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Effects of *Zataria multiflora* Essential Oil on Innate Immune Responses of Common Carp (*Cyprinus carpio*)

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Abstract: Influence of dietary administration of *Zataria multiflora* was evaluated on some immunological factors of common carp (*Cyprinus carpio*) under temperature less than optimum (16-17°C). Fish weighing 30-35 g were fed with *Z. multiflora* at different doses of 30, 60 and 120 ppm for a period of 8 continuous days. Serum lysozyme activity, bactericidal activity, total white blood cell population, total protein, globulin and albumin levels were measured on days 1, 2, 8, 15 and 23 after the essential oil administration. On day 23 post-administration the remaining fish from each group were intraperitoneally injected with formalin-killed whole cells of *Aeromonas hydrophila* (6×10^8 cells mL⁻¹) and antibody titer was measured 3 weeks later. The obtained results showed that this essential oil had some immunostimulatory effects on these immunological factors such as antibody titers, total white blood cells and serum bactericidal activity in some test groups especially at 30 and 60 ppm.

Key words: *Zataria multiflora*, common carp, essential oil, dietary administration, immunological factors

INTRODUCTION

The increase of productivity in fish culture has been accompanied by stressful conditions and problems related to fish diseases. Enhancement of the immune system seems to be the most promising method for preventing fish diseases. This enhancement can be achieved with immunostimulants. Some immunostimulants that have been used in aquaculture are: synthetic substances (like levamisole), biological substances (like bacterial derivatives), nutritional factors, animal and plant compounds (Sakai, 1999). Unlike chemical substances used in aquaculture, plant immunostimulants have no residuals in the environment and do not cause drug resistance. On the other hand, in traditional medicine plants have been used in order to prevent and treat a lot of diseases in human and animals. Recently, these plants have received more attention for their immune stimulating functions in fish culture too (Ardo *et al.*, 2008; Choi *et al.*, 2008; Dügenci *et al.*, 2003; Jian and Wu, 2003, 2004; Rao and Chakrabarti, 2005; Yin *et al.*, 2006). *Zataria multiflora* is one of the medicine plants that is a member of *Labiatae* family. Main compositions in the *Zataria* are

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Thymol, Carvacrol, Para-cymene, Terpinene and β -caryophyllene that provide 37.59, 33.65, 7.72, 3.88 and 2.06%, respectively in this essential oil (Sharififar *et al.*, 2007). In traditional medicine *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 this plant in aquaculture. Sharifrohani (2004) showed that *Zataria multiflora* is effective against fungal diseases in rainbow trout eggs till eyed egg stage and significant differences were observed particularly at 50-100 ppm compared with control group.

In spite of positive effects of this plant in controlling some microbial diseases and its immunostimulation effect in human and some animal models (Basti *et al.*, 2005; Dakhili *et al.*, 2006; Fazeli *et al.*, 2007; Khosravi *et al.*, 2007; Khosravim *et al.*, 2008; Sharififar *et al.*, 2007; Shokri *et al.*, 2006; Vigo *et al.*, 2004) 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 and specific immune responses of common carp (*Cyprinus carpio*) as one of the main worldwide farmed species. Particularly the aim was to study effect of the essential oil of this plant on non-specific immune system of common carp under lower water temperature similar to winter condition that fish are faced in the ponds.

MATERIALS AND METHODS

Experimental Conditions

Healthy common carp, *Cyprinus carpio*, weighing 30-35 g were obtained from a fish farm in Gilan province, Iran. The fish were acclimated with essential oil free feed in out door 300 L tanks for one month. Water quality parameters consisting of water temperature, dissolved oxygen, ammonia, nitrite, hardness were 16-17°C, >6, <0.01, 0.1, 180 mg L⁻¹ and 7.8, respectively through out the experiment. This study was conducted in 18 months from April 2007 to December 2008.

Administration of *Z. multiflora*

Fish were divided into 4 groups (20 fish/group) and fed diets containing different doses of 30, 60 and 120 ppm essential oil of *Z. multiflora* (Barij essence company, Iran) for a period of 8 continuous days. Fish were fed at the rate of 1% body weight. A control group was included without any treatment.

Blood Sampling

Four fish per treatment group were bled from caudal vein 1, 2, 8, 15 and 22 days post-treatment. A proportion of blood sample was put into an Eppendorf tube to prepare serum for assaying lysozyme, serum bactericidal activity, total protein, globulin and albumin and the remaining blood samples were taken into another tube containing 10% EDTA to measure white blood cell count.

Serum Lysozyme Activity

The turbidimetric assay for lysozyme content was carried out to measure according to Kumari (2006). A suspension of 150 μ L of *Micrococcus lysodeikticus* (0.2 mg mL⁻¹ in 0.02 M sodium acetate buffer, pH 5.5) was added to 96-well U-bottom microtitre plates containing a 15 μ L test serum in each well. Initial O.D was then obtained at 450 nm immediately after adding the substrate and final O.D was obtained after 1 h incubation at 24°C. Lyophilized hen egg white lysozyme, HEWL (Sigma), was used to develop a standard

curve. Serum lysozyme values were expressed as $\mu\text{g mL}^{-1}$ equivalent to hen egg white lysozyme activity.

Serum Bactericidal Activity

Serum bactericidal activity was estimated following the method of Barnes *et al.* (2003) with slight modification. *Aeromonas hydrophila* isolate was cultured overnight in blood agar and the grown colonies were collected in sterile PBS and centrifuged at 2000 rpm (1500x g) for 15 min. The pelleted bacterial cells were then washed and resuspended in sterile PBS to an OD (540 nm) equivalent to 5.32×10^7 cfu mL. This was diluted 50-fold in PBS and a volume of 25 μL was incubated with an equal volume of heat-inactivated (at 44°C for 20 min) serum sample for 1 h at room temperature. Complement activity was provided by the addition of 200 μL fresh normal serum. Following incubation at room temperature for 1.5 h, bacterial suspensions were serially diluted in PBS and spotted (10 μL) onto TSA plates, incubated at 25°C for 24 h and plates containing 30-300 viable colony forming unit were then counted.

Leukocyte Population Size

Leukocyte count was determined using a haemocytometer with Neubauer counting chamber.

Serum Total Protein, Albumin and Globulin

Serum protein was estimated by Biuret method and serum albumin by bromocresol green method using Pars Azmun kits (Iran) and Auto analyzer (Ependorf). Serum globulin was estimated by subtracting these two amounts.

Serum Antibody Titer

The remaining fish were intraperitoneally injected with formalin-killed whole cells of *Aeromonas hydrophila* (6×10^8 cells mL^{-1}) (Khoshbavar-Rostami *et al.*, 2007) and blood samples were obtained 3 weeks later after anesthetizing fish with clove oil (100 mg L^{-1}). Blood sera were then separated after overnight keeping at 4°C.

Micro agglutination titer was estimated by method described by Robertson (1990). A 25 μL of PBS was added to all wells of round-bottomed plates (except the first well) followed by adding 25 μL of sample serum to first and second plates. Serum of test and control fish were serially diluted from second to twelve plates and then 25 μL of *Aeromonas hydrophila* suspension prepared in PBS (9×10^8 cells mL^{-1}) was added to all plates, kept overnight at room temperature and the last well with positive agglutination was considered as the antibody titer in each treatment.

Statistical Analysis

All data were statistically analyzed by one-way ANOVA, Kruskal-Wallis and Mann-Whitney using SPSS version 16. The level of significance was $p < 0.05$.

RESULTS

The obtained data are shown in Fig. 1-6. No significant difference was observed between control fish and treatment groups ($p > 0.05$) in lysozyme content. Significant increase of serum bactericidal activity was just measured in groups treated with 60 ppm of *Z. multiflora* on day 15 and 22, respectively (Fig. 1). Mean while, white blood cell count was significantly higher in groups fed 30 and 60 ppm of *Z. multiflora* ($P < 0.05$) (Fig. 2) after 15 days post-treatment.

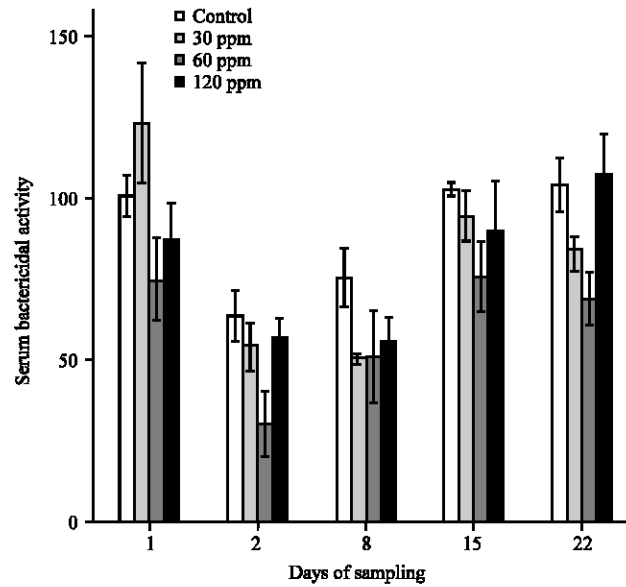


Fig. 1: Serum bactericidal activity in common carp fed diets containing different doses of *Zataria multiflora*. Data are expressed as the Mean±Standard error of mean

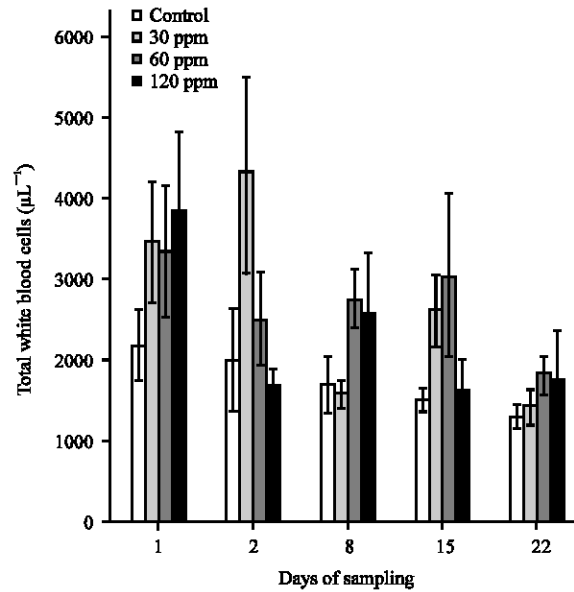


Fig. 2: White blood cells in common carp fed diets containing different doses of *Zataria multiflora*. Data are expressed as the Mean±Standard error of mean

During this study, there were no significant changes in plasma protein during the whole experiment ($p>0.05$) (Fig. 3). Globulin content did not show any significant differences too ($p>0.05$) (Fig. 4). No significant difference was also observed between control fish and treatment groups ($p>0.05$) in albumin content.

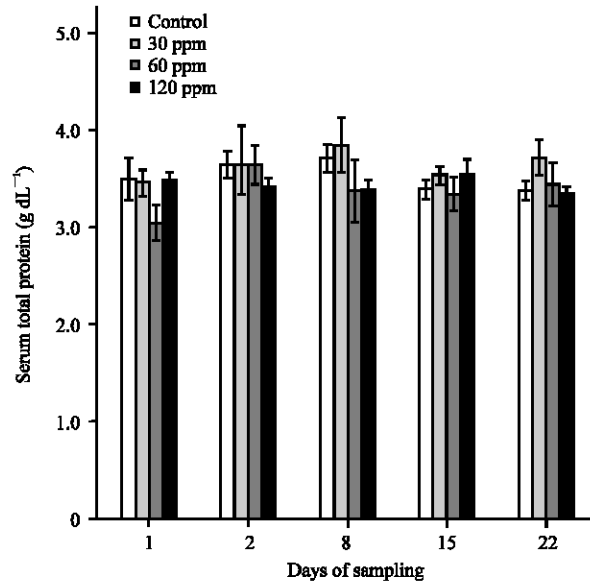


Fig. 3: Serum total protein in common carp fed diets containing different doses of *Zataria multiflora*. Data are expressed as the Mean±Standard error of mean

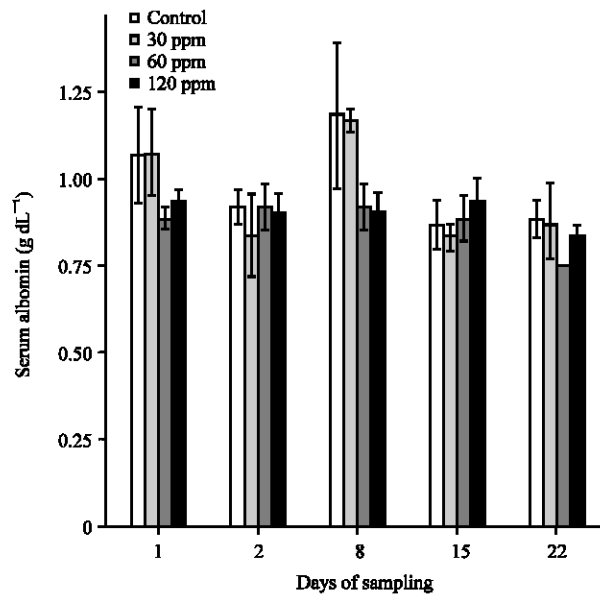


Fig. 4: Serum albumin in common carp fed diets containing different doses of *Zataria multiflora*. Data are expressed as the Mean±Standard error of mean

A significant increase in antibody titer was seen in the fish treated with 30 and 60 ppm of *Z. multiflora* 3 weeks post-immunization with *A. hydrophila* whole cell antigens ($p < 0.05$) (Fig. 6).

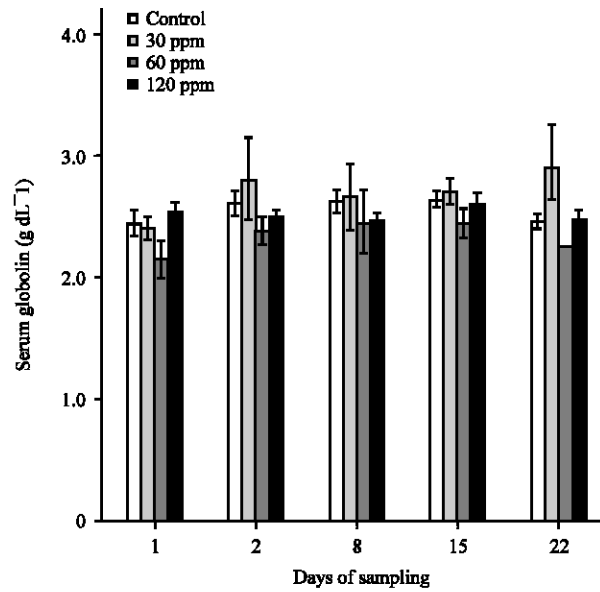


Fig. 5: Serum globulin in common carp fed diets containing different doses of *Zataria multiflora*. Data are expressed as the Mean±Standard error of mean

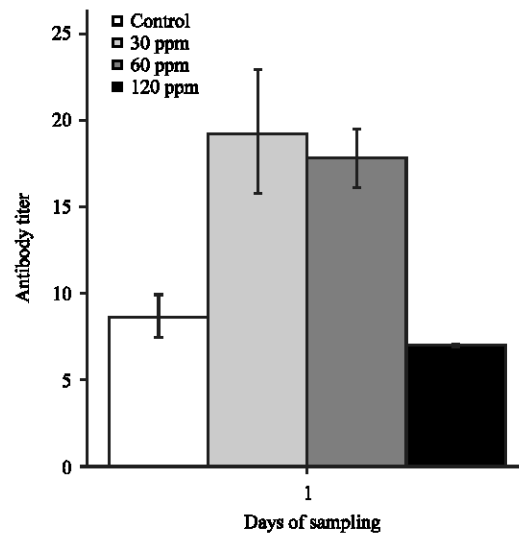


Fig. 6: Antibody titer estimated by bacterial micro agglutination test in common carp fed diets containing different doses of *Zataria multiflora*. Data are expressed as the Mean± Standard error of mean

DISCUSSION

In aquaculture industry fishes are inevitably subjected to different stresses during the period of culture thus use of immunostimulant can act as an alternative way for the

prevention and control of disease outbreaks. Diet supplemented with *Zataria multiflora* essential oil enhanced common carp immunity to some extent even though fish can not express their potential maximum immunity during low temperature. These lower immune responses may in part due to the lower water temperature used in this study (Bagni *et al.*, 2005; Tort *et al.*, 2004). In some regions such as Iran growing of carp encounters a severe stress during the cold weather in Winter because of lower water temperature of about 12-17°C. Thus enhancing the fish immune response may improve the fish health condition to some pathogenic microorganisms.

Several innate immune responses of the common carp were examined after oral administration of different doses of *Zataria multiflora* during low water temperature. Essential oil supplementation in common carp had no effects on serum lysozyme activity for the duration of the experiment. On the other hand, WBC significantly increased in 30 ppm and 60 ppm fed groups on day 15 with slight increase on day 2 in 30 ppm groups. Elevation in serum bactericidal activity was just noted on day 15 and 22 in 60 ppm fed groups when compared to control and a slight but not significant increase was observed on day 2 in this group too. In our experiment, total protein, albumin and globulin were measured, but neither of these parameters was affected by *Zataria* essential oil but antibody titer measured showed a significant increase in lower doses, 30 and 60 ppm, 3 weeks after injecting formalin killed *Aeromonas hydrophila*. It is note worthy to mention that even in blood smears prepared from different treatments in lower doses higher amount of lymphocytes were observed that is in agreement with the results in antibody titers (Data not shown).

Only few studies have been focused on the innate immune responses of *Z. multiflora* in some species of terrestrial animal models but till now there were no studies to determine the effects of this plant on fish immune system. The BALB/C mice respiratory burst activity was significantly influenced by intraperitoneally injection of *Z. multiflora* 4 and 7 days after treatment (Shokri *et al.*, 2006). In rabbit, intra cutaneous injection of this essential oil affected antibody titer against *Candida albicans*, neutrophil phagocytosis plus significant difference in lymphocyte transformation (Khosravi *et al.*, 2007).

Diet supplemented with this essential oil to some extent enhanced common carp immunity even though fish can not express their maximum immunity potential during low temperature. This conclusion is supported by the out come of the challenge experiments in sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) that reduction in total WBC, complement and lysozyme activities was observed during reduction of water temperature (Bagni *et al.*, 2005; Tort *et al.*, 2004).

Dose optimizing of an immunostimulant and its administration duration are also other parameters need to be considered. Li *et al.* (2006) showed that using 100 mg kg⁻¹ of levamisole, the best immunity responses was achieved while 1000 mg kg⁻¹ of levamisole caused chronic toxicity and higher FCR so further studies using even longer administration period for *Zataria* should be considered. Also, the essential oil of *Zataria multiflora* used in this study contains several ingredients such as Thymol, Carvacrol and Para-cymene. Therefore, the fish immune responses to a single component of this herbal oil need further studies.

In conclusion, this study showed that *Z. multiflora* essential oil had the potential for enhancing innate immune system of carp under temperature stress but further studies are required to evaluate the immunostimulatory effect of *Z. multiflora* essence in carp in different situations during the period of culture.

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