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Research Article

Pot Marigold (*Calendula officinalis*) Powder in Rainbow Trout (*Oncorhynchus mykiss*) Feed: Effects on Growth, Immunity, and *Yersinia ruckeri* Resistance

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The current research investigated the effects of pot marigold (*Calendula officinalis*) powder on growth, biochemical parameters, digestive enzymes, serum and mucus immune responses, antioxidant defense, and resistance of rainbow trout (*Oncorhynchus mykiss*) against *Yersinia ruckeri*. Fish (No. 450, 15.06 ± 0.11 g; mean ± SE) were randomly distributed to five groups fed with a diet containing 0 (control group), 0.5%, 1%, 1.5%, and 2% of pot marigold powder (MP) for 60 days. Then, fish were challenged with *Y. ruckeri* infection. Specific growth rate (SGR), weight gain (WG), final weight (FW), feed conversion ratio (FCR), mucus lysozyme (LYZ), mucus protease, serum nitroblue tetrazolium test (NBT), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) values in the 1%, 1.5%, and 2% MP groups significantly increased ($P < 0.05$) compared to the other groups. Regression analysis exhibited that rainbow trout growth indices were polynomially linked to dietary MP concentrations. In this regard, the optimal levels of MP, according to growth parameters (SGR and FCR), were 1.31 and 1.4% diet, respectively. The intestinal protease, serum complement component 3 (C3), complement component 4 (C4), and LYZ activities in fish fed with the 1% and 1.5% MP-supplemented regime were higher ($P < 0.05$) compared to the other groups. Also, fish fed with diets containing 1.5% MP had significantly higher intestinal lipase and mucus ALP activities than the other groups ($P < 0.05$). Aspartate aminotransferase (AST) activity in all MP groups remarkably increased ($P < 0.05$) compared to the control. Moreover, fish fed with a diet containing 1.5% MP had remarkably higher ($P < 0.05$) lactate dehydrogenase (LDH) and total protein (TP) values than the other groups. Serum myeloperoxidase (MPO), total immunoglobulin (Ig), and mucus peroxidase values in the 1% MP group remarkably increased ($P < 0.05$) compared to the other groups. In addition, catalase (CAT) and superoxide dismutase (SOD) activities in the 1.5% and 2% MP groups were significantly higher ($P < 0.05$) compared to the others. However, malondialdehyde (MDA) levels in fish fed with the 1%, 1.5%, and 2% MP-supplemented diet remarkably decreased than in the other groups ($P < 0.05$). The highest survival rate after a challenge with *Y. ruckeri* was recorded in the 1.5% group compared to the control group. Our findings revealed that using pot marigold powder in diets, especially at the 1.5% level, has positive effects on growth, digestive enzyme activities, antioxidant capacity, immune response, and disease resistance in *O. mykiss*.

1. Introduction

Aquaculture is considered one of the most productive activities in many countries. The rapid global population growth, increase in quality protein needs for human nutrition, and depleting water resources have led to the use of intensive and ultraintensive systems for aquaculture in many countries [1]. In intensive systems, aquatic animals are exposed to stressful conditions and disease, which cause mortality and consequent economic damage [2–4]. Conventional methods in preventing or treating the disease in fish are the use of antibiotics, vaccines, and some chemicals. The misuse of antibiotics can induce drug-resistant microorganisms, drug residues in fish tissues, many health problems in the human population, and environmental pollution. On the other hand, these chemicals in the aquatic ecosystems prevent the growth of beneficial bacterial flora in the fish's gut, which has remarkably many negative impacts on fish health [5, 6]. Hence, the identification and development of alternative methods to promote aquatic animal immunity and disease prevention in the aquaculture systems, especially in recent years, have been considered by many researchers [7, 8]. Immunostimulants are used as a new method in disease prevention and control, which can help maintain optimal fish conditions while also increasing production and profits [9]. Herbal additives are one of the most applicable immune stimulants.

In recent years, the use of herbal plants and their derivatives due to their few side effects, low toxicity, stability, eco-friendly, availability, reasonable price, and health have been considered in the aquaculture industry. Moreover, the addition of herbal additives to the diet, through selective antimicrobial activity or the creation of favorable conditions for some bacterial species, affects the microbial spectrum of the gut, which leads to greater utilization with better absorption of nutrients. Consequently, it promotes growth as well as stimulates the immune system function [10–13]. The usage of various herbal additives as a novel eco-friendly method in the different fish species' diets has successfully affected growth, appetite, feed utilization, oxidative stress response, immune system functions, and disease resistance [14–20]. As a part of the adaptive immune system, the skin mucus layer of aquatic animals is the crucial first line of host defense against different infections. This layer contains natural antibiotics and specialized immune cells such as total Ig, protease, peroxidase, ALP, and LYZ [21, 22]. It has been shown that herbal additives can cause a change in some of these immune molecules [12, 23].

One of the herbal immunostimulants is the pot marigold. Pot marigold (*Calendula officinalis*) is an industrial plant that belongs to the Asteraceae family. This species exists in tropical and subtropical regions worldwide and is rich in bioactive compounds such as polyphenols, terpenoids, saponins, carotenoids, flavonoids, mucilages, and steroids [24–26]. Some studies revealed that pot marigold dietary supplements have beneficial effects on survival rate, growth, antioxidant defense, immunity, and inflammation in humans and animals [27–31]. Nevertheless, information on the effect of pot marigold supplementation on different fish species is limited.

Rainbow trout (*Oncorhynchus. mykiss*) is a high-value cold-water fish species with high global production of more than 811000 tons in 2017 in the aquaculture industry [32]. Formulation of efficient food supplements, optimization of nutritional requirements, improvement of the immune system, and high survival rate against pathogens are among the critical elements required for the sustainable production of rainbow trout. Among pathogenic bacteria, *Yersinia ruckeri* is one of the most common groups (can cause enteric redmouth (ERM)) that cause considerable economic damage in rainbow trout farming [33–35]. The natural antimicrobial property of the pot marigold plant has been considered a powerful weapon against many infections in humans [26, 29]. Despite the beneficial effects of food regimes supplemented with various herbal additives in aquatic animals, there is still limited information regarding the impacts of the pot marigold plant (in any form as powders, extracts, and oils) on fish species, especially *O. mykiss* as a critical economic species.

Therefore, the purpose of the current research was to assess the effects of pot marigold (*Calendula officinalis*) powder on growth, biochemical parameters, digestive enzymes, antioxidant defense, serum and mucus immune responses, and resistance of *O. mykiss* against *Y. ruckeri*.

2. Materials and Methods

2.1. Essential Oil Extraction and Composition Assay. To extract the essential oil, pot marigold powder was subjected to distillation by a Clevenger device for up to 3 hours. The technique was done three times using 50 g of powder. Then, it was dried using anhydrous sodium sulfate [36]. The obtained essential oil was kept in the dark at 4°C until use. Then, the compounds of the pot marigold plant (Table 1) were determined using a gas chromatography-mass device (Agilent 6890) with a 30-meter long column, 0.25 mm inner diameter, and 0.25 μm layer thickness (HP-5MS type).

2.2. Fish and Experimental Setup. O. mykiss (No. 560, 10.2 ± 0.2 g; mean ± SE) was obtained from a private sector farm in Kohgiluyeh and Boyer-Ahmad Province, Iran. The fish were adapted to the trial conditions (temperature: 14-15°C) into fiberglass 1000 l tanks for fourteen days and were fed a control diet. Five experimental groups with three replications (at a density of 30 fish each replicate; total volume: 300 l; water volume: 150 l) were tested: control group (diet without marigold powder), MP 0.5% (a diet with 5 grams per kilogram of pot marigold powder), MP 1% (a diet with 10 grams per kilogram of marigold powder), MP 1.5% (a diet with 15 grams per kilogram of marigold powder), and MP 2% (a diet with 20 grams per kilogram of pot marigold powder). *O. mykiss* (No. 450, 15.06 ± 0.11 g; mean ± SE) was fed with the experimental diets to apparent satiation three times a day (08 : 00, 13 : 00, and 19 : 00 h) for 60 days, and the water flow was 0.5 l/min·kg. The water quality parameters were checked during the trial as follows: ammonia nitrogen (NH3−N): 0.03 ± 0.00 ppm; temperature: 15.5 ± 0.50°C; pH: 7.3 ± 0.18; and dissolved oxygen: 8.0 ± 0.20

Table 1: The compounds of the pot marigold plant (*C. officinalis*).

Chemical components	Frequency of components (%)
α-Pinene (%)	7.50
α-Copaene (%)	0.64
γ-Muurolene (%)	0.72
Germacrene D (%)	3.56
α-Muurolol (%)	7.33
Sigma-cadinene (%)	12.66
α-Calacorene (%)	0.60
β-Calacorene (%)	8.50
Ledol (%)	1.50
1-10-Epi-cubenol (%)	1.80
1-Epi-cubenol (%)	1.52
δ-Cadinol (%)	9.73
α-Cadinol (%)	39.00
Nonacosane (%)	2.90
n-Hexadecanoic acid (%)	0.70
Total	98.66

ppm. Four hours after each meal, uneaten food was harvested from the bottom each tank and dried at 60°C and subtracted from the given food, and the amount of food eaten was calculated, daily.

2.3. Pot Marigold Powder and Fish Diet. Pot marigold powder was obtained from Atarak Company (Tehran, Iran). Diets' chemical compositions in this study are presented in Table 2. The selection of doses in this research was according to the positive results of the previous studies [37]. For preparing experimental diets, the pot marigold powder was added to the control diet at 0, 5, 10, 15, and 20 grams per kilogram, respectively. Ingredients were mixed, and then, water (0.3 l/kg diet) was added to make dough. Afterward, the prepared dough was pelleted using an industrial meat grinder and kept at room temperature for 36 hours. Then, the pellets were dried and kept in plastic bags in a freezer (-20°C) until used.

The approximate biochemical composition of the diets was assayed according to the AOAC [38] method. The protein content was assessed by calculating the total nitrogen using the Kjeldahl method and based on the conversion factor (extracted $N \times 6.25$, method 992.15). The fat value was calculated using petroleum ether as a solvent and via the Soxhlet extraction protocol (method 930.15). Moisture value was computed by drying the samples in an oven at 105 for 24 hours (method 924.05). Determination of ash (method 924.05) was carried out by placing the samples in an electric furnace at a temperature of 550 until complete burning (12 hours).

2.4. Growth Indices. At the first and end of the trial, all fish from each replicate were unfed for a day and then anesthetized (eugenol, 100 mg/l). After that, their weight (g) was measured individually. Growth and feed utilization parameters were calculated for each group using the following formulas:

$$\text{Weight gain (WG, g)} = \text{final weight} - \text{initial weight},$$

$$\text{Specific growth rate (SGR, \%/day)} = 100 \times \left[\frac{\ln \text{ final weight} - \ln \text{ initial weight}}{\text{day}}\right],$$

$$\text{Feed intake (FI)} = \frac{\text{net dry feed consumed per tank}}{\text{fish number per tank}},$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{feed intake}}{\text{final weight} - \text{initial weight}},$$

$$\text{Survival rate (SR, \%)} = \left(\frac{\text{final fish number}}{\text{initial fish number}}\right) \times 100. \quad (1)$$

2.5. Sample Collection. After the 60-day feeding trial, three fish from each replicate were euthanized using an overdose concentration of clove powder to measure the activities of the digestive enzymes. After dissecting the intestines, intestinal samples were mechanically homogenized with 50 mM Tris buffer and centrifuged at 6,000 g (10 min, 4°C). After that, the supernatant was separated and subjected to analyze digestive enzyme activity. For serum analyses, three fish from each replicate (9 fish per group) were anesthetized using eugenol (100 mg/l). Blood samples were obtained using a 2 ml syringe from the caudal vein and centrifuged at 3000 g (10 min, 4°C). The acquired serum samples were kept at -70°C until use. Also, three fish were blindly selected from each replicate and put in polyethylene bags containing physiological serum (10 ml, 50 mM) for mucus sampling [39, 40]. After three min, the mucus samples were centrifuged (2500 g, 10 min, 4°C), and the supernatant was kept at -80°C until further analysis.

2.6. Digestive Enzyme Analyses. Amylase activity was assayed according to the method expressed by Worthington [41] using starch solution 1% as substrate in citrate phosphate buffer (0.1 M, pH = 7.5) for 5 min at 25°C. Then, 500 μl of DNS (dinitrosalicylic acid) was added to the reaction, and the OD was recorded at 550 nm [41]. Lipase enzyme was quantified based on the protocol of Iijima et al. [42] For this purpose, p-nitrophenyl myristate in chelate buffer (5 mM sodium cholate, pH = 9.0 + 0.25 mM 2 − methoxy ethanol + 0.25 mM Tris HCl) was incubated at 30°C for 15 min. Then, the reaction was inhibited using acetone/n-heptane (5 : 2, *v*/*v*). After that, the optical density of the solution was recorded at 405 nm. Total protease activity was quantified based on the protocol of García-Carreño [43]. For this purpose, azo-casein in 0.5 ml of Tris (Tris–HCl 0.1 M; pH = 8) and 5% trichloroacetic acid was incubated at 25°C for one hour and was then centrifuged (4000 g for 6 min at 4°C). Finally, the optimal density of the supernatant was measured at 440 nm. According to the Bradford [44] method, bovine serum albumin was subjected as a standard for measuring the total proteins in the crude enzyme extracts.

Table 2: Ingredients and proximate compositions of different trial diets.

Ingredients	Control	MP 0.5%	MP 1%	MP 1.5%	MP 2%
Fish meal[a] (g/kg)	495	495	495	495	495
Soybean meal [b](g/kg)	127	127	127	127	127
Wheat flour (g/kg)	150	150	150	150	150
Corn gluten meal[c] (g/kg)	70	70	70	70	70
Fish oil (g/kg)	45	45	45	45	45
Soybean oil (g/kg)	35	35	35	35	35
Mineral mix[d] (g/kg)	10	10	10	10	10
Vitamins mix[d] (g/kg)	10	10	10	10	10
Lecithin[e] (g/kg)	0.5	0.5	0.5	0.5	0.5
L-Threonine (g/kg)	0.4	0.4	0.4	0.4	0.4
DL-methionine (g/kg)	0.1	0.1	0.1	0.1	0.1
Mono calcium phosphate (g/kg)	5	5	5	5	5
Cane molasses (g/kg)	20	20	20	20	20
Antioxidant (BHT)[f] (g/kg)	0.2	0.2	0.2	0.2	0.2
Toxin binder (antimycotoxin) (g/kg)	1.8	1.8	1.8	1.8	1.8
Filler (g/kg)	30	25	20	15	10
Marigold powder (MP) (g/kg)	0	5	10	15	20
Proximate composition% in dry basis					
Crude protein (%)	45.18	45.10	44.88	44.60	44.50
Crude lipid (%)	14.14	14.10	14.00	13.95	13.90
Crude ash (%)	9.20	9.26	9.32	9.30	9.37
Crude carbohydrate (%)	22.5	22.76	23.3	23.77	24.09
Dry matter (%)	91.02	91.22	91.50	91.62	91.86
Gross energy (kcal/g)	487.60	487.84	487.87	487.74	488.02

[a]Zafar Fishmeal Factory, Guilan, Iran (crude protein 66.10%). [b]Soybean Co., Gorgan, Iran (crude protein 45.2%). [c]Glucosan Co., Qazvin, Iran. [d]The premix provided the following amounts per kilogram of feed: A: 1000 IU; D3: 5000 IU; E: 20 mg; B5: 100 mg; B2: 20 mg; B6: 20 mg; B1: 20 mg; H: 1 mg; B9: 6 mg; B12: 1 mg; B4: 600 mg; C:50 mg; Mg: 350 mg; Fe: 13 mg; Co: 2.5 mg; Cu: 3 mg; Zn: 60 mg; Se: 0.3 mg; I: 1.5 mg; and Mn: 10 mg. Chinechin Co., Tehran, Iran. [e]Pouyavision, Tehran, Iran. [f]Butylated hydroxytoluene (BHT) (Merck, Germany).

2.7. Biochemical Parameters. The serum levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were quantified spectrophotometrically via commercial kits (Pars Azmun Co., Tehran, Iran). Serum total protein (TP) concentration was assayed according to the protocol of Bradford [44]. Serum albumin (ALB) level was estimated photometrically at 620 nm according to the protocol of Nicholson [45]. Also, serum globulin (GLU) concentration was assessed after subtracting albumin from the total protein content.

2.8. Immunological Analyses. The turbidometric assay method expressed by Ellis [46] was used to assay serum and mucus lysozyme activity (U/ml) using 0.2 mg/ml *Micrococcus luteus* bacteria as a target in a 0.05 M sodium phosphate buffer. The levels of total serum and mucus immunoglobulin (mg/dl) were measured by quantifying protein amounts before and after the addition of polyethylene glycol. Serum alternative complement activity (ACH50) was calculated according to the method of Yano [47]. For this purpose, sheep red blood cells (SRBC) and EGTA-Mg++-gelatin-veronal buffer were subjected as the target and a reaction medium, respectively. The levels of serum complement components (C3 and C4) (mg/dl) were quantified using a commercial kit (Pars Azmun Co., Tehran, Iran) and an ELISA plate reader (ELX800, BioTek, Vermont, USA). Skin mucus protease was quantified according to the method of Hoseinifar et al. [48] Skin mucus peroxidase was measured according to the protocol of Quade and Roth [49] using Hank's buffer (HBSS), and the optical density (OD) was recorded at 450 nm. Skin mucus alkaline phosphatase was measured spectrophotometrically using commercial kits (Pars Azmun Co., Tehran, Iran) based on the manufacturer's protocol. Serum NBT activity was measured according to the method of Anderson and Siwicki [50]. The protocol of Quade and Roth [49] was adopted to assay serum myeloperoxidase activity (MPO).

2.9. Antioxidant Response. Serum superoxide dismutase (SOD) activity was quantified using a commercial kit (Zellbio, Hamburg, Germany) based on the reduction rate of cytochrome C. Also, serum catalase (CAT) enzyme activity was assayed according to the decomposition rate of hydrogen peroxidase according to the method of Goth [51]. Serum malondialdehyde (MDA) and glutathione peroxidase (GPx) levels were quantified according to the instruction of commercial kits (Zellbio, Hamburg, Germany).

2.10. Bacterial Resistance Challenge. At day 60, 15 fish from each replicate (KC291153) were injected intraperitoneally with 0.1 ml *Y. ruckeri*. According to this method, *Y. ruckeri* was incubated (for 48 hours at 27°C) in a broth culture medium. The bacterial suspension was centrifuged and then washed twice using phosphate buffer. After that, bacterial concentration was adjusted by serial dilution at 10^7 cells per millimeter [39]. Daily total mortality in each group was collected for 14 days post infection. Then, relative percent survival (RPS) values were obtained for each group as follows [52]:

$$\text{RPS}\,(\%) = \left[1 - \left(\frac{\%\text{fish mortality in the challenged group}}{\%\text{fish mortality in the control group}}\right)\right] \times 100. \quad (2)$$

2.11. Statistical Analysis. The results are given as means ± standard error. All the analyses were performed by SPSS software v.22 (Chicago, IL, USA). The Levene and Shapiro-Wilk tests, respectively, were subjected to assess variance homogeneity and normality of the data. One-way analysis of variance (ANOVA) followed by Tukey's test was subjected to evaluate significant differences (when $P < 0.05$) among groups. Broken line regression model was applied to specify the optimal level of pot marigold powder according to the measured parameters.

3. Results

3.1. Growth and Survival. Growth performances, feed utilization, and survival rate of *O. mykiss* fed with diets containing MP for 60 days are shown in Table 3. There was no notable variation ($P > 0.05$) in the survival rate and initial weight of *O. mykiss* among different groups. However, FW, WG, SGR, and FCR levels in the 1, 1.5, and 2% MP groups were significantly higher ($P < 0.05$) than the others. For growth parameters, 1.31 and 1.4% MP diets were chosen as the optimum dose of pot marigold powder for SGR and FCR, respectively, by the broken line regression analysis (Figure 1).

3.2. Digestive Enzyme Analyses. Digestive enzyme analyses of *O. mykiss* fed with diets containing MP are presented in Figure 2. The findings showed that amylase content did not change ($P > 0.05$) among different groups. However, the protease activity in fish fed with the 1 and 1.5% MP-supplemented regimes was higher ($P < 0.05$) than in the other groups. Also, fish fed with diets containing 1.5% MP had remarkably higher lipase activity ($P < 0.05$) than the other groups.

3.3. Immune Biomarkers. Serum and mucus immune biomarker analyses of *O. mykiss* fed with diets containing MP are presented in Tables 4 and 5. There was no notable variation ($P > 0.05$) in the serum ACH50 level of *O. mykiss* among different groups. However, serum LYZ, C3, and C4 activities in the 1 and 1.5% MP groups were remarkably higher ($P < 0.05$) compared to the other groups. Moreover, fish fed a diet containing 1% MP had higher serum MPO and total Ig levels than the other groups ($P < 0.05$). Furthermore, serum NBT activity in the 1, 1.5, and 2% MP groups were significantly higher ($P < 0.05$) compared to the other groups.

In the current study, there was no significant difference ($P > 0.05$) in the mucus total Ig level of *O. mykiss* among different groups. Mucus LYZ and protease activities in the 1, 1.5, and 2% MP groups were significantly higher ($P < 0.05$) compared to the other groups. In addition, mucus ALP level was remarkably elevated ($P < 0.05$) in fish fed with a diet containing 1 and 1. 5% MP compared to the control. Moreover, peroxidase activity was remarkably elevated ($P < 0.05$) in fish fed with a diet containing 1% MP compared to the control group.

3.4. Biochemical Indices. The measured indices regarding the effects of dietary supplementation with MP on the serum biochemical parameters of *O. mykiss* are shown in Table 6. There was no significant difference ($P > 0.05$) in the serum ALB and GLU levels of *O. mykiss* among different groups. However, fish fed with diets containing MP had remarkably lower AST levels than the control ($P < 0.05$). ALP and ALT levels in the 1, 1.5, and 2% MP groups were significantly lower ($P < 0.05$) compared to the other groups. Moreover, fish fed with a diet containing 1.5% MP had significantly higher TP levels while lower LDH levels ($P < 0.05$) than the other groups.

3.5. Antioxidant Response. Antioxidant responses of *O. mykiss* fed with diets containing MP for 60 days are displayed in Figure 3. There was no notable variation ($P > 0.05$) in the GPx activity of *O. mykiss* among different groups. However, CAT and SOD activities in the 1.5 and 2% MP groups were remarkably higher ($P < 0.05$) compared to the other groups. In addition, fish fed with diets containing 1, 1.5, and 2% MP had remarkably lower MDA levels ($P < 0.05$) than the other groups.

3.6. Bacterial Resistance Challenge. The mortality rate, survival rate, and RPS in *O. mykiss* fed with diets containing MP during and after 14 days of challenge with *Y. ruckeri* are revealed in Table 7. These results showed that diets containing MP enhanced *O. mykiss* resistance against *Y. ruckeri* ($P < 0.05$). The highest postchallenge mortality percent was recorded in the control group, while the lowest postchallenge mortality ratio was recorded in the 1.5% MP group. In addition, 14 days post challenge, the RPS rate in MP groups (0.5, 1, 1.5, and 2%) were 36.20, 50.42, 61.05, and 39.71, respectively.

4. Discussion

In recent years, herbal plants and their ingredients have appealed to the attention of numerous investigators due to their advantageous influences on metabolism, growth, immunological response, and disease resistance in different fish species [53–56]. The present study investigated the potential of including MP (*C. officinalis*) as a beneficial feed supplement in rainbow trout food regimes, focusing exclusively on the growth, intestinal enzyme activities, serum and skin mucus immunities, biochemical factors,

Table 3: Growth performance, feed utilization, and survival rate (means ± SE) of *O. mykiss* fed with various levels of pot marigold (*C. officinalis*) powder for 60 days.

Parameter	Different experimental diets				
	Control	MP 0.5%	MP 1%	MP 1.5%	MP 2%
IW (g)	15.43 ± 0.23	15.03 ± 0.26	14.80 ± 0.25	14.83 ± 0.38	15.23 ± 0.12
FW (g)	73.50 ± 1.04^{c}	76.46 ± 0.83^{bc}	77.83 ± 1.01^{b}	82.10 ± 0.78^{a}	77.73 ± 0.49^{b}
SGR (% d^{-1})	2.60 ± 0.04^{b}	2.70 ± 0.01^{ab}	2.76 ± 0.02^{a}	2.85 ± 0.04^{a}	2.72 ± 0.01^{ab}
WG (g)	58.06 ± 1.23^{c}	61.43 ± 0.57^{bc}	63.03 ± 0.84^{b}	67.26 ± 0.91^{a}	62.50 ± 0.50^{b}
FCR	1.34 ± 0.02^{a}	1.24 ± 0.03^{ab}	1.19 ± 0.01^{b}	1.10 ± 0.02^{c}	1.20 ± 0.02^{b}
FI (g)	77.83 ± 0.72^{a}	76.33 ± 0.88^{ab}	76.16 ± 0.44^{ab}	74.00 ± 0.57^{b}	75.00 ± 0.58^{ab}
SR (%)	93.00 ± 1.00	96.00 ± 2.64	98.00 ± 2.00	97.00 ± 1.73	95.00 ± 2.64

Different letters in a row denote significant difference ($P < 0.05$).

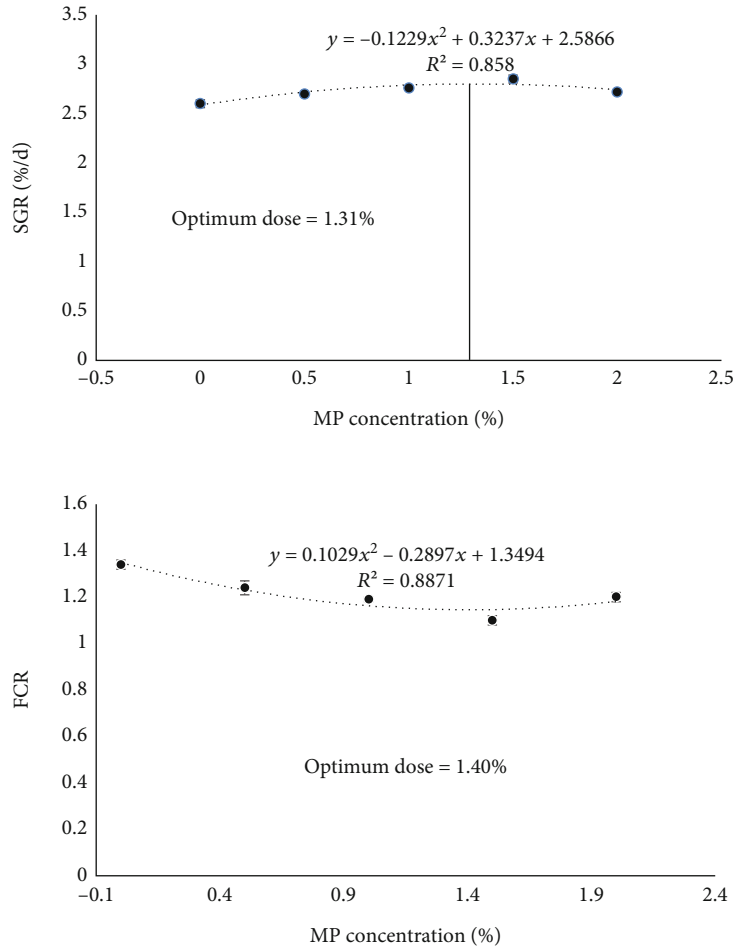


Figure 1: Relationships between the dietary MP levels and growth parameters of rainbow trout ($n = 3$).

antioxidant defense, and tolerance against *Y. ruckeri* infection. In our research, the growth performance of rainbow trout was remarkably affected after being fed with MP-supplemented food. The positive effects on growth performance may be linked to the wide range of bioactive compounds in MP as flavonoids, carotenoids, steroids, sterols, glycosides, volatile oil, quinines, and amino acids [31, 57]. It has been confirmed that these components increase the production of bile and the secretion of pancreatic digestive enzymes, resulting in better absorption of nutrients. Also, complex sugars such as polysaccharides found in plants can act as prebiotics and facilitate nutrient absorption by improving gut health conditions, morphology, microbial diversity, and digestive enzyme activities [58, 59]. Moreover, the findings of our study show that MP can probably enhance growth and feed utilization efficiency in *O. mykiss* by improving immune system function. According to the findings, dietary administration of MP remarkably reduced

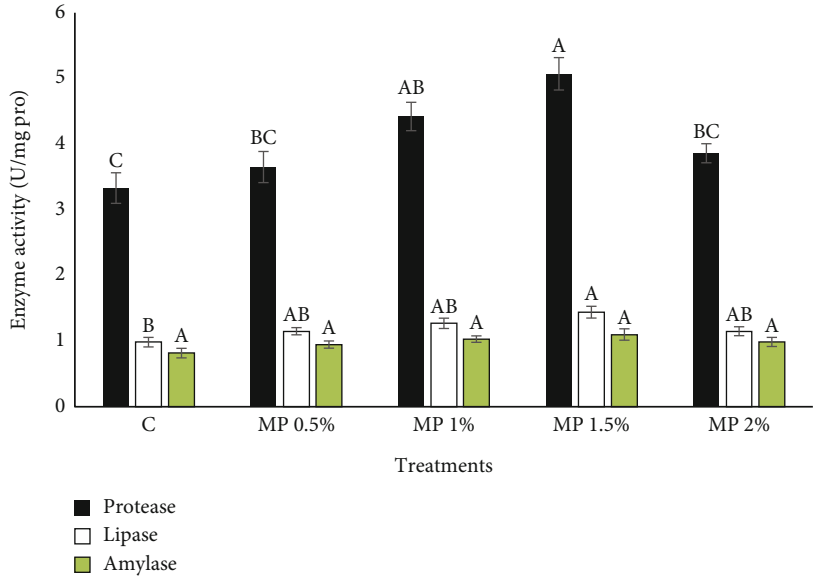


Figure 2: Digestive enzyme analyses (means ± SE, $N = 9$) of *O. mykiss* fed with various levels of pot marigold (*C. officinalis*) powder for 60 days.

Table 4: Serum immunological parameters (means ± SE, $N = 9$) of *O. mykiss* fed with various levels of pot marigold (*C. officinalis*) powder for 60 days.

Parameter	Different experimental diets				
	Control	MP 0.5%	MP 1%	MP 1.5%	MP 2%
LYZ (U/ml)	25.27 ± 1.12^{c}	27.40 ± 0.67^{bc}	31.99 ± 0.83^{a}	30.19 ± 0.60^{ab}	28.46 ± 0.54^{abc}
Total Ig (mg/ml)	18.89 ± 1.09^{b}	20.90 ± 0.51^{ab}	23.05 ± 0.88^{a}	22.70 ± 0.55^{ab}	20.20 ± 1.02^{ab}
C3 (g/dl)	15.45 ± 0.72^{c}	17.10 ± 0.51^{bc}	18.50 ± 0.75^{ab}	20.43 ± 0.53^{a}	17.50 ± 0.51^{bc}
C4 (g/dl)	6.43 ± 0.56^{c}	7.50 ± 0.68^{c}	10.30 ± 0.51^{ab}	11.70 ± 0.69^{a}	7.60 ± 0.52^{bc}
ACH50 (U/ml)	113.00 ± 3.60	118.00 ± 2.64	119.66 ± 2.33	124.66 ± 2.60	120.33 ± 3.52
NBT (540 nm)	0.48 ± 0.04^{c}	0.61 ± 0.04^{bc}	0.79 ± 0.03^{ab}	0.91 ± 0.05^{a}	0.72 ± 0.02^{b}
MPO (450 nm)	1.09 ± 0.05^{b}	1.23 ± 0.08^{ab}	1.43 ± 0.10^{ab}	1.57 ± 0.07^{a}	1.27 ± 0.08^{ab}

Different letters in a row denote significant difference ($P < 0.05$).

Table 5: Skin mucus immunological parameters (means ± SE, $N = 9$) of *O. mykiss* fed with various levels of pot marigold (*C. officinalis*) powder for 60 days.

Parameter	Different experimental diets				
	Control	MP 0.5%	MP 1%	MP 1.5%	MP 2%
LYZ (U/ml)	11.40 ± 0.60^{b}	13.48 ± 0.62^{ab}	15.15 ± 0.60^{a}	14.18 ± 0.57^{a}	14.10 ± 0.43^{a}
ALP (U/l)	25.51 ± 0.45^{c}	26.43 ± 0.69^{bc}	29.83 ± 0.92^{a}	29.23 ± 0.53^{ab}	27.40 ± 0.49^{abc}
Peroxidase (U/ml)	10.66 ± 0.44^{b}	12.76 ± 0.66^{ab}	13.53 ± 0.64^{a}	12.70 ± 0.62^{ab}	11.66 ± 0.56^{ab}
Protease (%)	5.2 ± 0.71^{b}	7.0 ± 0.57^{ab}	8.73 ± 0.58^{a}	8.36 ± 0.47^{a}	8.43 ± 0.80^{a}
Total Ig (mg/ml)	12.36 ± 0.75	12.66 ± 0.97	14.40 ± 0.50	13.73 ± 0.68	13.60 ± 0.52

Different letters in a row denote significant difference ($P < 0.05$).

the FCR value, especially in fish fed with the MP 1.5% diet. Therefore, this may decrease the cost of feed for rainbow trout culture. The main goals of aquaculture are to achieve maximum growth and reduce FCR. In the present study, the weight gain in rainbow trout fed with MP1.5%-containing feed was the highest, while the FCR and FI were low in this group. The reason for this could be related to the increased digestibility of food in fish fed with MP1.5%-containing feed, as well as the presence of nutrients in MP that can reduce fish nutritional requirements and consequently result in less food consumption by fish. Our findings are similar to previous studies on the positive effects of diets supplemented with herbal additives on growth performance in *O. mykiss* [60–62].

It has been reported that herbal additives as immunostimulants improve fish biochemical indices and health status

Table 6: Biochemical parameters (means ± SE, $N = 9$) of *O. mykiss* fed with various levels of pot marigold (*C. officinalis*) powder for 60 days.

Parameter	Different experimental diets				
	Control	MP 0.5%	MP 1%	MP 1.5%	MP 2%
AST (U/l)	171.66 ± 4.40^{a}	152.83 ± 4.10^{b}	135.33 ± 2.60^{c}	126.00 ± 3.78^{c}	132.66 ± 2.33^{c}
ALP (U/l)	310.33 ± 5.78^{a}	302.33 ± 6.11^{a}	270.66 ± 5.81^{b}	241.00 ± 8.33^{c}	263.00 ± 4.04^{bc}
ALT (U/l)	45.00 ± 1.14^{a}	42.50 ± 0.87^{ab}	39.50 ± 1.04^{b}	33.30 ± 1.75^{c}	38.50 ± 0.76^{bc}
LDH (U/l)	868.33 ± 13.64^{a}	822.00 ± 13.31^{ab}	824.33 ± 12.33^{ab}	788.33 ± 11.66^{b}	812.66 ± 11.85^{ab}
TP (g/dl)	3.36 ± 0.17^{b}	3.68 ± 0.19^{ab}	3.93 ± 0.18^{ab}	4.33 ± 0.23^{a}	3.48 ± 0.15^{ab}
ALB (g/dl)	2.16 ± 0.13	2.17 ± 0.16	2.43 ± 0.12	2.53 ± 0.29	1.90 ± 0.05
GLU (g/dl)	1.20 ± 0.11	1.52 ± 0.19	1.50 ± 0.07	1.80 ± 0.20	1.58 ± 0.13

Different letters in a row denote significant difference ($P < 0.05$).

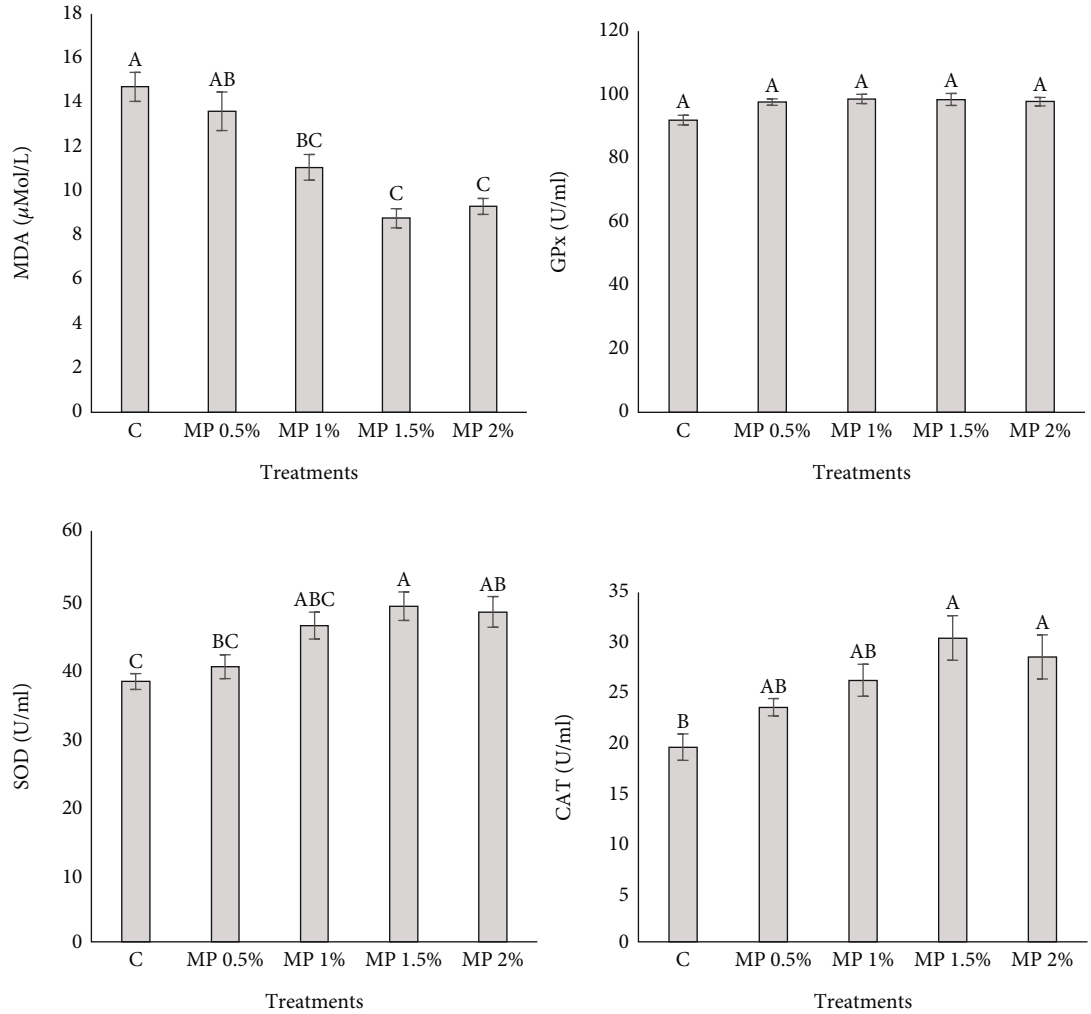


Figure 3: Antioxidant response (means ± SE, $N = 9$) of *O. mykiss* fed with various levels of pot marigold (*C. officinalis*) powder for 60 days.

[10]. Our findings showed that diets containing MP, especially in fish fed with the MP 1.5% diet, have beneficial effects on the serum biochemical parameters such as AST, ALP, ALT, LDH, and total protein in *O. mykiss*. Our observations are similar to the research of Yousefi et al. [63] that reported decreased plasma AST, ALP, and ALT levels in *Cyprinus carpio* receiving diets supplemented with different garlic levels (0.5, 1, and 1.5%) for 35 days. AST, ALP, and ALT are indicators of hepatocyte damage [64, 65]. Therefore, such positive effects in the serum biochemical parameters are probably associated with elevating antioxidant capacity, which reduces free radical attacks on the liver. This hypothesis is confirmed by serum activities of oxidative stress biomarkers, which showed similar patterns compared

Table 7: Disease resistance of *O. mykiss* fed with various levels of pot marigold (*C. officinalis*) powder after 14 days of bacterial challenge with *Y. ruckeri*.

Different experimental diets	SR (%)	RPS (%)
C	37.34 ± 2.00^{c}	
MP 0.5%	60.02 ± 3.83^{b}	36.20 ± 6.11
MP 1%	68.93 ± 2.23^{ab}	50.42 ± 3.56
MP 1.5%	75.60 ± 2.20^{a}	61.05 ± 3.51
MP 2%	62.22 ± 2.22^{b}	39.71 ± 3.54

Different letters in the same column denote significant difference ($P < 0.05$).

to the levels of biochemical parameters. As an organism's response to internal and external circumstances, blood serum proteins are a highly responsive biochemical system. Serum TP, ALB, and GLU are indicators of deteriorated fish health and immune function [66]. The present results indicated that fish treated with MP 1.5%-supplemented foods showed higher serum TP levels than the control group. However, no notable variations were detected in the ALB and GLU levels between the MP-supplemented and control groups. An increase in the serum total protein values could be a strong indicator of the immune promoter influence of the herbal additives [62]. Hence, our results show that pot marigold powders can enhance adaptive immune system function in *O. mykiss*. Similarly, Chelemal Dezfoulnejad and Molayemraftar [15] reported that diets containing rosemary (*Rosmarinus officinalis* L.) extract significantly increased total protein levels in *C. carpio*.

According to the results, diets containing MP have beneficial effects on the intestinal protease and lipase amounts in *O. mykiss*. Using many nutritional supplements and additives can change the production of endogenous and exogenous digestive enzymes [67]. Likewise, it has been well documented that dietary herbal plants increase digestion efficiency through digestive enzyme improvement [8, 10, 58]. Thus, the enhancement of digestive enzyme activities presumably was one of the primary causes for the growth stimulatory results of the MP used in the current study. Moreover, increased intestinal protease and lipase levels are probably attributed to the bioactive compounds of pot marigolds. Similar to our findings, stimulated activities of intestinal protease and lipase after fed with dietary herbal plants have been observed in *O. mykiss*, *Oreochromis niloticus*, and *C. carpio* [61, 68, 69].

The nonspecific immune system is the first defense barrier in aquatic animals which has an essential role against pathogens. Serum immunological statuses such as LYZ, ACH50, NBT, MPO, total Ig, C3, and C4, as the nonspecific immune status of fish, are practical biomarkers that may reflect immune response, and homeostasis changes result from several factors such as nutrition position, environmental pollution, stress, and physical damage [70–73]. In the current study, the enhancement of LYZ, NBT, MPO, C3, and C4 levels, especially in the 1.5% group, in fish fed with MP-supplemented foods may indicate the positive impact of MP on the serum immunological responses of *O. mykiss*. This response could be because of the high antioxidant and polyphenolic levels in MP that activate the mechanisms of the immune system. Also, herbal additives can affect the nonspecific immune status through different mechanisms as blocking the enzymes involved in the arachidonic acid pathway, activating the enzymes involved in inflammatory reactions, and the production of proinflammatory cytokines, especially TNF-α [74]. Though, no significant differences were detected in the ACH50 activities between the MP-supplemented and control groups. Similarly, other herbal additives such as rosemary extract [15], jojoba extract [75], clove basil leaf extract [76], and purslane leave powder [77] improved the nonspecific immune system activities in different fish species.

The mucosal immune system is the first line of the innate immune system against infection. Hence, skin mucus immunological status such as LYZ, ALP, total Ig, and protease, as the nonspecific immune status of fish, are practical biomarkers that may reflect fish's immune system activities [78, 79]. In our research, it has been noticed that mucosal immunological status such as LYZ, ALP, and protease activities enhanced in fish fed on diets containing MP. Moreover, peroxidase value remarkably increased in fish fed with a diet containing 1% MP. Though no notable variations were detected in the mucus total Ig levels between the MP-supplemented and control groups, it has been proved that MP is an effective mediator of the specific and nonspecific immune systems [80]. Also, it has been well documented that herbal plants could reinforce nonspecific immunity and diminish the vulnerability of fish to various diseases [13]. Similar to our results, Hoseinifar et al. [59] revealed that food regimes containing fern (*Adiantum capillus-veneris*) leave powder significantly increased mucus LYZ value of *C. carpio* while it did not affect the mucus Ig levels between the supplemented and control groups. Moreover, Adel et al. [81] stated that diets containing peppermint (*Mentha piperita*) extract significantly increased mucus LYZ and ALP activities of *Rutilus frisii kutum*.

Overall, MP contains bioactive compounds, which may be the main reason for its effectiveness in the nonspecific immune system of *O. mykiss*. Although further investigation is needed to determine the exact mechanism of MP's immunomodulatory effect in fish species, our findings confirmed that the serum and skin mucus immunological status were positively modulated by MP supplementation in fish diets that were parallel to an increase in fish resistance to the bacterial challenge.

Oxidative damage in organisms is directly associated with the capacity to generate antioxidants and their efficiency in defending against oxidative stress. SOD, CAT, GPx, and MDA are also biomarkers in fish for assessing antioxidant defense. Many of the antioxidants that contribute to the antioxidant capacity of aquatic animals come from their food [82]. It is well known that herbal additives have a high antioxidant power because of their flavonoid and phenolic compounds [58]. Based on the results, serum oxidative stress biomarkers were improved in the supplemented groups, particularly in fish fed with the MP 1.5% diet. However, no differences were observed in the GPx activities

between the MP-supplemented and control groups. Hence, our findings showed that MP-supplemented diets stimulated the activities of two pioneer antioxidant enzymes, SOD and CAT. Eventually, these cumulatively suppress lipid peroxidation (MDA level) and increase the health index. Our results propose that the activation of antioxidant defense in the fish may be linked to the presence of bioactive compounds, especially α-cadinol, σ-cadinene, δ-cadinol, β-calacorene, α-pinene, and α-muurolol, in MP that induces radical scavenging activity. Also, bioactive compounds may affect the antioxidant defense by various mechanisms such as the following: (a) ability to chelate metal ions, especially copper and iron; (b) increment of the level of antioxidative enzymes like catalase and superoxide dismutase; (c) prevention of ROS or RNS production by inhibiting enzymes like NADH oxidase, mitochondrial succinoxidase, and microsomal monooxygenase; and (d) addition of hydrogen atoms to reactive free radicals and declining the potential to oxidize lipid membranes [74]. The findings of our study are consistent with those of the previous research investigating the beneficial effects of dietary supplements with herbal plants for antioxidant defense in *O. mykiss* [83], *Salmo salar* [84], and *C. carpio* [53].

Y. ruckeri was used as a model to investigate the effectiveness of the MP antibacterial activity. The findings of this research showed that dietary rainbow trout with diets containing MP remarkably reduced the mortality rate after the challenge with *Y. ruckeri*, and the highest protection was recorded in fish fed with the MP 1.5% diet. Hence, this plant can be considered a biological and eco-friendly means of controlling *Y. ruckeri* in the culture of this species. In the current study, reducing fish mortality rates might be due to the beneficial effects of MP-supplemented diets on the immune process as well as antioxidant capability. Moreover, marigold contains many valuable components such as α-cadinol, σ-cadinene, δ-cadinol, β-calacorene, α-pinene, and α-muurolol that have been found to have antimicrobial activities. Many studies confirmed that herbal plants, because of their bioactive compounds, have antimicrobial effects on different fish species [85]. Similar to our results, Adel et al. [60] stated that *O. mykiss* fed with diets containing *Polygonum minus* extracts showed enhanced resistance protection against *Y. ruckeri*, with the RPS ranging from 50.24 to 84.88%.

5. Conclusion

Initially, the research demonstrates that diets containing MP have positive effects on growth performance, digestive enzyme activities, immunological responses, and antioxidative capacity in *O. mykiss.* Moreover, the best dietary MP level in this species is 1.5%. Furthermore, our findings indicate that the enriched diet with MP positively affects fish health and disease resistance. Therefore, MP could perform a key role in preventing disease outbreaks in aquaculture systems.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Ethical Approval

All applicable guidelines for the care and use of animals were followed according the National Ethical Framework for Animal Research in Iran. All experiments were performed following the protocol approved by the Committee of Ethics of the Faculty of Sciences, University of Tehran (357; 8 November 2000).

Conflicts of Interest

The authors state that they have no conflict of interest.

Authors' Contributions

Hamed Ghafarifarsani was responsible for the conceptualization, formal analysis, and methodology. Seyed Hossein Hoseinifar was responsible for the supervision and wrote, reviewed, and edited the manuscript. Taravat Molayemraftar wrote the original draft. Mahdieh Raeeszadeh wrote, reviewed, and edited the manuscript. Hien Van Doan was responsible for the supervision and resources.

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