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ORIGINAL ARTICLE

Non-specific Immune Responses and Immune related Genes Expression of Rainbow trout (*Oncorhynchusmykiss*,Walbaum) fed *Zataria multiflora* Boiss extract

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ABSTRACT
Influence of dietary administration of Zataria multiflora Boissextract was evaluated on some non- specific immune response and immune related genesof rainbow trout, Oncorhynchusmykiss . Trout with an average initial weight of 100±10 g were fed diets containing three doses of Z. multiflora (20, 50 and 100 mg/kg feed) in addition to a control diet (without the herb extract) for 2 weeks. The results of this study showed that feeding rainbow trout with 50 mg/ kg feed Z. multiflora significantly enhanced phagocytosis index and respiratory burst activity of phagocytic kidney cells. The highest total WBC/RBC and lysozyme activity were observed in rainbow trout fed with 50-100 mg/ kg feed of Z. multiflora extract. Feeding rainbow trout with 100 mg/ kg feed of Z. multiflora extract significantly (P < 0.0 5) upregulated lysozyme and tumor necrosis factor α (TNF α) gene expression. The present study indicated the role of optimal doses Z. multiflora extract (50 and 100 mg/ kg feed) onnon-specific immune response and immune related genes of rainbow trout (O.mykiss) .
Key words: Immuno stimulants; Zataria multiflora extract; immunological parameters; gene expression; Oncorhynhusmykiss

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INTRODUCTION

In fish aquaculture, culturing different species of marine and fresh water fish continues to increase each year. Also, the aquaculture industry is recently facing a serious setback due to infectious[1]. Enhancement of the immune system seems to be the most promising method for preventing fish diseases. Fish usually depend on a much greater extent on the non-specific immune mechanisms [2]. Immuno-stimulants lead to an increase in various compounds of immunity, for example, phagocytic levels, lysozyme, respiratory burst and total white blood and red blood cells (WBC/RBC).In aquaculture, there are many studies reporting a variety of substances including synthetic [3] bacterial [4], animal and plant compounds [1, 5, 6] can be used as immune-stimulants. Up to now, a large number of traditional medicinal plants have been used in order to prevent and treat of several diseases in Fish [6-11]. These natural plant products have various activities like anti stress, appetizer, tonic, anti microbial and immune-stimulants[12]. *Zatariamultiflora* Boiss is one of the medicine plants that is members of Lamiaceae family. *Z. multiflora* is used for intestinal and liver inflation, regulating the menstruation, removing different parasites, bloating and even disinfecting the environment. There are few studies on effect of *Z. multiflora* in aquaculture. Soltani*et al*. [13] showed that *Z.multiflora* essential oil has some immunostimulatory effect on immunological factors such as antibody titers, total white blood cells and serum bactericidal activities. Howeve, many investigations have focused on identifying cytokines and other immune related genes (IL-1B, TNF-α) in rainbow trout fed with lupin, mango, stringin nettle[3], *Cyprinuscarpio* L fed with blue green alga spriulina (*Spirulinaplantensis*[3], No information is available concerning effect of *Z.multiflora* extract on immune related genes expression (Lysozyme lyz2) and tumor necrosis factor a (TNFα) in fish. Therefore the aim of present study was to investigate the effect of *Z.multiflora* extract on some non

specific immune responses and immune related genes expression (Lysozyme lyz2) and tumor necrosis factor a (TNFα)of rainbow trout (*O.mykiss*) as one of the main cultured species.

MATERIALS AND METHODS

Fish

Two hundred and ten rainbow trout (*O.mykiss*) with an average initial weight of 100± 10 g were obtained in mid January 2012 from a cultured fish farm in Sepidan, Shiraz, Iran. Fish were transported alive in plastic bags containing water enriched with oxygen. They were kept in 100 l fiber glass tanks filled with chlorine free tap water and provided with continuous aeration using electric air pumping compressors. During the experiment water temperature 15 ±0.5 °C, pH 7.8± 0.18, Salinity 0.75± 0.05 ppt and dissolved oxygen concentration 5.8± 7.7 mg/ l were maintained. All the fish were fed ad libitum 3 times a day at the rate of 3% of their body weight with a commercial feed (Beyza 121 Feed Mill (BFM) Co, Ltd., Iran).

Herbal extract

The leaves *Zatariamultiflora* medicinal plant were collected from herbal medicine shop and its identity was confirmed using monographs by Mozaffarian[14] . The leaves of the plant were shade dried and ground into a powder (50 g), macerated in 400 ml of methanol, filtered and dried at 35 °C using a rotary vacuum. The extract of sample was stored in the bottle and refrigerated at 4 °C prior to further analyses[15].

Experimental design

After 7 days of acclimation to the condition, to study the innate immune mechanisms, fish were allocated into 4 groups (30 fish/ group) and fed diets containing three doses of *Z. multiflora* (20, 50 and 100 mg/ kg feed) extract. Also control group only was fed with commercial feed. Cod oil (10 ml /kg feed) was used to bind the powdered herbal extract to the fish feed. The prepared feed was maintained at room temperature.

Blood collection

Ten fish were randomly sampled from each group at the end of experiment. Approximately 2 cc blood was collected from the caudal vein of each fish, anticoagulant heparin was added, and the blood was placed in Vacutainer tubes(500 U sodium heparinate /ml). Red and white blood cells (RBC/WBC) were counted according to Atamanalp*et al*. [16].The remaining whole blood samples were centrifuged at 3000 g for 5 minutes and plasma was stored at -80 ^{o}C to be used for plasma lysozyme assay.

Collection of macrophages from head kidneys

After the blood collection of each fish, fish were killed by decapitation. After decapitation, the head kidneys were removed and immediately transferred to a container with Leibovitz L- 15 culture medium (pH adjusted to 7.8) (Merck, Germany). Then Head kidney macrophages, collected according to the procedures described byKim and Austin [17] with some modifications, were used for analysis of RBA and PA. Briefly, head kidneys were processed individually by disruption across a nylon mesh (100µm) with L-15 medium containing 2% (v/v) fetal calf serum (FCS), 100 µl/ml gentamycin (Sigma) and 10 µl/ml heparin (Sigma). The resulting suspensionswere layered onto a 34 to 51% (v/v) Percoll (Sigma) gradient diluted in Hank's Balanced Salt Solution(HBSS, Sigma) before tubes were centrifuged at 400×g for 25 min at 4°C. The band of cells located at the34% to 51% interface was collected and washed twicewith HBSS. The cell density was adjusted to 10^6cells/ml in L-15 medium supplemented with 0.1 %(v/v) FCS and 100 µl/ml gentamycin. Viability was evaluated by the trypan blue exclusion method.

Phagocytic activity

Phagocytic activity of headkidney macrophages was evaluated using apreviously described method [17]with some modifications. One ml of the macrophage cell suspension (10^6 cells/ml) obtained from eachindividual fish was allowed to adhere onto a methanolcleaned glass slide for 1 h at 18°C in ahumid chamber. Non-adherent cells were removedby washing with HBSS before adding 1.0 mlautoclaved congo red-colored yeast cells (10^8cells/ml). Phagocytosis was allowed to proceed for 1h. Air-dried slides were fixed in absolute methanol for3 min and stained by Giemsa's method for 15 min.Approximately 200 cells were counted randomly andPAwas expressed as:

PA = number of phagocytosing cells/number oftotal cells × 100

The phagocytic index was determined by the numberof yeast cells phagocytosed per macrophage cell.

Macrophage production of reactive oxygen species (respiratory burst activity, RBA)

Superoxide anion production by head kidneymacrophages was determined by the reduction ofnitrobluetetrazolium (NBT, Sigma) following a previously described method [18].

Plasma lysozyme

Plasma lysozyme was determined by the turbid metric assay according to Demers and Boyne [19]Briefly, the lysozyme substrate was 0.75 mg/ml of gram positive bacterium Micrococcus lysodeikticus lyophilized

cells (Sigma, St. Louis, MO). The substrate was suspended in 0.1 M sodium phosphate/citric acid buffer, pH 5.8. Plasma (25 µl) was placed, in triplicate, into a microtiter plate and 175 µl of substrate solution was added to each well at 25 ^{o}C. The reduction in absorbance at 450 nm was read after 0 and 20 minutes using microplate ELISA reader (Bio TEC, ELX800G, USA). The units of lysozyme present in plasma (µg/ml) were obtained from standard curve made with lyophilized hen-egg-white lysozyme (Sigma).

Immune relates genes expression

Total RNA extraction and cDNA synthesis RNA extraction of head kidneys were carried out by the acid guanidiniumthiocyanate-phenolchloroform method described by Chomczynski and Sacchi [20]. Total RNA extraction was performed according Awad *et a*l., [21]with slight modifications. Head kidney tissue samples were obtained and immediately transferred to liquid nitrogen tank and then kept in _80 _C until further use. For total RNA extraction 50e100 mg of tissue homogenated in 1.0-mL Biozole reagent for 15 min in room temperature following the manufacturer protocol. The quantity and concentration of RNA were measured by spectrophotometer at 260/280 nm. Also, the RNA quality was evaluated by electrophoresis on a 1.5% agarose gel and staining with ethidium bromide. 1 mg of total RNA was used to synthesize first-strand cDNAs using a FermentascDNA synthesis Kit for RT-PCR, following the manufacturer's instructions and a mixture of oligo-dT as primer.

Primer design

The qPCR primers for Lyzo2 and TNFa genes were designed based on the conserved regions extracted from DNA sequences in GenBank and the genomic contigs of the NCBI database. Multiple qPCR primer combinations were designed for each gene using PRIMER3.0 software program (available at http://wwwgenome. wi.mit.edu) (Table 1). The qPCR efficiency was also taken into account for selection of the best qPCR primer pair with specific and correct size. bactine gene was used as reference gene for standardization of expression levels.

Quantitative real-time PCR (qPCR)

Real-time PCR analysis was carried out using an iCycler (Bio- Rad) with Fermentas Maxima SYBR Green qPCR Master Mix (2_) (Fermentas) and all primers at [100 nM]. The fold change in Lyzo2 and TNF relative mRNA expression was calculated by the 2_DDCt method ., [22]. The obtained data were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad).

Table 1: Sequences of oligonucleotide primers and the conditions used for real-time PCR.

Gene	Accession number	qPCR primers, forward/reverse	Amplicon(bp)
Lysozyme			
C-type	NM_001124374.1	ACAGCCGCTACTGGTGTGACG GCTGCTGCCGCACATAGAC	203
TNF1α	NM_001124374.1	CAAGAGTTTGAACCTTGTTCAA GCTGCTGCCGCACATAGAC	180
βactine	NM_001124235.1	ATGGGCCAGAAAGACAGCTACGTG CTTCTCCATGTCGTCCCAGTTGGT	140

Statistical analysis

All measurements were repeated twice. Comparisons of resultsbetween different treated groups were carried outusing one-way analysis of variance (ANOVA, SPSS for windows version 16). A value of $P<0.05$ was considered.The differences between all groups were tested by using Duncan multiple comparisons test.

RESULTS

The effect of *Zatariamultiflora* extract on total white and Red blood cells (WBC/RBC) and plasma lysozyme activity is shown Table 2. Total WBC and plasma lysozyme activity were significantly higher after weeks in group fed with the highest (100mg.kg feed) and medium (50 mg/ kg feed) dose of *Z.multiflora* extract. There were no significant differences in total RBC among all groups.

Changes of phagocytosis index and respiratory burst activity of macrophages isolated of head kidney in rainbow trout fed with different doses of *Z.multiflora* were shown in Fig 1.

In the group fed with medium (50 mg/kg feed) dose of *Z. multiflora* extract, the highest respiratory burst activity and phagocytosis index of head kidney macrophages were observed

However in high (100mg.kg feed) dose of *Z. multiflora* extract did not changerespiratory burst activity and phagocytosis index of head kidney macrophages when compared with that of the control group.

Table 2: Total White and Red blood cells (WBC/RBC) and, plasma lysozyme (µg/ ml) activity in rainbow trout (*Oncorhynchusmykiss*) fed diets containing different doses of *Zatariamultiflora* extract.

Groups	WBC ×10^3	RBC ×10^6	plasma Lysozyme activity (µg/ml)
Control	44.62^{a}± 2.55	63.62^{a} ± 1.28	7^{a} ± 1.51
1	50.50^{b}± 2.44	64.50^{a} ± 2.44	7.50^{a} ± 1.60
2	51.62^{b} ± 3.73	64.75^{a} ±3.41	9.25^{b} ± 1.38
3	52.50^{b}± 1.18	64.37^{a} ± 2.87	12.37^{c} ± 1.06

Data are expressed as the mean of five fish±SEM. Group 1,2 and 3 fed with 20, 50 and 100 mg/kg feed of *Z.multiflora* extract respectively. Identical superscript letters indicate no significant differences between groups.

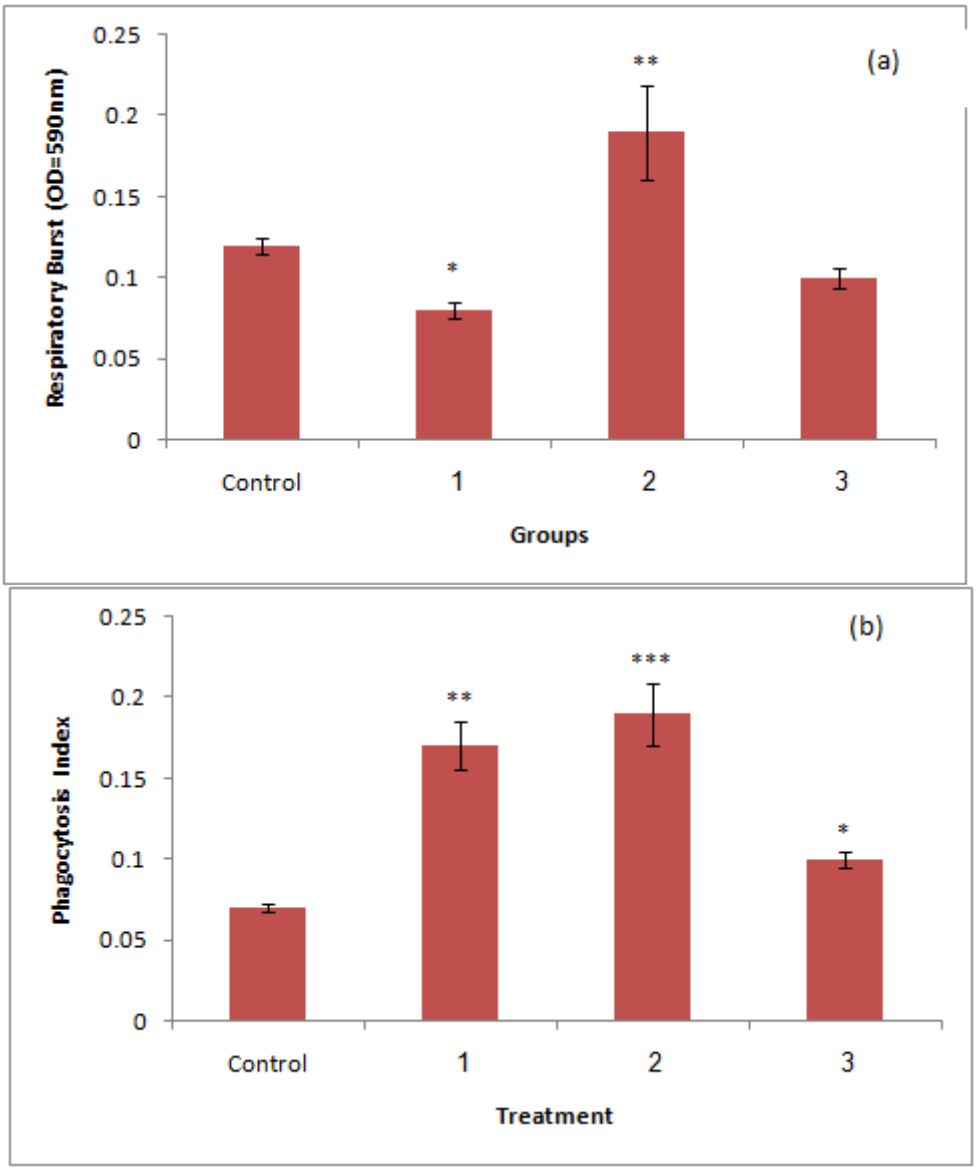

Fig 1. Changes of respiratory burst activity(a) and phagocytosis index of macrophages isolated from head kidney(b) in rainbow trout (*O.mykiss*) fed diets containing different doses of *Zatariamultiflora* extract. Data are expressed as the mean of ten fish ± SEM. Significant differences (P<0.05) from the untreated control and among treated groups are indicated by asterisks.Group 1, 2 and 3 fed with 20, 50 and 100 mg/kg feed of *Z. multiflora*extract respectively.

The effects of *Zatariamultiflora* extract on the expression of immune related genes; lysozyme and TNFa of rainbow trout are as follows: The present study revealed that feeding on 100 mg/ kg feed dietary (after two weeks) significantly ($P < 0.05$) increased the expression of immune related genes; lysozyme (two times of the control) and TNF α (3 times of the control).

DISCUSSIONS

Recently, use of natural products, like plant extract, in aquaculture is developing venture which needs further research in fish [8,12]. The immunostimulating effect of *Zatariamultiflora*was investigated in this study.

The results of this study showed that feeding *O.mykiss* with low (20 mg/ kg feed) and medium (50 mg/kg feed) doses of *Z.multiflora* extract enhanced WBC (but not RBC) count of rainbow trout compared to control group. In agreement with the present findings, Gopalakannan and Arul [23] reported that there was an increase in the WBC count after feeding the common carp with immunostimulants like chitin.Soltani et al[13] also reported that WBC count was higher in common carp fed *Zatariamultiflora* essential oil .

Lysozyme is a component of the non- specific defense mechanism that has the ability to prevent the growth of infectious microorganism by splitting β-1,4 glycosidic bonds between N- acetylmuramic acid and N- acetyl glucosamine in the peptidoglycan of bacterial cell wall [11, 23]. In our experiment, dosesof *Z.multiflora* extract (50 and 100 mg/kg) had significant effect on plasma lysozyme activity compared with the control group.

In fish, phagocytic cells have been recognised as the most important cellular components of the innate immune system of fish in the host's defenses against invading microorganisms[24].The present results showed that *Z.multiflora* extract significantly enhanced the phgocytic index of macrophages isolated from rainbow trout 2 weeks after the start of feeding in the groups fed with feed containing low (20mg/ kg feed) and medium(50 mg/kg feed) doses of *Z.multiflora* extract.The present results come close to those reported in other fish species such as tilapia[10]. They reported that feeding tilapia with 0.1 and 0.5% doses of*Astragalus radix* for 3 weeks enhanced phagicytic activity of phagocytic blood cells.

Fish phagocytes are able to produce superoxide anion (O_2^-) during a process called respiratory burst [10, 24]. The respiratory burst activity can be quantified by the nitrobluetetrazolium (NBT) assay, which measures the quantity of intracellular superoxide radicals produced by leukocyte [25]. For instance, Robertson et al. [26] showed that injection with glucan increased head kidney macrophages extracellular respiratory burst activity.Dùgenci et al. [27] also reported that rainbow trout fed with *Zingiberofficinale* extract had significantly higher extracellular activity of phagocytic cells in blood. In our study, we detected significant differences in respiratory burst activity in rainbow trout fed with medium dose (50 mg/kg feed) of *Z. multiflora* extract. It was shown however that in tilapia fed with Astragalus extract [10] and trout fed with nettle and mistletoe extracts [27] the production of extracellular superoxide anion was on the same level as in the control fish.

*Zatariamultiflora*increased the expression of immune related genes; lysozyme and TNFα in this survey. It seems that *Zatariamultiflora*act as an immunostimulant on the innate immune system through the same mechanism of other prebiotics and probiotics. This is good correlated with increase of serum lysozyme with 50-100 mg/kg of *Z.multiflora* in the fish feed. Sealey et al. [28]stated that inclusion of Grobiotic-A, a commercial prebiotic contains a mixture of partially autolyzed brewer's yeast, dairy components and dried fermentation products, up-regulated TNFα gene expression in rainbow trout. However, b-glucan (derived from S. cerevisiae) had no significant effects on the expression of TNFα in zebra fish (Daniorerio) [29]. These differences can be attributed to the prebiotic source, dosage and the species. Also, in a probiotic study, Panigrahi et al. [30]observed up regulation of TNFα gene expression in rainbow trout after feeding with *Lactobacillus rhamnosus*. Tumor necrosis factor *a* (TNF*a*) is a pleiotropic cytokine that plays pivotal roles in the organization and functions of the immune system. TNF*a*exerts its functions by interacting with two distinct receptors [31].

CONCLUSION

The optimal doses of *Z.multiflora* extract for enhancing WBC count, respiratory burst and phagocytic index were 20 and 50 mg/kg feed for 2 weeks after the start of feeding. Thus it can be concluded that *Z. multiflora* extract could increasephagocytic index of head kidneymacrophages, the expression of immune related genes; lysozyme. It seems that *Zatariamultiflora*act as an imunostimulant on the innate immune system through the same mechanism of other prebiotics and probiotics.

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