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Effects of Laurel (*Laurus nobilis*) on the Non-Specific Immune Responses of Rainbow Trout (*Oncorhynchus mykiss*, Walbaum)

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Abstract: In this study, it is investigated that immunostimulant effects of laurel powder by dietary intake on rainbow trout. After 14 days of adaptation on a control diet 3 groups of rainbow trout were fed the experimental diets containing 0.5 and 1% laurel leaf powder for 21 days. The fish were then switched back to the control diet. Non-specific immunity was investigated at the end of the 21 day experimental feeding period and then again 42nd, 63rd days later. The non-specific immune parameters, extracellular and intracellular respiratory burst activities, phagocytosis in blood leukocytes, lysozyme and total plasma protein level were evaluated. The result of this study showed no effect on respiratory burst, lysozyme activity and total plasma protein levels. Phagocytic activity of fish fed diet supplemented 0.5 and %1 laurel, respectively was significantly higher than other groups. However, there is no correlation between dosages of laurel.

Key words: Laurel, *laurus nobilis,* rainbow trout, immunostimulant, experimental feeding, plasma protien

INTRODUCTION

In cultured fish industry, diseases are limiting factors and non-specific defense mechanisms are most important determinant factor on mobilizing to invasion of pathogens. When pathogens such as viruses, bacteria and parasites enter the fish body non-specific defense mechanism are activated. Immunostimulants using in fish culture is a prophylactic measure and improve non-specific defense of the fish. It has been widely accepted by fish farmers.

Many immunostimulants are currently in use in the aquaculture industry. However, some disadvantages of immunostimulants such as high cost, uselessness, low effectiveness are limiting factors. To date, many immunostimulant were determinated for fish (Santarem *et al*., 1997; Gatta *et al*., 2001; Dügenci and Candan, 2003; Cook *et al*., 2003; Puangkaew *et al*., 2004; Ashida and Okimasu, 2005; Misra *et al*., 2006; Rairakhwada *et al*., 2007; Gupta *et al*., 2008; Tewary and Patra, 2008; Cerezuela *et al*., 2009; Kunttu *et al*., 2009). Therefore, effect of some medicinal plants on non-spesific immune response of fish were examined (Dügenci *et al*., 2003; Yin *et al*., 2009; Divyagnaneswari *et al*., 2007).

In the present study laurel leaf were chosen because of their recorded ability antimicrobial and antibacterial effects (Digrak *et al*., 2001; Dadalioglu and Evrendilek, 2004). In this study, it is investigated that immunostimulant effect on rainbow trout fed with different doses of laurel leaf (*Laurus nobilis*) powder. Non-specific defense parameters such as extracellular and intracellular respiratory burst activities, phagocytosis in blood leukocytes, lysozyme and total plasma protein level were observed.

MATERIALS AND METHODS

Fish and experimental design: Experimental fishes of average body weight of 89.25+0.12 g obtained from the farm of Filiz Su Urunleri Limited Company and retained for acclimatization in raceways tanks of 24 m^3 in the same farm for 2 weeks. After acclimatization, the fish were divided into two major treatment groups for administration of laurel. About 4500 fishes were kept in each of the triplicate raceways designed for every treatment group. Laurel was dried under usual conditions and to be grounded in grinding machine. Laurel powder were solubilized in the water and then added to the food at a dose of 0.5 and 1% of the food. The basal diet obtained from commercial company was fed to the fish of both the triplicate raceways of every treatment group at to satiety. The fish were fed containing laurel at dose of 0.5 and 1% during 21 days. After that the fish were fed by basal diet not included laurel. Changes on immune system of rainbow trout were monitored on 21st, 42nd and 63rd days.

Blood and serum collection: The 9 fish were sampled at random from each experimental group on every sampling days on 21st, 42nd and 63rd anaesthetized by 0.01 mg L^{-1}

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of fenoxyethanol. The blood samples of the fish were collected from caudal vein of 9 fish and serum samples were added in different tubes.

Isolation of leukocytes: As collected blood from each fish was used isolation for isolated of leucocytes by density-gradient centrifugation method (Rowley, 1990; Jeney *et al*., 1997). About 1 mL of histopaque 1.119 (Sigma, St. Louis, MO) containing bacto hemagglutination buffer, pH 7.3 was dispensed into a siliconized centrifuge tube. About 1 mL of histopaque 1.077 containing bacto hemagglutination buffer and 1 mL of blood were carefully layered on top. The gradient was centrifuged at 500×g for 20 min at +4°C by using a swing out bucket rotor. The white cell band at the interface of the blood plasma was gently collected with a Pasteur pipette and dispensed into a siliconized tube. The resulting cells were washed twice in HBSS (HBSS, Sigma) and adjusted to 2×10^6 viable cells mL^{-1}.

Determination of respiratory burst activity: Bactericidal activity of phagocytic cells was measured by two methods (detection of extracellular and intracellular activities) as described by Chung and Scombes (1988). About 100 μL of cytochrome c solution (2 mg mL^{-1} in phenol red-free HBSS) containing phorbol 12-myristate 13-acetate (PMA, Sigma, 1 μg mL^{-1}) was added to a 100 μL of leukocyte suspension. To determine of the specificity, a 100 μL of cytochrome c solution containing PMA and superoxide dismutase (SOD, Sigma, 300 U mL^{-1}) was added to the leukocyte suspension (100 μL).

Samples were mixed and incubated at room temperature for 15 min. Samples were read in a multiscan spectrophotometer operating at 550 nm using cytochrome c solution as blank. OD values were converted to nanomoles O_2^- and final results were expressed as nanomoles O_2^- produced per 10^5 blood leukocytes. The intracellular superoxide anion production was identified by the formation of formazan crystals. About 100 μL of blood leukocyte solution was mixed with 100 μL of NBT (0.2% in PBS) containing PMA (1 μg mL^{-1}) and SOD (300 U mL^{-1}). After incubation at room temperature for 60 min, samples were centrifuged at 500×g for 3 min and the supernatants were discarded. Cells were washed twice with HBSS. The formazan crystals were solubilized in 120 μL of 2M KOH and 140 μL DMSO (100%). Absorbance values were read in a multiscan spectrophotometer operating at 620 nm using KOH/DMSO as blank.

Determination of phagocytosis: Phagocytosis was determined by the method of Seeley *et al*. (1990). Briefly, 3 mL of a Congo red solution (0.87% in PBS) was added to yeast cell suspension (1.5 g) and waited for 15 min at room temperature. After that 7 mL of distilled water was added, mixed and autoclaved for 15 min. These cells were then washed three times in HBSS and stored at +4°C till use. Cells were resuspended at 4×10^7 cells/mL in HBSS.

About 250 μL of the leukocyte solution was mixed with 500 μL of the Congo red stained yeast cell suspension (yeast cell/leukocyte, 40/1) then incubated at room temperature for 60 min. About 1 mL HBSS was added and 1 mL of histopaque (1.077) was layered to the bottom by a syringe. To separate macrophages from free yeast cells, samples were centrifuged at 850×g for 5 min. Macrophages were harvested and washed three times in HBSS. The cells were renewed in 1 mL trypsin-EDTA solution (5 g L^{-1} trypsin and 2 g L^{-1} EDTA, Sigma) and incubated at 37°C nightlong. The absorbance values were measured at 510 nm using trypsin-EDTA as blank.

Lysozyme activity: The assay was designated by Osserman and Lawlor (1966) and modificated by Bagni *et al*. (2000) was used to investigate of lysozyme activity. About 50 μL of serum was added triplicate into the plate then mixed with 50 μL of PBS (pH 5.8). About 50 μL of samples were taken from this mixtures and mixed with 125 μL of *Micrococcus lutius* (75 mg mL^{-1} fosfat buffer). Samples were incubated at 0-15 min room temperature and read in a ELISA reader at 450 nm. The hen egg white (Sigma USA) was used as blank.

Statistical analysis: Two-way analysis of variance and PLS multiple range test were run to compare the dietary treatment values at any one sampling time using the SPSS for Windows Release 10.0 program. Statistical analysis was run in the same way for all parameters tested. The accepted levels of significance were 0.05. Results of statistical analysis are corresponding to the study conducted.

RESULTS AND DISCUSSION

The fish fed laurel leaf powder supplemented diets for 21 days demonstrated effect on respiratory burst activities (Intracellular and extracellular) of blood leukocytes were shown in Table 1. Intracellular production of superoxide anion was detected by formation of formazan crystals, displayed that there was not any enhancement production of superoxide anion compared to control groups. The results of 42nd and 63rd the fish not fed by laurel supplemented diets were not shown any differences

Table 1: Non-specific immune parameters in rainbow trout

Days	Parameters	Intracellular activiy	Extracellular activiy	Phagocytic activity	Lysozyme activity	Total protein level
	Control	0.99±0.05	0.1±0.01	1.37±0.04	1412.69±18.7	2.71±0.08
21th	0.5% Laurel	0.94±0.08	0.12±0.01	1.71±0.05	1381.20±69.5	2.54±0.18
	1% Laurel	1.01±0.2	0.10±0.02	1.54±0.06	1475.75±57.2	2.43±0.06
	Control	1.06±0.02	0.12±0.02	1.79±0.07	1575.44±18.95	2.88±0.1
42nd	0.5% Laurel	1.16±0.09	0.15±0.02	2.07±0.06	1643.22±74.7	3.03±0.2
	1% Laurel	1.23±0.22	0.12±0.01	1.91±0.04	1744.89±61.6	2.89±0.07
	Control	1.10±0.05	0.12±0.01	1.60±0.04	1559.69±18.76	2.90±0.08
63rd	0.5% Laurel	1.05±0.08	0.15±0.01	1.94±0.05	1528.20±69.5	2.73±0.18
	1% Laurel	1.12±0.2	0.12±0.03	1.77±0.06	1622.75±57.2	2.62±0.06

Values are mean+SE

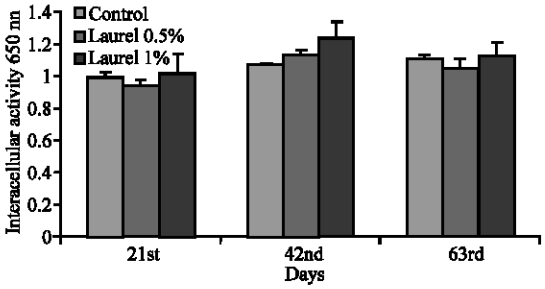

Fig. 1: Intracellular activity of blood leukcytes. Vertical lines are mean±SE

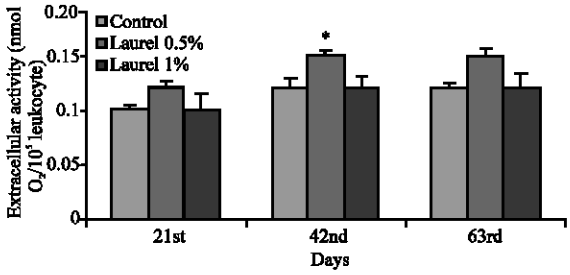

Fig. 2: Extracellular activity of blood leukocytes. *p<0.05; vertical lines are mean±SE

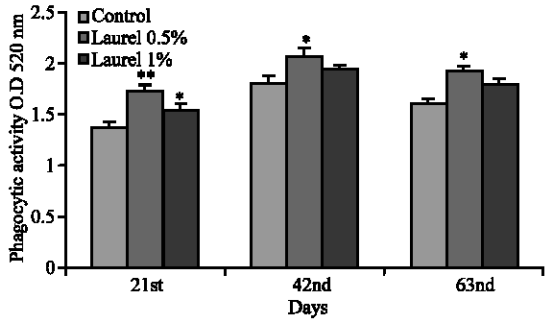

Fig. 3: Phagocytic activity observed on different days. Values are mean±S.E. Mean values bearing same superscript are not statistically significant, p>0.05

compared to day 21st (Fig. 1). In addition to extracellular oxidative radical production was not shown any effects on day 21st compared to the control group. But on day

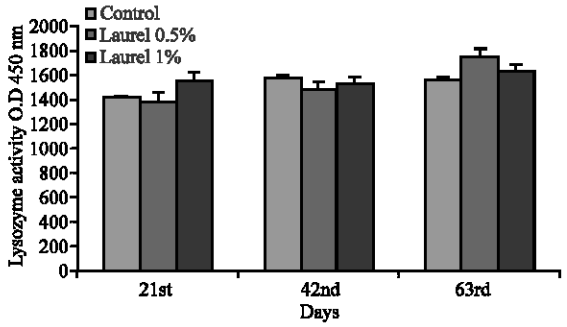

Fig. 4: Lysozyme activity observed on different days. Values are mean±SE

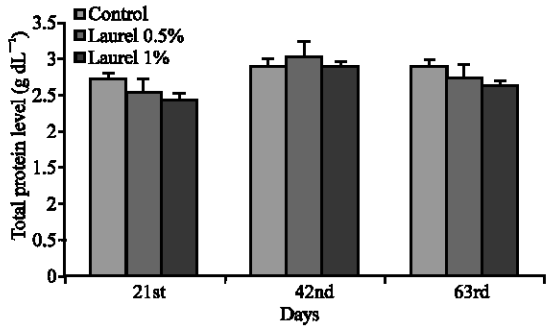

Fig. 5: Total protein levels. Values are mean±SE

42nd extracellular activity of laurel 0.5% was significantly higher than laurel 1% and control group (p>0.05) (Fig. 2).

Phagocytosis of blood leukocytes increased in all groups and this increase respectively was fed with 0.5 and 1% laurel group on 21st day (Fig. 3). On 42nd and 63rd days, there was no differences between 1% laurel and control group. Although, 0.5% laurel group was shown the highest value.

Changes in serum lysozyme activity after fed the fish laurel leaf powder supplemented diet were not shown sany significant differences between groups any time of the research (p<0.05) (Fig. 4). Total protein levels in plasma was not shown significant differences in all groups (p<0.05) (Fig. 5).

CONCLUSION

In the prevention of fish diseases, non-specific immune systems is an important factor in protecting fish. Several immunostimulatory chemicals have been used for this purpose (Sakai, 1999). Respiratory burst activity mostly used immunological technique to determine changes in defense mechanism. In this study two methods used to determine respiratory burst. The reduction of the redox dye NBT to determine intracellular O_2^- and the reduction ferricytochrome c to determine extracellular O_2^-. Many researcher reported that dietary immunostimulants such us glukan (Dalmo and Bogwald, 1996; Couso *et al*., 2003), zeranol (Keles *et al*., 2002), fermented vegetable product (Ashida and Okimasu, 2005), *Saccharomyces cerevisiae* derivative and vitamin C (Verlhac *et al*., 1998) were increased intracellular activity. Unlike in the study, we could not detect any effect of laurel on respiratory burst activity.

The phagocytic activity of the blood leucocytes from rainbow trout treated with laurel powder was significantly higher than the control and especially increased 0.5% laurel containing food. It is well-known that fish treated with immunostimulants show increased phagocytic activity (Sakai, 1999) and the results support that Chung and Secombes (1987) indicate that humoral factors may enhance phagocytosis in fish. But it is difficult to explain for the results because of our results showed that there is no differences between plasma protein level of laurel and control group.

Serum lysozyme levels were used as parameters to assess the influence of dietary laurel supplementation on the humoral components of the non-specific defense mechanism of the experimental fish. Serum lysozyme activity of this research showed no differences between control group.

Lysozyme activity of trout maintained for 4 weeks on diets of differing glucan concentrations (0, 0.1, 0.5 and 1.0%) (Jeney *et al*., 1997) and oral administration of glucans on *Scophthalmus maximus* (De Baulny *et al*., 1996) did not find any difference in the serum. Those results are in line with this research. The results showed that laurel powder is not able to enhance non-specific and specific defense mechanisms of rainbow trout. However, further studies including determination of optimal dose, extraction and treatment method could change this results.

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