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Original Article

Mortality rate and immune responses of rainbow trout (*Oncorhynchus mykiss*) infected with *Yersinia ruckeri* subsequent to feeding on diet supplemented with *Ducrosia anethifolia* essential oil

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Abstract: Application of the immunostimulant is the most promising method for controlling diseases in aquaculture. In this study, the mortality rate and immune responses of rainbow trout (*Oncorhynchus mykiss*) fed on diet supplemented with *Ducrosia anethifolia* essential oil was investigated after challenging with *Yersinia ruckeri*. The essential oil mixed with sunflower oil at different concentrations (0.001, 0.01 and 0.1%) and the commercial food was coated with this oil. Fish were fed with diets for 8 weeks and infected with *Y. ruckeri* at the ending of feeding trial. Serum protein, albumin, globulin and lysozyme and bactericidal activity of challenged fish were evaluated one week after injection and mortality were counted till day 10. The results showed that albumin had not differed among treatments. The highest level of the protein and globulin were found in control group. Serum lysozyme activity showed no difference between groups. The highest and lowest serum bactericidal activity was observed in 0.001% and control group, respectively. The mortality rates in infected fish were as 55% in control group, 40% in 0.001%, 70% in 0.01% and 70% in 0.1% treatment. Lowest rate of mortality was observed in group 0.001%, while began two days earlier than other groups.

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Introduction

The increase in fish culture productivity is accompanied with stressful conditions that provide a susceptible environment for infectious diseases (Soltani et al., 2010; Sheikhzadeh et al., 2011). Improving the immune system using immune-stimulants seems to be the most promising method to control diseases in aquaculture (Harikrishnan et al., 2011).

Yersinia ruckeri is an opportunistic gram-negative bacterium that causes enteric red mouth disease (ERM). Antibiotic treatments would be useless if they induce antibiotic resistance in pathogen (Ispir et al., 2009). Hence, administration of the herbal immunostimulants is considered as an alternative method to chemotherapy and vaccination

(Harikrishnan et al., 2011a, b). Herbal products are safer both for consumer and environment, cheaper and more available (Hajibeglou and Sudagar, 2010; Soltani et al., 2010).

Ducrosia anethifolia is a medicinal plant that belongs to the family Apiaceae. It widely grows in Afghanistan, Pakistan, Syria, Lebanon, Iraq and west and south of Iran. In Iran, it is known as Moshgak, Roshgak or Moshkbu (Hajhashemi et al., 2010; Shahabipour et al., 2013). In previous studies, the antibacterial, antimicrobial and antianxiety effects of *D. anethifolia* were examined in laboratory or in homoeothermic animals like rat and the results were positive (Hajhashemi et al., 2010; Shahabipour et al., 2013), but the effects of this plant in a poikilothermic animals such as fish have not been studied yet.

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Therefore, the objective of this study was to investigate the mortality rate and changes in immune parameters of rainbow trout (*Oncorhynchus mykiss*) infected by *Y. ruckeri* subsequent to fed on diet containing *D. anethifolia* to discover its immune-stimulant potential.

Materials and Methods

Examined fish: Rainbow trout (with average weight of 32.6 ± 8.1 g.) were obtained from a local fish farm (22 Bahman farm, Sepidan, Iran), transported to the laboratory and acclimatized for 2 weeks in a recirculating aquaculture system (RAS). The RAS were included 12 700-L tanks. Water quality parameters, including dissolved oxygen, temperature, pH, TSS and EC were 5.91 ± 1.08 ppm, $8.22 \pm 2.32^\circ\text{C}$, 8.31 ± 0.26 , 0.27 ± 0.01 ppm and 0.53 ± 0.02 μS during the experiment, respectively.

Preparation of immunostimulant diets: *Ducrosia anethifolia* was collected from the Jahrom city (southern Iran), air dried in room temperature and its essential oil was extracted by Clevenger based on Hajhashemi et al. (2010). The essential oil mixed with sunflower oil in concentrations of 0.001, 0.01 and 0.1% and commercial rainbow trout diets (Beyza Feed Mill, Shiraz, Iran) were coated with this suspension. Test diets were stored in the freezer until use. In control group, sunflower oil without essential oil was used.

Culture of *Y. ruckeri*: The source of bacterium was obtained from the fish health and diseases laboratory, Shiraz University. The bacterium was injected to 4 fish intraperitoneally. After 3 days, new plates were prepared from the kidney of infected fish. The colonies of new culture were proliferated for challenge test.

Experimental design: Fish were randomly divided into 12 groups (4 treatments in triplicates) and fed with the test diets for 8 weeks. After the experiment period, 20 fish of each treatment with average weight of 63.64 ± 12.68 g. were intraperitoneally injected with 2.24 ± 10^6 *Y. ruckeri*. Five fish of each treatment were bled a week after challenge test and sera were separated. Serum lysozyme activity, bactericidal

activity, total protein, albumin and globulin were evaluated and the mortality of fish was monitored for 10 days.

Serum lysozyme activity: The serum lysozyme activity was measured spectrophotometrically based on Parry et al. (1965). *Micrococcus lysodeikticus* (0.2 mg ml^{-1}) dissolved in PBS (0.04 M, pH 5.75). Two μl serum was added to the bacterium suspension for the final volume of 3 ml. The absorbance was read in 530 nm after 0.5 and 4.5 min in room temperature. The level of enzyme activity was defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per min using following formula:

$$U/ml = \frac{OD1 - OD2}{4 \times 0.001 \times 2} \times 1000$$

Serum bactericidal activity: Serum bactericidal activity was determined as described by Rao et al. (2006). *Aeromonas hydrophila* bacterial culture was centrifuged and the pellet was washed and suspended in PBS. OD of the suspension was adjusted to 0.5 at 546 nm. This suspension was serially diluted (1:10) with PBS 5 times and the least dilution was used for assay. Ten μl suspension was added to 100 μl serum and incubated at 20°C for 1 hour. After incubation, the combination of bacteria and sera were cultured on the nutrient agar plates in triplicates and colonies were counted after 24 hours incubation at 25°C . PBS was used instead of serum for control samples.

Total protein, albumin and globulin: Serum total protein and albumin were determined using a diagnostic kit (Pars Azmun, Iran) following the protocol recommended by factory (Hajibeglou and Sudagar, 2010). Serum globulin was determined by subtracting the amount of albumin from total protein (Rao et al., 2006).

Statistical analysis: Data were analyzed using SAS 9.1.3 software. The normality of data and homogeneity of variance were performed by kolmogorov-smirnov and Leven test, respectively. Groups were compared by one-way analysis of variance (ANOVA) and if there were any significant differences between treatments, the means were compared by Tukey's test to determine differences

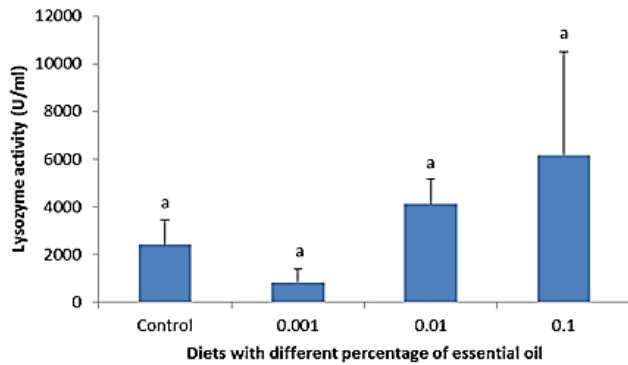


Figure 1. Changes in serum lysozyme activity of rainbow trout one week after challenging with *Yersinia ruckeri*. Fish were fed on diets containing different amount of *Ducrosia anethifolia* essential oil for 8 weeks before challenge. Data are expressed as mean \pm SD. No significant differences were observed among treatments ($P>0.05$).

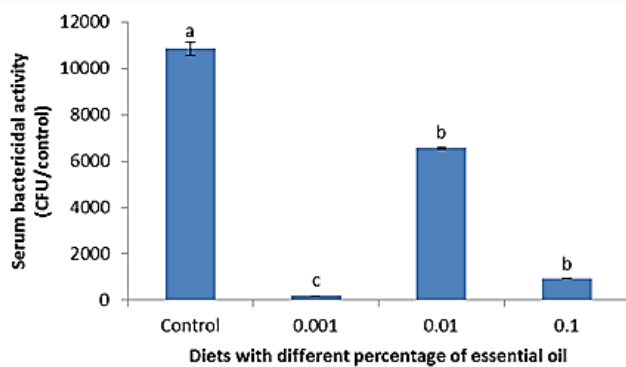


Figure 2. Changes in serum bactericidal activity level of rainbow trout one week after challenge with *Yersinia ruckeri*. Fish were fed on diets containing different amount of *Ducrosia anethifolia* essential oil for 8 weeks before challenge. Data are expressed as mean \pm SD. Bars with Different letters are significantly different ($P<0.05$).

among treatments at a significant level of $P<0.05$.

Results

Serum lysozyme activity: The levels of serum lysozyme in different groups are shown in Fig. 1. The serum lysozyme activity showed no significant differences among the groups ($P\geq 0.05$).

Serum bactericidal activity: The highest serum bactericidal activity was observed in fish fed diet containing 0.001% *D. anethifolia* essential oil and the least bactericidal activity was observed in fish in control group (Fig. 2).

Total protein, albumin and globulin: The highest protein level was observed in control group which was significantly different from those in 0.001 and

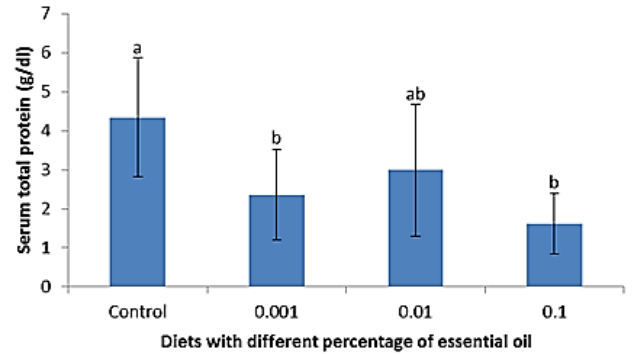


Figure 3. Changes in serum total protein level of rainbow trout one week after challenge with *Yersinia ruckeri*. Fish were fed on diets containing different amount of *Ducrosia anethifolia* essential oil for 8 weeks before challenge. Data are expressed as mean \pm SD. Bars with Different letters are significantly different ($P<0.05$).

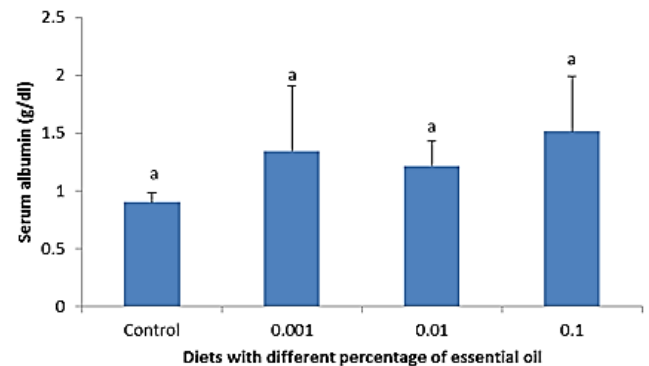


Figure 4. Changes in serum total albumin level of rainbow trout one week after challenge with *Yersinia ruckeri*. Fish were fed on diets containing different amount of *Ducrosia anethifolia* essential oil for 8 weeks before challenge. Data are expressed as mean \pm SD. No significant differences were observed among treatments ($P>0.05$).

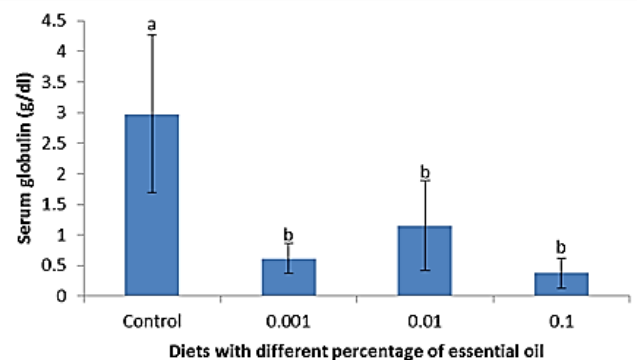


Figure 5. Changes in serum globulin level of rainbow trout a week after challenge with *Yersinia ruckeri*. Fish were fed on diets containing different amount of *Ducrosia anethifolia* essential oil for 8 weeks before challenge. Data are expressed as mean \pm SD. Bars with Different letters are significantly different ($P<0.05$).

0.1% treatments (Fig. 3). No significant differences were observed in albumin levels among treatments after challenging with *Y. ruckeri* (Fig. 4). The

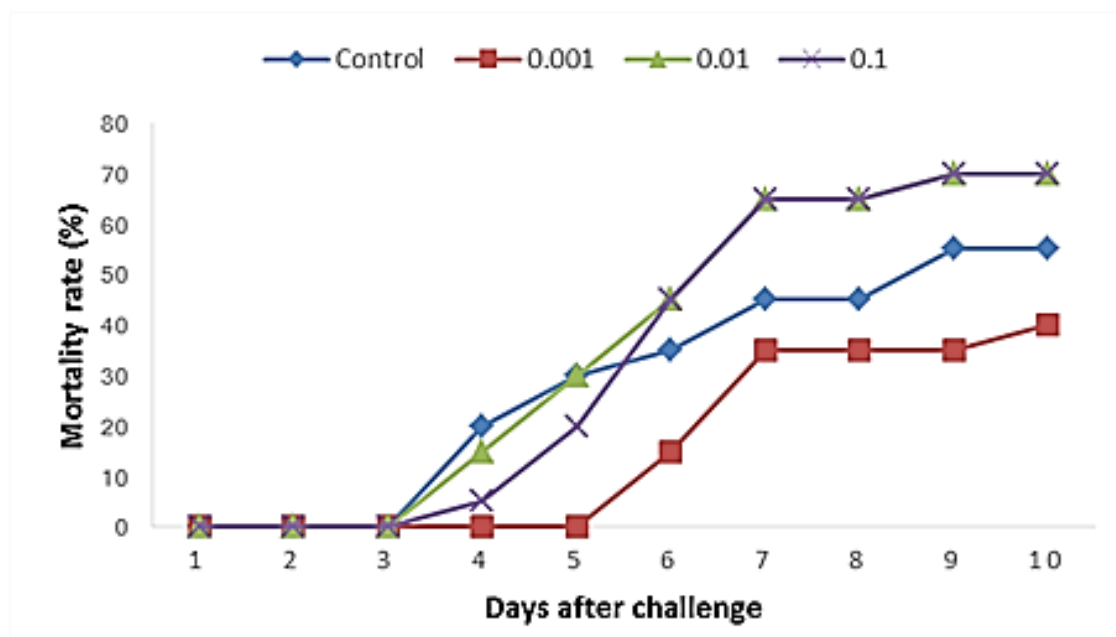


Figure 6. Cumulative mortality rate (%) of rainbow trout monitored for 10 days after challenging with *Yersinia ruckeri*. Fish were fed on diets containing different amount of *Ducrosia anethifolia* essential oil for 8 weeks before challenge.

highest level of globulin was observed in control group which was significantly different compared to other treatments (Fig. 5).

Mortality rate: Fish were monitored for 10 days after challenge test and mortality were counted. Mortality in 0.001 treatment was started on day 6, but fish started to die on day 4 after challenge in other treatments. The mortality rates in infected fish were 55% in control group, 40% in 0.001%, 70% in 0.01% and 70% in 0.1% treatment. Therefore, mortality started earlier than other treatments in 0.001% treatment but its rate was lowest (Fig. 6).

Discussion

The result showed no differences in lysozyme activity among all treatments. Bowden et al. (2004) stated that lysozyme activity affected by seasonal changes; this means that lysozyme activity increases in summer and decreases in winter. Since this study carried out in winter, lack of difference among groups might be due to seasonal changes.

The results also showed increasing of the serum bactericidal activity in treatments fed on the diet supplemented with immunostimulant compared to the control group. Increasing serum bactericidal activity represents enhancement of the protective

proteins that is occurred after natural infections like outbreaks or artificial ones like vaccination or challenge tests (Biller-Takahashi et al., 2013). In the line of this study, an increase of serum bactericidal activity was observed after administration of *Aegle marmelos* extract or combination of different herbal extracts (Hajibeglou and Sudagar, 2010; Pratheepa et al., 2010). Janssen et al. (1984) also stated that - *D. anethifolia* essential oil has antibacterial effects against gram-positive bacteria. Since *Y. ruckeri* is a gram-negative bacterium (Ispir et al., 2009), it is possible that *D. anethifolia* cannot induce all the immune responses as an immunostimulant.

In this study, the levels of proteins *viz.* albumin and globulin were evaluated after challenge and the results showed that these indices did not enhance in groups that used the herbal essential oil as immunostimulant. In contrast, Hajibeglou and Sudagar (2010) reported the enhancement of the protein, albumin and globulin after challenge in common carp (*Cyprinus carpio*) subsequent to using mixed herbal extracts as immunostimulant. Although increasing of all these three indices is of surprising, because serum globulin is estimated by subtracting the amount of protein and albumin.

Mortality rate was lower in 0.001% group and

higher in 0.01 and 0.1% treatments compared to that of the control group. Similarly, Harikrishnan et al. (2011) reported that higher doses of herbal extracts could have immunosuppressive effects and cause more mortality rates.

As conclusion, it can conclude that the best and most effective dose of *D. anethifolia* essential oil is 0.001% to enhance the immune response and prevent mortality and higher doses of this essential oil can cause more damages and suppress the immunity of fish.

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