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**THE IMMUNOMODULATORY EFFECT OF ELDER BERRY (*SAMBACUSNIGRA* L.)
EXTRACT ON RAINBOW TROUT, *ONCORHYNCHUSMYKISS***

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ABSTRACT

The immunomodulatory effects of elder berry (*Sambacusanigra* L.) orally administered orally to rainbow trout-*Oncorhynchusmykiss*-were investigated against the streptococcal infection. A significant increase was observed in the chemiluminescent responses of the kidney phagocytes after the treatment with extract were significantly increased. Compared to the control group, the fish administered elder berry extract showed high phagocytic activities. In addition, the immunomodulatory effects were found to be dose dependent. The treated fish displayed an increased resistance to both natural and experimental beta-hemolytic streptococcal infection.

Key words: Immunomodulatory, Elder Berry Extract, Rainbow Trout, *Sambacusanigra* L.

INTRODUCTION

Culture of both freshwater and marine fish has been developed successfully, which has eventually resulted in productivity. However, in intensive culture systems the environment

can become highly stressful for the fish with resultant increased susceptibility to fish pathogens. Overcrowding also affects the health of the cultured fish. These conditions

tend to produce a poor physiological environment for the fish and increase the susceptibility to infections (Sakai 1999). Chemotherapy may be useful for controlling the infectious diseases, although it is becoming less effective due to the increase of drug-resistant bacteria (Aoki, Kanazawa et al. 1985; Takashima, Aoki et al. 1985). Immunostimulants and vaccines are used to prevent infectious diseases. More ever immunostimulants increase host resistance to infectious diseases, by enhancing non-specific defense mechanisms rather than enhancing specific immune responses (Sakai 1999). Using these immunostimulants is an effective means of increasing the immunocompetency as well as disease resistance of the fish. Therefore a great number of researches have evaluated the effect of immune stimulating materials on the rainbow trout's immune system (Blazer and Wolke 1984; Grayson, Williams et al. 1987; Kajita, Sakai et al. 1990; Sakai, Kamiya et al. 1991; Hardie, Marsden et al. 1993; Jeney and Anderson 1993; Jeney and Anderson 1993; Jorgensen, Sharp et al. 1993; Jang, Marsden et al. 1995). Research on fish immunostimulants is developing and many agents are currently in use in the aquaculture industry. The present study aims to evaluate the immunomodulatory effect of

Sambucus nigra L. extract on the rainbow trout against the streptococcal pathogens.

MATERIALS AND METHODS

Plant extracts

Elder berry was collected from the plains and jungles of Malayer, Hamadan, Iran. The plant was duly authenticated at the College of Agriculture of Shiraz University, Shiraz, Iran. The method of Okogun (2000) was used in order to obtain the berry extract. Fifty grams (50g) of freeze-dried berry material were extracted with 200 ml of solvent (in the ratio of 9:1ml distilled methanol: water respectively). Extraction was allowed to proceed for 48 hours and then the extract was decanted afterwards; the solvent was removed by evaporation at the room temperature ($28 \pm 2^\circ\text{C}$) in order to obtain the extract. The air dried extract was stored for 48 hours in sterile universal bottles at room temperature. The sterility of the extract was tested before use.

Fish

Two hundred and fifty Rainbow trout-*Oncorhynchus mykiss*-(average body weight 70 g) was obtained from the Sheshpir farm of rainbow trout, Shiraz, Iran. The fish were kept at 18°C and were fed with commercial dry pellets.

Bacterial pathogen

Beta-haemolytic *Streptococcus* sp. SG8004 was isolated from the kidney of the diseased rainbow trout in 2010. The strain was frozen at -80°C in a nutrient broth (Difco) containing 10% glycerol and was inoculated into the blood agar before the experiment. Afterward it was incubated at 30°C until an optical density of 0.4 at 620 nm was reached. Bacterial suspensions were centrifuged and adjusted to a final concentration of 10^1 to 10^8 colony-forming units (cfu) per ml for the LD_{50} assay.

Administration of Extract

Extract was suspended in 0.2 ml of physiological saline (PS) and was orally given to the fish using a catheter. The control fish were given bovine serum albumin (BSA) (Sigma, USA). The groups of live fish were administered 10, 20, 50, and 100 mg/kg elder berry extract or bovine serum albumin. The chemiluminescent response of phagocytes was determined one day after the treatment in order to decide the optimal dose. Immunostimulation of phagocytosis and chemiluminescent response by *Sambucus nigra* L. extracts was also determined 1, 3, 5 and seven days after the fish were treated with 50 mg/kg of extract for three days.

Chemiluminescence (CL) assay

The kidney tissue was removed from each fish and was minced in the RPMI-1640 Medium (Sigma-Aldrich, USA). Cell suspensions were centrifuged at 500 g for five minutes and were washed with the medium. Viable phagocytes, including neutrophils and macrophages, were counted by the trypan blue exclusion test. Also, the cell density was adjusted to 10^7 cells/ml in the medium without phenol red. Twenty mg of zymosan (Sigma, USA) was mixed with 2 ml of collected rainbow trout sera from each group, and reaction continued at 20°C for 30 minutes. The sera were removed by centrifugation and the opsonized zymosan was suspended in an appropriate RPMI medium. Luminol (Katayama Chemical Co. Ltd., Japan) was used to enhance the CL reaction which was examined according to the method of *Kajita et al. (1990)*. L activity was measured with a 1250 Luminometer (Wallic) for 30 min at the room temperature. The peak response in each experimental group was compared with that of the control group.

Phagocytosis assay

Phagocytosis was estimated using the cells adhering to the coverslips. Latex particles (0.85 μm , Difco, USA) suspended at 10^7 per ml in the RPMI 1640 medium supplemented with 5% fetal bovine serum (Gibco®) were

added to the cells adhering to the glass and the coverslip was incubated for 2 hours at 20°C. After the incubation, the glass-adherent cells were rinsed with Hanks' balanced salt solution (Gibco®) to remove free latex beads and were stained with Giemsa solution. The number of the cells with phagocytized latex particles per 300 cells was counted by phase contrast microscopy and the phagocytic activities expressed as the percentage of cells which had phagocytized latex beads.

LD₅₀ determination

Ten fish of each group of rainbow trouts, which had been given 50 mg/kg extract for 3 days were intraperitoneally injected by with live *Streptococcus* sp. at doses ranging from 10¹ to 10⁸cfu/fish one day later. After the injection, the fish were maintained at 18°C and the number of the dead fish was daily recorded for 14 days. All the dead fish were necropsied and the kidney samples were inoculated onto BHI agar (Bactiflask™) in order to verify the cause of the deaths. The LD₅₀ was calculated by the method of Reed and Muench (1938).

Field experiments

Two hundred and fifty rainbow trouts, average body weight 70 g, were acclimated to

17°C for 5 days in three separate ponds until the normal feeding behavior was re-established. Using food oil (Riken Co., Ltd., Japan) the dry feed pellets were coated with elder berry extract, to deliver 0, 10 and 50 mg/kg extract per day for 30 days. The accumulative dead fish were recorded and the kidney smears were inoculated onto the BHI agar (Bactiflask™) for bacteriological examination.

Statistical analysis

The CL and the phagocytic responses of the experimental and the control group were statistically analyzed through the Student's t test. In addition χ^2 test was used in order to analyze the field experiments of natural infections.

RESULTS

CL responses

The dose response relationship of CL in kidney phagocytes of the rainbow trout which were orally administered elderberry extract is shown in **Figure 1**. The phagocytes from the fish which were administered 50 and 100 mg/kg of the extract showed higher CL responses than the other groups ($P < 0.05$). Compared to the control group, no significant differences were observed in CL responses of fish administered 10 or 20 mg/kg extract. Three days after the treatment maximum

mean level of the CL responses in *Sambacusnigra* L.-treated fish was 372 mV which is twice as much as that of the control cells. Significant increases were observed in the CL responses at 1 and 3 days after the treatment ($P < 0.05$); however, no difference was observed 5 days after the treatment (**Figure 2**).

Phagocytosis assay

The phagocytic activity in the fish treated with 50 mg/kg elder berry significantly increased 1 day after the treatment (37.3% compared to 15% in the control group ($P < 0.05$)). It also increased significantly 3 and 5 days after the administration ($P < 0.05$) while it was decreased after 7 days (**Figure 3**).

LD₅₀ against *Streptococcus* sp.

The LD₅₀ value of 1.38×10^5 in the extract-treated fish was significantly different from the LD₅₀ value of $1.38 \times 10^{2.8}$ of the control fish ($P < 0.01$).

Efficacy of *Sambacusnigra* L. extract in the treatment of natural infection with *Streptococcus* SP.

The total numbers of the dead fish were 69 and 91 for the fish fed with 10 mg/kg and 50 mg/kg respectively, which was significantly different from the number of dead fish in the controls (134) ($P < 0.05$) (**Figure 4**).

DISCUSSION

This study demonstrated that elder berry extract had the ability to stimulate the nonspecific immunity of the rainbow trout, and induce protection against the infection with *Streptococcus* sp. It also suggested that the fish phagocytes play an important role in non-specific defense mechanisms against pathogens. The extract significantly stimulated the activity of the phagocytes in the rainbow trout. Previously reported immunostimulants such as FK565 (**Kitao and Yoshida 1986**), levamisole (**Kajita, Sakai et al. 1990**), and chitin (**Sakai 1992**) are also proved to increase the phagocytosis in rainbow trouts. Thus, the increase in phagocytosis may provide a useful in vitro test to investigate the efficacy of the immunostimulants. The CL response of the phagocytes is related to the respiratory burst and the bactericidal activity and was previously used in order to measure the activities of the fish phagocytes (**Scott and Klesius 1981; Stave, Roberson et al. 1983**). This assay was also used to determine the efficacy of a fish vaccine (**Kajita, Sakai et al. 1990; Sakai, Kamiya et al. 1991**). In this study, the CL response in the fish which fed with elder berry extract was significantly stimulated and the peak level was twice as much as that of the control fish.

The maximum CL response of the phagocytes was found in the fish which were administered 10 mg/kg of *Sambacusnigra* L. extract while no significant difference was observed in the fish given 10 mg/kg. Immune suppression was observed in the fish which were treated with high levels of levamisole (Siwicki, Anderson *et al.* 1990). However the elder berry extract in the examined doses was not found to cause suppression and may consequently, be safer for the fish.

The rainbow trouts which received the extract displayed resistance to the experimental infection with *Streptococcus* sp. compared to the control fish. Furthermore, the fish which were fed with elder berry showed protection against the natural infection. In a field trial, the accumulated mortalities were similar in all the groups which were fed with 10 mg/kg or 50 mg/kg elder berry extract. The CL response of the phagocytes was not elevated in the rainbow trouts which received 10 mg/kg extract. These seemingly incompatible results may be explained by the differences in the food consumption between the individual fish, as well as the period of the administration (1 day in CL assays and 30 days in the field experiment).

In this study, the ability of *Sambacusnigra* L. extract to stimulate the non-specific cellular immunity and to induce resistance against the

streptococcal infection has been demonstrated. Elder berry is considered to be the immunostimulant which can be orally administered. Therefore may also be useful in enhancing the non-specific defense mechanisms as well as the potency of vaccines. Further studies are needed to determine the duration of the effect of *Sambacusnigra* L. extract, optimal methods of administration, the minimal active component, the stimulation of humoral defense factors including complement and lysozyme, and its effect when being administered with vaccines.

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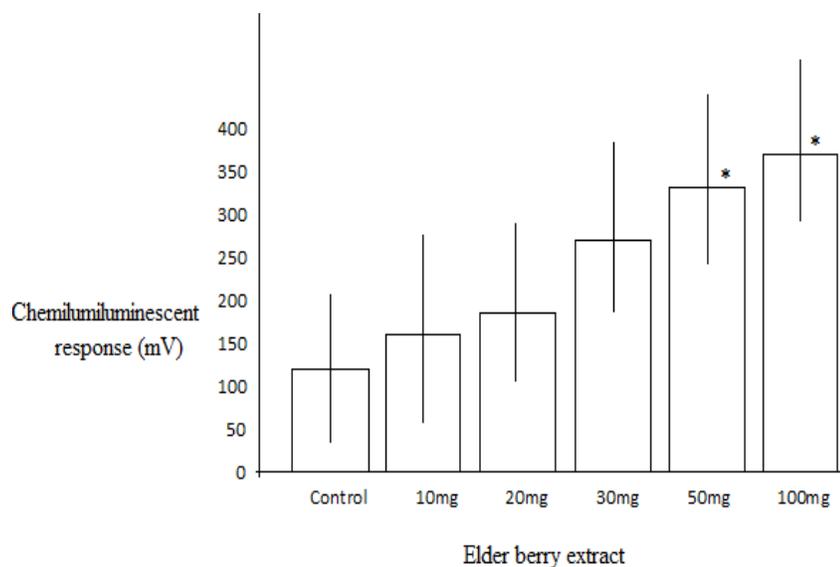


Figure 1: Dose-dependent chemiluminescent responses of the trout phagocytes to the immunostimulant elder berry extract. Difference of the means is significant compared to the control group.*P<0.05

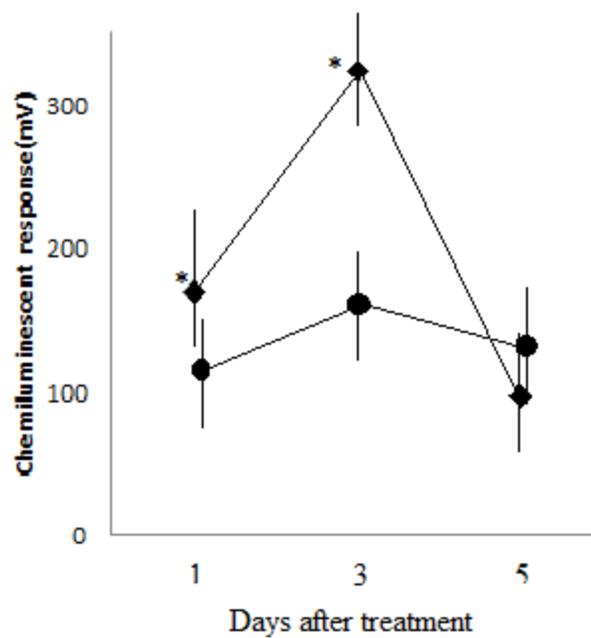


Figure 2: Chemiluminescent responses of the trout phagocytes at varying times after the treatment with elderberry extract (50 mg/kg) (♦) and control (●) for 3 days. Difference of the means is significant compared to the control, *P<0.05

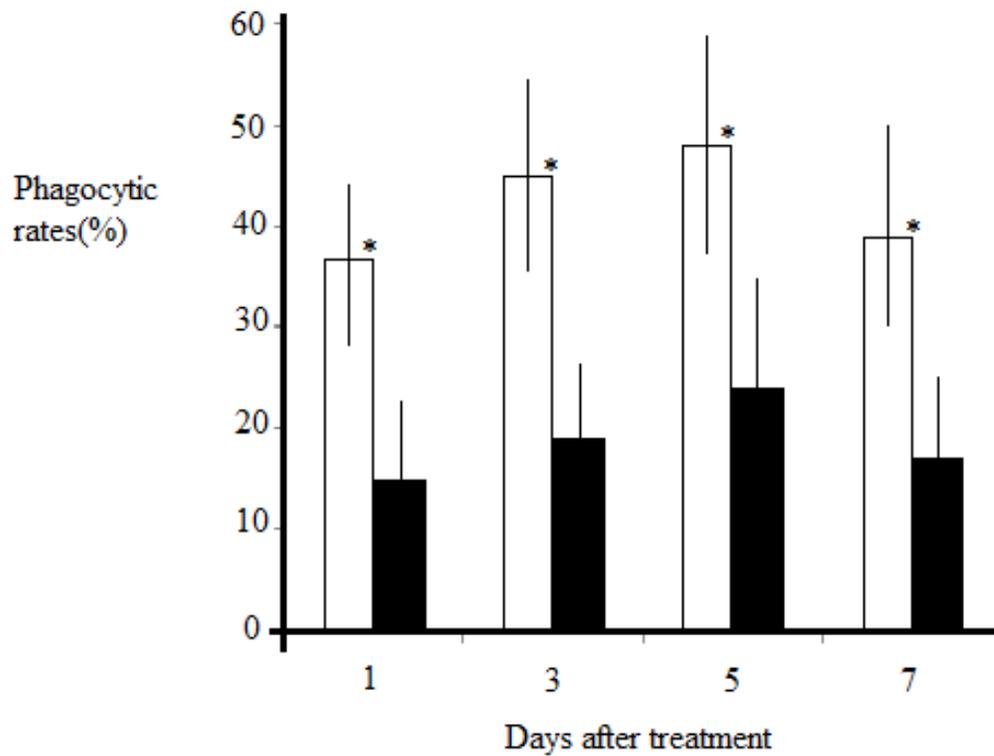


Figure 3: The phagocytic activity of the trout adherent kidney cells 1, 3, 5 and 7 days after the treatment with elder berry extract (□) and control (■) for 3 days. Difference of the means is significant compared to the control ($P < 0.05$)

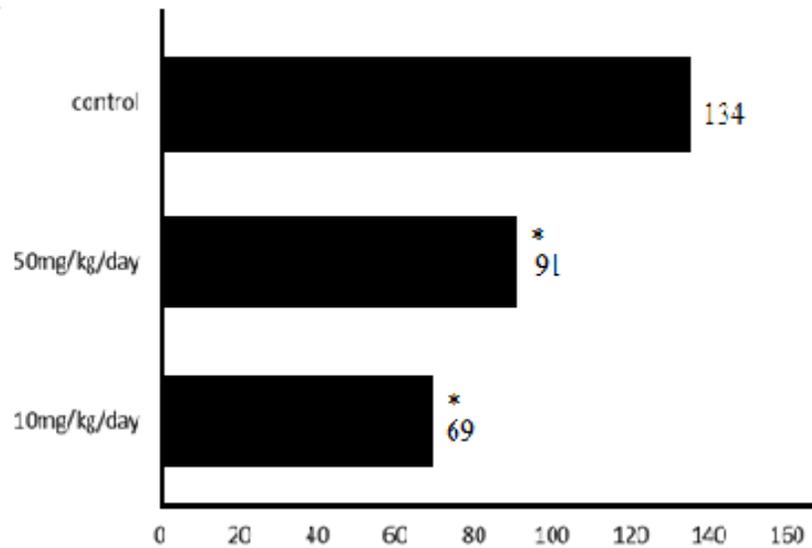


Figure 4: Cumulative mortalities of the fish given elder berry extract (10 or 50 mg/kg per day) from the natural infection of haemolytic *Streptococcus* sp. for 30 days. Difference of the values is significant compared to the control group, ($P < 0.05$)