Research Article

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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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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The authors declare that there is no conflict of interest.

Abstract

The development and implementation of nutritionally balanced diets are regarded as essential biological factors in improving growth and sustaining the health of fish. In the current study, the effects of *Mentha longifolia* hydroalcoholic extract were investigated on growth performance, Hemato-immunological responses and skin mucus protein in *Oncorhynchus mykiss*. A total of 360 fish (with an average weight of 10.17 ± 0.18 g) were acclimated to the experimental environment and distributed across 12 tanks (four groups in triplicate), maintaining a density of 30 fish per tank. Various concentrations of *M. longifolia* hydroalcoholic extract (0, 1, 2, and 3 g/kg) were incorporated into a basal diet. Following a 60-day feeding trial, the fish were challenged with *Saprolegnia parasitica*. All fish administered *M. longifolia* exhibited significant improvements in specific growth rate and weight gain, alongside a notable reduction in feed conversion ratio after compared to the control treatment. Moreover, the counts of red and white blood cells, serum respiratory burst activity, albumin levels, and total protein were significantly higher in the *M. longifolia* group in evaluation to the control group. Fish in the treatments receiving *M. longifolia* extract demonstrated a significant increase in survival rate following exposure to *S. parasitica* compared to the control group. Based on these findings, the incorporation of *M. longifolia* hydroalcoholic extract at a dosage of 1 g/kg in the diet of rainbow trout is recommended to enhance immune response, promote growth performance, and improve resistance to *S. parasitica*.

Keywords
Mint; Growth; Immunity; Blood; Oomycete; *Oncorhynchus mykiss*

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

Introduction

Intensive culture of Rainbow trout is associated with many stresses that predispose the fish to a variety of infectious diseases. Oomycetes are found in various freshwater ecosystems and can impact both farmed and wild fish populations [1]. Among them, *S. parasitica* is a significant species that affects numerous fish types, including salmonids, leading to a disease known as saprolegniasis, which is considered one of the most crucial oomycete-related diseases in fish. [1].

The use of chemical disinfectants is among the most common methods for controlling saprolegniasis. However, the impact of chemical usage in declined use of pesticides and harmful chemical compounds has persuaded researchers to seek for achieving more natural and eco-friendly compounds [2]. Plants have always been considered as an important source of biologically active compounds, and plant metabolites as a huge and valuable reserve can be helpful among various immune stimuli [3,4]. In-depth research on the utilization of medicinal plants for enhancing the immune system in animals confirms the beneficial effects of numerous medicinal plants in promoting both immunity and growth in these animals [5,6]. Due to the effectiveness of immunostimulants for stimulation of non-specific immune, the use of these stimuli in aquatic animals is more preferable than in warm-blooded animals [7,8].

Numerous studies have attentive on the use of medicinal plants to enhance growth and boost fish immunity, as well as their resistance to diseases [5,9]. For example, Mehrabi et al. [10] reported that aloe vera (*Aloe brabadensis*) stimulated the immune system and resistance to *S. parasitica* in rainbow trout. Increased resistance of rainbow trout against the bacterium *Yersinia ruckeri* has recently been demonstrated by feeding *Mentha longifolia* hydroalcoholic extract [11]. Mehrabi et al. [12] reported that nettle *(Urtica dioica)* powder stimulated the immune system of rainbow trout and increased fish survival in challenge with the *S. parasitica.*

M. 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, attributed to their 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 with other vertebrates, the immune system in fish includes innate and acquired immune system, with the former playing a crucial role in defence against pathogens in aquatic animals [15]. As the primary line of defence touching pathogens, mucus is a component of the innate

immune mechanism that prevents pathogens from binding due to continuous secretion and excretion of dead tissues and stimuli [16,17].

So far, no studies have been conducted on the effect of horsemint hydroalcoholic extract on fish resistance to saprolegniasis. *Mentha longifolia* contains compounds with antibacterial and antifungal properties, which help prevent microbial and fungal infections in fish [18]. Therefore, this study was conducted for the first time to investigate the effects of *M. longifolia* extract on the growth performance and resistance of rainbow trout against *S. parasitica*, as well as its impact on immune performance.

Results

Growth parameters

The results of measuring the growth parameters of trout fed diets containing *M. longifolia* extract after 60 days are presented in Table 1. The changes in the mean final weight indicate that the final weight significantly increased in all groups fed with *M. longifolia* ($P < 0.05$) compared to the control group, with the highest final mean weight observed in fish that were fed a diet containing 1 g/kg of *M. longifolia*. The same treatment gained the uppermost SGR ($P < 0.05$). Additionally, all fish fed the extract demonstrated a significantly increased specific growth rate (SGR) ($P < 0.05$) compared to the control group. The feed conversion ratio (FCR) values decreased significantly in the groups that received *M. longifolia* extract compared to the control group ($P < 0.05$), with the lowest FCR observed in the treatment group fed a diet containing 1 g/kg of the extract ($P < 0.05$). All the fish in the experimental treatments, as well as the control group, exhibited a survival rate of 100% at the end of the 60-day feeding period.

Blood parameters

As shown in Table 2, significant increases were observed in red blood cell (RBC) and white blood cell (WBC) counts, hemoglobin concentrations, and hematocrit percentages in all treatments fed with *M. longifolia* extract for 60 days compared to the control group ($P < 0.05$). Feeding with the minimum effective dose of the extract (1 g/kg of diet) resulted in significant increases in all blood parameters compared to the control group. However, the experimental treatments were not significantly different in terms of the number of RBC and WBC counts, hematocrit percentages, and hemoglobin concentrations ($P < 0.05$). Monocyte and lymphocyte counts were significantly higher in the groups fed with 0.2% and 0.3% extract of mint ($P < 0.05$). Conversely, neutrophil counts significantly decreased in the fish fed with the extract ($P < 0.05$). Additionally, mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) values significantly decreased in fish that received the extract compared to the control

group ($P < 0.05$). After challenging with *S. parasitica*, all the measured blood parameters significantly increased in the group fed with the plant extract compared to the control group ($P < 0.05$). White blood cell (WBC) counts increased with higher doses of *M. longifolia* extract, with the highest counts observed in the treatments receiving 2 g/kg and 3 g/kg of diet. These counts differed significantly from those in the control group and the group fed with 1 g/kg of the extract. ($P < 0.05$). However, no significant differences were found among the treatments fed the extract regarding red blood cell (RBC) counts, hemoglobin concentrations, and hematocrit percentages ($P > 0.05$). Furthermore, fish fed with the extract showed significant increases in lymphocyte counts, mean corpuscular hemoglobin concentration (MCHC), monocyte and neutrophil counts, as well as MCHand MCV, compared to the control group ($P < 0.05$).

Serum biochemical indices

According to the serum biochemical indices measured after 60 days of feeding with different doses of *M. longifolia* extract and following the challenge with *S. parasitica* (Table 3), albumin values and total protein levels increased significantly in all extract-fed treatments compared to the control group ($P < 0.05$). These variables were highest in fish fed with the extract at 1 g/kg diet, showing statistically significant differences from the other groups ($P < 0.05$).

Immune response of fish

As shown in Table 4, neutrophil respiratory burst, lysozyme activity, and ACH50 of Serum levels in rainbow trout showed significant increases related to the control treatment ($P < 0.05$) after 60 days of feeding with *M. longifolia*. However, there were no notable differences between the extract-fed treatments concerning lysozyme activity and ACH50 ($P < 0.05$). The neutrophil respiratory burst was maximized in the 1 g/kg treatment, showing significant differences from the other groups ($P < 0.05$). Feeding with the extract at minimum effective dose (1 g/kg of diet) resulted in significant rises of all immune factors as opposed to control fish. After the experimental challenge with *S. parasitica*, all measured immune factors significantly increased in fish fed with *M. longifolia* extract at all three levels compared to the control ($P < 0.05$). The highest neutrophil respiratory burst was observed in the treatment group that received the extract at 2 g/kg of diet, which differed significantly from the other groups ($P < 0.05$).

Mucus protein pattern

Figure 1 illustrates the protein profile of skin mucus in rainbow trout fed M. longifolia hydroalcoholic extract for 60 days, along with observations made 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 ranging from 17 to 75 kDa (Kilodalton). 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. Following the fungal challenge, the count and density of protein bands in the groups fed the extract remained stable compared to the pre-challenge assessment, whereas a significant reduction was observed in the control group.Overall, the number and density of bands in the fish that received the extract were greater than those in the control group. After 15 days of exposure to *S. parasitica*, the survival rates of fish (Fig 2) showed a significant increase in groups that received *M. longifolia* extract compared to the control group ($p < 0.05$). However, there were no significant differences in survival rates among the groups fed with *M. longifolia* after the challenge with *S. parasitica* ($p < 0.05$).

Discussion

Nowadays, immune system stimulants are generally used in the aquaculture industry to improve and stimulate the activity of non-specific immune system in fish [19,20]. Meanwhile, the use of plants to control pathogens by stimulating the immune system and antioxidant activity has received further attention on behalf of researchers [5,8,9].

In the current investigation, administering the lowest dose (1 g/kg) of *M. longifolia* extract to fish resulted in significant differences in specific growth rate (SGR), weight gain, and feed conversion ratio (FCR) values, as well as with other treatments containing *M. longifolia* when compared to the control group. Several studies have documented the effects of plant extracts on various growth parameters in fish. For example, positive effects of *M. longifolia* as a growth stimulant were observed in a significant weight increase in feeding Caspian kutum (*Rutilus frisii kutum*) with 1 g/kg *M. longifolia* in comparison to the control [21]. Raissy et al. [22] observations indicated that a combination of medicinal plants (*Mentha longifolia*, *Thymus carmanicus*, and *Trachyspermum copticum*) can be used to enhance the weight gain and FCR of rainbow trout for 45 days. Ghanbary et al. [23] investigated the effect of incorporating *Thymbra spicata* hydroalcoholic extract into the diet of rainbow trout over an 8-week period and observed a significant increase in final weight. 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 fish fed 5 g/kg of dietary nettle showed significant enhancements in specific growth rate (SGR) and final growth, along with a reduction in feed conversion ratio (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]. The study found that dietary levels of M. longifolia extract improved blood parameters such as RBCs, WBCs, hemoglobin, and hematocrit. All experimental treatments exhibited a significant increasing trend in these parameters compared to the control group. It is likely that vitamin C in *M. longifolia* can increase intestinal absorption of vitamins and minerals from consumption of this plant and lead to improvement in blood parameters of fish [26]. Feeding rainbow trout with diets containing *M. longifolia* [11], common mallow (*Malvae sylvestris)* [27], and *Thymbra spicata* [23] resulted in improvements in hematological parameters similar to the present research. Moreover, positive effects of *Aloe barbadensis* as an immunostimulants were observed in significant increases of RBC count and hemoglobin concentrations in experimental groups of rainbow trout compared with the control group [10]. In another study, feeding rainbow trout with *Urtica dioica*, extract for 4 weeks led to significant elevations of WBC and RBC counts [12]. White blood cells (WBCs), along with biochemical factors such as lysozyme, serum proteins, and alternative complement hemolytic activity (ACH50), are crucial components of the fish innate immune system, particularly 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]. An increase in serum protein levels indicates an elevation in the non-specific immune stimulation, and in fact in the 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 increased significantly lysozyme activity in rainbow trout [3]. Also, feeding rainbow trout with a diet containing *Stachys lavandulifolia* and greek juniper (*Juniperus excelsa*) resulted in

elevated lysozyme activity [33,34], as was also observed here. Phagocytic cells are able to produce 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, the assessment of leukocyte respiratory burst after 60 days of feeding indicated a significant increase 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. 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]. Our observations indicated that the inclusion of *M. longifolia* hydroalcoholic extract in the diet of rainbow trout led to a significant increase in complement system activity by the end of the rearing period. 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 relative to the control group.

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. Our findings demonstrated that both the number and density of bands significantly increased in the skin mucus of fish fed the extract compared to the control group. Additionally, these fish exhibited the highest number and density of bands following the fungal challenge when compared to the control group.

Heydari et al. [11] recently studied the impact of hydroalcoholic extract of *M. longifolia* on the protein patterns of skin mucus in rainbow trout. Fish fed a diet containing 3% plant extract for 30 days exhibited the highest concentration and number of bands in their skin mucus, showing

significant differences compared to the control treatment.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. Our results of fish challenge with *S. parasitica* indicated a significantly increased relative survival rate of fish in various treatments receiving *M. longifolia* hydroalcoholic extract. The results indicated that incorporating *M. longifolia* extract into the diet of rainbow trout could enhance the immune system by increasing blood and serum immunity factors, subsequently improving the fish's resistance to *S. parasitica*. 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 group [8], which aligns with the present results.

Conclusions

Medicinal plants are acknowledged as a viable alternative to pharmaceutical compounds for enhancing health and promoting fish growth in the aquaculture industry. Overall, the results of this study suggest that incorporating *M. longifolia* hydroalcoholic extract into the diet of *O. mykiss* can positively influence 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

In this study, 2 kg of fresh *M. longifolia* leaves 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 plant material was dried at room temperature (25 °C) in the shade and subsequently ground using a mill. Following this, the hydroalcoholic extract of *M. longifolia* was prepared according to the method described by Heydari et al. [11].

Diet preparation

In order to prepare diet, the plant hydroalcoholic extracts were first dissolved and homogenized in 6 cc of ethanol according to the amount of each treatment (1, 2, and 3 g of *M. longifolia* per kg diet) and then sprayed on a commercial diet (Bayza Company, Fars, Iran) formulated for rainbow trout. An extract-free diet containing only 6 cc of the solvent was supplemented to the diet of the control group [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 application.

Experimental fish

All examinations were conducted in accordance with standard ethical guidelines, protocols, and the approval (No.1274320) obtained from the Animal Ethics Committee of the Sari Agricultural Sciences and Natural Resources University. In the present study, rainbow trout were obtained from a farm in Sari and clinically assessed 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 was performed with four treatments of diets containing 1, 2, and 3 g of *M. longifolia* hydroalcoholic extract per kg, and an extract-free control treatment, all with three replications each containing 30 juvenile fish (10.17 ± 0.18 g, 10.27 ± 0.63 cm) in 12 tanks of 250 L. The fish were fed three times a day at 8:00, 12:00, and 16:00, with the feed amounting to 3% of their body weight [10,4038] for a duration of 60 days. Water was supplied from a well for the culture of juvenile fish. All tanks were oxygenated using a central aeration system. The physicochemical parameters of the water were measured daily and maintained at the following levels: 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 siphoned out and replaced daily.

Challenge with *S. parasitica*

Pure *S. parasitica* (accession number KC992717) was obtained from Sari Agricultural Sciences and Natural Resources University (SANRU) and used to perform the challenge test. First, the oomycete was grown in a solid culture medium (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 for sporogenesis 18-20 °C for one week. The contents of the tubes were centrifuged at 3000 rpm for 10 minutes to separate the zoospores, which were subsequently counted using a hemocytometer slide [41]. At the conclusion of the experimental period, the fish were challenged with *S. parasitica*. To perform challenge test, 30 fish were selected from each 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 3×10^5 zoospores per liter for 8 hours to undergo the bath. [12]. Fungal-infected fish were confirmed by direct microscopic testing.

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 measured using a digital scale with accuracies of 1 mm and 0.01 g, respectively. Fish growth indices, specifically mean weight gain (WG), feed conversion ratio (FCR), specific growth rate (SGR), and survival rate (% SR), were evaluated according to the following formulas:

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 challenge with *S. parasitica*, blood samples were collected from the peduncles of 12 fish (4 per replicate) to evaluate blood indices. The fish were starved for 24 hours prior to each blood sampling. Part of the blood sample was infused into tubes containing heparin (500 U heparin/ml) to measure blood indices [11]. Hematocrit and hemoglobin were measured using the microhematocrit [42] and the cyan methemoglobin [43] methods, respectively. After

dilution with a Natt-Herrick solution, red blood cells (RBCs) and white blood cells (WBCs) were counted using a hemocytometer [42]. The remaining blood was transferred to heparin-free tubes, and after centrifugation, the separated serum was stored at −20 °C until further analysis.

Lysozyme levels were measured through turbidimetry at 450 nm [44]. Alternative complement pathway (ACH50) was assessed with photometric method at 414 nm [45]. Neutrophil respiratory burst was determined by the nitroblue tetrazolium (NBT) index [46]. Whole protein and albumin were quantified respectively using commercial kits (Parsa Azmoon) and an Autolyser (bs120, Manda, China).

Collection of mucus

Fish mucus were collected as described by Subramanian et al. [16]. At the finish of 60 days of feeding with mint extract and 15 days after the challenge with *S. parasitic*, 12 specimens were sampled from each group, anesthetized with triacaine methanesulfonate (MS222) [41] and placed in cold, clean water to minimize body surface bacteria and eliminate other contaminants. The fish were then placed separately in plastic bags containing 10 mL of 50 mM sodium chloride for 2 minutes. The mucus collected from the bags was stored at −80 °C until further experiments [11].

Protein profile

The protein pattern was analyzed using the SDS-PAGE technique [47]. Mucus samples were combined with a sample buffer consisting of 4% SDS, 5 mM tris hydrochloric acid, 2% mercaptoethanol, 12% glycerol, and 5% bromophenol blue in a 4:1 ratio, and heated at 95 °C for 5 minutes. Following this, the mixture was centrifuged at 1000 rpm for 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 conducted at 120 V until the bromophenol blue indicator migrated past the stacking gel, after which the voltage was increased to 200 V and maintained for 7 hours using a 5X electrophoresis buffer. To visualize the protein bands, the gels were stained with 5% Coomassie Blue (250 mg) for 5 hours, followed by a destaining process using Coomassie Blue solution in two steps over a period of 2 hours.

Statistical analysis

Data for the factors were analyzed using SPSS statistical software version 23. Normality of the data was assessed using the Kolmogorov-Smirnov test. A one-way analysis of variance (ANOVA) was conducted to evaluate variations between groups, and the Tukey test was applied to identify significant differences among the treatments at a significance level of 0.05.

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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}	100^{a}
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}	100^{a}
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}	100^{a}
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}	100^{a}

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 (×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 (×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}
Hemoglobin (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}
Hematocrit (%)	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}
Lymphocyte (%)	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}
Neutrophil (%)	22.10±*0.82*^{b}	13.25±*0.17*^{a}	11.18±*0.41*^{a}	11.01±*0. 74*^{a}	28.66±*0. 49*^{c}	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±*0.79*^{b}	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).

Table 3

The biochemical 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	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}	1.88±*0.49*^{b}
	3 g/kg	3.41±*0.21*^{b}	1.93±*0.39*^{b}

In each column, 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±*0.25*^{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}
Post-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 ($P < 0.05$).

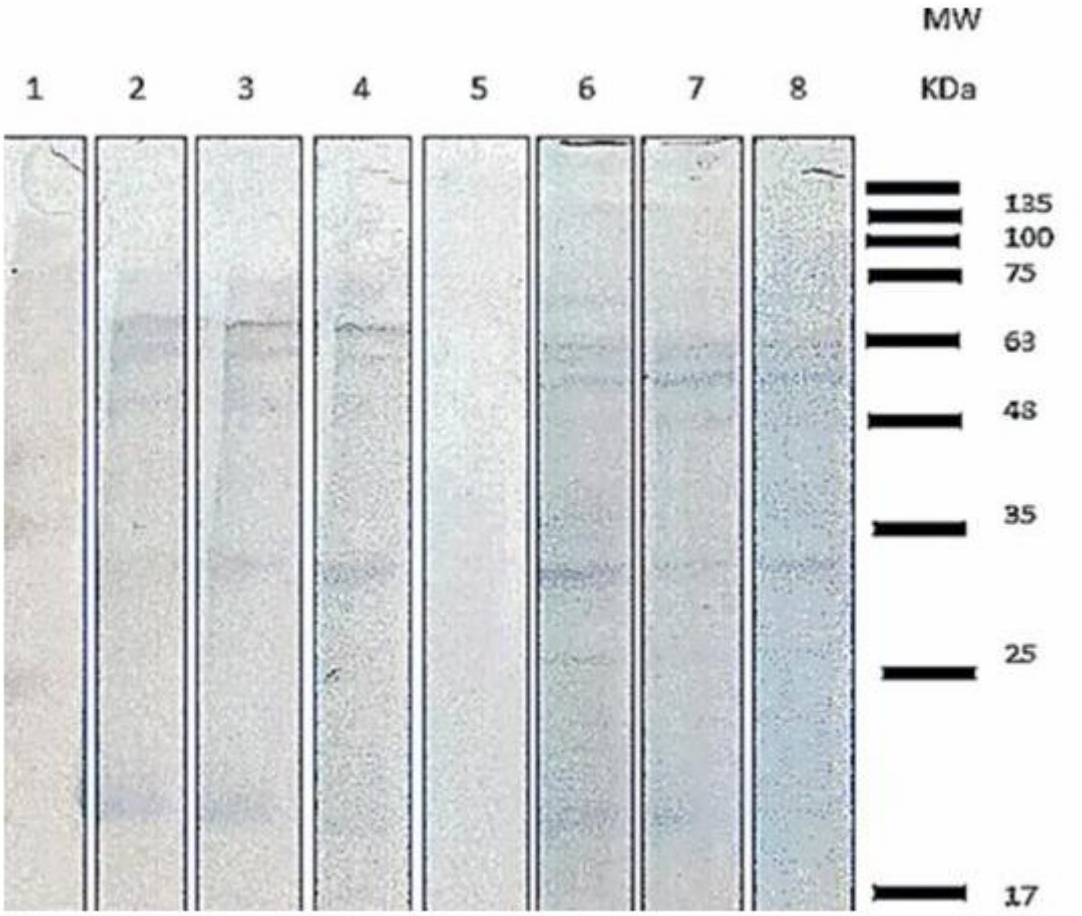

Fig.1 Mucus protein pattern of rainbow trout after feeding with *M. longifolia* for 60 days (pre-challenge) and 15 days after challenging with *S. parasitica* (post-challenge)

(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%)

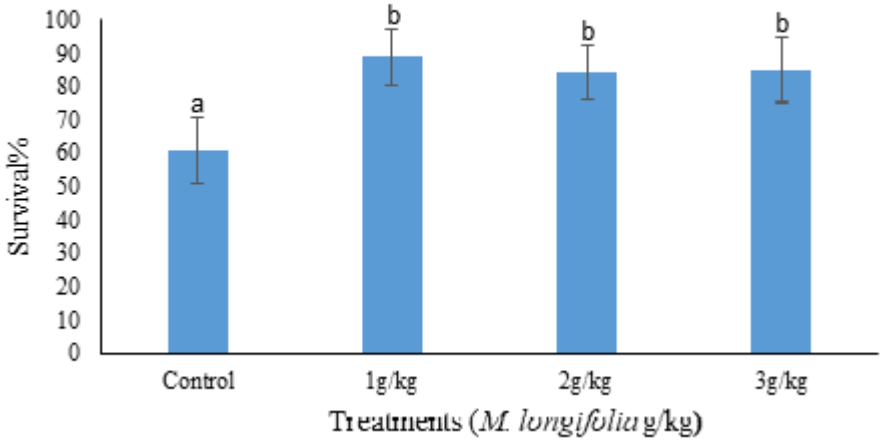

Fig 2. Survival rate of rainbow trout fed with different levels of *M. longifolia* after 15 days of challenging with *S. parasitica.*

the values with different superscript letters are significantly different (P < 0.05).