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Research Article

Effect of dwarf elder (*Sambucus ebulus*) to improve hemato-immunological and biochemical parameters and resistance against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The present study examined the effects of dietary dwarf elder (*Sambucus ebulus*) on hemato- immunological and biochemical parameters and protection against *Yersinia ruckeri* in rainbow trout. The fish were divided into four groups (in triplicate) and fed diets supplemented with 0, 2.5, 5, and 10% of the powdered leaf of dwarf elder for 8 weeks. The blood samples were taken on weeks 4 and 8 of the trial. The use of dietary dwarf elder did not significantly affect the red bloo cells (RBC), hematocrit, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), lymphocytes percentage, glucose, and alanine transaminase (ALT) levels of all the treated groups compared to the control group on weeks 4 and 8. At the same time, the total count of white blood cells (WBC) and the neutrophil percentage in the fish receiving the herb powder were significantly higher than the control group. The highest total protein, albumin, immunoglobulin levels, and respiratory burst activity were observed in 5% group after 4 and 8 weeks. On the 8th week, the lysozyme activity was significantly increased and the triglyceride was significantly decreased in 5 and 10% groups compared to the control group. After 4 and 8 weeks, the treated groups exhibited a significant decrease in the cholesterol and alanine aminotransferase levels compared to the control group. The lowest number of bacterial colonies in the serum antibacterial activity belonged to 5 %, 10 %, 2.5 %, and control groups, respectively. At the end of 8 weeks, the fish were challenged with *Yersinia ruckeri* and the survival rates of the control, 2.5 %, 5 %, and 10 % groups that were 22.2 %, 60.3 %, 79.5 %, and 68.5 %, respectively. The present findings indicate dwarf elder possesses beneficial dietary effects on immune responses and resistance against *Y. ruckeri* in rainbow trout.

Keywords: Dwarf elder, *Sambucus ebulus*, *Yersinia ruckeri*, Immunity, Total white blood cell, Lysozyme activity

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Introduction

Rainbow trout (*Oncorhynchus mykiss*) is the second freshwater cultivated species in Iran, preceded by Carp, with total production increasing from 91519 metric tons in 2010 to 190287 metric tons in 2020 (FAO, 2020; IFO, 2021). The aquaculture production has shifted from extensive systems towards more intensified systems due to the increasing demand for seafood. However, the intensification of aquaculture poses many problems including stress in aquatic species, outbreaks of infectious diseases, and inappropriate applications of antibiotics (Moradyan *et al.,* 2012; Yarahmadi *et al.,* 2016). Unfortunately, various investigations have shown that the excessive use of antibiotics has led to a generation of bacteria that are resistant to drugs (Raissy and Moumeni 2016; Aflakian *et al.,* 2022) and the accumulation of antibiotics in rainbow trout muscle causes serious concern for public health in Iran (Soltani *et al.,* 2014; Adel *et al.,* 2017; Rafati *et al.,* 2018). The enteric red-mouth disease (ERM) or Yersiniosis is one of the most important bacterial diseases of rainbow trout in Iran caused by *Yersinia ruckeri (*Tulaby Dezfuly *et al.,* 2022). It causes subcutaneous hemorrhages at the corners of the mouth, gums, and tongue, exophthalmia, skin darkness, inflammation, hemorrhage of the lower intestine, and splenomegaly (Kumar *et al.,* 2015; Zorriehzahra *et al.,* 2017). For the first time, yersiniosis-like infection was reported from Iranian farmed rainbow trout in 1999 (Soltani *et al.,* 1999), and then, Akhlaghi and Sharifi Yazdi (2008), recognized *Y. ruckeri* by molecular techniques. In recent years, several studies have been conducted to produce a vaccine against yersiniosis by use of the native bacterial species (auto vaccine) in Iran (Soltani *et al.,* 2016; Erfanmanesh *et al.,* 2022), but this method is still expensive for farmers, and researchers are looking for cheaper and more appropriate ways to control the disease in Iran.

The use of herbal plants has recently deserved growing attention in the aquaculture industry due to their biological activities related to secondary metabolites, low costs, and minimal side effects compared to conventional antibiotics (Reverter *et al.,* 2021). Plant-enriched diets have been reported to increase disease resistance due to their phytochemical substances and immunomodulatory activities. Therefore, there has been increased interest in the possibility of using medicinal herbs as immunostimulants to enhance immune responses and disease resistance in cultured fish species (Ahmadifar *et al.,* 2021; Alagawany *et al.,* 2020; Elumalai *et al.,* 2020; Firmino *et al.,* 2021).

Several studies mentioned the positive effects of many Iranian medicinal plants such as *Mentha piperita* (Adel *et al.,* 2016) *Ducrosia anethifolia* (Dehghan *et al.,* 2016), *Urtica dioica* (Saeidi asl *et al.,* 2017), *Carthamus tinctorius* (Zargari *et al.,* 2018), *Polygonum minus* (Adel *et al.,* 2019), *Coriandrum sativum* (Naderi Farsani *et al.,* 2019), *Zingiber officinale* (Soltanian *et al.,* 2019), *Quercuse*

brantii (Ghafarifarsani *et al.,* 2020), *Rhus coriaria* (Gharaei *et al.,* 2020), *Mentha longifolia* (Heydari *et al.,* 2020), *Malvae sylvestris* (Rashidian *et al.,* 2020), *Ziziphora clinopodioides* (Oroji *et al.,* 2021), and *Capsicum annuum* (Firouzbakhsh *et al.,* 2021) on hemato-biochemical parameters, immune responses and immune-related gene expression, and growth performance as well as positive effect on oxidative stress and resistance against *Y. ruckeri* infection in rainbow trout.

Elderberry or dwarf elder (*Sambucus ebulus*) is an herbaceous plant, well-known in Iranian folk medicine and extensively grows in the northern regions of Iran (Aghajanzadeh *et al.,* 2021). It has been reported to possess marked antiemetic and neuroprotective (Fathi *et al.,* 2015), antiviral (Ghaffari *et al.,* 2021), antibacterial (Mahboubi *et al.,* 2012; Rodino *et al.,* 2015; Hashemi *et al.,* 2022), antifungal (Rezaei-Moshaei *et al.,* 2021; Mirmazloomi *et al.,* 2022), anti-parasite (Heidari-Kharaji *et al.,* 2019), antioxidant (Karami *et al.,* 2015), anti-mutagenic and anticancer (Saeedi Saravi *et al.,* 2013; Rezaei-Moshaei *et al.,* 2021; Hashemi *et al.,* 2022), anti-inflammatory and anti-nociceptive activities (Shokrzadeh *et al.,* 2010; Schwaiger *et al.,* 2011; Jabbari *et al.,* 2016), and wound healing effects (Süntar *et al.,* 2010; Babaei *et al.,* 2017).

The most important phytochemical compounds have been found in dwarf elder including phytosterols, anthocyanins, quercetin, phenolic compounds, flavonoids, alkaloids, minerals (especially Ca, Mn, and Fe), vitamin C, chlorogenic and ursolic acids, carotenoids, and chlorophylls (Ebadi and Hisoriev 2011; Fathi *et al.,* 2015; Jabbari *et al.,* 2017; Kaya *et al.,* 2019; Tasinov *et al.,* 2013, 2021). All these compounds made dwarf elder an appropriate candidate for use in folk veterinary medicine. However, there are no reports on the dietary effect of dwarf elder on immune-hematological profiles in rainbow trout. Therefore, this study aimed to investigate the impact of dietary dwarf elder powder on biochemical and hemato-immunological parameters and disease resistance against *Y. ruckeri* in rainbow trout.

Materials and methods

Diet preparation

Four experimental diets were obtained by adding the dwarf elder (dried powdered leaves and stem obtained from Sari, north of Iran) at 0, 2.5, 5 and 10% into a rainbow trout commercial basal diet (Beyza21™ Feed Com, Shiraz, Iran) containing 44.50% protein, 14.20% lipid, 7.62% ash, and 21.90 MJ/kg gross energy. The dietary ingredients were blended with water (100 mL of water per 1 Kg of diet) to form a stiff paste, which passed through a meat grinder (Techno Sanat, Iran) and pelleted to produce 5.0 mm pellets. The experimental diets were air-dried at room temperature (25˚C) for 24 h and stored in plastic bags at -4˚C until use.

Fish and experimental design

Rainbow trout (92.2±2.2 g initial weight) were obtained from a commercial fish farm in Babol

(Mazandaran province, north of Iran). The health of the fish (changes in behavior and physical appearance) was checked two weeks before starting the experiment. The fish were randomly distributed into 12 fiberglass tanks (1000 L) at 30 fish per tank density (3 tanks per treatment). During the acclimatization period (7 days), the fish were fed with the basal diet. During the experimental time (8 weeks), the feeding rate was 3.5-4% of the body weight and the fish were fed four times a day (07:00, 12:00, 17:00, and 22:00). The water temperature, dissolved oxygen, pH, and electrical conductivity were monitored daily and maintained at 14.4±1.3°C, 7.9±0.5 mg/L, 7.3, and 5733.1±137.1 MM/cm, respectively. Continuous aeration was provided to each tank through an air stone connected to a central air compressor.

Blood sampling

On weeks 4 and 8 after the feeding, 9 fish were randomly sampled from each treatment and were anesthetized by clove extract, a natural alcoholic extract of *Eugenia aromatic*, at 250 µL/L which was developed at Caspian Sea Ecology Research Center. The blood (~ 2 ml) was drawn from the caudal vein, using a non-heparinized syringe, after they were starved for 24 h. One half of each blood sample was transferred to microtube containing heparin as an anti-coagulant (50 µL) and immediately used for respiratory burst assay and hematological examinations, while the other half was transferred to non-heparinized microtube, placed at room temperature and allowed to clot for 2 h. The sera were separated by centrifugation at 1500 ×*g* for 20 min and stored at -20ºC until use.

Hematological and biochemical assays

The red blood cells (RBC) and white blood cells (WBC) counts were determined using a Neubauer hemocytometer. The hematocrit (Hct) was measured by microcentrifuge technique, using standard heparinized microhaematocrit capillary tubes (75 mm at 7000 ×*g* for 10 min). The haemoglobin level was analyzed spectrophotometrically at 540 nm by the cyamethaemoglobin method (Blaxhall and Daisley, 1973). The blood indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Seiverd (1964) method. To estimate the differential leukocyte percent (lymphocytes and neutrophils), blood smears were prepared, air-dried, fixed in methanol, and stained using May-Giemsa solution (Lee *et al.,* 1998). Biochemical analyses were performed for albumin, glucose, triglyceride, cholesterol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) by commercial kits (Pars Azmoon, Tehran, Iran) and a biochemical auto analyzer instrument (Eurolyser, Belgium) (Binaii *et al.*, 2014).

Immunological assays

Lysozyme activity

Serum lysozyme activity was measured using a modified turbidimetric method described by Ellis (1990). Briefly, aliquots (1.75 mL) of *Micrococcus lysodeikticus* (Sigma, USA) suspension (0.375 mg/mL, 0.05 M PBS, pH 6.2) were mixed with 250 mL of each sample and optical density was measured after 15 and 180 s by spectrophotometer (Biophotometer Eppendorf) at 670 nm. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001/min. The units of lysozyme present in sera were obtained from a standard curve made with hen egg white lysozyme (Sigma, USA).

Respiratory burst activity

The oxidative burst produced by leukocytes of the blood samples was measured by Chemiluminescent assay (CL) (measuring of light emission) as described by Khoshbavar-Rostami *et al.* (2006) using an automated system for CL analysis (LUMI scan Ascent T392, Finland). The results of light emission are expressed in the form of relative light units per second (RLU/s) recorded by the CL assay. CL assays were performed in duplicate and the mean of replicate assays was used in subsequent evaluations.

Serum total protein and total immunoglobulin

The serum total immunoglobulin (Ig) levels were determined according to the method described by Siwicki and Anderson (1993). Briefly, the serum total protein (TP) content was measured using a microprotein determination method (C-690; Sigma), before and after precipitating down the immunoglobulin molecules, using a 12% solution of polyethylene glycol (Sigma).

Serum antibacterial activity

In vitro bactericidal activity of the serum samples was examined against *Y. ruckeri* (KC291153, obtained from Faculty of Veterinary Medicine, University of Tehran, Iran) which was prepared as lyophilized stock. The isolate was cultured overnight in nutrient broth (Merck, Germany) and centrifuged at 1500 × *g* for 20 min. The pellets were washed and re-suspended in PBS to an OD540 of 1.0 (5.32×10^7 CFU/mL). This bacterial suspension was serially diluted (1:10) with PBS five times. Serum bactericidal activity was determined by incubating 2 mL of this diluted *Y. ruckeri* suspension with 20 ml of serum in a micro-vial for 1 h at 37 °C. In the bacterial control group, PBS replaced the serum. After incubation, the number of viable bacteria was determined by counting the colonies grown on a nutrient agar plate (duplicate) for 24 h at 37°C (Rao *et al.*, 2006).

Bacteria and challenge

Y. ruckeri (KC291153) was used for the challenge experiment. The bacteria were grown in LB-medium (Oxoid LP0042, Tryptone 10 g, Oxoid LP0021Yeast-extract 5 g, NaCl 5 g, H2O to 1000 ml, pH 7.4) at 20°C for 36 h and enumerated as colony forming units (CFU) by the

spread plate method on blood agar (40 g/L Oxoid blood agar base CM0055) with 5% bovine blood) (Raida and Buchmann, 2009). At the end of 8 weeks, eighty fish (ten fish in duplicate from each experimental group) was randomly selected and anesthetized by the clove extract (250 µL/L). The fish were infected by intraperitoneal injection with 6×10^7 CFU/fish in 100 µL ringer. The mortalities were monitored for 14 days, and dead fish were dissected to confirm the presence of the pathogen through microbiological examination of the head kidney. The survival rate (SR) was calculated to evaluate the efficacy of the plant powder as SR=(Nt / N0)×100. Here, N0 is the initial number of fish and Nt is the final number of fish (Nya and Austin, 2009).

Statistical analysis

The effects of dietary dwarf elder on the hemato-biochemical parameters and immunity of rainbow trout were analyzed using SPSS (Ver. 20) and significant differences between the groups were determined by one-way analysis of variance (ANOVA). Duncan's multiple range tests were performed when the differences were significant. Mean values were considered significantly different at $p<0.05$. Data are expressed as mean values±SE.

Results

Hematological and biochemical profiles

The effect of dietary dwarf elder on hematological and biochemical parameters in weeks 4 and 8 is summarized in Tables 1 and 2. The statistical analysis of data showed that there were no statistically significant changes in the RBC, Hb, Hct, MCV, MCH, MCHC, lymphocytes, glucose, and AST values in all treated groups compared to the control group on weeks 4 and 8 ($p>0.05$). At the end of week 4th, the WBC count significantly increased in the fish fed with 5 and 10 % dwarf elder when compared to the control and 2.5% groups ($p<0.05$). Also at the same time, there was a significant change in the neutrophil percentage of all treated groups compared to the control group ($p<0.05$). At the end of week 8th, the WBC counts of all herbal-enriched diets were significantly higher than the control group ($p<0.05$) and the highest WBC count was obtained in 5% group. At the same time, neutrophil percentage was also higher in the fish fed with the herbal enriched diets compared to the control group.

The highest levels of cholesterol and ALT were noticed in the control group at the end of weeks 4 and 8 ($p<0.05$). No significant difference was observed between the treated and control groups at the end of the 4 weeks in the triglyceride level ($p>0.05$), whereas it was significantly decreased in 5 and 10% herbal enriched diets on week 8th ($p<0.05$). Also, the TP and albumin levels were significantly increased in the fish fed with 5% dwarf elder when compared to the control group at the end of weeks 4 and 8 ($p<0.05$).

Immunological profile

The results showed that the serum total Ig value of 5% dwarf elder group was significantly higher than the control and 2.5% groups at the end of the week 4th. After 8 weeks, the serum total Ig value was increased in the fish fed with the herbal-enriched diets compared to the control group, but just 5% group had a significant difference compared to the control group (Fig. 1).

Table 1: Average hematological parameters for rainbow trout fed with 0, 2.5, 5 and 10% dwarf elder of feed for 4 and 8 weeks.

Sampling time	Percent dwarf elder of feed	RBC (10^6 mL^{-1})	WBC (10^3 mL^{-1})	Htc (%)	Hb (gdL^{-1})	MCV (fL)	MCH (pg)	MCHC (gdL^{-1})	Lymphocytes (%)	Neutrophils (%)
After 4 weeks	10	1.11± 0.13^{a}	13.08± 0.41^{b}	36.44± 2.69^{a}	6.11± 0.60^{a}	343.92± 18.53^{a}	53.90± 1.95^{a}	15.73± 0.53^{a}	93.66± 1.32^{a}	6.33± 1.32^{b}
	5	1.08± 0.15^{a}	13.64± 0.62^{b}	37.01± 2.54^{a}	6.13± 0.36^{a}	350.83± 9.16^{a}	55.24± 1.23^{a}	15.20 ± 0.66^{a}	92.44± 1.28^{a}	7.55± 1.28^{b}
	2.5	1.15± 0.9^{a}	10.42± 0.87^{a}	36.33± 2.95^{a}	6.06± 0.63^{a}	337.58± 8.41^{a}	56.11± 1.35^{a}	15.34 ± 0.43^{a}	94.33± 0.94^{a}	5.66± 0.94^{b}
	0	0.99± 0.1^{a}	9.56± 0.58^{a}	35.66± 1.87^{a}	5.87± 0.68^{a}	343.76± 8.06^{a}	57.81± 1.54^{a}	15.78 ± 0.75^{a}	98.22± 0.46^{a}	1.77± 0.46^{a}
After 8 weeks	10	1.17± 0.14^{a}	16.66± 0.85^{bc}	42.33± 4.12^{a}	6.58± 0.75^{a}	362.43± 11.41^{a}	60.30± 4.10^{a}	16.92± 1.13^{a}	92.66± 1.46^{a}	7.33± 1.46^{b}
	5	1.26± 0.24^{a}	18.02± 1.37^{c}	42.44± 3.46^{a}	6.65± 0.62^{a}	364.36± 9.77^{a}	58.01± 1.84^{a}	16.53± 0.81^{a}	91.6± 9.16^{a}	7.35± 1.05^{b}
	2.5	1.17± 0.21^{a}	11.54± 0.77^{b}	41.55± 2.87^{a}	6.45± 0.65^{a}	366.36± 6.77^{a}	56.67± 1.09^{a}	16.83± 0.46^{a}	93.66± 1.14^{a}	6.33± 1.12^{b}
	0	1.10± 0.14^{a}	0.95± 0.25^{a}	40.22± 3.59^{a}	6.17± 0.84^{a}	367.90± 14.96^{a}	57.08± 1.66^{a}	16.60± 0.61^{a}	96.66± 4.6^{a}	3.33± 1.02^{a}

RBC, red blood cells; WBC, white blood cells; Ht, hematocrit; Hb, hemoglobin concentration; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration. Data represent as mean±SE. ata in the same columns with different superscript are significantly different (p<0.05).

Table 2: Biochemical indices of rainbow trout after feeding with different percent of dwarf elder for 4 and 8 weeks.

Sampling time	Percent dwarf elder of feed	Total protein (gdL^{-1})	Albumin (gdL^{-1})	Glucose (gdL^{-1})	Triglyceride ($mgdL^{-1}$)	Cholesterol ($mgdL^{-1}$)	ALT ($IUdL^{-1}$)	AST ($IUdL^{-1}$)
After 4 weeks	10	3.74± 0.77^{ab}	2.14± 0.13^{ab}	94.26± 14.01^{a}	243.62± 25.25^{a}	224.38± 10.15^{a}	9.27± 0.93^{a}	223.22± 21.16^{a}
	5	4.15± 1.07^{b}	2.96± 0.11^{b}	95.02± 10.67^{a}	229.84± 17.16^{a}	221.84± 11.99^{a}	11.93± 1.24^{a}	223.97± 15.01^{a}
	2.5	3.72± 0.78^{ab}	2.23± 0.14^{ab}	95.84± 8.56^{a}	209.77± 12.26^{a}	237.14± 15.17^{a}	10.38± 1.32^{a}	226.85± 21.68^{a}
	0	3.47± 0.32^{a}	2.02± 0.18^{a}	100.08± 9.39^{a}	242.24± 17.41^{a}	283.93± 8.95^{b}	16.33± 1.81^{b}	258.12± 15.07^{a}

Table 2 continued:

After 8 weeks	10	4.31± 0.31^{ab}	2.74± 0.33^{b}	92.35± 6.94^{a}	215.45± 16.05^{a}	234.02± 11.82^{a}	6.21± 1.43^{a}	212.88± 15.19^{a}
	5	4.85± 0.29^{b}	2.97± 0.31^{b}	93.33± 2.41^{a}	211.35± 13.08^{a}	258.61± 11.24^{a}	6.61± 0.46^{a}	221.71± 18.88^{a}
	2.5	4.22± 0.35^{ab}	2.63± 0.42^{b}	94.06± 4.21^{a}	282.05± 8.42^{b}	243.82± 12.33^{a}	6.38± 0.58^{a}	229.92± 16.38^{a}
	0	3.71± 0.12^{a}	2.08± 0.45^{a}	101. 21± 4.24^{a}	294.81± 21.51^{b}	309.74± 7.78^{b}	16.75± 1.62^{b}	243.25± 11.93^{a}

ALT, alanine aminotransferase; AST, aspartate aminotransferase. Data represent as mean ± SE. ata in the same columns with different superscript are significantly different (p<0.05).

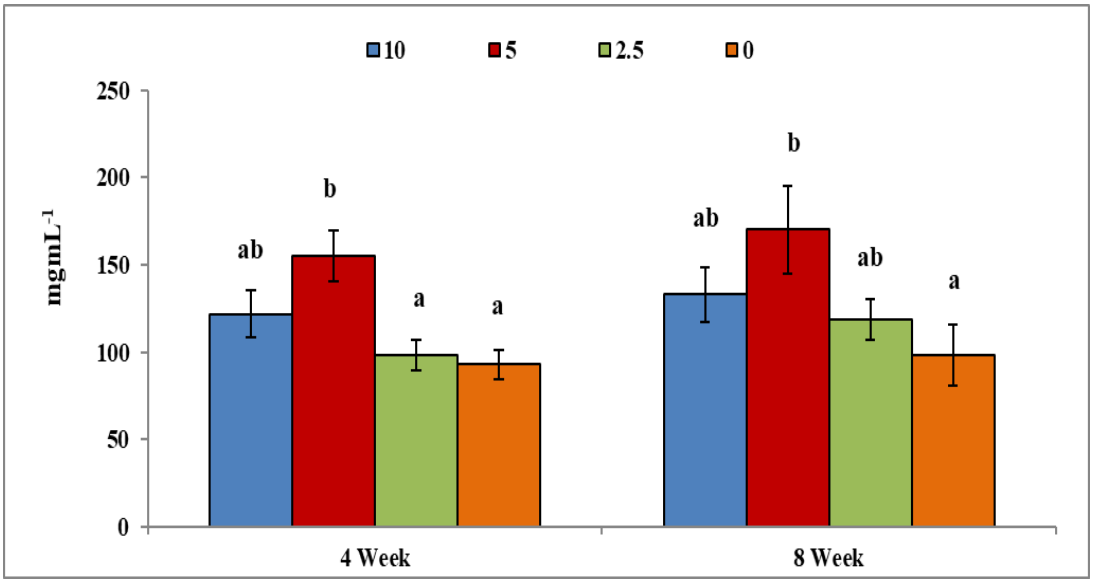

Figure 1: Serum total immunoglobulin (Ig) levels of rainbow trout fed with 0, 2.5, 5 and 10% dwarf elder. Data represent the mean±S.D. Data in the same row with different superscript are significantly different (p<0.05).

The lysozyme activity of rainbow trout was not affected by different doses of dietary dwarf elder powder until the week 4th when compared to the control group (p>0.05), but it was significantly enhanced in the fish fed with 5% and 10% dwarf elder powder on week 8th when compared to the control group (p<0.05) (Fig. 2).

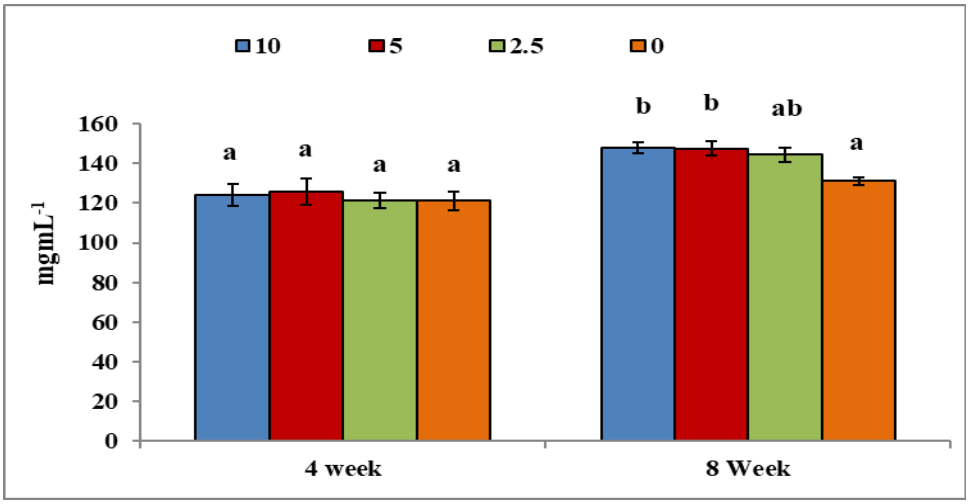

Figure 2: Lysozyme activity of rainbow trout fed with 0, 2.5, 5 and 10% dwarf elder. Data represent the mean±S.D. Data in the same row with different superscript are significantly different (p<0.05).

The respiratory burst activity was significantly enhanced in the fish fed with 5 and 10% dwarf elder powder on week 4th when compared to the control and 2.5% groups (p<0.05), but it was significantly enhanced in all treated groups on week 8th when compared to the control group (p<0.05). The highest respiratory burst activity was found in 5% group (Fig. 3).

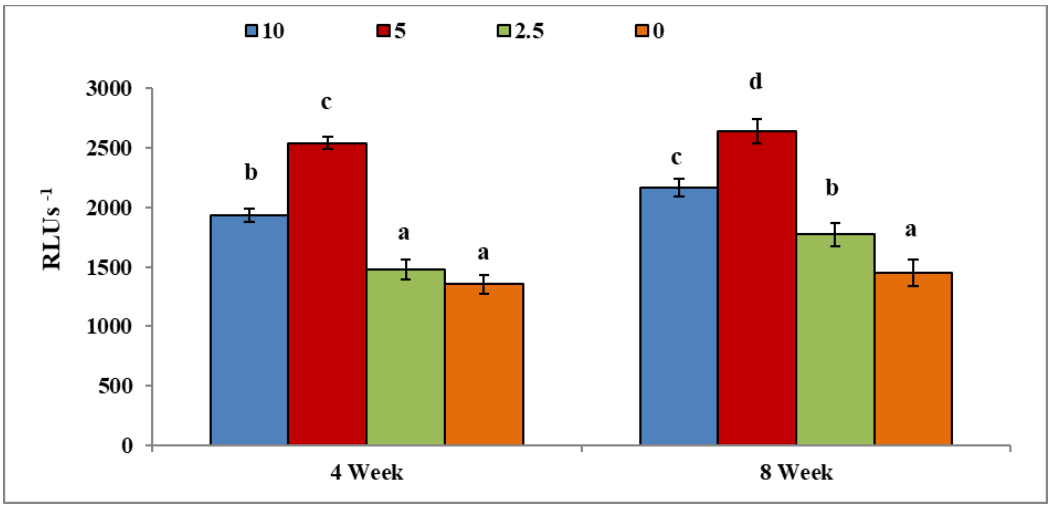

Figure 3: Respiratory burst activity of rainbow fed with 0, 2.5, 5 and 10% dwarf elder. Data represent the mean±S.D. Data in the same row with different superscript are significantly different (p<0.05).

The results of the serum bactericidal activity (based on colony count) showed that 5% group had the highest value compared to the other groups and the control group had the lowest serum bactericidal activity (p<0.05) (Fig. 4).

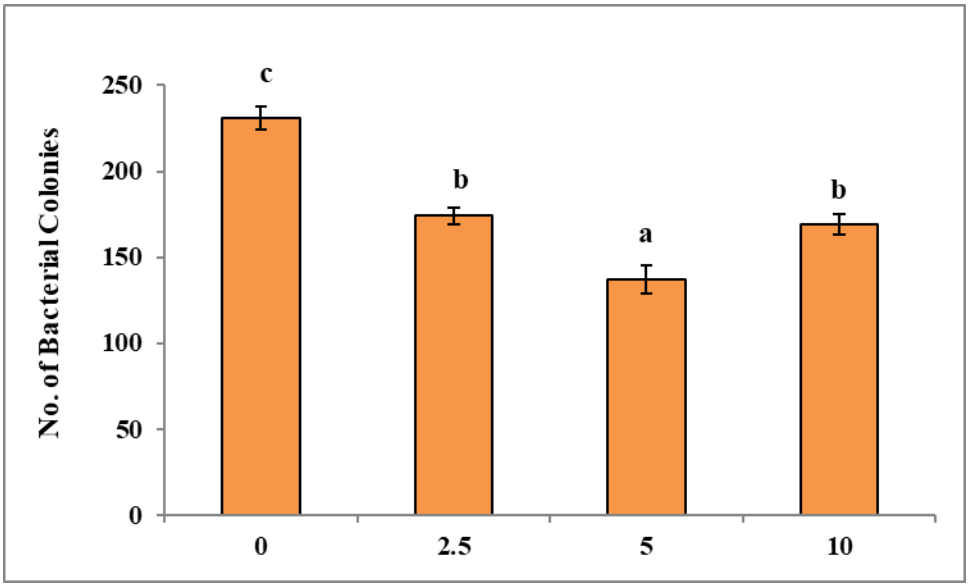

Figure 4: Serum antibacterial activity of rainbow trout after 8 weeks feeding of dietary dwarf elder. Data represent the mean±S.D. Data in the same row with different superscript are significantly different (p<0.05).

Disease resistance

The use of dietary dwarf elder powder led to a marked reduction in the mortality rate after challenging with *Y. ruckeri* infection. The survival percentages were 22.2%, 60%, 79.5%, and 68.53% in the control group, 2.5, 5, and 10% dwarf elder powder, respectively. The survival rate of the treated groups did not show any sign of disease at the end of the feeding experiment.

Discussion

Immunostimulants have an important role to regulate fish health and prevent fish losses due to infectious diseases and medicinal herbs have been extensively used as an immunostimulant to enhance immunity and fish increase resistance against infectious diseases (Liu *et al.*, 2022). In this study, we determined the effect of dwarf elder (*Sambucus ebulus*) powdered leaf supplementation of rainbow trout diet on immuno – hematological responses and resistance against *Yersinia ruckeri*. In our study, the results showed that WBC levels in the 5% and 10% dwarf elder enriched diet treated group significantly increased in rainbow trout on week 4th, but at the end of 8 weeks, WBC levels significantly rose in all groups treated by dwarf elder. WBCs monitoring can reflect the general immune system status in fish and many investigations have shown that medicinal plants could improve and increase the total WBC of rainbow trout (Adel *et al.*, 2016, 2019; Farsani *et al.*, 2019; Gharaei *et al.*, 2020; Heydari *et al.*, 2020; Rashidian *et al.*, 2020; Firouzbakhsh *et al.*, 2021) probably as a result of the stimulation of erythropoiesis and leukopoiesis by plant active compounds (Ahmed, 2020).

Teleost neutrophils are professional phagocytes and the key components of the innate immune response. They ingest

pathogens and produce reactive oxygen species and release toxic substances from intracellular granules, thus becoming very potent killers in fish blood (Kordon *et al.,* 2018). The use of dwarf elder enriched diet significantly rose the neutrophil percentage of all the treatment groups on weeks 4th and 8th. Joshi *et al.* (2022) found that phytosterol components (especially β-sitosterol) increase the neutrophils count in humans. Shokrzadeh and Saeedi Saravi (2010) described that β-sitosterol is one of the dwarf elder leaf's phytosterol components and it could be explained by the rise of neutrophil count in our investigation.

Lysozyme is normally used as an indicator of fish's non-specific immune response in defense against bacterial pathogens. Its bactericidal action is due to destroy of Gram-positive and some Gram-negative bacteria. Moreover, it promotes phagocytosis of macrophages and polymorphonuclear leucocytes (by acting as an opsonin) and the complement system in marine and freshwater fish (Uribe *et al.*, 2011; Alagawany *et al.,* 2020). In the present study, the lysozyme activity of fish was not changed after feeding with different doses of dwarf elder at the end of week 4th, but it was significantly increased in the 5% and 10% groups after 8 weeks when compared to the control. The increase in lysozyme activity within the dwarf elder groups of rainbow trout had been confirmed in previous studies with other Iranian herbs including *Mentha piperita*, *Polygonum minus* (Adel *et al.,* 2016, 2019), *Capsicum annuum* (Firouzbakhsh *et al.,* 2021), *Aloe vera* (Alishahi *et al.,* 2017), *Nigella sativa*, *Echinacea angustifolia* (Fadeifard *et al.,* 2018), *Zingiber officinale* (Fadeifard *et al.,* 2018; Soltanian *et al.,* 2019) *Verbascum speciosum* (Nofouzi *et al.,* 2017), *Urtica dioica* (Saeidi asl *et al.,* 2017), *Echinacea purpurea* (Sharif Rohani *et al.,* 2016) *Allium sativum* (Zaefarian *et al.,* 2017). Various studies on other Iranian medicinal plants have shown that the flavonoid compounds are effective in elevating of lysozyme activity of rainbow trout (Firouzbakhsh *et al.,* 2021; Hosseini Shekarabi *et al.,* 2021) and there is information that dwarf elder leaves are rich in these compounds (Fathi *et al.,* 2015).

The respiratory burst is a potent antimicrobial defense mechanism of phagocytes. It is activated after the stimulation of the plasma membrane during phagocytosis and triggers the production of reactive oxygen species (Bulfon *et al.,* 2018). The results showed that the respiratory burst activity in fish fed by the 5% and 10% dwarf elder diet significantly rose compared to two other groups at the end of 4 weeks, but at the end of 8 weeks, the respiratory burst activity significantly rose in all groups treated by dwarf elder and the highest respiratory burst activity were observed in 5% group at both times. The same results were reported in rainbow trout after receiving the other Iranian herbs (Adel *et al.,* 2016, 2019; Saeidi asl *et al.,* 2017; Heydari *et al.,* 2020). Akbay *et al.,* (2003) have shown that the flavonoid glycosides of nettle can cause to modulate the degranulation and

oxidative burst of appropriately stimulated human neutrophils. Five flavonoid glycosides were recognized in dwarf elder (Zahmanov *et al.,* 2015) and it seems that the same mode of action could happen in the respiratory burst activity of rainbow trout after receiving dwarf elder.

The components of TP (globulin and albumin) are mainly secreted by the liver and can be used as an indicator of fish health to determine physiological changes following different feeding herbal plants (Hosseini Shekarabi *et al.,* 2021; Farsani *et al.,* 2019; Adel *et al.,* 2016, 2019). At the end of the 4 and 8 weeks, the total serum protein and albumin concentrations were increased in all treatment groups, but this increase was only significant in the 5% group compared to the control. The total Ig of the fish after receiving dwarf elder was significantly increased in the 5% group compared to the 2.5% group and control on the week 4th and just control on the week 8th. Albumin is the most prevalent protein in the blood circulation of fish and functions as a carrier protein for a variety of nutrients, metabolites, and xenobiotics. Moreover, the increase in the levels of globulins in fish is thought to be associated with a stronger immune response and it is among the most frequently tested immune parameters in herbal-supplemented diets in fish (Wang *et al.,* 2017; Awad and Awaad, 2017).

The glucose and AST of rainbow trout were not affected by different levels of dwarf elder, but its usage significantly decreased the cholesterol, triglyceride, and ALT levels in the treatment groups compared to the control. Various studies have shown that dwarf elder is rich in phenolic compounds (Jabbari *et al.,* 2017; Tasinov *et al.,* 2013, 2021). Phenols and polyphenolic compounds can serve as antioxidants (Fathi *et al.,* 2015) and improve human blood lipid profile (Ivanova *et al.,* 2014). In our study, it seems that the phenols and polyphenolic compounds have affected and changed cholesterol, triglyceride, and ALT in the treated groups.

Serum bactericidal activity was introduced as an indicator of innate immunity in teleost fish. The lowest number of bacterial colonies indicated the efficiency of immune cells in serum to kill the pathogen (Uribe *et al.,* 2011). The results showed that the serum bactericidal activity against *Y. ruckeri* was significantly increased in the group supplemented with dwarf elder. The enhancement of serum bactericidal activity has been reported previously with other herbs in rainbow trout (Awad *et al.,* 2013; Alishahi *et al.,* 2017). These findings indicate that the bioactive compounds of medicinal herbs may provide some non-specific and specific immune responses in fish.

The results of the survival rate of fish fed with dwarf elder supplementation feeds were higher than those of the control after infection with *Y. ruckeri* and the maximum protection was detected in the group fed with 5% of dwarf elder. Previous works have shown that rainbow trout which received dietary herbs revealed protection against *Y. ruckeri* (Adel *et al.,* 2016, 2019;

Dehghan *et al.*, 2016; Soltanian *et al.*, 2019; Bilen *et al.*, 2020; Ghafarifarsani *et al.*, 2020; Heydari *et al.*, 2020; Firouzbakhsh *et al.*, 2021). The improved immune indices that were tested in the current research could be correlated with the improved resistance against *Y. ruckeri.*

In conclusion, the present results suggested that dwarf elder was found beneficial for rainbow trout when applied as a feed additive. Several hemato-serological and immunological parameters were enhanced in fish that received diets containing dwarf elder at different levels and the best results were found in 5% group compared to the other groups. Thus, it seems that dwarf elder could be introduced as an immunomodulator and improver of hemato-biochemical parameters to aquaculture. But there is still limited information available regarding the possible effects of dwarf elder on fish, and therefore, further research is required.

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