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RESEARCH ARTICLE

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Growth indices, non-specific immune parameters, skin mucus protein profile and resistance to *Saprolegnia parasitica* in rainbow trout fed *Mentha longifolia*

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ABSTRACT

The development and implementation of nutritionally balanced diets is regarded as essential biological factors in improving growth and maintaining the health of aquaculture species. This study evaluated the effects of *Mentha longifolia* hydroalcoholic extract on growth performance, Hemato-immunological responses and skin mucus protein in rainbow trout (*Oncorhynchus mykiss*). A total of 360 fish (10.17 ± 0.18 g) were acclimated to the experimental environment and randomly distributed across 12 tanks (four treatment in triplicate; 30 fish per tank). Experimental diets were prepared by incorporating *M. longifolia* hydroalcoholic extract at 0 (control), 1, 2, and 3 g/kg into a basal diet and fed to the fish for a 60 day, after which they were challenged with *Saprolegnia parasitica*. Fish fed *M. longifolia* diets exhibited significantly higher specific growth rate and weight gain, along with reduced feed conversion ratio compared with the control. Red and white blood cells counts, serum respiratory burst activity, albumin, and total protein levels were also significantly higher in extract fed groups. Following challenge with *S. parasitica*, survival rate was markedly higher in treated fish compared to control. Overall, dietary supplementation with 1 g/kg *M. longifolia* hydroalcoholic extract is recommended to stimulate immune response, enhance growth performance, and improve resistance of rainbow trout to *S. parasitica.*

Keywords

Mentha longifolia; rainbow trout; Growth; Immunity; Blood; Oomycete; Oncorhynchus mykiss

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Abbreviations

M. longifolia: Mentha longifolia
S. parasitica: Saprolegnia parasitica
O. mykiss: Oncorhynchus mykiss
FCR: Feed conversion rate
WG: weight gain
SGR: specific growth rate
WBC: White Blood Cell
RBC: Red Blood Cell

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Introduction

Intensive Rainbow trout aquaculture is aften associated with many stresses that predispose the fish to a variety of infectious diseases. Among the most important pathogens in freshwater systems are oomycetes, which can be found in various freshwater ecosystems and can pose risks to both farmed and wild fish populations [1]. Among them, *Saprolegnia parasitica* is a significant oomycete species that affects salmonids and other fish, leading to a disease known as saprolegniasis, which is considered one of the most crucial oomycete-related diseases in aquaculture. [1].

Chemical disinfectants are commonly used to control saprolegniasis. However, concerns regarding the negative impact of pesticides and harmful chemical compounds have encouraged researchers to seek for safer, natural and eco-friendly alternatives [2]. Plants have always been considered as a valuable source of biologically active compounds, with potential immunostimulatory properties [3,4]. Extensive research has highlighted the ability of medicinal plants to improve immunity and growth in aquatic animals [5,6]. Because immunostimulants primarily enhance the non-specific immune system, they are often considered more effective in aquatic animals than in warm-blooded animals [7,8].

Numerous studies have attentive on the use of medicinal plants to enhance fish growth, immunity, and resistance to pathogens [5,9]. For instance, Mehrabi et al. [10] reported that aloe vera (*Aloe brabadensis*) stimulated the immune system and improved resistance to *S. parasitica* in rainbow trout. Feeding *Mentha longifolia* hydroalcoholic extract to rainbow trout increased resistance to Yersinia ruckeri infection [11]. Similarly, Mehrabi et al. [12] reported that nettle (Urtica dioica) powder was shown to stimulate the immune system and increased survival in rainbow trout challenged with the *S. parasitica.*

Mentha longifolia, commonly known as horsemint, is one of the most important species in the Lamiacea family. The fresh buds and leaves possess antifungal, antimicrobial, anti-inflammatory, and antioxidant properties. These activities are attributed to its diverse biochemical compounds, including cinnamic acid, aglycone, acetylated flavonoids, glycoside steroids, carvone, menthol, piperitone oxide, limonene, 1.8-quinol, polgun, beta-caryophyllene, and trans-piperitol epoxide. [13,14].

As in other vertebrates, the immune system in fish includes innate and acquired immune system, with innate responses serving as the first line of defence against pathogens [15]. Mucus, a critical innate immune barrier, prevents pathogen binding through continuous secretion and excretion of dead tissues and stimuli [16,17].

To date, no study has investigated the effect of horsemint (*M. longifolia*) hydroalcoholic extract on rainbow trout resistance to saprolegniasis. Given *Mentha longifolia* contains compounds with antibacterial and antifungal properties, which help prevent microbial and fungal infections in fish [18]. Therefore, the present study was conducted to investigate, for the first time, the effects of *M. longifolia* extract on growth performance, immune responses and resistance of rainbow trout against *S. parasitica.*

Result

Growth parameters

The effects of feeding diets containing *M. longifolia* extract on the growth performance of rainbow trout after 60 days are presented in Table 1. The final mean weight increased significantly in all groups fed with *M. longifolia* ($p < 0.05$). The highest weight gain was observed in fish fed the diet containing 1 g/kg of *M. longifolia*. Similarly, this treatment also gained the highest SGR ($p < 0.05$). Overall, all extract fed groups showed significantly improved SGR compared to the control ($p < 0.05$). FCR decreased significantly in all groups receiving *M. longifolia* extract ($p < 0.05$), with the lowest FCR observed in the 1 g/kg treatment group ($p < 0.05$). Survival remained 100% across all treatments and the control after 60 days.

Blood parameters

Table 2 shows the hematological responses of trout after feeding *M. longifolia* extract for 60 days. RBC and WBC counts, hemoglobin concentrations, and hematocrit percentages all increased significantly in extract fed groups compared to the control ($p <$ 0.05). Feeding with the minimum effective dose of the extract (1 g/kg) resulted in significant improvements

Table 1.
The growth factors (mean ± SD) of rainbow trout after feeding with M. longifolia for 60 days

groups	Initial weight (g)	Final weight (g)	Weight gain (g)	SGR (%)	FCR (%)	Survival rate (%)
0g/kg	10.25 ± 0.20^{a}	27.74 ± 1.68^{a}	17.49 ± 0.76^{a}	1.65 ± 0.11^{a}	1.49 ± 0.08^{c}	100a
1g/kg	10.20 ± 0.10^{a}	44.03 ± 2.16^{c}	33.83 ± 1.08^{c}	2.43 ± 0.19^{c}	0.89 ± 0.06^{a}	100a
2 g/kg	10.08 ± 0.23^{a}	38.77 ± 2.08^{b}	28.69 ± 1.02^{b}	2.24 ± 0.14^{b}	1.18 ± 0.07^{b}	100a
3 g/kg	10.16 ± 0.25^{a}	40.34 ± 1.98^{b}	30.18 ± 0.98^{b}	2.29 ± 0.10^{b}	1.21 ± 0.05^{b}	100a

In each column, values with different superscript letters are significantly different (P < 0.05).

Table 2.
The hematological factors (mean ± SD) of rainbow trout after feeding with *M. longifolia* for 60 days (pre-challenge) and 15 days after challenging with *S. parasitica* (post-challenge) are presented below

Factors	Time							
	Pre- challenge				Post- challenge			
	0.0%	0.1%	0.2%	0.3%	0.0%	0.1%	0.2%	0.3%
WBC ($\times 10^3$ µL)	15.62 ± 0.88^{a}	19.89 ± 0.34^{b}	20.06 ± 0.52^{b}	20.23 ± 0.31^{b}	19.28 ± 0.46^{a}	20.39 ± 0.61^{b}	21.12 ± 0.44^{c}	21.38 ± 0.63^{c}
RBC ($\times 10^6$ µL)	0.98 ± 0.19^{a}	1.47 ± 0.65^{b}	1.50 ± 0.26^{b}	1.53 ± 0.15^{b}	0.68 ± 0.49^{a}	1.28 ± 0.12^{b}	1.30 ± 0.17^{b}	1.31 ± 0.14^{b}
Hemo-globin (g dL^{-1})	9.13 ± 0.76^{a}	11.98 ± 0.32^{b}	12.21 ± 0.62^{b}	12.25 ± 0.49^{b}	6.41 ± 0.42^{a}	10.93 ± 0.59^{b}	11.10 ± 0.78^{b}	11.14 ± 0.61^{b}
Hemato-crit (%)	28.20 ± 1.38^{a}	35.93 ± 1.00^{b}	36.19 ± 0.84^{b}	36.64 ± 1.10^{b}	28.90 ± 1.77^{a}	32.71 ± 1.41^{b}	33.13 ± 1.38^{b}	33.62 ± 1.65^{b}
MCV (fl)	287.75 ± 8.23^{b}	244.42 ± 7.51^{a}	241.26 ± 10.45^{a}	239.47 ± 8.62^{a}	425.00 ± 8.94^{b}	255.54 ± 8.24^{a}	254.84 ± 8.72^{a}	256.64 ± 9.81^{a}
MCH (pg)	93.16 ± 1.16^{b}	81.49 ± 2.24^{a}	81.4 ± 1.54^{a}	80.06 ± 1.356^{a}	94.26 ± 2.19^{b}	85.39 ± 2.28 ^{a}	85.38 ± 1.10^{a}	85.03 ± 2.45^{a}
MCHC (g dL^{-1})	32.37 ± 2. 42^{a}	33.34 ± 1. 37^{a}	33.73 ± 1. 80^{a}	33.43 ± 2. 48^{a}	22.17 ± 1. 56^{a}	33.41 ± 1.23^{b}	33.50 ± 1.12^{b}	33.13 ± 1.86^{b}
Lympho-cyte (%)	73.22 ± 2. 28^{a}	81.63 ± 3.4^{b}	81.88 ± 2.94^{b}	82.10 ± 2.766^{b}	61.59 ± 2. 32^{a}	70.36 ± 1.14^{b}	75.91 ± 1.28^{c}	76.58 ± 1.49^{c}
Neutro-phil (%)	22.10 ± 0.82^{b}	13.25 ± 0.17^{a}	11.18 ± 0.41^{a}	11.01 ± 0. 74^{a}	28.66 ± 0. 49c	23.32 ± 0. 35^{b}	17.21 ± 0. 21^{a}	16.73 ± 0. 40^{a}
Monocyte (%)	4.68 ± 0.75^{a}	5.12 ± 0. 33^{a}	6.94 ± 0.7^{9b}	6.89 ± 0. 65^{b}	9.75 ± 0. 28^{b}	6.32 ± 0.15^{a}	6.88 ± 0.36^{a}	6.69 ± 0.21^{a}

Red blood cells (RBC), white blood cells (WBC), Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). In each column, values with different superscript letters are significantly different ($P < 0.05$).

compared to the control. However, no significant difference were detected among the different treatments in RBC and WBC counts, hematocrit, and hemoglobin ($p < 0.05$). Monocyte and lymphocyte counts were significantly higher in the groups fed with 0.2% and 0.3% extract fed groups ($p < 0.05$), while neutrophil counts decreased significantly in all extract fed groups ($p < 0.05$). Additionally, MCH and MCV also decreased significantly in the extract fed groups ($p < 0.05$). After challenging with *S. parasitica*, blood parameters significantly increased in the extract fed groups compared to the control ($p < 0.05$). WBC counts increased with higher doses of *M. longifolia* extract, reaching their peak in the 2 g/kg and 3 g/kg groups. These values differed significantly from both the control and the 1 g/kg treatment. ($p < 0.05$). However, no significant differences were observed among the extract fed treatments regarding RBC counts, hemoglobin concentrations, and hematocrit percentages ($p > 0.05$). Furthermore, lymphocyte counts, MCHC, monocyte and neutrophil counts, as well as MCH and MCV, all showed significant increases in extract fed fish compared to the control ($p < 0.05$).

Serum biochemical indices

Serum biochemical parameters after 60 days of feeding with different doses of *M. longifolia* extract and post challenge with *S. parasitica*, are summarized in Table 3. Both albumin and total protein levels increased significantly in all extract fed treatments compared to the control ($p < 0.05$). The highest values were observed in the 1 g/kg treatment, which different significantly from the other groups ($p < 0.05$).

Immune response of fish

As shown in Table 4, neutrophil respiratory burst, lysozyme activity, and ACH50 of levels were significantly increased in extract fed groups compared to the control after 60 days ($p < 0.05$). However, no significant differences were detected between the extract fed treatments in lysozyme activity and ACH50 ($p < 0.05$). The highest neutrophil respiratory burst occurred in the 1 g/kg treatment, which differed significantly from the other groups ($p < 0.05$). Feeding fish with the minimum effective dose (1 g/kg) resulted in significant rises of all immune parameters compared to the control. Following the *S. parasitica* challenge, immune factors significantly increased in all extract fed groups, at all three levels, compared to the control ($p < 0.05$). The highest neutrophil respiratory burst was observed in the 2 g/kg treatment, which differed significantly from the other groups ($p < 0.05$).

Table 3.
The growth factors (mean ± SD) of rainbow trout after feeding with *M. longifolia* for 60 days

	Groups	Total protein (g dL-1)	Albumin (g dL-1)
Pre-challenge	0 g/kg	3.49 ± 0.12^{a}	1.30 ± 0.10^{a}
	1 g/kg	4.18 ± 0.25^{c}	2.60 ± 0.35^{c}
	2 g/kg	3.81 ± 0.19^{b}	2. 20 ± 0.29^{b}
	3 g/kg	3.80 ± 0.20^{b}	2.10 ± 0.55^{b}
Post-challenge	0 g/kg	3.10 ± 0.33^{a}	1.00 ± 0.31^{a}
	1 g/kg	3.80 ± 0.26^{c}	2.35 ± 0.26^{c}
	2 g/kg	3.44 ± 0.41^{b}	1.93 ± 0.39^{b}
	3 g/kg	3.41 ± 0.21^{b}	1.88 ± 0.49^{b}

In each column, values with different superscript letters are significantly different ($P < 0.05$).

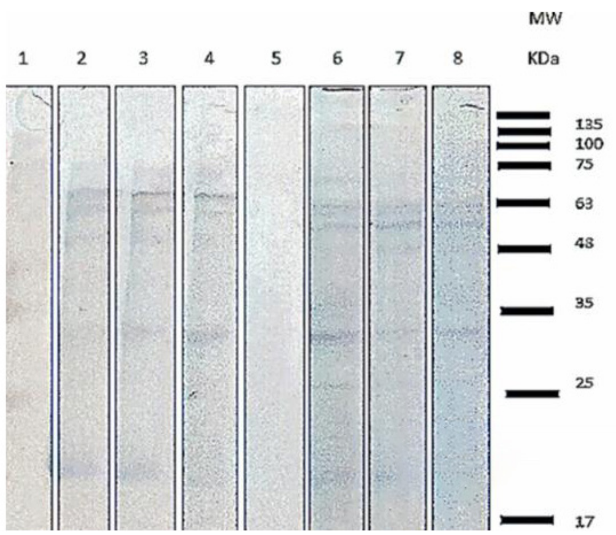

Figure1.
Mucus protein pattern of rainbow trout after feeding with *M. longifolia* for 60 days (pre-challenge) and 15 days after challenging with S. parasitica (post-challenge) (End of 60 days: 1 = Control, 2 = 0.1%, 3 = 0.2%, 4 = 0.3% and 15 days post-challenge: 5 = Control, 6 = 0.1%, 7 = 0.2%, 8 = 0.3%)

Mucus protein pattern

Figure 1 shows the protein profile of skin mucus in rainbow trout fed *M. longifolia* extract for 60 days, both before and 15 days after exposure to *S. parasitica*. The band density exhibited significant variations among the protein profiles of the different groups, with the identified proteins bands ranged from 17 to 75 kDa. The quantity and mass of bands within the 17-75 kDa molecular weight range were found to be greater in the groups receiving the extract compared to the control group. After the fungal challenge, the count and density of protein bands remained stable in extract fed groups, while the control group showed significant reduction. Survival results following the challenge are presented in Figure 2. After 15 days of exposure to *S. parasitica*, the fish that received *M. longifolia* extract exhibited significantly higher survival rates compared to the control ($p < 0.05$). However, no significant differences were detected among the groups fed with *M. longifolia* after the challenge with *S. parasitica* ($p < 0.05$).

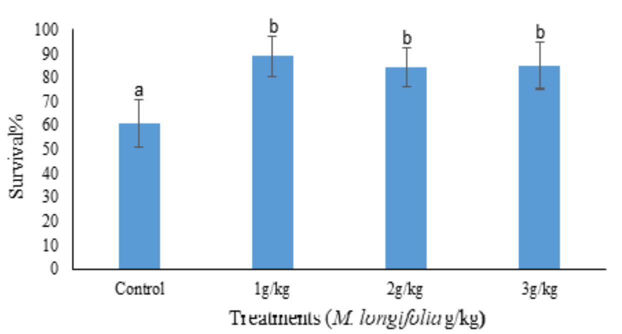

Figure 2.
Survival rate of rainbow trout fed with different levels of *M. longifolia* after 15 days of challenging with *S. parasitica*. The values with different superscript letters are significantly different ($p < 0.05$).

Table 4.
The immune factors (mean ± SD) of rainbow trout after feeding with *M. longifolia* for 60 days (pre-challenge) and 15 days after challenging with *S. parasitica* (post-challenge)

Time	Groups	Respiratory burst (OD at 540 nm)	Lysozyme (U/ml)	ACH50 (U/ml)
Pre-challenge	0 g/kg	0.493 ± 0.06^{a}	429.21 ± 0.29^{a}	38.09 ± 0.16^{a}
	1 g/kg	0.551 ± 0.09^{c}	430.17 ± 0.29^{b}	42.12 ± 0.69^{b}
	2 g/kg	0.530 ± 0.03^{b}	430.00 ± 0.32^{b}	42.44 ± 1.18^{b}
	3 g/kg	0.529 ± 0.04^{b}	430.45 ± 0.26^{b}	42.31 ± 1.14^{b}
Pre-challenge	0 g/kg	0.464 ± 0.05^{a}	430.00 ± 21^{a}	128 ± 14^{a}
	1 g/kg	0.572 ± 0.01^{b}	431.17 ± 47^{b}	138 ± 12^{b}
	2 g/kg	0.739 ± 0.07^{d}	431.14 ± 35^{b}	139 ± 17^{b}
	3 g/kg	0.680 ± 0.04^{c}	431.62 ± 39^{b}	140 ± 19^{b}

Alternative complement activity (ACH50). In each column, values with different superscript letters are significantly different ($p < 0.05$).

Discussion

The use of immune system stimulants in aquaculture has become increasingly important for improving and stimulating the activity of non-specific immune system in fish [19,20]. Among these, the use of plants to control pathogens by stimulating the immune system and antioxidant activity have attracted considerable attention [5,8,9].

In the present study, supplementation with the lowest test dose (1 g/kg) of *M. longifolia* extract significantly improved SGR, weight gain, and FCR, as well as with other treatments containing *M. longifolia*, compared to the control. Several studies have documented the effects of plant extracts on various growth parameters in fish. Feeding Caspian kutum (*Rutilus frisii kutum*) with 1 g/kg *M. longifolia* led to increased body weight compare to controls [21]. Raissy et al. [22] showed that a combination of *Mentha longifo*lia, *Thymus carmanicus*, and *Trachyspermum copticum* improved weight gain and FCR in rainbow trout for 45 days. Likewise, Ghanbary et al. [23] demonstrated that incorporating *Thymbra spicata* hydroalcoholic extract significantly increased the final weight in rainbow trout on 8-week period. While, Mehrabi et al. [3] administered 5, 10, and 15 g of nettle (Urtica dioica) powder per kilogram of diet to rainbow trout over an 8-week period and found that fish fed 5 g/kg of dietary nettle showed significant enhancements in SGR and final growth, and reduction in FCR.

Blood parameters in fish change due to physiological and various external factors, such as diet [24]. Blood tissue, determination of blood factors, hematological tests, and biochemical analysis of blood plasma can be suitable indicators for diagnosis, determination of health, and infectious diseases in fish [25]. In this study, M. longifolia extract significantly improved blood parameters such as RBC, WBC, hemoglobin, and hematocrit levels compared to the control. These improvements may be linked to the vitamin C content of *M. longifolia*, which can increase intestinal absorption of vitamins and minerals from consumption of this plant, and thereby lead to improvement of hematological parameters [26]. Comparable results have been reported with M. *longifolia* [11], common mallow (*Malvae sylvestris*) [27], and *Thymbra spicata* [23], all of which improved hematological parameters in trout. Moreover, *Aloe barbadensis* also showed positive effects as an immunostimulants and significantly increased RBC count and hemoglobin concentrations in experimental groups of rainbow trout compared to the control [10]. In another study, feeding rainbow trout with Urtica *dioica*, extract for 4 weeks led to significant elevations of WBC and RBC counts [12]. Since WBCs, along with biochemical factors such as lysozyme, serum proteins, and ACH50, are crucial components of the fish innate immune system, their modulation is particularly relevant under stressful conditions such as illness, dietary imbalances, high stocking density, and environmental stressors [28]. In general, the evaluation of total protein and albumin is considered as an important indicator in response to environmental stresses. Stress from oxidative compounds in the liver, the primary organ for producing albumin and globulins, reduces serum total protein due to liver damage [29]. In contrary, elevated serum protein levels reflect enhanced non-specific immune stimulation, and in fact enhanced defensive response [30]. Our findings indicated that feeding rainbow trout with varying levels of *M. longifolia* hydroalcoholic extract significantly increased albumin and total protein levels. This aligns with the findings of Mehrabi et al. [10], who reported significant increases in serum albumin and total protein in rainbow trout fed aloe vera extract. Comparable results have also been reported regarding enhanced serum biochemical factors in rainbow trout when fed extracts of sweet basil (*Ocimum basilicum*) and Sargassum (*Sargassum angustifolium*) hot water extract [8,31].

Increasing serum lysozyme activity is indicative of an improvement in fish immunity and will help the immune system to cope better with infectious and stressful factors [32]. The results showed a significant rise in lysozyme activity after 60 days of feeding with *M. longifolia* extract. Similarly, the use of dietary aloe vera (*Barbados aloe*) hydroalcoholic extract for 30 days [3], *Stachys lavandulifolia* and greek juniper (*Juniperus excelsa)* significantly increased lysozyme activity in rainbow trout [33,34]. Phagocytic cells are able to produce

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superoxide anions during their respiratory burst activity, which kill toxic oxygen, killing bacteria [35]. In fish, the respiratory burst is associated with the release of cytokines and the activation of inflammatory reactions [36].

In the present study, *leukocyte* respiratory burst after 60 days of feeding, increased significantly in the extract fed treatments compared to the control. This is similar to increased leukocyte respiratory burst activity as a result of feeding rainbow trout with ginseng (*Ginseng panax*) compared with the control group [37]. Similarly, feeding sea bass (*Dicentrarchus labrax*) with extracts from okra (*Abelmoschus esculentus*) seeds, fruits, and leaves [38], as well as Nile tilapia (*Oreochromis niloticus*) with *Artemisia annua* alcohol extract [39], and rainbow trout with *Aloe barbadensis* extract [10], has been associated with elevated leukocyte respiratory burst activity. Increase in complement system activity was also observed in this study, complement proteins are important non-specific immune factors that play a significant role in the immune response of fish. Increased complement activity has frequently been reported following the use of non-specific immunostimulants [7]. In general, the ability of medicinal plants has been proven in the activation and stimulation of complement activity, and it can be claimed that the present results are consistent with those of other researchers. Naderi Farsani et al. [40] studied the nutritional effects of coriander (Coriandrum sativum) on the immune system of rainbow trout and found that fish fed with this plant at 2% for 8 weeks exhibited significantly increased complement activity compare to the control.

The fish's skin is covered by a constantly replaced mucus layer, which acts as the first barrier against pathogens. Fish mucus is a vital source of components involved in the non-specific immune system, including lectins, immunoglobulins, antibacterial lipids, lysozyme, various proteins, and complement proteins [16]. Therefore, changes in mucus protein levels are considered a suitable indicator for assessing the non-specific immune status in fish. In this study, extract-fed trout exhibited an increased number and density of skin mucus bands compared to the control, both before and after fungal challenge.

Similar outcomes were reported by Heydari et al. [11], they recently studied the impact of hydroalcoholic extract of *M. longifolia* on the protein patterns of skin mucus in rainbow trout. Based on their study, feeding with a diet containing 3% *M. longifolia* extract for 30 days increased concentration and number of skin mucus bands compare to the control. In farmed aquatic animals, most bacterial, viral, fungal, and parasitic pathogens are secondary pathogens that present with stressful conditions and impaired aquatic health and immunity of aquaculture species. Therefore, strengthening the immune system is of paramount importance in maintaining the health of aquaculture species and preventing the spread of diseases among the population of farmed fish, thereby preventing disease induced economic losses and mortalities. In our study, results of challenge test with *S. parasitica* confirmed the protective effects of dietary *M. longifolia*, as survival rates significantly increased in all extract fed treatments compared to controls, *M. longifolia* extract enhanced the immune system by increasing blood and serum immunity factors, and subsequently improved the fish's resistance to *S. parasitica*. Comparable improvements in disease resistance have been also reported. The use of *Aloe barbadensis* powder at 15 g/kg [10] and 0.5% *Urtica dioica* [12] of rainbow trout diet for 8 weeks increased fish survival in challenge with *S. parasitica*, in addition to improvements in hematological and serum biochemical parameters. The dietary hot-water extract of *S. angustifolium* at a concentration of 400 mg/kg for 56 days resulted in a cessation of rainbow trout losses, improved serum and blood parameters, and significantly increased fish survival against *Yersinia ruckeri* compared to the control [8].

Conclusions

Medicinal plants represent as a viable alternative to pharmaceutical compounds for improving health and promoting growth in aquaculture species. The present study demonstrates that dietary inclusion of *M. longifolia* hydroalcoholic extract into the diet of *O. mykiss*, positively influences growth, hematological parameters, serum biochemistry, immune response, and mucus protein patterns. Different levels of dietary *M. longifolia* extract had obvious impacts during 60 days of feeding, and on immunity and enhanced resistance of this species in challenge with *S. parasitica*. From an economic perspective, utilizing minimal doses of medicinal plants lowers production costs, including those associated with plant procurement and management. As a result, the most favourable outcomes in weight gain (33.83 ± 1.08g), final weight (44.03 ± 2.16g), SGR (2.43 ± 0.19%), FCR (0.89±0.06%), total protein (4.18 ± 0.25 g dL-1) and albumin (2.60 ± 0.35 g dL-1) of fish after the challenge with *S. parasitica* were observed in the treatment group receiving 1 g/kg *M. longifolia* in the basal diet. This difference was statistically significant for most parameters compared to other experimental groups. Therefore, *M. longifolia* hydroalcoholic extract, in particular 1 g/kg of diet, as the lowest effective dose can be recommended as an oral supplement to stimulate the growth and immune system of *O. mykiss*.

Materials and Methods

Extraction Solvent Preparation

Fresh leaves of *M. longifolia* (2 kg) were collected from mountainous areas of Semnan city, Iran, and approved by botanists at the University of Agricultural Sciences and Natural Resources, Sari, Iran. The leaves were shade dried at room temperature (25 °C), ground into powder using a mill, and used for the hydroalcoholic extraction following the method described by Heydari et al. [11].

Diet preparation

In order to prepare diet, the hydroalcoholic extracts were dissolved and homogenized in 6 mL of ethanol according to required concentrations (1, 2, and 3 g/kg diet) and then sprayed onto a commercial rainbow trout diet (Bayza Company, Fars, Iran). A control diet was prepared by adding only 6 mL of the solvent without extract [11]. The experimental diets were then dried for 24 h, after which the oil was sprayed on the feed in all groups to cover and preserve the plant extract lastly, the diets were stored at 4 °C until use.

Experimental fish

All procedures were conducted in accordance with standard ethical guidelines, protocols, and were approved by Animal Ethics Committee of the Sari Agricultural Sciences and Natural Resources University (Approval No.1274320). In the present study, rainbow trout were obtained from a local farm in Sari, clinically screened for potential diseases and the absence of any sores. The fish were subsequently maintained in a fish culture facility at the university for a two-week period to acclimatize to their new environment. This experiment consisted of four dietary treatments: 1, 2, and 3 g/kg of *M. longifolia* hydroalcoholic extract, and a control, each with three replications (30 juvenile fish per replicate; average initial weight 10.17 ± 0.18 g; length 10.27 ± 0.63 cm). Fish were stocked into 12 tanks (250 L) and fed three times daily (8:00, 12:00, and 16:00) at 3% of body weight [10,4038] for 60 days. All tanks were oxygenated using a central aeration system, and water was supplied from a well. The water physicochemical parameters were monitored daily and maintained as follows: temperature 14.01 ± 0.78 °C, dissolved oxygen 6.32 ± 0.33 mg/L, ammonia 0.05 mg/L, hardness 600.52 ± 22.26 mg/L, salinity 0.61 ± 0.01 ppt, electrical conductivity (EC) 1202.72 ± 46.84 µS/cm, and pH 7.2 ± 0.22. To ensure water quality, 50% of the water was replaced daily.

Challenge with S. parasitica

A pure culture of *S. parasitica* (accession NO. KC992717) was obtained from Sari Agricultural Sciences and Natural Resources Universit)SANRU(and used to perform the challenge test. First, the oomycete was grown on Potato Dextrose Agar and then circular tablets (1 cm in diameter) were prepared from the fungus with the culture medium and placed in sterile tubes containing distilled water at 18-20 °C for one week to induce sporogenesis. Zoospores were harvested by centrifuged (3000 rpm for 10 minutes) to separate the zoospores and counted using a hemocytometer slide [41]. At the end of the experimental period, the fish were challenged with *S. parasitica*. To perform challenge test, 30 fish were selected per treatment (10 pieces per replication). Before the fish were exposed to the zoospores, they were first anesthetized. Then, to increase the permeability of the zoospores to the fish's skin, the scales in the tail area (3cm) were removed. Afterward, to induce stress, the fish were placed in a net and gently shaken in the water for one minute. Finally, excess mucus was washed off with water, and the fish were immersed in tanks containing dose of 3 × 105 zoospores/L for 8 hours to undergo the bath. [12]. Fungal infected fish were confirmed microscopically.

Measurements of growth parameters

To investigate the result of *M. longifolia* on the growth performance of rainbow trout, biometry was performed measuring fish length and weight were recorded using a digital caliper (± 1 mm) and scale (± 0.01 g). Growth performance indices were calculated as follows:

Weight gain (g) = Final weight (g) - initial weight (g)

Specific growth rate (SGR) = [ln (final weight) - ln (initial weight)/days] × 100

Feed conversion rate (FCR) = Feed intake (g) / Weight gain (g)

Survival rate = [Number of survived fish/initial number of fish] × 100

Haematological and immune factors

At the end of 60 days of feeding the fish with *M. longifolia* extract and 15 days after the S. parasitica challenge, blood samples were collected from the peduncles of 12 fish (4 per replicate) to evaluate blood indices. The fish were fasted for 24 hours prior to sampling. Blood was collected into heparinized tubes (500 U/ml) for hematological analysis [11]. Hematocrit was measured using the microhematocrit method [42] and hemoglobin concentration was determined by the cyanmethemoglobin method [43]. RBC and WBC counts were obtained using a hemocytometer, after dilution with Natt–Herrick solution [42]. Serum was separated by centrifugation from non heparinized samples and stored at −20 °C until analysis.

Lysozyme levels were measured turbidimetrically at 450 nm [44]. ACH50 was determined photometrically at 414 nm [45]. Neutrophil respiratory burst was quantified using the nitroblue tetrazolium (NBT) assay [46]. Total protein and albumin were measured using commercial kits (Parsa Azmoon) and an Autolyser (bs120, Manda, China), respectively.

Collection of mucus

Skin mucus was collected following Subramanian et al. [16]. After 60 days of feeding and 15 days post challenge, 12 specimens were sampled from each group, anesthetized with triacaine methanesulfonate (MS222) [41] and placed in cold water to remove contaminants. Each fish was placed separately in plastic bags containing 10 mL of 50 mM sodium chloride (NaCl) for 2 minutes. The mucus was collected and stored at −80 °C until analysis [11].

Protein profile

Protein patterns were analyzed using SDS-PAGE technique [47]. Mucus samples were combined with buffer (4% SDS, 5 mM tris hydrochloric acid, 2% mercaptoethanol, 12% glycerol, and 5% bromophenol blue) in a 4:1 ratio, heated at 95 °C for 5 minutes, centrifuged (1000 rpm, 3 minutes) at room temperature, and the filtered supernatant was utilized for electrophoresis. Subsequently, 25 µL of each sample, along with a molecular weight marker, was loaded onto an 18% polyacrylamide gel equipped with a 5% stacking gel. Electrophoresis was run at 120 V until the bromophenol blue indicator migrated past the stacking gel, then continued at 200 V for 7 hours using 5X electrophoresis buffer. To visualize the protein bands, the gels were stained with 5% Coomassie Blue (250 mg) for 5 hours, and destained using Coomassie Blue solution in two steps over 2 hours.

Statistical analysis

Data were analyzed using SPSS statistical software version 23. Normality of the data was assessed with the Kolmogorov-Smirnov test. A one-way ANOVA was used to evaluate treatment effects, followed by Tukey's to compare means. Differences were considered significant at $p < 0.05$.

Authors' Contributions

Mohadeseh Heydari and Farid Firouzbakhsh conceived and planned the experiments. Mohadeseh Heydari carried out the experiments. Mohadeseh Heydari contributed to sample preparation. Farid Firouzbakhsh contributed to the interpretation of the results. Farid Firouzbakhsh took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Abbreviations-Cont'd

ACH: Alternative hemolytic complement activity
MCV: Mean Corpuscular Volume
MCH: Mean Corpuscular Hemoglobin
MCHC: Mean Corpuscular Hemoglobin Concentration

Competing Interests

The authors declare that there is no conflict of interest.

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