EA-50 group ($p < 0.05$). However, there was no significant difference between the EA-100 and EA-200 groups ($p > 0.05$). Accordingly, the highest RPS was observed in the EA-200 group (73.33%), followed by the EA-100 group (70.00%), the EA-50 group (56.67%) and the control group (40.00%).

Some of infected fish showed one or more typical signs of yersiniosis, including haemorrhages in the oral cavity, dark coloration, reddening at the base of the fins, abnormal swimming behaviour, slight growth in the spleen, haemorrhages in the liver and inflammation in the tissues.

3.5. Effect of EA on antioxidant capacity

The effects of different experimental diets on the oxidative stress and antioxidant capacity in the liver, head kidney and spleen are shown in Table 4.

At the end of the trial, the groups fed diets containing EA had significantly lower the tissue MDA levels than the control group ($p < 0.05$). Among the groups fed diets containing EA, the tissue MDA levels of EA-100 and EA-200 groups were significantly lower than the EA-50 group ($p < 0.05$). However, there was no significant difference between the tissue MDA levels of EA-100 and EA-200 groups ($p > 0.05$).

After EA feeding for 8 weeks, the groups fed diets containing EA had significantly higher the tissue SOD, CAT and GSH-Px activities than the control group ($p < 0.05$). Among the groups fed diets containing EA, the tissue SOD, CAT and GSH-Px enzyme activities of EA-100 and EA-200 groups were significantly higher than the EA-50 group ($p < 0.05$). However, there was no significant difference between the EA-100 and EA-200 groups ($p > 0.05$).

4. Discussion

Among immunostimulants, natural immunostimulants can be considered as good candidates for prevention and control of fish diseases in the aquaculture. They are bioactive components which have biocompatible and biodegradable properties. The use of natural immunostimulants has offer an eco-friendly prophylactic approach and provides a safe environment for the host body. Also, they are a significant potential alternative to chemotherapy and vaccination [33]. In recent years, many studies have described the good results obtained with use of different natural immunostimulants and their in vivo effects on fish. In the present study, the growth performance, haematology, cellular and humoral immune response, and antioxidant capacity of rainbow trout were evaluated with the dietary EA. The results showed

Table 2
Haematological values of the control group and experimental groups fed with different levels of EA for 8 weeks.

Parameters	Groups			
	C	EA-50	EA-100	EA-200
RBC	1.56 ± 0.07[a]	1.70 ± 0.10[b]	1.79 ± 0.08[c]	1.80 ± 0.09[c]
Hb	6.98 ± 0.39[a]	7.45 ± 0.49[b]	7.96 ± 0.63[c]	8.02 ± 0.60[c]
Ht	32.58 ± 2.29[a]	36.70 ± 2.61[b]	39.18 ± 2.93[c]	39.92 ± 2.20[c]
MCV	209.61 ± 12.46[a]	213.19 ± 15.72[a]	217.25 ± 18.90[a]	220.57 ± 16.14[a]
MCH	43.72 ± 2.53[a]	43.89 ± 3.24[a]	44.29 ± 3.66[a]	44.85 ± 3.49[a]
MCHC	22.24 ± 1.86[a]	21.98 ± 2.53[a]	20.86 ± 1.71[a]	21.03 ± 2.04[a]

RBC: Red blood cell (erythrocyte) count (x10^6), Hb: Haemoglobin concentration (g/dL), Ht: Haematocrit level (%), MCV: Mean corpuscular volume (μm^3), MCH: Mean corpuscular haemoglobin (pg), MCHC: Mean corpuscular haemoglobin concentration (%).
The values (n = 15 for haematological analysis) are expressed as mean ± standard error. The mean values in same line with different superscripts are significantly different ($p < 0.05$).

Table 3
Immunological values of the control group and experimental groups fed with different levels of EA for 8 weeks and relative percentage survivals of rainbow trout challenged with *Y. ruckeri* in the control group and experimental groups.

Parameters	Groups			
	C	EA-50	EA-100	EA-200
WBC	26.45 ± 2.42[a]	32.57 ± 2.13[b]	34.95 ± 2.76[c]	35.26 ± 3.08[c]
NBT	1.17 ± 0.11[a]	1.28 ± 0.15[b]	1.37 ± 0.20[c]	1.38 ± 0.18[c]
PA	34.75 ± 2.33[a]	38.45 ± 3.75[b]	41.25 ± 2.80[c]	40.60 ± 2.73[c]
PI	3.40 ± 0.55[a]	4.25 ± 0.70[b]	4.90 ± 0.95[c]	4.95 ± 1.00[c]
TP	27.09 ± 2.32[a]	33.90 ± 3.68[b]	37.46 ± 3.11[c]	37.73 ± 3.44[c]
IgM	13.52 ± 1.24[a]	16.03 ± 1.72[b]	17.89 ± 2.20[c]	18.08 ± 1.69[c]
BA	28.39 ± 1.40[a]	43.81 ± 2.08[b]	57.22 ± 2.36[c]	56.89 ± 2.63[c]
LYZ	115.27 ± 6.49[a]	136.91 ± 13.65[b]	151.66 ± 12.37[c]	154.08 ± 15.10[c]
MPO	0.72 ± 0.05[a]	0.85 ± 0.08[b]	0.94 ± 0.10[c]	0.95 ± 0.09[c]
RPS	40.00[a]	56.67[b]	70.00[c]	73.33[c]

WBC: White blood cell counts (x10^3), NBT: Nitroblue tetrazolium assay (mg/ml), PA: Phagocytic activity (%), PI: Phagocytic index, TP: Total protein level (mg/ml), IgM: Total immunoglobulin M level (mg/ml), BA: Serum bactericidal activity (% cfu/control), LYZ: Lysozyme activity (U/ml), MPO: Myeloperoxidase activity (Optic density), RPS: Relative percentage survivals (%).
The values (n = 15 for immunological analysis, n = 30 for RPS) are expressed as mean ± standard error. The mean values in same line with different superscripts are significantly different ($p < 0.05$).

that the dietary EA positively affected the haematological values, immune response, disease resistance against *Y. ruckeri*, and tissue antioxidant status of rainbow trout.

Growth enhancement is a trait of particular interest in the aquaculture, as it is inherently linked to the productivity and profitability of enterprises [34]. Positive effects of plant extracts on the growth parameters in different aquatic species have been reported by other authors [35–44]. However, in the present study, no significant difference in the growth parameters was observed among the groups after feeding EA. This result showed that EA had no effect on the growth performance of rainbow trout. This may be due to anti-nutritional factors in EA which has tannin. But the same time, the present results also indicated that EA did not negatively affect the growth.

The haematological parameters are widely used to evaluate health

Table 4
Antioxidant capacity of the control group and experimental groups fed with different levels of EA for 8 weeks.

Tissue	Parameter	Groups			
		C	EA-50	EA-100	EA-200
Liver	MDA	2.34 ± 0.41[c]	2.13 ± 0.54[b]	1.98 ± 0.66[a]	1.97 ± 0.49[a]
	SOD	3.19 ± 0.48[a]	3.41 ± 0.62[b]	3.65 ± 0.50[c]	3.68 ± 0.69[c]
	CAT	3.35 ± 0.59[a]	3.60 ± 0.73[b]	3.82 ± 0.48[c]	3.86 ± 0.71[c]
	GSH-Px	2.52 ± 0.34[a]	2.81 ± 0.63[b]	3.05 ± 0.60[c]	3.03 ± 0.84[c]
Head kidney	MDA	3.31 ± 0.55[c]	2.98 ± 0.66[b]	2.81 ± 0.37[a]	2.82 ± 0.49[a]
	SOD	2.65 ± 0.33[a]	2.89 ± 0.52[b]	3.08 ± 0.69[c]	3.10 ± 0.54[c]
	CAT	3.12 ± 0.39[a]	3.38 ± 0.73[b]	3.54 ± 0.80[c]	3.56 ± 0.92[c]
	GSH-Px	2.25 ± 0.49[a]	2.38 ± 0.42[b]	2.59 ± 0.66[c]	2.57 ± 0.51[c]
Spleen	MDA	3.04 ± 0.48[c]	2.88 ± 0.63[b]	2.70 ± 0.51[a]	2.67 ± 0.65[a]
	SOD	2.56 ± 0.39[a]	2.75 ± 0.46[b]	2.99 ± 0.72[c]	3.01 ± 0.58[c]
	CAT	2.92 ± 0.61[a]	3.11 ± 0.77[b]	3.32 ± 0.85[c]	3.36 ± 0.70[c]
	GSH-Px	2.06 ± 0.29[a]	2.20 ± 0.38[b]	2.36 ± 0.45[c]	2.37 ± 0.41[c]

MDA: Malondialdehyde level (nmol/mg protein), SOD: Superoxide dismutase activity (U/mg protein), CAT: Catalase activity (k/mg protein, k: the first-order rate constant) and GSH-Px: Glutathione peroxidase activity (U/mg protein).
The values (n = 15) are expressed as mean ± standard error. The mean values in same line with different superscripts are significantly different ($p < 0.05$).

status, stress and disease conditions of fish [9,45]. These parameters are important tools that could be widely used in evaluating of functional and nutritional status and the capacity for fish adaptation to the external environment [46]. In addition, the haematological parameters give an indication to the health status of fish by determining any abnormality occurring owing to the use of immunostimulants [47]. The results of present study showed that the RBC count, Hb concentration and Ht level of EA-50, EA-100 and EA-200 groups significantly increased in juvenile rainbow trout after 8 weeks. Mişe Yonar et al. [9] recorded an increase in the RBC count of rainbow trout fed diets supplemented with different levels of EA for 21 days. Also, they stated a statistically insignificant increase in the Hb and Ht levels. The differences from the present study can be explained by different doses in the feed, the length of studies and fish weights. On the other hand, the erythrocyte indices are useful in elucidating the etiology and morphological classification of anemias. In the present study no change was observed in the erythrocyte indices (MCV, MCH and MCHC). Data from the present study showed that the dietary EA had a positive effect on the haematological parameters in juvenile rainbow trout and EA did not cause anemia. Also, the change in erythrocyte indices was not significant from the control, which indicated the fish was not under stress.

Leucocytes are involved in the control of immunological functions. Leucocytes are considered as one of the important factors of nonspecific or innate immunity and the leucocyte count/activity can be indicated as indicators of health of aquatic animals [48]. The results of present study demonstrated that the WBC counts of groups fed diets containing EA significantly increased after 8 weeks. This is in agreement with Mişe Yonar et al. [9] who stated that EA induced the WBC count of rainbow trout after 21 days of feeding. Adel et al. [49] also stated herbal plants could be act as immunostimulants and increased the total WBC of rainbow trout.

A respiratory burst, or oxidative burst, is an indication of the oxidative potential of reactive oxygen species produced by activated phagocytic cells. Respiratory bursts from innate immune cells, including blood neutrophils, are measured using NBT or MPO assays [50]. The NBT assay measured in terms of respiratory burst activity of phagocytes is used to detect oxidative radical production which is considered an important innate immune mechanism [51]. MPO is a haem based peroxidase enzyme released by neutrophils in fish and plays an important role in the nonspecific cellular immunity. It produces reactive oxygen species, which help in the killing of bacteria [52]. Phagocytic cells including the monocyte/macrophages and granulocytes in teleost fish are the main components of innate immune response, which regulate immune function and play a major role in combating diseases. Their phagocytic activity is a primitive defence mechanism and an important characteristic of the nonspecific immune system [53]. In addition, phagocytosis associated with respiratory burst activity is an important indicator of innate immunity in fish [54]. Previous studies have showed that the use of herbal immunostimulants led to stimulation of phagocytosis in different fish species [5,34,42,43,47,55–60]. Data from the present study also showed that the groups fed with EA had significantly higher respiratory bursts and phagocytosis than the control group, confirming that nonspecific cellular immunity was enhanced in fish fed diets containing EA. However, the study of Mişe Yonar et al., [2014] showed that EA administration insignificantly induced the NBT activity of rainbow trout after 21 days; this may be due to shorter experiment duration.

Proteins including albumin and globulin are the most important compounds in serum and its level is considered as a basic index for sustaining healthy immune system. Increases in serum protein contents are usually thought to be associated with a stronger innate immune response in fish. Among the serum proteins, globulin is the major proteins, which plays a significant role in the immune response [61]. Immunoglobulins are glycoproteins that have antibody activity and are found in the blood, lymph, and vascularized tissues. IgM, the main antibody of fish, is a major component of the teleost humoral immune system [62]. This molecule is believed to play a vital role in several immune processes such as phagocytosis, opsonisation, and neutralization of pathogenic bacteria, viruses, and toxins in the host body [63,64]. Prior researches have reported the relationship between total protein and immunoglobulin levels [54,65]. Several previous studies have reported elevation of the TP or Ig levels in fish fed with herbal immunostimulant such as garlic [5,42], ginger [43,56], black cumin [66], and curcumin [41]. Similarly, the results of present study showed that the TP and IgM levels increased significantly in fish fed with EA. This outcome may be explained by induction of humoral immunity after the administration of EA. In this sense, the results of present study demonstrate that EA may induce humoral immunity by increasing the TP or IgM levels in fish.

The presence of protective proteins in fish blood including complements, acute phase proteins, LYZ, transferrin and anti-proteases might be analysed by serum BA, which considered as nonspecific response to inhibit the growth of pathogenic microbes [67]. LYZ present in the mucus, plasma, and other body fluids of fish plays a significant role in the protection from infectious microorganisms in fish [54]. It is an opsonin, and thus activates the complement system and phagocytes [68]. Immunostimulants are known to increase LYZ activity [5,69], and rising of LYZ following immunostimulation has been demonstrated in different fish species [47]. So far, no information about the effect of EA on the LYZ activity and BA in rainbow trout has been reported. In this study, fish fed diets supplemented with different levels of EA showed significantly higher LYZ activity and BA when compared with the control group. Moreover, the highest activities depended mainly on the dose administration. The elevated LYZ activity observed in this study could be correlated with enhanced phagocytic activity and serum bactericidal activity [70]. Also, previous studies have demonstrated that elevation in LYZ activity may be associated with increase in leucocyte number [71] or antibody titre [72].

An experimental infection is one of the most valuable tests to evaluate the efficiency of immunostimulant in the resistance against pathogens [52]. In literature there was no study about the effect of EA on disease resistance in fish. However, previous studies showed reduction in mortality against *Y. ruckeri* in fish after using herbal immunostimulant [44,49,68,73]. In the present study, the results revealed that dietary supplement with EA relatively increased the resistance of rainbow trout against *Y. ruckeri*, where the highest doses (200 and 100 mg EA kg^{-1} diet, respectively) recorded the highest protection or survival rate. This result may be explained by stimulation of the nonspecific humoral and cellular immune parameters after the administration of EA.

The equilibrium between antioxidant defences and the generation of oxygen reactive species (ROS) is fundamental for the animal's homeostasis. Under normal physiological conditions, animal cells produce ROS. However, the cells have antioxidant defence mechanisms to cope with it [61,74]. When there is an imbalance between ROS and antioxidant defences, the situation known as oxidative stress can be established, in which the excessive generation of ROS may lead to irreversible impairment of DNA and other macromolecules and even to death [74–76]. Lipid peroxidation is considered to be a valuable indicator of the oxidative stress in cellular components [77–79]. MDA as a main end product of lipid peroxidation can be used as one of the indices of lipid peroxidation [10,80,81]. As the first line of enzymatic antioxidant defence, SOD, CAT and GSH-Px are the important biochemical parameters for antioxidant defence and they protect cells from oxidative stress due to excessive generation of ROS. In general, the measurement of those activities can provide an indication of the antioxidant status of fish, as can also serve as biomarkers of oxidative stress [61]. On the other hand, the balance between oxidant and antioxidant is fundamental for immune cell function since it preserves cell membrane integrity and functionality, cellular proteins, and nucleic acids [82]. Mişe Yonar et al. [9] showed that EA supplementation in rainbow trout diet resulted in an obvious elevation of both enzymatic (SOD, CAT

and GSH-Px activities) and nonenzymatic (reduced glutathione (GSH) level) antioxidant with a significant decline in the MDA levels. Similarly, in the present study, the dietary EA significantly increased the SOD, CAT, and GSH-Px activities while decreased the tissue MDA levels of rainbow trout. The increase determined in this study may be explained with possibly enhancement of antioxidant capacity. The enhanced antioxidant capacity was further supported by the relatively low MDA levels observed in the tissues of fish fed diets supplemented with EA. García-Niño and Zazueta [83] stated that EA could increase GSH levels through upregulation of GSH synthetase, glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase regulatory subunit (GCLR). Also, EA could induce the expression of NADPH:quinone oxidoreductase 1 (NQO1), haem oxygenase-1 (HO-1), SOD, CAT, GSH-Px and glutathione-*S*-transferase (GST).

In conclusion, the results of this study demonstrated that dietary supplement with EA to rainbow trout for 8 weeks improves the growth performance, haematological values, immune response, antioxidant capacity, and disease resistance against *Y. ruckeri*. The optimal dietary EA level for rainbow trout could be considered as 100 or 200 mg kg^{-1} diet, based on the effectiveness of EA on the growth, haematological indices, immunity and antioxidant capacity of rainbow trout, *O. mykiss*. These results indicated that EA can be considered as a beneficial dietary supplement for rainbow trout in the aquaculture.

Declaration of competing interest

There is no conflict of interest to declare.

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