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Dietary Myrtle (*Myrtus communis* L.) improved non-specific immune parameters and bactericidal activity of skin mucus in rainbow trout (*Oncorhynchus mykiss*) fingerlings

Running head: Myrtle effects on mucosal immune response

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Summary

The present study examined the effects of dietary Myrtle (*Myrtus communis* L.) on non-specific immune parameters and bactericidal activity of skin mucus in rainbow trout (*Oncorhynchus mykiss*) fingerlings. Three hundred and sixty fingerlings (6.50 ± 0.55 g) were distributed in twelve cages (65 × 65 × 65 cm) with a metal framework. The study included four treatments repeated in triplicates. The treatments were feeding trouts with experimental diets containing different levels (0, 0.5, 1 and 1.5%) of Myrtle powder. The fingerlings were fed on experimental diet for sixty days and then skin mucus non-specific immune parameters as well as bactericidal activity were measured. At the end of the trial, the highest skin mucus soluble protein level was observed in group fed with 1.5% Myrtle ($P < 0.05$). The alkaline phosphatase (ALP) activity was significantly increased in fish groups fed 1 and 1.5% Myrtle compared with the control group ($P < 0.05$). However, evaluation of skin mucus lysozyme activity showed no significant difference between treatments and control group ($P > 0.05$). Also, no antibacterial activity was detected against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella enterica* in all treatments and control group. Whereas skin mucus of rainbow trout showed antimicrobial activity against fish pathogens (*Aeromonas hydrophila* and *Yersinia ruckeri*) in 1 and 1.5% Myrtle treatments. These results indicated beneficial effects of dietary Myrtle on mucosal immune parameters of fingerling rainbow trout.

Keywords: Rainbow trout, Myrtle, mucosal immunity, non-specific immunity, Skin, Bactericidal activity

Introduction

In the aquatic environment, fish consistently faces with pathogenic and non-pathogenic microorganisms. The innate immune system provides required defence against those harmful

organisms and contributes to host survival [1]. A key part of nonspecific immune defenses in fish is the mucus layer on the skin, secreted by epidermal goblet cells, that is the first line of defense against invading pathogens [2-5]. Epidermal mucus contains widely varied biologically active substances that playing important roles in inhibiting the pathogens entrance [6].

The intensive production systems exposes cultured organism to stress which may consequently elevates the risk of disease outbreak [7]. The application of immunostimulants in diet has been suggested as a promising mean for improving the health status and disease prevention [8]. The usefulness of immunostimulants has been demonstrated in modern aquaculture. There is a wide range of immunostimulants such as vitamins, microorganisms, animal and plant extracts, by-products of other industries, etc. [9]. The medicinal plants and their derivate classified as environment friendly immunostimulants which are cost-effective and without any side-effects [10]. Myrtle (*Myrtus communis* L.) is an important medicinal plants native to Mediterranean regions and Middle East especially in Iran. It belongs to Myrtaceae family (Myrtales order) and in literatures many medicinal properties attributed to this plant [11, 12]. Myrtle leaves contain various chemical and polyphenolic compounds such as, flavonoids, tannins, saponins, vitamin C and essential oils [13,14]. Previous studies revealed antimicrobial and antibacterial [15-19], antiviral [20, 21], antifungal [22-24] and antioxidant properties [19, 25-28] of Myrtle. This medicinal plant reported to be safe and without harmful and toxic effects [29-32].

It has been reported that administration of herbal dietary supplements such as Garlic [33], Peppermint (*Mentha piperita*) [34] and palm fruit [35] improved the mucosal immune responses of Caspian roach (*Rutilus rutilus*), Caspian white fish (*Rutilus frisii kutum*) and common carp (*Cyprinus carpio*), respectively. However, to the best of our knowledge there is no information on the effects of *M. communis* on the fish mucosal parameters. Thus, the aim of this study was

69 determination of the effects of dietary administration of Myrtle on some epidermal mucosal
70 immunity parameters in the rainbow trout (*Oncorhynchus mykiss*) fingerling.

71 **1. Materials and methods**

72 **2.1. Fish and experimental diets**

73 Rainbow trout (*Oncorhynchus mykiss*) fingerling weighing 6.50±0.55g obtained from a fish farm
74 in Lorestan, Iran. The fishes were allowed to acclimatize for two weeks prior to experiment.
75 During the acclimatization period, fish were fed five times a day with a commercial food
76 (Faradaneh, Iran). The analysis of this commercial diet is given in Table 1. Thereafter, the fishes
77 were randomly stocked into 12 square cage (65 × 65 × 65 cm) with a metal framework at a
78 density of 30 fish per cage. Cages were kept in a Raceway pond (30×3×2 m) with the flow
79 through system. The average temperature, dissolved oxygen level and pH were 15 ± 1.2 °C, 6.4
80 ± 0.1 mg L^{-1} and 7.7 ± 0.2, respectively.

81 *M. communis* samples were supplied, dried at room temperature and then powdered as suggested
82 elsewhere [55-56]. The obtained powder kept at 4^{o}C until preparation of experimental diets. For
83 preparation of experimental diets, a commercial diet (Table 1) was considered as basal diet
84 (control diet, 0% Myrtle) and three experimental diets were prepared by supplementation of the
85 basal diet with three levels (0.5, 1 and 1.5%) of Myrtle. Experimental diets were kept in plastic
86 bags at -4 °C until used. During the feeding trial (60 days), fish were hand-fed (3.8 % of body
87 weight) four times a day. The feeding ration was corrected every 2 weeks following a 24-h
88 starvation period and batch weighing.

89 **2.2. Skin mucus collection**

90 Fish were starved for 24 h and then six specimens were randomly selected from each cage. Prior
91 to collection of skin mucus after Ross et al. [36] fish were anesthetized with 5 mg l^{-1} clove

92 powder. Polyethylene bags contain 10 ml NaCl (50 mM) were prepared and sampled fish were
93 transferred to bags. Fish were removed after 2 min, the obtained mucus samples were transferred
94 to 15-ml sterile tubes and centrifuged at 1500×g for 10 min at 4°C. The supernatants were kept at
95 -80 °C until use.

96 **2.2. Skin mucus protein and enzyme activities**

97 The mucus total protein was determined using bovine serum albumin as standard according to
98 the method of Lowry et al. [37]. The absorbance was read using a spectrophotometer (Biochrom,
99 Libra S12) at 750 nm. Alkaline phosphatase (ALP) was estimated by using the Ziest Chem
100 Diagnostics kit (Tehran Company, Iran). The absorption read at 405 nm in a spectrophotometer
101 and ALP activity was calculated according to the manufacturer protocol [38]. Lysozyme activity
102 in mucus samples of rainbow trout was determined using the method described by Ellis [39] with
103 some modifications. Briefly, the suspension of lysozyme sensitive bacterium (*Micrococcus*
104 *luteus*) prepared in 0.05 M sodium phosphate buffer (pH 6.2). 50 mL of skin mucus samples
105 were added to 2 ml of a suspension. Then, after three min the absorbance was read by a
106 spectrophotometer (Biophotometer Eppendorf). The decrease in absorbance of 0.001 min^{-1} was
107 considered as one unit of lysozyme activity.

108 **2.4. Skin mucus antibacterial activity**

109 The antimicrobial activities of rainbow trout mucus were evaluated against five bacterial strains
110 including two common fish pathogens [*Aeromonas hydrophila* (ATCC 7966) and *Yersinia*
111 *ruckeri* (BCCM5/LMG3279)] and three other bacteria [*Escherichia coli* (ATCC 1554),
112 *Staphylococcus aureus* (ATCC 1113) and *Salmonella enterica* (ATCC)]. The later were selected
113 as they were used in previous studies on fish skin mucus antibacterial activity. The antibacterial
114 activity was determined using the standard disc diffusion method described by Hellio et al. [40].

The bacteria were cultured in nutrient broth medium overnight (at 37 °C) and then 0.1 ml of broth culture (1.5×10^8 CFU ml^{-1}) was cultured on nutrient agar. Then, paper discs (6 mm) contain 100 µl mucus were placed on the medium and incubated overnight (at 37 °C). The growth inhibition zone was calculated based on visual observation and by using a Caliper.

For minimum inhibitory concentration (MIC), a serial dilution of the mucus samples were prepared in well as suggested elsewhere [40]. 1.5×10^8 CFU ml^{-1} bacteria were added to wells and incubated overnight at 37 °C. The MIC of samples was determined by visual observation [40].

2.5. Statistical analysis

After checking the normality of data and homogeneity of variance, one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests was used for data analysis. Mean values were considered significantly different at $P < 0.05$. All analyses were performed using statistical software SPSS (version, 17) and Excel.

3. Results

Regarding skin mucus protein level, a Myrtle dose-dependent increase was observed. The highest increments of protein level was noticed in those fish fed 1.5% Myrtle enriched diets ($P < 0.05$) (Figure 1). Also, the ALP activity significantly increased ($P < 0.05$) in fish fed 1 and 1.5% Myrtle compared with the control group. In addition, the highest ALP level was observed in the 1.5% Myrtle group (Figure 3). No significant difference ($P>0.05$) was observed between treatments and control group at the end of the 60 days (Figure 2) in case of skin mucus lysozyme activity.

The antibacterial activity against *E. coli*, *S. aureus* and *S. enterica* was not observed in all treatments and control group. Whereas skin mucus of 1 and 1.5% Myrtle fed rainbow trout showed antimicrobial activity against *A. hydrophila* and *Y. ruckeri* pathogens (Tables 2). Also, the antibacterial activity against *A. hydrophila* was significantly higher in fish fed 1.5% Myrtle compared to those in 1% Myrtle treatment ($P < 0.05$). In case of MIC of skin mucus similar results obtained as mentioned above in case of antibacterial activity (Table 3).

4. Discussion

This study is the first attempt to investigate the effects of dietary Myrtle on mucosal immune parameters of rainbow trout fingerling. Our results indicated that *Myrtus communis* as feed additive increased skin mucus soluble protein level; the highest increments in fish fed 1.5% Myrtle. Similar to the present study, the increase of skin mucus protein levels were recorded in Caspian white fish (*Rutilus kutum*) fed peppermint (*Mentha piperita*) [34], Caspian roach (*Rutilus rutilus*) fed garlic (*Allium sativum*) [33] and Porthole livebearer (*Poecilopsis gracilis*) fed commercial probiotic (*Lactobacillus casei*) [41]. It has been suggested that the increase of soluble protein level can be considered as a sign of mucus secretion which is one of the defence mechanism [42]. In the present study, the elevation of skin mucus protein levels possibly indicates 1.5% Myrtle supplementation in the diet enhance this defense mechanisms in rainbow trout. However, determination of the exact mode of action merit further research.

Alkaline phosphatase is an important lysozomal enzyme which acts as an antibacterial agent and plays protective roles in fish during wound healing, parasitic infection and stress [6, 43, 44]. Our results showed that rainbow trouts fed 1 and 1.5% Myrtle exhibited significant increase in alkaline phosphatase activity compared with the control group. These finding was in agreements with those results reported by Adel et al. [34] and Salmanian-Ghahderijani et al. [33]. The

increase in protein levels and ALP activity along with the elevation of Myrtle supplementation level can be due to elevation of mucus secretion or stimulation of mucosal immune responses [45].

Among antimicrobial substances in mucus, lysozyme is a ubiquitous bactericidal enzyme which is responsible for destroying pathogens through cleaves the glycosidic bonds of the peptidoglycan layer bacteria. Besides, lysozyme also responsible for activation of other important defense molecules include the complement system and phagocytic cells [46-48].

The present results, revealed that feeding on Myrtle supplemented diet had no remarkable effects on skin mucus lysozyme activity. There is no studies regarding the effects of medicinal herbs as immunostimulant on mucus lysozyme activity. However, in contrast with present results, feeding rainbow trout and common carp with Ergosan and palm fruit significantly increased skin mucus lysozyme activity compared to the control group [1, 35]. It has been demonstrated that lysozyme activity to significantly vary depending on many environmental (such as water temperature, pH, photoperiod, season and toxins) and intrinsic (including size, age, sex, infections and stressors) parameters [49-51].

Evaluation of skin mucus in several fish species revealed antibacterial activity against different types of Gram-positive and Gram-negative bacteria [52]. Likewise, antimicrobial activity has been reported for skin mucus of rainbow trout [53]. In the present study, antibacterial activity against the fish pathogen *A. hydrophila* and *Y. ruckeri* was observed in 1 and 1.5% Myrtle treatments but no activity were observed against *E. coli*, *S. aureus* and *S. enterica*. These results were in agreement with previous findings where aqueous and crude extract of Snakehead fish (*Channa striatus*) skin mucus had no antimicrobial activity against the human pathogens [54]. Also, in accordance with present finding, the antibacterial activity of fish skin mucus against *Y.*

ruckeri has been demonstrated in Ergosan fed rainbow trout [1]. Furthermore, in agreement with our results, antimicrobial activity of Caspian white fish skin mucus against several species of bacteria such as *Y. ruckeri* increased along with elevation of peppermint (*Mentha piperita*) inclusion level [34]. These findings denote elevation of components with bactericidal properties (e.g. immunoglobulin, complement, lysozyme and lectin) in fish skin mucus following dietary administration immunostimulants [53].

In conclusion, the present results revealed that the dietary administration of Myrtle powder in rainbow trout fingerlings may led to enhanced mucosal immune response. This study encourages extensive research for determination of Myrtle as immunostimulants in aquaculture with special focus on determination of mode of action.

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347

Table 1. Analysis of the commercial feed for rainbow trout (Faradaneh, Iran- SFT)

Analysis (%)	
Moisture	11
Crude protein	46
Total lipid	14
Ash	10
Fiber	3

Table 2. The antibacterial activity of the skin mucus (growth inhibition zone diameter [mm]) of rainbow trout fed diets enriched with different levels of *Myrtus communis* (0% [control], 0.5%, 1% and 1.5%) for 60 days. Values are presented as the mean ± SD. Values in a row with different superscripts denote significant difference (P<0.05).

	Control	0.5% M	1% M	1.5% M
A. hydrophila	-	-	8.58±0.03^{b}	10.08±1.04^{a}
Y. ruckeri	-	-	8.22±0.28^{a}	8.25±0.04^{a}

Table 3. The Minimum inhibitory concentration (µl ml^{-1}) of skin mucus of rainbow trout fed diets enriched with different levels of *Myrtus communis* (0% [control], 0.5%, 1% and 1.5%) for 60 days.

	Control	0.5% M	1% M	1.5% M
A. hydrophila	>200	>200	200	100
Y. ruckeri	>200	>200	50	50

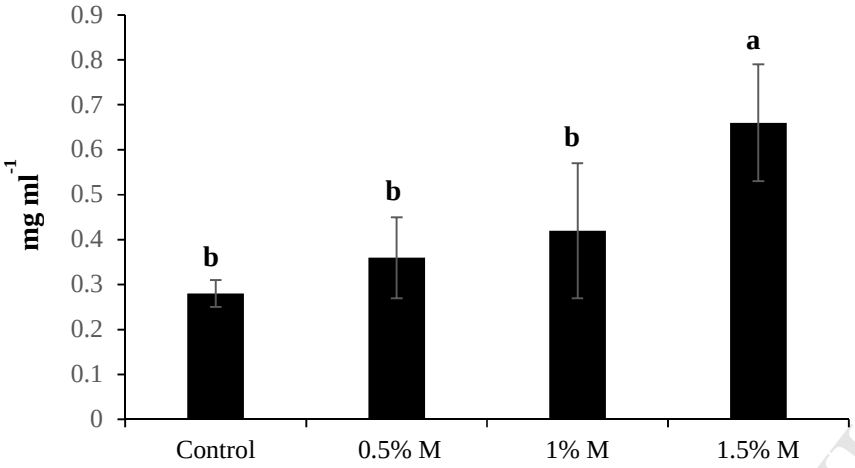

Fig. 1. The skin mucus total protein levels (mg ml^{-1}) of rainbow trout fed diets enriched with different levels of *Myrtus communis* (0% [control], 0.5%, 1% and 1.5%) for 60 days. Values are presented as the mean ± S.D. Values in a row with different superscripts denote significant difference ($P<0.05$).

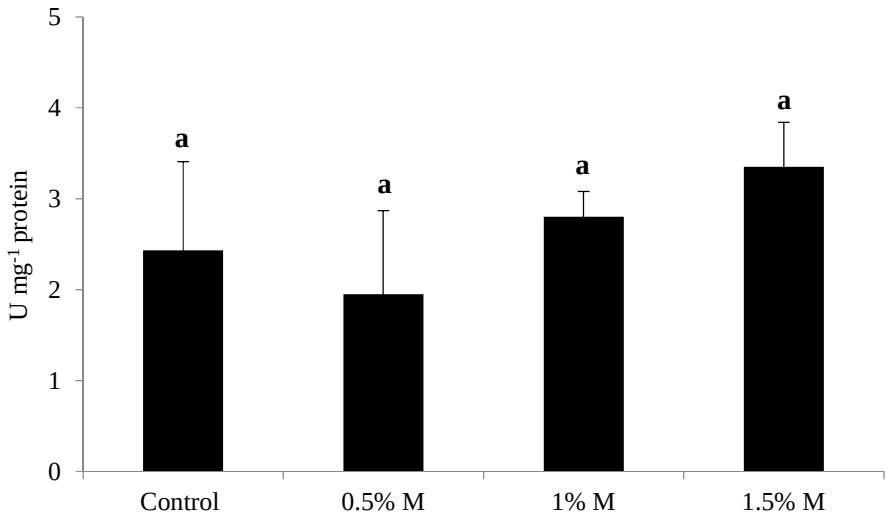

Fig. 2. The skin mucus lysozyme activity of rainbow trout fed diets enriched with different levels of *Myrtus communis* (0% [control], 0.5%, 1% and 1.5%) for 60 days. Values are presented as the mean ± S.D. Values in a row with different superscripts denote significant difference ($P<0.05$).

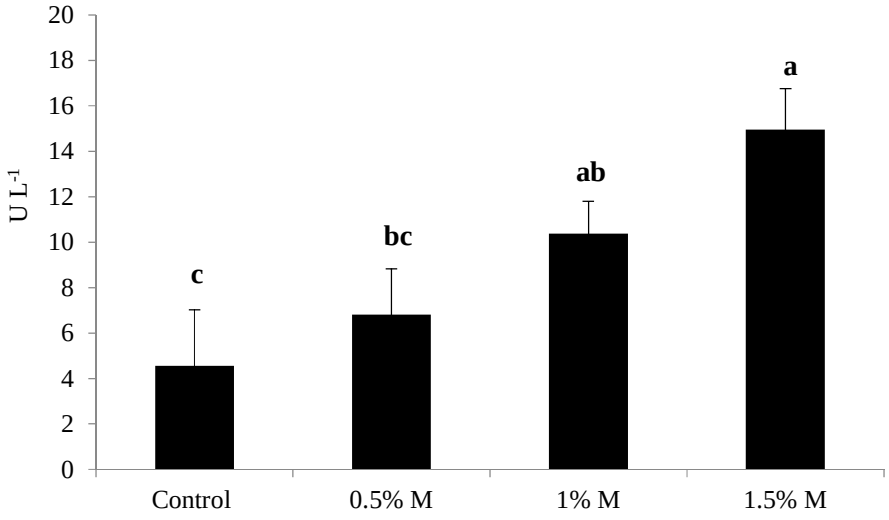

Fig. 3. The skin mucus alkaline phosphatase activity of rainbow trout fed diets enriched with different levels of *Myrtus communis* (0% [control], 0.5%, 1% and 1.5%) for 60 days. Values are presented as the mean ± S.D. Values in a row with different superscripts denote significant difference (P<0.05).

- Dietary Myrtle increased mucosal immune parameters in Rainbow trout
- Myrtle powder fed Rainbow trout showed increased skin mucus antimicrobial activity.
- Feeding on Myrtle powder supplemented diet had no effects on skin mucus lysozyme activity