The effects of short- and long-term diet supplementation with Iranian propolis on the growth and immunity in rainbow trout (*Oncorhynchus mykiss*)

Tukmechi, A.[1*]; Karimi Rad, F.[2]; Farrokhi, F.[3]; Agh, N.[4] and Jalili, R.[5]

[1]Department of Pathobiology and Quality Control, Artemia and Aquatic Animals Research Institute, Urmia University, Urmia, Iran; [2]Graduated from Faculty of Science, Urmia University, Urmia, Iran; [3]Department of Biology, Faculty of Science, Urmia University, Urmia, Iran; [4]Department of Ecology and Nutrition, Artemia and Aquatic Animals Research Institute, Urmia University, Urmia, Iran; [5]Graduated from Faculty of Natural Resources, Urmia University, Urmia, Iran

[*]***Correspondence:*** *A. Tukmechi, Department of Pathobiology and Quality Control, Artemia and Aquatic Animals Research Institute, Urmia University, Urmia, Iran. E-mail: atokmachi@gmail.com*

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Summary

Propolis, a resinous substance collected by *Apis mellifera* bees from various plant sources is transformed in the presence of bee enzymes. Short- and long-term effects of diet supplementation with ethanol extract of Iranian propolis (EEIP) was investigated on growth and immunity in juvenile rainbow trout. The fish (mean body weight 30 ± 3.2 g) were fed a commercial diet supplemented with 0 (control), 1, 2 and 5 g/kg EEIP for 96 h and 0, 0.5, 1 and 2 g/kg EEIP for a 45-days. Rainbow trout growth performance significantly ($P<0.05$) increased by the dietary supplementation of Iranian propolis. Our results showed that significant increase in serum lysozyme activity, complement activity and total immunoglobulin were seen in all treatment groups during short- and long-term feeding trial when compared to the control group. On the basis of our findings, propolis improved rainbow trout growth performance and some immune parameters.

Key words: Rainbow trout, Iranian propolis, Growth performance, Immunity

Introduction

The usefulness of immunostimulants has been demonstrated in aquaculture (Nagai *et al*., 2001), and fish farmers use a wide range of immunostimulants which may or may not need to be purified (vitamins, chitin, glucans, etc.) (microorganisms, animal and plant extracts, sub products of other industries, etc.). The second category of immunostimulants have recently received more attention since they are cheaper, easy to incorporate into the diet and have low impact on the environment. Besides, they have many additional effects on fish physiology because they act as a "cocktail" containing many nutrients, micronutrients as well as immunostimulant substances (Ji *et al*., 2007).

Propolis (bee glue) is a complex resinous mixture collected by bees from bud and plants secretions, which is transformed in the presence of bee enzymes. Its color varies from green, red to dark brown. Propolis is adhesive and has a characteristic smell and reacts strongly with skin lipid and proteins. Due to its numerous pharmacological properties, it has been used in folk medicine since ancient times. In general, propolis is composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollen, and other substances (Burdock, 1998).

Propolis is a non-toxic natural product with multiple pharmacological effects and complex chemical composition (Burdock, 1998). Nowadays, propolis is used extensively in poultry and fish as a growth promoter (Meurer *et al*., 2009; Beyraghdar Kashkooli *et al*., 2011), as adjuvant for mammals and poultry or as immunostimulant for fish (Cuesta *et al*., 2005; Chu, 2006; Abd-el-Rhman, 2009; Talas and Gulhan, 2009; Zhang *et al*., 2009).

Because of the importance of rainbow trout (*Oncorhynchus mykiss*) as the most important aquacultured fresh water fish in Iran (Akhlaghi and Sharifi Yazdi, 2008) and the proven immunostimulatory effects of propolis in mammals, and recent finding on effects of Iranian propolis against some fish pathogens (Tukmechi *et al*., 2010), we decided to examine the impact of short- and long-term dietary supplementation of ethanol extracted propolis on growth, innate immune response and disease resistance of this fish. Further, the goal was to assay the safety of Iranian propolis (as a natural product) for application in aquatic animals as growth and immune stimulator.

Materials and Methods

Preparation of propolis

Crude propolis samples were collected from the honey bee, *Apis mellifera carnica*, apiaries of a local bee

farm near Urmia city. Hand collected propolis was kept in a dry place and stored at 4°C until processing. The sample was cut into small pieces and 25 g of ground propolis was extracted by 250 ml of 80% ethanol using an orbital shaker at 150 rpm at 25°C for 48 h. The ethanol extract was then filtered through a Whatman 42 filter paper. Propolis samples were dried by evaporation, weighed and then diluted in ethanol to obtain a 10% (w/v) solution. Samples were stored in the dark at 4°C. Its biochemical composition was determined by gas chromatography-mass spectrometry (Tukmechi *et al*., 2010) and used within 2 months (Bosio *et al*., 2000; Yang *et al*., 2007).

Experimental design

Eight hundred and forty rainbow trout (30 ± 3.2 g) were purchased from a commercial fish farm in Urmia, Iran. Fish were acclimatized to the laboratory condition for 10 days in 300 L tanks using aerated deep well water. The culture conditions included: temperature (15 ± 1°C); pH (7.5); dissolved oxygen (8 ± 0.2 mg/ml); natural photoperiod (10 h light/14 h dark); flow rate (1.25 l/s). Fish were fed an average of 3% initial body weight per day (at 8 am, 14 and 20 pm) with commercial rainbow trout feed (40% protein and 4300 Kcal/kg digestible energy).

Diet preparation and feeding trial

Commercial basal diet (Faradaneh, Iran) was crushed and mixed with water and sufficient amount of ethanol extract of Iranian propolis (EEIP) to obtain supplemented diet with 0 (control), 1, 2 and 5 g/kg for a short-term and 0.5, 1 and 2 g/kg of diet for a long-term period. The diets were repelleted, allowed to dry and coated with fish oil. The diets were stored at 4°C until use. At short-term feeding trial, three hundred and sixty fish were divided into 4 groups (in triplicate), 30 animals per tank and were fed EEIP for a 96-h period (Talas and Gulhan, 2009) and sampling was done for immunological assay at 0 and 96 h. For long-term period, four hundred and eighty fish were divided into 4 groups (in triplicate), 40 animals per tank and were fed EEIP for 45days. The culture of long-term feeding trial group was continued for another 15 days and during this time all fish were fed control diet without propolis supplementation. Samples (3 individuals/tank, 9/treatment) were collected on days 0, 15, 30, 45 and 60, to measure immunological parameters.

Growth parameters

Five fish were randomly harvested from each tank on day 45 to measure the following growth indices (Mohammadpour, 2011):

Percentage weight gain (WG) = $(W_2-W_1) \times 100/W_1$

where,
W_1: Initial weight (g)
W_2: Final weight (g)

Condition factor (CF) = $W/L^3 \times 100$

where,
W: Final weight (g)
L: Total length (cm)

Specific growth rate (SGR) = $100 (\ln W_2-W_1) / T$

where,
W_1: Initial weight (g)
W_2: Final weight (g)
T: Number of days in feeding period

Immunological parameters

Fish were anesthetized with 200 mg/L clove oil; blood was collected from caudal vein using heparin coated syringe and transferred into sterile tubes. Blood was allowed to clot at room temperature for 1 h and stored in a refrigerator overnight. The clot was then centrifuged at 1500 × g for 5 min. The serum was collected and stored in sterile eppendorf tubes at -20°C until immunological assays.

Serum lysozyme activity

The serum lysozyme activity was measured by the method of Tukmechi *et al*. (2011) based on the lysis of the lysozyme sensitive gram positive bacterium, *Micrococcus lysodiekticus* (Sigma, USA). The dilutions of hen egg white lysozyme (Sigma, USA) ranging from 0 to 20 mg/ml (in 0.1 M phosphate-citrate buffer, pH = 5.8) were considered as the standard. This along with the undiluted serum sample (25 ml) was placed into wells of a 96-well plate in triplicate. One hundred seventy five µL of *M. lysodiekticus* suspension (75 µg/ml) prepared in the same buffer was then added to each well. After rapid mixing, the change in turbidity was measured every 30 s for 5 min at 450 nm at approximately 20°C using a microplate reader. The equivalent unit of activity of the sample as compared to the standard was determined and expressed as mg/ml serum.

Serum hemolytic complement activity (ACH50)

The hemolytic complement activity was assayed using rabbit red blood cells (RRBC) as targets (Tukmechi *et al*., 2011). Rabbit red blood cells were washed three times in ethylene glycol tetraacetic acid-magnesium-gelatin veronal buffer (0.01 M EGTA-MgeGVB, pH = 7) and the cell numbers were adjusted to 2×10^8 cells ml^{-1} in the same buffer. First, the 100% lysis value was obtained by adding 100 ml of the above RaRBC to 3.4 ml distilled water. The hemolysate was centrifuged and the optical density (OD) of the supernatant was determined at 414 nm using a spectrophotometer (Awareness, USA). Following this, the test sera were diluted (100 times), different volumes ranging from 100 to 250 ml (total volume was adjusted to 250 ml with the buffer) wereallowed to react with 100 ml of RaRBC in small test tubes. This mixture was incubated at 20°C for 90 min with intermittent mixing, following which 3.15 ml of 0.85% NaCl solution was added and the tubes were centrifuged at 1600 × g for 10 min at 4°C and the OD of the supernatant was measured as mentioned above. A lysis curve was obtained by plotting the percentage of hemolysis against the volume

of serum added on a log-log graph. The volume yielding 50% hemolysis was used for determining the complement activity of the sample as follows:

ACH50 (units/ml) = K × (reciprocal of the serum dilution) × 0.5

where,
K: The amount of serum (ml) giving 50% lysis
0.5: The correction factor since the assay was performed on half scale of the original method

Serum total antibody level

Serum total immunoglobulin was determined following the method of Siwicki *et al*. (1994). After dilution of serum samples with 0.85% NaCl (100 times), total protein content was determined by Bradford method (Kruger, 1996). Briefly, 100 ml of total serum samples were mixed with an equal volume of 12% solution of polyethyleneglycol (Sigma, USA) in a 96-well microtiter plate. After 2 h of incubation at room temperature, plate was centrifuged at 5000 × g at 4°C. The supernatant was diluted 50 times with 0.85% of NaCl and the protein content was determined by Bradford method. This value was subtracted from the total protein level and the result was equal to the total immunoglobulin concentration of the serum that was expressed as mg/ml.

Statistical analysis

The results were subjected to analysis of variance (ANOVA) followed by Tukey's post hoc to compare different treatments using SPSS version 19. Correlation coefficients were considered significant at P<0.05.

Results

At the end of the experiment, total length, final weight, WG (%), specific growth rate (SGR) and CF of rainbow trout fed on the experimental diets for 45 days were determined and presented in Table 1. No mortality was observed in any of the groups over the entire periods of the experiment. Long period dietary administration of EEIP into the rainbow trout diet significantly (P<0.05) improved growth performance when compared to the control group.

Before the experiment, no significant changes in all immunological parameters were observed between groups. Significant increase in lysozyme activity was shown in all treatment groups after 96-h feeding (Fig. 1). The serum complement activity of fish exposed to 2 g/kg of EEIP was considerably higher than that of fish in control group (Fig. 2). Serum total immunoglobulin level showed a significant increase in fish fed 2 g/kg of EEIP compared to the control group (Fig. 3).

Statistical analysis also showed that the lysozyme activity was significantly increased on days 30 and 45 in fish that received 2 g/kg of EEIP in diet (Fig. 4). On days 30 and 45, complement activity was significantly higher in the same fish than the control fish (Fig. 5). On days 15, 30 and 45, this group also showed a significant increase in total immunoglobulin level (Fig. 6).

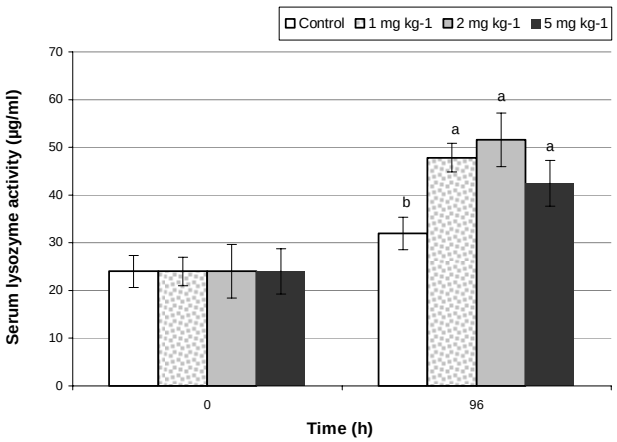

Fig. 1: The lysozyme activity of rainbow trout fed with EEIP. Each value (mean±SE) is the average performance of nine fish per treatment for a period of 96 h. Different letters represent significant differences between bars (P<0.05)

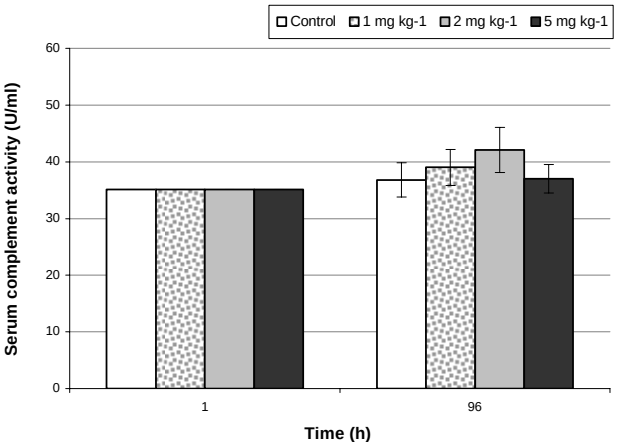

Fig. 2: The complement activity of rainbow trout fed with EEIP. Each value (mean±SE) is the average performance of nine fish per treatment for a period of 96 h

Table 1: Growth performance of rainbow trout fed with EEIP. Each value (mean±SD) is the average performance of fifteen fish per treatment for a period of 45 days

Items	Control	Ethanol extract of Iranian propolis concentration (g/kg)		
		0.5	1	2
Final weight (g)	65.23 ± 3.4[a]	67.12 ± 2.76[a]	68.01 ± 4.53[a]	68.99 ± 3.33[a]
Total length (cm)	18.21 ± 1.9[a]	19.01 ± 2.1[a]	19.23 ± 2.7[a]	19.41 ± 1.4[a]
Percentage weight gain	157.65 ± 12.4[a]	159.43 ± 15.4[a]	162.28 ± 16.5[a]	163.14 ± 15.4[a]
Specific growth rate (%)	0.86 ± 0.05[a]	0.87 ± 0.05[a]	0.91 ± 0.04[a]	0.91 ± 0.06[a]
Condition factor	0.86 ± 0.05[a]	0.87 ± 0.05[a]	0.9 ± 0.02[a]	0.9 ± 0.03[a]

The same superscript alphabets in the same row are not significantly different at P<0.05

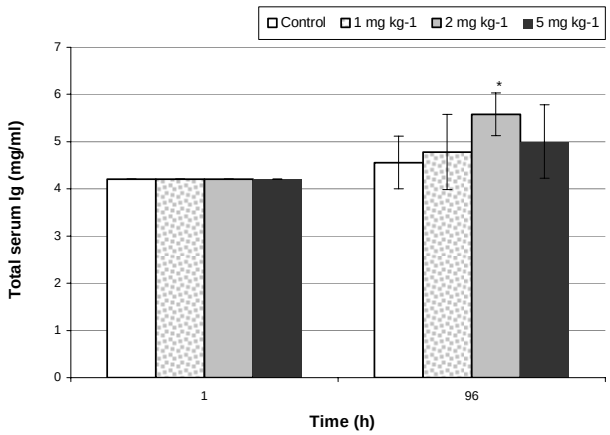

Fig. 3: The total antibody level of rainbow trout fed with EEIP. Each value (mean±SE) is the average performance of nine fish per treatment for a period of 96 h. Asterisk indicates statistically significant differences (P<0.05)

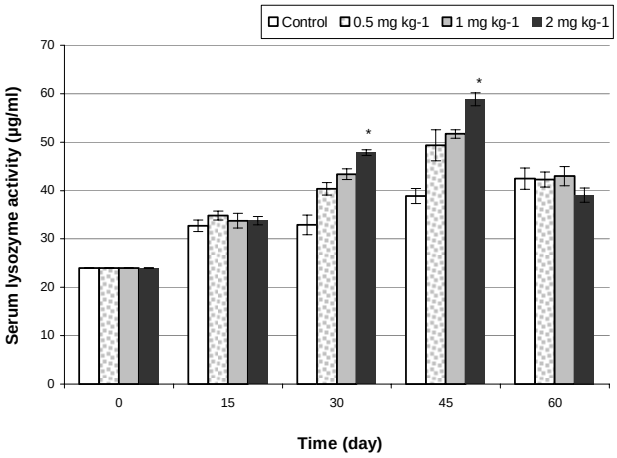

Fig. 4: The lysozyme activity of rainbow trout fed with EEIP. Each value (mean±SE) is the average performance of nine fish per treatment for a period of 45 days. Statistically significant differences are indicated by asterisks (P<0.05)

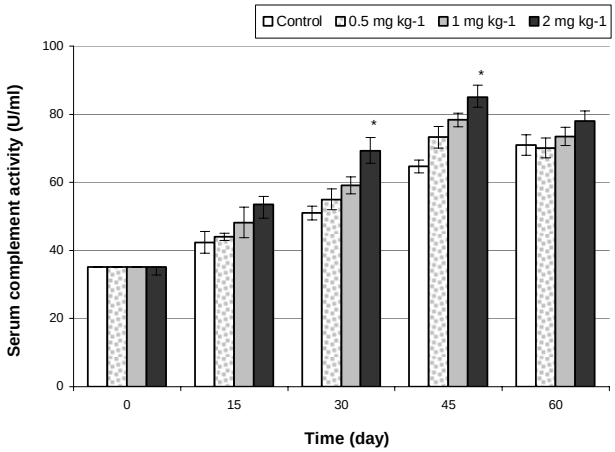

Fig. 5: The complement activity of rainbow trout fed with EEIP. Each value (mean±SE) is the average performance of nine fish per treatment for a period of 30 days. Statistically significant differences are indicated by asterisks (P<0.05)

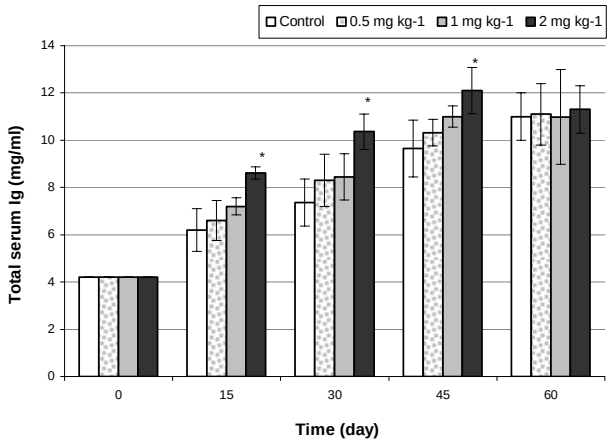

Fig. 6: The total antibody level of rainbow trout fed with EEIP. Each value (mean±SE) is the average performance of nine fish per treatment for a period of 45 days. Statistically significant differences are indicated by asterisks (P<0.05)

Discussion

Nowadays, large quantities of antibiotics are administered to human and animals to treat disease and is also commonly used at sub therapeutic levels for livestock to prevent disease and promote growth (Sugita *et al*., 1996). Fish are protected from infectious diseases by vaccination or chemotherapeutic treatments. However, because of extensive use of antimicrobial agents, the occurrence of resistance among pathogens and the associated environmental problems have been well documented (Esiobu *et al*., 2002; Andani *et al*., 2012). Therefore, several alternative strategies including the use of immunostimulants have been proposed. Fish defense mechanisms are improved through prophylactic administration of immunostimulants (Dugenci *et al*., 2003). In the present study, the supplementation of Iranian propolis improved the growth in rainbow trout. Similarly, recent work with rainbow trout (Gulhan *et al*., 2012) demonstrated that the administration of propolis increased total counts of psychrophilic and mesophilic bacteria in digestive tract. Similarly, Abd-El-Rhman (2009) and Meurer *et al*. (2009) showed that incorporation of brown propolis extract and crude propolis increased the growth performance of Nile tilapia (*Oreochromis niloticus*). According to their results, these agents decreased the feed conversion ratio too. Our results indicated that EEIP or crude propolis has a growth stimulating action for fish. The large number of chemical components in Iranian propolis (Tukmechi *et al*., 2010) may justify its numerous biological activities. Talas *et al*. (2012) showed that dietary administration of propolis increased blood parameters such as triglyceride, urea, total cholesterol, cobalt and ALT (alanine amino transferase), AST (asparate alanine amino transferase) and LDH (lactate dehydrogenase) values in carp (*Cyprinus carpio*). However, it is possible hypothesize that propolis complex composition may lead to better intestinal health, blood parameters, enzymes and improved digestion and absorption, and thereby improved the growth performance (Deng *et al*., 2011).

Lysozyme is a fish defense compartment that causes hydrolysis of the N-acetylmuramic acid and N-acetylglucosamine which are constituents of the peptidoglycan layer bacterial cell wall, and activation of the complement system and phagocytes by acting as an opsonin (Magnadottir, 2006). Thus, lysozyme activity is an important indicator of the immune defense of both invertebrates and vertebrates (Abd-El-Rhman, 2009). In the current study, we observed that the short- and long-term administration of EEIP significantly increased the serum lysozyme activity in rainbow trout. The increased lysozyme activity was seen after supplementing the fish-feed with 2 g/kg of EEIP at both periods (Figs. 1 and 4).

The bacteriolytic activity of complement constitutes an important part of natural humoral immunity of fish and has an effective role towards a range of microorganisms, except those containing large quantities of sialic acid (Kreutz *et al*., 2011). In the present work, complement hemolytic activity was higher in fish that received 2 g/kg of EEIP in short- and long-term period compared to the respective control groups. This increase can be attributed to the effect of propolis on liver and leukocyte production, the important sites for the synthesis of complement system components. In previous studies in mice model, stimulating effect of propolis was clearly shown on complement activity (Orsolic *et al*., 2003). However, Cuesta *et al*. (2005) reported that intraperitoneal administration of propolis and dietary EEP inclusion (0.1 or 10 g/kg EEP) had no effect on serum complement activity in gilthead seabream (*Sparus aurata*). The apparent discrepancy among these studies may be attributed to the propolis source, dose, and fish species.

Plasma proteins include the humoral elements of the nonspecific immune system such as immunoglobulin. In this study, the total immunoglobulin level of serum in fish that received 2 g/kg of EEIP during short- and long-term feeding trial increased significantly. In humans, oral administration of propolis did not affect plasma immunoglobulin and cytokine (TNF-alpha, IL-1beta, IL-8, IL-6) levels, though peripheral blood leucocytes were *ex vivo* primed to produce and release them (Bratter *et al*., 1999). The importance of humoral immunity and the scarce available data concerning the effect of propolis deserve further study.

Our results indicated that diet supplemented with ethanol extract propolis enhanced the growth and immunity in rainbow trout. Due to the presence of some effective compounds such as flavonoids (flavones and flavanones), phenolic acids and their esters in propolis and propolis extracts, it could be used as a fish immune stimulant if its positive physiological properties and non-toxicity to fish are proved. Due to the immune stimulating properties of propolis, it may not only prolong the physiological functions of some aquatic living organisms, but also contribute to the health benefit of consumers who consume aquatic animals (Talas and Gulhan, 2009).

It can be concluded that short- and long-term administration of EEIP in the diet of juvenile rainbow trout significantly improved the growth performance. In addition, supplementation with EEIP for long-term period generally increased the serum lysozyme, complement activity and total immunoglobulin. These results indicate the potential of EEIP to be used as a growth promoter and non-specific immunostimulant for rainbow trout. However, there is still a need for further studies regarding the use of propolis extracts or its constituents and environmental distribution as natural antioxidant, possible food supplement and natural protective agent.

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