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Effects of dietary purslane (*Portulaca oleracea*) extract on growth performance, hematological indices and immune responses of rainbow trout (*Oncorhynchus mykiss*) fry

Mohammadalikhani M.[1]; Shamsaie Mehrgan M.[1*]; Haghighi M.[2]; Soltani M.[3]; Kamali A.[1]

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Abstract

Inclusion of dietary herbal supplements in aquafeed can be considered as a growth promoter and enhancer of immunity system in preventing various fish infections. Therefore, this experiment was conducted to evaluate the effects of different levels of purslane (*Portulaca oleracea*) extract (POE) on growth performance, hematological indices and immune responses of rainbow trout fry. The fries with initial weight of 3.3±0.1g were divided into four groups with feeding diets supplemented containing 0% (control, T_0), 0.5% (T_1), 1% (T_2), 1.5% (T3) and 2% (T4) of POE for 56 days. After eight weeks of feeding trail, enriched POE diets significantly increased red blood cell count, white blood cell count, hematocrit, hemoglobin concentration, mean corpuscular hemoglobin (MCHC), neutrophil and monocyte percentages, especially in T_3 group compared to the control group (p<0.05). Although no significant differences were observed between eosinophil percentage and MCHC, but lymphocyte percentage decreased significantly (p<0.05). Also supplemented diets with POE significantly increased immune parameters including total immunoglobulin (Ig), IgM, complementary activity, total protein and lysozyme activity in all experimental groups, especially in T_3 (1.5% POE) compared with the control group (p<0.05). This study demonstrates POE as a potential immunostimulant to stimulate and improve immune system of rainbow trout fry.

Keywords: *Portulaca oleracea*, *Oncorhynchus mykiss*, Growth performance, Hematological indices, Immune responses.

1-Department of Fisheries Science, Science and Research Branch, Islamic Azad University, Tehran, Iran.

2-Cold-water Fishes Research Center, Iranian Fisheries Science Research Institute, Agricultural Research Education and Extension Organization, Tonekabon, Iran

3-Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Iran.

* Corresponding author's Email: m.shamsaie@srbiau.ac.ir

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Introduction

In recent years using safe substances, such as herbal plants, help improve growth performance and immune system in feeding of farmed fish (Dadar *et al*., 2016; Mohammadi *et al*., 2018, Samavat *et al*.; 2019). Immunostimulants are substances which enhance non-specific defense mechanism and provide resistance against pathogen organisms (Citarasu *et al*., 2002). In recent years, there is a growing attention to the effects of herbal products on immune system of farmed fish due to several reasons, such as eco-friendly, cost-effective and also being a potential alternative for chemotherapy and chemical drugs (Jeney *et al*., 2009; Mohammadi *et al*., 2018). Many plant-derived compounds are found to have positive effects on hematological and immune system of farm-raised fish, such as *Aloe vera* extract, ginger (*Zingiber officinale*), peppermint (*Mentha piperita*), date palm (*Phoenix dactylifera*) seed, and grapefruit (*Citrus paradisi*) peel extracts (Haghighi and Sharif Rohani 2013; Adel *et al*., 2016; Haghighi *et al*., 2017; Bazari Moghaddam *et al*., 2017; Mohammadi *et al*., 2018; Samavat *et al*., 2019).

Portulaca oleracea belonging to the family Portulacaceae is a kind of common plant that grows in many parts of the world especially in Iran (Mousavi *et al*., 2015). Purslane is a warm-climate plant and is widely distributed in tropical and subtropical areas of the world (Levey, 1993). Its contents include: flavonoids, alkaloids, fatty acids, terpenoids, polysaccharides, coumarins, vitamins, sterols, proteins, and mineral compounds (Abdel Moneim *et al*., 2013; Kamal Uddin *et al*., 2014). Several types of flavonoid compounds were identified in *P. oleracea*, including kaempferol, myricetin, luteolin, apigenin, quercetin, genistein, and genistin (Zhu *et al*., 2010).

Purslane extract has various bioactivities such as antibacterial (Banerjee and Mukherjee, 2003), antioxidant (Arruda *et al*., 2004), antitumor (Azarifar *et al*., 2018) and anti-inflammation (Chan *et al*., 2000). The positive effects of *P. oleracea* extract in growth performance and microflora composition was confirmed in broilers by Zhao *et al*. (2013). Arruda *et al*. (2004) also suggested that dietary administration of purslane improve immune (both systemic and mucosal) parameters of gilthead seabream.

The main objective of this study was to evaluate the effects of different levels of purslane (*Portulaca oleracea*) extract (POE) on hematological parameters and immune responses of rainbow trout (*Oncorhynchus mykiss*) fry.

Materials and methods

Preparation of Portulaca oleracea extract

Purslane (*Portulaca oleracea*) was prepared in Perpin Company in Tehran and was identified and confirmed by botany section of Institute of Medicinal Plants (Tehran, Iran). Aerial parts of *P. oleracea* were collected and washed in sterile distilled water. Samples were separately shade-dried for 10 days till

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weight constancy was achieved. Then they were powdered in an electric blender. The extract was prepared according to the method recommended by Askari *et al*. (2016). For preparation of hydro-ethanolic extract 100g of aerial parts of plant powder in was placed in 1000ml ethanol 80% for 72 hours. Then the solution was dried by rotary evaporator and the solvent was isolated. Finally crude extract was stored in dark bottle at 4ºC until use.

Fish conditions

Six hundred rainbow trout (*Oncorhynchus mykiss*) fry with mean weigh of 3.3±0.1g were obtained from a local fish farm in Tonekabon city and transferred to the Coldwater Fishes Research Center (Tonekabon, Iran). The Fish were distributed randomly in 15 fiberglass tanks (300 liter) each with 40 fish. The water temperature was maintained at 14± 2ºC with a flow rate of 900Lh^{-1}. The pH was 7.5±0.3 and photoperiod was 12h light: 12h dark cycle. Fish acclimatized for 2 weeks before start of the experimental trial. During acclimatization procedure fish were fed with commercial diet (Faradaneh, Shahrekord, Iran) at a rate of 3-4% body weight, six times a day.

Experimental design

Fish were randomly distributed into 5 groups each containing 120 fish (40 fish per tank). The basal diet was mixed with obtained POE in an appropriate concentration, to get five different experimental diets: with 0 g (control group, T0), 0.5% (T_1), 1% (T_2), 1.5% (T3) and 2% (T4) of POE. The diets were allowed to dry and stored at 4°C until use. During this study fish were fed (3-4% of body weight) six times a day for 56 days.

Growth performance

The fish were deprived of food for 24 hours before weighing and sampling and the following parameters were measured at the end of feeding trial after 56 days. Growth parameters were calculated using below equations (Hushangi and Hosseini Shekarabi, 2018):

Specific growth rate (SGR, %)= $(\frac{\ln(W2)-\ln(w1)}{T}) \times 100$

Where: W_2=Final weight at time t_2, W_1=Initial weight at time t_1, and T=days of the experiment.

Feed conversion ratio (FCR) $=\frac{Feed\ consumption(g)}{Weight\ gain\ (g)}$

Survival rate (SR, %) $=\frac{Final\ number\ of\ fish}{Initial\ number\ of\ fish} \times 100$

Blood sampling

At the end of the feeding trial, on day 56, five fish were selected from each tank and anaesthetized with MS222 (100 mgL^{-1}). Then blood samples (2 mL) were obtained from the caudal vein of the fish. Blood samples were immediately divided into two parts: 1 mL was transferred to a tube containing anti-coagulant (heparin) for hematological analysis, while the other half was transferred to non-heparinized tubes for immunological analysis. Serum samples were obtained by blood

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centrifugation (5000 g, 5 min) and stored at -80ºC until use.

Hematological assay

Total red blood cells (RBC: $10^6 mm^{-3}$) and white blood cells (WBC: $10^3 mm^{-3}$) were enumerated in an improved Neubaeur hemocytometer using Hayem and Turck diluting fluids (Blaxhall and Daisley, 1973). Haematocrit (Ht%) was determined by standard microhematocrit (D-78532 Tuttlingen, Hettich, Germany) method after centrifugation at 14000g for 7 min and expressed as percentage (Houston, 1990). The haemoglobin (Hb, g dl^{-1}) level was determined according to cyanmethemoglobin procedure (Klontz, 1994). Also differential leukocyte cells were measured by preparing Giemsa stained smears. Blood smears were studied by light microscopy in order to make blood cell counts (Gao *et al*., 2007). Erythrocyte indices (MCV, MCH and MCHC) were calculated using the formulae mentioned by Klinger *et al*. (1996).

Immunological parameters

The concentration of total serum protein (TP) was determined using a Prestige 24i automated biochemical analyzer (Tokyo Boeki, Japan) and commercial kits (Pars Azmun, Iran) according to the manufacturers' instruction. Activity of lysozyme was measured according to turbidometric assay method described by Ellis (1990). Briefly a 50µL sera was added to 950µL of a suspension of *Micrococcus luteus* (0.2mg mL^{-1} in a 0.05M sodium phosphate buffer (Sigma, USA, pH 6.2) and absorbance was measured at 520-560nm and 22°C after 30s and 180s by spectrophotometer (2100-VIS model Unico, USA).

Serum immunoglobulin (IgM) content was measured according to the method recommended by Adel *et al*. (2015) by using a microprotein determination method (C-690; Sigma), prior and after precipitating down the immunoglobulin molecules by means of a 12% solution of polyethylene glycol (Sigma). The difference in protein content was considered as IgM content.

Complementary activity (ACH50) was measured using DiaMetra kit (Diametra, Milan, Italy), based on manufacturer's instructions to calculate the value of ACH50 of the samples as follows (Migliorini *et al*., 1985):

$$\text{ACH50 (UmL}^{-1}) = \frac{1}{K} \times r \times \frac{1}{2}$$

Where: K is the amount of serum yielding 50% haemolysis, R is the reciprocal of serum dilution, and 1/2 is the correction factor. The assay was performed on a 1/2 scale of the original method.

Statistical analysis

The statistical analyses were carried out using SPSS software version no 20 (SPSS Inc., Chicago, IL, USA). The statistical analysis was done using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. *P*-value of <0.05 was considered significant. All experiments were performed in three replicate.

Results

Growth performance

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Based on the result final weight and SGR were significantly increased in POE 1.5% and 2% treatments compared with other treatments, especially control group (p<0.05). Furthermore the highest survival rate (%) was observed in POE 1.5% treatment. Regarding FCR values, control and 0.5% POE enriched diets showed similar values, while FCR value decreased in 1%, 1.5% and 2% POE enriched diets (Table 1).

Table 1: Growth performance and survival of rainbow trout fed with different levels of dietary *Portulaca oleracea* extract (POE). SGR: specific growth rate, FCR: fed conversion ratio, %SR: percentage survival rate. Values in a row with different superscripts show significant differences (p<0.05).

Parameters	Dietary levels of POE (%)				
	0 (control)	0.5	1	1.5	2
Initial weight (g)	9.37±0.1^{a}	9.40±0.2^{a}	9.37±0.4^{a}	9.37±0.2^{a}	9.37±0.3^{a}
Final weight (g)	47.65±1.16^{a}	52.42±0.9^{b}	55.76±1.1^{c}	57.42±0.9^{c}	55.50±1.3^{c}
SGR (%/day)	1.04±0.09^{a}	1.14±0.10^{b}	1.39±0.09^{c}	1.58±0.14^{d}	1.50±0.10^{d}
FCR	1.30±0.19^{a}	1.12±0.16^{b}	0.91±0.18^{c}	0.86±0.11^{c}	0.90±0.11^{c}
SR (%)	94.16±0.6^{b}	94.2±0.8^{b}	95.00±0.1^{b}	98.3±0.3^{a}	93.3±1.1^{b}

Hematological indices

The number of red blood cells significantly increased (p<0.05) in fish fed with the enriched diets with 1.5% and 2% of POE (Table 2). POE enriched diets (0.5 to 2%) significantly increased red blood cell count (RBC), white blood cell count (WBC), hematocrit (HCT), hemoglobin (HB) values, mean corpuscular hemoglobin (MCH), neutrophil and monocyte percentage especially in T_3 group compared with the control group (p<0.05). Although there were no significant difference in eosinophil percentage and mean corpuscular hemoglobin concentration (MCHC), but lymphocyte percentage decreased significantly (p<0.05).

Table 2: Hematological indices of rainbow trout fed with different levels of dietary *Portulaca oleracea* extract (POE) for 8 weeks. *Data are presented as mean (or average form) ± S.E (n =9 fish from each group). Means in the same rows with different superscript are significantly different (P< 0.05).

Parameters	Dietary levels of POE (%)				
	0 (control)	0.5	1	1.5	2
RBC (×10^{3}cells mm^{-3})	1.0±0.03^{b}	1.1±0.04^{b}	1.1±0.0.02^{b}	1.6±0.05^{a}	1.5±0.06^{a}
WBC (×10^{6}cells mm^{-3})	13.0±0.3^{b}	13.2±0.1^{b}	14.3±0.6^{a}	14.6±0.10^{a}	13.3±0.3^{b}
Lymphocyte (%)	76±1.2^{a}	74±0.9^{b}	71±0.8^{c}	72±0.8^{c}	74±0.8^{b}
Monocyte (%)	3.66±0.57^{b}	5.00±1.00^{a}	5.33±1.15^{a}	5.66±0.59^{a}	4.66±0.6^{a}
Eosinophil (%)	1±0.09 ^{a}	1.32±0.57^{a}	1.2±0.15^{a}	1.5 ±0.79 ^{a}	1±0.08^{a}
Neutrophil (%)	20.0±1.00^{b}	19.33±0.52^{b}	22.33±0.57^{a}	22.33±0.53^{a}	19.66±0.47^{b}
HTC (%)	32.0±1.00^{c}	35.0±0.60^{b}	40.0±1.20^{a}	36.0±1.29^{b}	35.0±1.00^{b}
Hb (g dl^{-1})	8.1 ±0.02^{b}	8.3 ±0.04^{b}	9.0 ±0.05^{a}	9.3 ±0.05^{a}	9.1±0.01^{a}
MCHC (g dl^{-1})	16.6±0.57^{a}	16.0±0.10^{a}	16±0.10^{a}	16.6±0.57^{a}	16.6±0.57^{a}
MCH (Pg)	7.16±0.35^{c}	8.16±0.14^{b}	9±0.45^{a}	9.23±0.68^{a}	9.33 ±0.47^{a}
MCV (Fl)	56.6±1.5^{a}	53.6±0. 57^{b}	56.3 ±0.57^{a}	56.0± 2.64^{a}	56.0±1.0^{a}

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Immunological responses

At the end of the study total immunoglobulin, IgM, lysozyme, total protein levels and complementary activity (ACH_{50}) were increased in groups that received POE (1%, 1.5% and 2%), compared with the control sdsd group (p<0.05). After 56 days, immune indices significantly increased (p<0.05) in 1.5% POE group compared with other groups, especially control. Also the group received 1.5% POE had highest level of all measured immunological parameters (Table 3).

Table 3: Immune parameters of rainbow trout fed with different levels of dietary *Portulaca oleracea* extract (POE) after 8 weeks. Data are presented as mean ± S.E (*n* =9 fish from each group). Means in the same rows with different superscript are significantly different (*P*< 0.05).

Parameters	Dietary levels of POE (%)				
	0 (control)	0.5	1	1.5	2
Total immunoglobulin (Ig, mg mL^{-1})	13.96 ± 1.36^{c}	15.93 ± 1.19^{b}	15.06 ± 1.07^{b}	17.36 ± 0.9^{a}	16.2 ± 0.55^{b}
Immunoglobulin M (IgM, mg dl^{-1})	13.0 ± 1^{c}	18.0 ± 0.64^{b}	19.3 ± 0.21^{b}	20.6 ± 0.51^{a}	20.6 ± 0.51^{a}
Complementary activity (ACH50, U mL^{-1})	114.3 ± 3.2^{b}	118.66 ± 2.0^{b}	123.3 ± 1.3^{a}	127 ± 3.4^{a}	115.3 ± 2.1^{b}
Total protein (g dl^{-1})	2.55 ± 0.22^{b}	2.99 ± 0.23^{ab}	2.95 ± 0.73^{ab}	3.41 ± 0.41^{a}	3.03 ± 0.17^{ab}
Lysozyme (U ml^{-1} min^{-1})	43.0 ± 2^{b}	45.66 ± 3.21^{b}	46.66 ± 3.21^{b}	51.0 ± 6^{a}	42.0 ± 3^{b}

Discussion

The results of this study showed that dietary purslane (*P. oleracea*) extract have positive effects on growth performance as well as immune responses of rainbow trout fry. The results of growth performance showed that the highest final body weight and SGR were in fish groups fed with 1.5% and 2% POE diets, simultaneously. Okafor *et al*. (2014) reported that high growth performance in broiler chickens fed with dietary purslane extract could be explained due to high antioxidant capacity of POE. Moreover Zhao *et al*. (2013) reported that POE can lead to an increase population size of beneficial bacteria, such as lactic acid bacteria, in broiler chickens. It is proved that some medicinal herbs and their extracts have positive effect on fish growth indices (Immanuel *et al*., 2004, Sivaram *et al*., 2004, Mohammadi *et al*. 2018), while others consider them as immunostimulant instead (Dugenci *et al*., 2003; Bohlouli Oskoii *et al*., 2012).

Emerging some antimicrobial resistances in aquaculture industry is one of the major problems in intensive fish farming (Lazado *et al*., 2015). Therefore, medicinal herbs can be used as an effective alternative for antibiotics, chemicals, and other artificial therapeutics (Baser, 2008). Based on the present results total immunoglobulin, IgM, lysozyme, total protein levels and complementary activity (ACH_{50}) of fish fry were significantly increased in groups that received different levels of POE

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compared with the control group. Meanwhile the fish that received 1.5% POE had the highest value of studied immunological parameters. Ruiz (2017) evaluated potential immunostimulant effect of purslane (*P. oleracea*) alone and/or in combination with probiotic *Shewanella* on gilthead seabream (*Sparus aurata* L.). She demonstrated that dietary administration of purslane (*P. oleracea*) for 30 days increased total immunoglobulin M levels in skin mucus which is consistent with our results. Moreover, interestingly IgM expression was up-regulated in head kidney after 15 days of diet administration. She concluded that purslane has the ability to modulate and improve several immune parameters, including systemic and mucosal immunity of seabream. In a similar study Allahmoradi *et al*. (2018) proved the immunomodulatory effects of *P. oleracea* extract by suppression of pro-inflammatory cytokines such as TNF-α and IL-6. Park *et al*. (2019) also showed that POE exerted has immunostimulatory effect in vivo and in vitro condition. It is proven that administration of some dietary herbal supplements, such as peppermint, *Mentha piperita* (Adel *et al*., 2015), fenugreek, *Trigonella foenum graecum* (Guardiola *et al*., 2017) and myrtle, *Myrtus communis* (Taee *et al*., 2017) were capable of improving the immune system of several fish species.

It could be mentioned that measurement of serum total proteins, especially globulin, is a good indicator to survey the status of immune system (Siwicki *et al*., 1994). After 56 days of feeding trial total protein level significantly increased in the 1.5% POE group compared with other groups (p<0.05). It is described that elevation of serum globulin following the use of herbal immunostimulants was justifiable by increasing total protein and albumin levels (Vasudeva *et al*., 2004).

Lysozyme is one of the components of the body's non-specific defense system, which is responsible for destroying pathogens by breaking down the glycosylated bonds of the peptidoglycan layer of the bacteria. In addition lysozyme is responsible for activating other important molecules of defense, including the complement system and phagocytic cells (Ellis, 1990). The level of lysozyme activity depends on environmental parameters (water temperature, pH, light period, season, and toxins) and intrinsic factors (size, age, sex, infections and stress) (Tukmechi and Bandboni, 2014). After 56 days of feeding trial, lysozyme activity increased in three groups (1%, 1.5% and 2%) which received POE compared with the control group. Similar results were observed on rainbow trout after feeding with feed containing *Mentha piperita* extract (Adel *et al*., 2016) and stinging nettle, *Urtica dioica* (Saeidi Asl *et al*., 2017). Studies conducted by researchers showed that the amount of lysozyme increased following the use of herbal extracts in fish diet. This increase was also significant in some cases depending on the species of fish and concentration of plant extract and type of extract used (Jeney *et al*., 2009).

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In principle complement pathway is stimulated and activated by immunostimulants (Engstad *et al*., 1992). Among them performance of medicinal plants is proven to activate and stimulate complement activity. Results of present study are in line with the observations of other researchers, Awad and Austin (2010) showed that use of *Lupinus perennis* indica *Managifera Urtica* dioica, especially in concentrations of 2 and 1 percent in rainbow trout rations after 14 days increases complement activity significantly. Haghighi *et al*. (2018) showed that dietary inclusion of *O. vulgare* extract at a rate of 1% improve non-specific immune parameters (respiratory burst activity, phagocytic activity and serum lysozyme activity) of juvenile rainbow trout. Azizi *et al*. (2016) further demonstrated the effect of diet containing essential oil of thyme (*Thymus vulgaris*) on blood and biochemical parameters of rainbow trout serum. The essential oil of this plant increased both serum lysozyme and white blood cell count significantly. These properties were mainly attributed to flavonoids components. Iravan *et al*. (2003) believe that levels of vitamin in POE could increase the immune system.

Results of current study showed that POE enriched diets (0.5 to 2%) significantly increased blood parameters, such as RBC, WBC, HCT, and HB in T_3 compared with the control group. Although no significant difference was observed in eosinophil percentage and MCHC, but lymphocyte percentage decreased significantly. In a similar study total WBC number and neutrophil percentages were significantly increased but lymphocyte percentages was decreased in received POE groups compared with the control groups in rat diets (Kaveh *et al*., 2017). These effects are related to phenolic acids, flavonoids, vitamin E, and vitamin C (Varmaghany *et al*., 2015) and also high content of n-3 fatty acids in POE (Okafor *et al*., 2014).

According to the present results it could be concluded that oral administration of POE up to 1.5% by feed in rainbow trout was effective and beneficial. However, further studies on specific mechanisms for immune modulation and disease resistance should be conducted to explore the feasibility of commercial application of POE in rainbow trout diet.

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