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Original Article

The effects of dietary Myrtle (*Myrtus communis* L.) supplementations on growth performance and some innate immune responses in rainbow trout (*Oncorhynchus mykiss*)

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Abstract: This study investigated the effect of dietary Myrtle (*Myrtus communis* L.) powder on the growth performance, immune responses and haematological parameters of rainbow trout fingerlings. Twelve cages were assigned to four treatments in triplicate and thirty fish (6.50±0.55 g) were stocked. Treatments were different levels 0 (control), 0.5 ($M_{0.5}$), 1 (M_1) and 1.5% ($M_{1.5}$) of Myrtle powder. The highest weight gain and specific growth rate and lowest feed conversion ratio were observed in fish fed with 1% Myrtle. The RBC, WBC and haematocrit were higher in the Myrtle fed treatments compared to the control group. The results showed increased total protein in $M_{1.5}$ and M_1 treatments compared to control and $M_{0.5}$ treatment. Also, the highest and lowest albumin were observed in $M_{1.5}$ treatment and control group. Furthermore, serum ALP decreased along with the increasing Myrtle levels in diet, and lowest level was observed in M_1 treatment. Lysozyme activity increased with increasing dietary Myrtle inclusion levels, although no significant difference was noticed when compared with control. These results revealed the potential growth enhancing and health promoting effects of Myrtle powder.

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Introduction

Rainbow trout, *Oncorhynchus mykiss*, is one of the most important fish species that commercially farmed in many countries (FAO, 2009). This species is cultured in both semi-intensive or intensive systems. Although these systems increase the production level, at the same time can cause stressful condition which negatively affect immune system and elevate the risk of disease outbreak (Mona et al., 2015; Hoseinifar et al., 2015; Dawood and Koshio, 2016). A practical approach for resolving the issue, especially in anti-biotic free aquaculture, is by potentially stimulating the immune system of the host animal by the inclusion of natural immunostimulants (Barman et al., 2013; Wang et al., 2016). Animal or plant originated immunostimulants contain various bioactive compounds which can elevate immune responses and disease resistance (Bairwa et al., 2012). To date, many studies have focused on the administration of dietary medicinal herbs and their extracts as potential immunstimulants in aquaculture (Asadi et al., 2012; Haghighi et al., 2014; Reverter et al., 2014; Hai, 2015; Adel et al., 2015; Akrami et al., 2015; Sonmez et al., 2015; Safari et al., 2016; Hoseinifar et al., 2016) and positive results reported on growth performance, feed utilization, immune response and disease resistance of various fish species.

Myrtle (*Myrtus communis* L.) belongs to Myrtaceae family and Myrtales has been considered as beneficial medicinal plants in Mediterranean regions and Middle East (Amensour et al., 2009; Asgarpanah and Ariamanesh, 2015). The foliage of this medicinal plant contains different types of biologically active compounds like flavonoids, tannins, saponins, vitamin C and essential oils (Martin et al., 1990; Yoshimura et al., 2008; Habiballah et al., 2014). In two studies, the positive effects of Myrtle powder on skin mucus immune parameters in rainbow

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trout and zebrafish (*Danio rerio*) has been shown (Mansouri Taee et al., 2017; Safari et al., 2017). In another experiment, the effect of savory and Myrtle essential oils on growth performance, hematological and biochemical parameters in rainbow trout has been studied (Mohamadi Saei et al., 2016). There are no study to reveal the effects of powder Myrtle on some innate immune parameters in fish serum. Therefore, the present study was performed to investigate the effects of dietary administration of *M. communis* powder as immunostimulant on growth, Hematological parameters and immune responses of rainbow trout fingerlings.

Materials and Methods

Fish and experimental diets: Three hundred and sixty healthy rainbow trout fingerling were purchased and transferred to a selected farm located in Lorestan Province, Iran. The fishes were acclimatized to experimental condition for two weeks. After this period, fish (6.50±0.55 g) were randomly stocked into 12 square cages (65×65×65 cm) and assigned to their respective experimental treatments in triplicate. Each replicate consisted of 30 fish and the cages were kept in a raceway pond (30×3×2 m) with the flow through water system. During the trial, the water temperature, dissolved oxygen and pH were 15±1.2°C, 6.4±0.1 $mg.L^{-1}$ and 7.7±0.2, respectively.

Prior to preparation of the experimental diet, Myrtle herb was supplied, dried and powdered as described by Safari et al. (2016). Then, a commercial diet (Faradaneh, Iran) formulated for trout fingerlings (Table 1) was considered as basal diet and experimental diet were prepared by supplementation of basal diet with different levels (0.5, 1 and 1.5%) of Myrtle powder after. The prepared diets were kept in plastic bags at -4°C until used. The feeding rate was 3.8% of body weight which corrected every 2 week after biometry.

Growth Performance and survival rate: To measure growth performance, weight and length of 20 fish in each tank was monitored once every 15 days following a 12 hrs starvation until the end of experiment. Growth performance and survival rate of the fingerlings were calculated using the following formula (Mohamadi Saei et al., 2016):

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

Analysis	
Moisture	11%
Crude protein	46%
Total lipid	14%
Ash	10%
Phosphorus	1.2%
Fiber	3%

FCR (Feed conversion ratio) = $F/(Bt - B_0)$

SGR% d^{-1} (Specific growth rate) = $(Ln\ Wt - Ln\ W_0) \times 100/t$

CF or K (Condition factor) = $100 \times (Wt/TL^3)$

GBM (Gain of body mass) = $Wt - W_0$

Survival rate = $100 \times (Nt/N_0)$.

Where: F: relative food intake (g), Bt and B_0: final and initial fish biomass (g); Wt and W_0: final and initial body weight (g); t: time of rearing (days); TL: total length; Nt and N_0: final and initial fish number.

Sample collection: Five specimens were randomly selected from each cage and anesthetized with clove solution for blood sampling. The blood samples were obtained from the caudal vein and divided into two parts. For haematological parameters, the samples were transferred to heparinized tubes and the remainings to non-heparinized tubes for serum isolation. The samples were centrifuged (3000 g for 15 min) and the obtained serums were stored at -80°C until use (Akrami et al., 2015).

Hematological Parameters: Red blood cells and white blood cells were counted using a Neubauer haemocytometer according to Martins et al. (2004). Haemoglobin concentration (Hb: g/dl) was measured spectrophotometrically at 540 nm using the cyanmethenoglobin method Drobkin (1945). Haematocrit (Hct) was measured with microcentrifuge method, using standard heparinized microhaematocrit capillary tubes (75 mm at 7000 g for 10 min) (Blaxhall and Daisley, 1973).

Serum biochemical and non-specific immunity parameters: The serum total proteins were analysed using a Biuret assay as described by Doumas et al. (1981). The serum Albumin level was determined according to the Bromocresol Green Method

Table 2. Growth parameters of rainbow trout fed diets enriched with different levels of *Myrtus communis* (control, 0.5%, 1% and 1.5%) for 60 days. Data are presented as mean ±S.D. Values in each row with different superscripts shows significant difference (P<0.05). WG, weight gain; SGR, specific growth rate.

	Control	$M_{0.5}$	M_1	$M_{1.5}$
WG (g)	42.23±7.02^{b}	55.02±11.50^{ab}	62.42±3.83^{a}	53.08±4.37^{ab}
SGR (%)	3.34±0.25^{b}	3.73±0.30^{ab}	3.94±0.09^{a}	3.69±0.13^{ab}
FCR	0.75±0.13^{a}	0.60±0.11^{ab}	0.55±0.03^{b}	0.64±0.06^{ab}

Table 3. Hematological parameters of rainbow trout fed diets enriched with different levels of *Myrtus communis* (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	$M_{0.5}$	M_1	$M_{1.5}$
RBC ($\times 10^6$ mm^{-1})	0.7±0.03^{b}	0.84±0.07^{ab}	1.05±0.18^{a}	0.91±0.25^{ab}
WBC ($\times 10^3$ mm^{-1})	13.86±0.22^{b}	22.59±1.4^{a}	23.65±3.43^{a}	20.9±2.67^{a}
Hb (gr dl^{-1})	6.77±0.67^{a}	7.0±1.12^{a}	7.15±0.5^{a}	7.51±1.2^{a}
Hct (%)	25.67±4.04^{b}	28.33±1.53^{ab}	33.67±2.08^{a}	30.0±2.0^{ab}

following the method of Doumas et al. (1971), using a diagnostic kit (ZiestChem, Diagnostics Co., Iran). Globulin content (subtracting albumin from the total protein) was calculated basedon Kumar et al. (2005). Alkaline phosphatase (ALP) was estimated using the Ziest Chem Diagnostics kit (Tehran Company, Iran) and the absorbance was read at 405 nm in a spectrophotometer (Sanchooli et al., 2012). Serum Lysozyme activity was determined according to Ellis et al. (1990).

Data analysis: For statistical analysis, the normality of data were checked. Then, the data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. In case of all statistics analysis, the mean values were considered significantly different at P<0.05. The statistical were performed by SPSS software (version, 17) and the figures were drawn by Microsoft Excel.

Results

The weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) of fish fed the different experimental diets are shown in Table 2. Regarding, WG and SGR a dose dependent increase was noticed following feeding on Myrtle supplemented diets (P<0.05); with the highest increments in those fish fed 1% Myrtle. Feed conversion ratio (FCR) of fish fed 1% Myrtle was lower than that of control group (P<0.05).

The number of red blood cells (RBC) and Haematocrit (Hct) in Myrtle (0.5, 1 and 1.5%) fed fish was higher than the control group. However, RBC and Haematocrit was also significantly higher (P<0.05) in fish fed 1% compared to the control group (Table 3). Similarly, the haemoglobin level (Hb) was significantly higher in all the groups fed Myrtle enriched diets, but no significant difference was noticed between the treatments (P>0.05). Also, feeding on the Myrtle supplemented diets significantly increased (P<0.05) WBC compared with the control group. In addition, at the end of feeding trial, the highest WBC level was observed in the 1% Myrtle group (Table 3).

A remarkable dose-dependent increase was observed in total protein and albumin, and the highest increments was recorded in those fish fed 1.5% Myrtle enriched diets (P<0.05). Also, the globulin levels was significantly higher in 1% and 1.5% Myrtle treatments compared to the control group (P<0.05). Feeding on Myrtle supplemented diet (1 and 1.5%) decreased serum ALP activity (P<0.05) respect to the control group. Lysozyme activity increased with increasing dietary Myrtle inclusion levels, although no statistically significant difference was noticed compared to control (Table 4) (P>0.05).

Discussion

The use of immunostimulants in aqua-feed is highly recommended in aquaculture (Barman et al., 2013; Wang et al., 2016). However, due to their high costs,

Table 3. The total protein level, albumin, globulin (gr dL^{-1}), ALP (U L^{-1}) and lysozyme activity (U mg^{-1}) in rainbow trout fed diets enriched with different levels of *Myrtus communis* (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	**$M_{0.5}$**	**M_1**	**$M_{1.5}$**
Total protein level (gr dL^{-1})	4.63±0.25^{b}	5.08±0.57^{b}	5.75±0.86^{ab}	7.02±0.85^{a}
Albumin (gr dL^{-1})	2.09±0.03^{b}	2.28±0.26^{b}	2.46±0.59^{b}	3.74±0.94^{a}
Globulin (gr dL^{-1})	2.55±0.28^{b}	2.79±0.59^{ab}	3.29±0.28^{a}	3.28±0.12^{a}
ALP (U L^{-1})	982.50±102.53^{a}	770±132.94^{ab}	427±33.94^{c}	617±70.71^{bc}
Lysozyme activity (U mg^{-1})	13±4.24^{a}	22±3.46^{a}	25±7.07^{a}	29±12.73^{a}

a large number of these additives are not economical. There are numerous medicinal herbs that cheaper source can be considered as promising immuno-stimulants and growth enhancer in aquaculture (Reverter et al., 2014; Hai, 2015; Gholipour Kanani et al., 2014; Caipang and Lazado, 2015; Jeney et al., 2015; Newaj-Fyzul and Austin, 2015). The present results revealed that *M. communis* powder as feed additive enhanced growth performance. Similar to the present study, the increase of growth performance were recorded in rainbow trout fed savory (*Satureja khuzestanica*), sage (*Salvia officinalis*), thyme (*Thymus vulgaris*) and ginger (*Zingiber officinale*) (Sonmez et al., 2015; Mohamadi Saei et al., 2016; Nya and Austin, 2009), Nile Tilapia (*Oreochromis niloticus*) fed garlic (*Allium sativum*) powder (Metwally, 2009), Caspian brown trout (*Salmo caspius*) fed peppermint (*Mentha piperita*) (Adel et al., 2015), Beluga (*Huso huso*) fed onion (*Allium cepa*) (Akrami et al., 2015), common carp (*Cyprinus carpio*) fed Persian hogweed (*Heracleum persicum*) (Hoseinifar et al., 2016). Regarding the mode of action of medicinal plants on growth promotion, it has been suggested that it may be due to appetite stimulation as well as elevation of digestive enzymes activity which per se increase protein synthesis and improve feed utilization (Hai, 2015; Adel et al., 2015).

The results of previous studies revealed that evaluation of haematological parameters can be considered as means of monitoring fish health (Banaee et al., 2008). In the present study, the haematological parameters such as haemoglobin was not affected by dietary Myrtle inclusion. This finding is in agreement with previous findings where feeding with other herbal supplementary food in rainbow trout (Haghighi et al., 2014; Farahi et al., 2012). However, our results suggest that oral administration of 1% Myrtle increased RBC (erythrocyte) count and haematocrit. This findings were in accordance with those of previous studies which suggested medicinal herbs could affect immune system through their effects on the blood cells (Nya and Austin, 2009; Adel et al., 2015). There are several published works showing the medicinal herbs could act as immuno-stimulants and increase the total WBC (Hai, 2015; Newaj-Fyzul and Austin, 2015; Mohamadi Saei et al., 2016). The increase in WBC, and other blood cells, following feeding of *M. communis*, may be due to positive effect of Myrtle on health status. This immunomodulatory effect has been attributed to presence of biologically active substance such as tannins, flavonoids, saponins and vitamin C found in *M. communis* prevented fish from infection by triggering immune system (Scalbert and Williamson, 2000).

In the present study, total protein in serum increased in fish fed 1% and 1.5% Myrtle. Similar results were reported in rainbow trout fed with *A. sativum* (Nya, 2009), *Z. officinale* (Nya and Austin, 2009), *Laurus nobilis* (Bilen and Bulut, 2010), *Nasturtium nasturtium* (Asadi et al., 2012) and *Aloe vera* (Haghighi et al., 2014). It has been well-documented that the increase of serum total protein is associated with enhanced non-specific immune response of fish (Akrami et al., 2015). This is mainly due to the fact that serum proteins comprises various elements of the non-specific immune system.

The results also revealed notable increase of globulin level in fish fed 1% and 1.5% Myrtle. Similar to the present study, the increase of globulin were

recorded in rainbow trout after feeding *N. nasturtium* and *A. vera* (Asadi et al., 2012; Haghighi et al., 2014). Likewise, Gholipour kanani et al. (2014) and Binaii et al. (2014) reported elevation of globulin in Beluga (*H. huso*) fed ginger and nettle, respectively. Indeed, the serum globulin level is associated with proteins involved in humoral immune responses (Biller-Takahashi et al., 2013). Therefore, the elevation of globulin level can be attributed to immunomulatory effects of Myrtle powder inclusion in rainbow trout diet. Also, regarding albumin level a dose-dependent increase was observed and the highest increments in those fish fed 1.5% Myrtle.

The activity of ALP in blood serum showed significantly decrease in fish fed 1.5% and 1% Myrtle. These results are in accordance with previous results reported by Metwally (2009) and Metwally et al. (2001). Since anti-radical and anti-oxidant properties of the medicinal plants may prevent lipid peroxidation of cell membranes and the release of foresaid enzymes into the plasma (Haghighi et al., 2014), Myrtle might inhibit those diseases caused by oxidative stress. Lysozyme is an important enzyme in non-specific immune defences which eliminates pathogenic bacteria through hydrolysis of their cell wall (Saurabh and Sahoo, 2008). In this study, an increasing trend in lysozyme activity observed following dietary administration of Myrtle which is in agreement with several reports indicating the role of herbal immuno-stimulants in enhancing lysozyme activity (Rao et al., 2006; Choi et al., 2008; Haghighi et al., 2014; Adel et al., 2015). This increase can be attributed to modulation of non-specific immune system following dietary administration of medicinal plants. However, determination of the exact mechanisms underlies the effects of medicinal herbs on lysozyme activity merit future research.

As conclusion, the present results showed beneficial effects of dietary Myrtle as medicinal herb on growth performance, immune response and antioxidant enzymes activity in rainbow trout. This study encourages additional research on different aspect of Myrtle application in aquaculture.

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چکیده فارسی

اثرات افزودن گیاه دارویی مورد (*Myrtus communis* L.) به جیره بر عملکرد رشد و برخی پارامترهای ایمنی غیراختصاصی ماهی قزل آلای رنگین کمان

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چکیده:

این مطالعه به بررسی اثرات پودر گیاه مورد روی عملکرد رشد، پاسخ ایمنی و پارامترهای خون‌شناسی بچه ماهی قزل آلای رنگین کمان می‌پردازد. برای این منظور تعداد ۱۲ قفس (با ابعاد ۶۵×۶۵×۶۵ سانتی‌متر) با ۴ تیمار و تکرار استفاده شد و ۳۰ عدد ماهی (۵۰/۵۵±۶/۰ گرم) در هر قفس ذخیره گردید. تیمارها شامل سطوح مختلف صفر (شاهد)، ۰/۵ ($M_{0/5}$)، ۱ (M_1) و ۱/۵ ($M_{1/5}$) درصد پودر گیاه مورد بودند. بیشترین وزن حاصله و ضریب رشد ویژه و کمترین میزان ضریب تبدیل غذایی در ماهی‌هایی که ۱ درصد گیاه مورد دریافت کرده بودند، مشاهده گردید ($P<0/05$). تعداد گلبول‌های قرمز، گلبول‌های سفید خون و هماتوکریت در تیمارهایی که در جیره مورد دریافت کرده بودند نسبت به گروه شاهد بیشتر بود ($P<0/05$). میزان پروتئین کل در تیمارهای $M_{1/5}$ و M_1 در مقایسه با شاهد و تیمار $M_{0/5}$ افزایش نشان داد ($P<0/05$). همچنین بیشترین میزان آلبومین در تیمار $M_{1/5}$ و کمترین میزان آن در شاهد مشاهده گردید ($P<0/05$). علاوه بر این آنزیم آلکالین فسفاتاز با افزایش دوز گیاه مورد در جیره کاهش یافت و کمترین میزان این آنزیم در تیمار M_1 مشاهده گردید ($P<0/05$). میزان فعالیت آنزیم لیزوزیم با افزایش سطوح مورد در جیره افزایش نشان داد، این در حالی است که اختلاف این تیمارها با شاهد معنی‌دار نمی‌باشد ($P<0/05$). این نتایج اثرات تقویت رشد و سلامت پودر گیاه مورد نشان می‌دهد.

کلمات کلیدی: مورد، ماهی قزل آلای رنگین کمان، محرک ایمنی، پاسخ ایمنی غیراختصاصی.