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Research Article

NON SPECIFIC IMMUNE RESPONSE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*, WALBAUM) FED WITH SEED EXTRACT OF *PEGANUM HARMALA* L.

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ABSTRACT
Influence of dietary administration of *Peganum harmal*a was evaluated on some non specific immune responses of rainbow trout (*Oncorhynchus mykiss*) in winter season. Fish with an average initial weight of 100±10 g were fed with *P.harmala* at different doses of 100,150 and 300 mg/kg food for a period of 14 continues days. Plasma lysozyme activity, total white/ Red blood cell population (WBC/RBC). Respiratory burst activity (extracellular superoxide anion production) and phagocytosis by isolated phagocytic kidney cells were investigated on day 14 after feeding. The obtained results showed that feeding rainbow trout with 100 and 150 mg/kg food for 2 weeks enhanced lysozyme activity, total WBC and phagocytosis index by phagocytic kidney cells, however respiratory burst activity of phagocytic cells was not increased. On the other hand, inhibition of lysozyme and respiratory burst activities were found when rainbow trout fed with 300 mg/kg *of P.harmal*. It can be concluded that P. harmala extract with optimal dose of 100 mg/ kg added to fish feed can act as immunostimulants and enhance the immune response of cultured fish.

Keywords: *Immunostimulant, Oncorhynchus Mykiss, Peganum Harmala Extract, Immunological Parameters*

INTRODUCTION
During last decades there has been a continuous growth of aquaculture industries all over the world and such intensive production would experience disease problems. Infectious diseases that occur as sporadic events in wild fish populations may cause high mortalities when appearing in intensive fish farming (Gudding *et al.*, 1999). 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 (Chakrabarti and Vasudeva, 2006). Immunostimulants 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 (Vasudeva *et al.*, 2006) bacterial (Goetz *et al.*, 2004), animal and plant compounds(Sakai, 1999; Ardó *et al.*, 2008; Baba *et al.*, 2014) can be used as immunostimulants. Up to now, a large number of traditional medicinal plants have been used in order to prevent and treat of several diseases in Fish (Dügenci *et al.*, 2003; Jian and Wu, 2004; Vasudeva Rao and Chakrabarti, 2005; Yin *et al.*, 2006; Ardó *et al.*, 2008; Choi *et al.*, 2008). These natural plant products have various activities like anti stress, appetizer, tonic, anti microbial and immunostimulants (Citarasu *et al.*, 2002).
Peganum harmala L. (Zygophyllaceae), that is also called Harmal, Suryin Rue, is a perennial, bushy, and wild-growing flowering plant with short creeping root which may grow to 30-100 cm high (Mahmoodian *et al.*, 2002 ; Shamsa *et al.*, 2007; Goel *et al.*, 2009) is known as "Espand'' in Iran and Harmal in North Africa and African Rue, Mexican Rue, Syrian Rue or Turkish Rue in United States (Mahmoodian *et al.*, 2002). This plant is widely distributed in North Africa, Mediterranean, the Middle East, Pakistan, India and Iran and has been introduced in America and Australia (Asghari and Lockwood, 2002; Ehsanpour and Saadat, 2002; Yousefi *et al.*, 2009). *P. harmala* traditionally has been used in Iran as an antiseptic and disinfectant agent by burning its seeds (Fathiazada *et al.*, 2006; Arshad *et al.*, 2008.). This plant has been

considered for the treatment of a variety of human ailments, such as lumbago, asthma, colic and jaundice (Bukhari *et al.,* 2008). The most pharmacological active compounds of *P. harmala* are several alkaloids which are found in the seeds and roots (Mirzaie *et al.,* 2007). Also, it has been reported that this plant had antibacterial, antifungal and antiviral effects. No information is available concerning its effect on the fish immunity. Therefore the aim of present study was to investigate the effect of this plant on some non specific immune responses of rainbow trout (*O.mykiss*) as one of the main cultured species.

MATERIALS AND METHODS

Fish

One hundred and twenty rainbow trout (*O.mykiss*) with an average initial weight of 100± 10g 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

Pegamum harmala medicinal plant was collected from herbal medicine shop and its identity was confirmed using monographs by Mozaffarian (1996) . The seeds of the plant were shade dried and ground into a powder (50g), 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 (Harikrishnan and Balasundaram, 2005).

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 *P. harmala* (100, 150 and 300 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 2cc 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.,* (2008). The remaining whole blood samples were centrifuged at 3000g 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 by Kim and Austin (2006) 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 suspensions were 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 twice with HBSS. The cell density was adjusted to 10^{6}cells/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 head kidney macrophages was evaluated using a previously described method (Kim and Austin, 2006) with some modifications. One ml of the macrophage cell suspension (10^{6}

cells/ml) obtained from each individual fish was allowed to adhere onto a methanol cleaned glass slide for 1 h at 18°C in a humid chamber. Non-adherent cells were removed by washing with HBSS before adding 1.0 ml autoclaved congo red-colored yeast cells (10^8cells/ml). Phagocytosis was allowed to proceed for 1h. Air-dried slides were fixed in absolute methanol for 3 min and stained by Giemsa's method for 15 min. Approximately 200 cells were counted randomly and PA was expressed as:

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

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

Macrophage Production of Reactive Oxygen Species (Respiratory Burst Activity, RBA)

Superoxide anion production by head kidney macrophages was determined by the reduction of nitroblue tetrazolium (NBT, Sigma) following a previously described method (Chung and Secombs, 1988).

Plasma Lysozyme

Plasma lysozyme was determined by the turbid metric assay according to Demers and Boyne (1996) 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).

Statistical Analysis

All measurements were repeated twice. Comparisons of results between different treated groups were carried out using 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 AND DISCUSSION

Results

The effects of the seed extract *Parganum harmala* on total white and red blood cell (WBC/RBC) and respiratory burst activity of head kidney macrophages are shown in Table 1. Total WBC/ RBC were significantly higher after 2 weeks in group fed with the lowest (100mg.kg feed) dose of *P.harmala* extract. There were no significant differences in both medium (150 mg/kg feed) and the highest (300 mg/kg feed) dose *of P.harmala* extract compared to control group. Different doses of *P.harmala* extract in rainbow trout had no effect on respiratory burst activity of head kidney macrophages after 2 weeks of the first feeding fish with this herb extract (Table 1). Elevation in phgocytosis index and plasma lysozyme activity was noted in all groups when compared to control (Figure 1). The highest phgocytosis index and lysozyme activity were observed in the group fed with the lowest level of herb extract (100 mg/ kg feed). No significant difference was observed between the highest (300 mg/kg feed) dose and control group.

Table 1: Total White and Red blood cells (WBC/RBC) and optical density (OD) of respiratory burst activities in rainbow trout (*Oncorhynchus mykiss*) fed diets containing different doses of *Peganum* harmala extract.

Groups	**WBC ×10^3**	**RBC ×10^4**	**OD of Respiratory burst activity**
Control	44.62^{a}±2.55	63.62^{a}± 1.28	0.12^{a}±0
1	57.55^{b} ± 3.24	71.33^{b}± 0.09	0.12^{a}±0
2	48.66^{a}± 2.64	63.33^{a}±1.50	0.11^{a}± 0
3	46^{a}±4.87	59.11^{a}± 4.28	0.07^{a}±0

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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 *P.harmala* extract respectively. Identical superscript letters indicate no significant differences between groups.

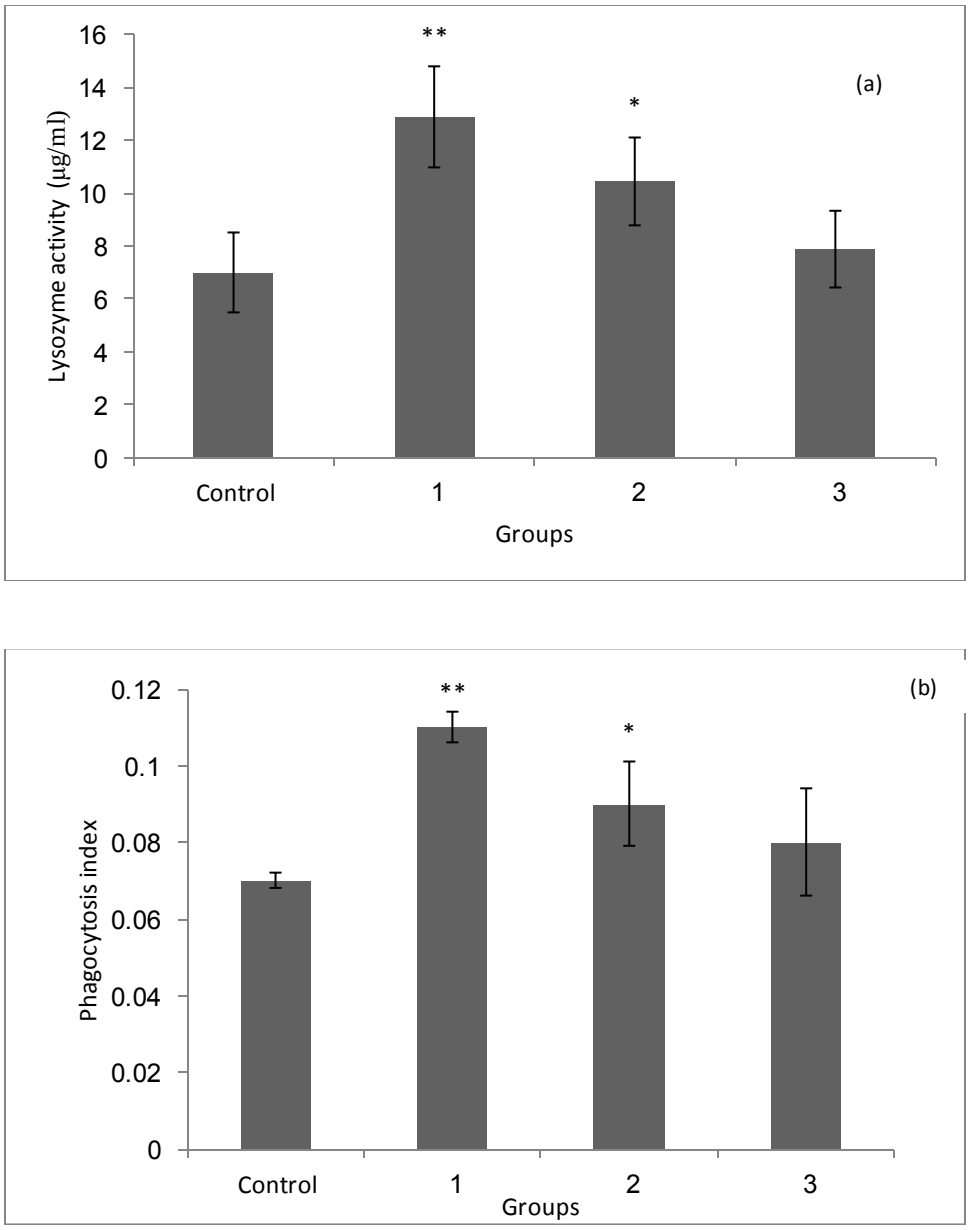

Figure 1 Changes of plasma lysozyme activity (a) and phagocytosis index of macrophages isolated from head kidney (b) in rainbow trout (*O.mykiss*) fed diets containing different doses of *Peganum harmala* extract. Data are expressed as the mean of ten fish ± SEM. Significant differences (P<0.05) from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

Discussion

Recently, use of natural products, like plant extracts, in aquaculture is developing venture which needs further research in fish (Citarasu *et al.*, 2002; Jian and Wu, 2004). The immunostimulating effect of the seed extract of *Peganum harmala L.* was investigated in this study.

The results of this study showed that feeding *O.mykiss* with low (100 mg/ kg feed) dose of the seed extract of this plant enhanced WBC and RBC counts of rainbow trout compared to control group. In agreement with the present findings, Sahu *et al.,* (2007) reported that WBC and RBC counts were higher in *Labe rohita* fingerlings fed *Magnifera indica* kernel when compared to control. Gopalakannan and Arul (2006) reported that there was an increase in the WBC count after feeding the common carp with immunostimulants like chitin Soltani *et al.,* (2010) also reported that WBC count was higher in common carp fed *Zataria multiflora* essential oil .

Lysozyme is a humoral 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 (Gopalakannan and Arul, 2006; Choi *et al.,* 2008). In our experiment, the low (100 mg/kg feed dose of the seed extract of *P. harmala* had effect on plasma lysozyme activity compared with control group. Similar to present observations were obtained by Chen *et al.,* (2003) who reported that plasma lysozyme activity was increased in crucian carp by feeding four Chinese herbs (*Rheum officinale* and *Rographis paniculata , Isatis indigotica*,*Lonicera japonica*) Elevated lysozyme was also observed in *Labeo rohita* after feeding the fish with *Achyranthes aspera* seed (Vasudeva *et al.,* 2006). Our results concerning lysozyme activity support the observations that humoral factors may enhance phagocytosis in fish (Dügenci *et al.,* 2003).

In fish, phagocytic cells have been recognised as the most important cellular components of the innate immune system of fish. Their phagocytic activity is an important mechanism in the host's defenses against invading microorganisms (Neumann *et al.,* 2001). The present results showed that *P. harmala* seed significantly enhanced the phagocytic index of macrophages isolated from rainbow trout 2 weeks after the start of feeding in the group fed with feed containing low (100mg/ kg feed) dose. The present results come close to those reported in other fish species such as tilapia (Yin *et al.,* 2006). They reported that feeding tilapia with 0.1 and 0.5% doses of *Astragalus radix* for 3 weeks enhanced phagocytic activity of phagocytic blood cells.

Fish phagocytes are able to produce superoxide anion (O_2^-) during a process called respiratory burst (Neumann *et al.,* 2001; Yin *et al.,* 2006). The respiratory burst activity can be quantified by the nitroblue tetrazolium (NBT) assay, which measures the quantity of intracellular superoxide radicals produced by leukocyte (Sahu *et al.,* 2007). For instance, Robertson *et al.,* (1990) showed that injection with glucan increased head kidney macrophages extracellular respiratory burst activity. Dügenci *et al.,* (2003) also reported that rainbow trout fed with *Zingiber officinale* extract had significantly higher extracellular activity of phagocytic cells in blood. In our study, we could not detect such differences in respiratory burst activity in rainbow trout fed with *P. harmala*. Similarity, It was shown that in tilapia fed with Astragalus extract (Yin *et al.,* 2006), and trout fed with nettle and mistletoe extracts (Dügenci *et al.,* 2003) the production of extracellular superoxide anion was on the same level as in the control fish.

Conclusion

In conclusion, The optimal dose of the seed extract of *P.harmala* for enhancing WBC and RBC counts, plasma lysozyme activity and phagocytic index of head kidney macrophages was 100 mg/kg feed for 2 weeks after the start of feeding. Thus, it can be concluded that this herbal extract can be used as immunostimulants to enhance immune response of cultured fish species.

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