Persian bee propolis and pollen extracts enhanced the non-specific immune response of rainbow trout (*Onchorhynchus mykiss*) and resistance against *Aeromonas hydrophila*

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Abstract

The effect of Persian bee propolis and pollen extracts on innate immune response of rainbow trout (*Onchorhynchus mykiss*) was investigated. Fish were fed diets containing 1% Pollen, 1% propolis and combination of the two immune-stimulants (0.5% Pollen and 0.5% propolis) as treatment groups and fed without the two immune-stimulants as positive and negative control groups for 4 weeks. The effects of different stimulants at different weeks (Time × stimulants) were analyzed with the Repeated Measures ANOVA. The first-four groups were then subjected to *Aeromonas hydrophila* for 5 days. The phagocytic activity, respiratory burst activity, Plasma Total Protein and cumulative mortality were monitored. The results showed that fish fed diet containing 1% propolos or 0.5% Pollen+ 0.5% propolis enhanced non-specific immunity including respiratory burst activity, phagocytosis but decreases the mortality in challenging with *A. hydrophila* by 45 and 65%, respectively.

Keywords: Propolis, Pollen, Non-specific immunity, *Onchorhynchus mykiss*

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Introduction

Promotion of the immune system of the wild and cultivated fish has been studied and it seems strength the innate defense against the pathogens as well as the acquired immune mechanism is impressive. Using immunostimulants individually or in combination with others is an effective method for enhancing the defense capabilities of fish.

The acquired immune response can be accomplished with vaccines, which promote the specific immune system of fish. Nevertheless, with regard to many pathogens such as bacterium *Aeromonas hydrophila*, are not yet commercially available (Yin *et al*., 2009) owing to the complicated antigenic structure (Ardó *et al.*, 2008). Contrarily, immunostimulants like herbs enhance the innate immune system which contains a series of blood cells, macrophages, granulocytes as well as humoral components such as lysosymes (Magnadóttir, 2006).

In the last decade, there has been an incredible rise in scientific activities about bee pollen, which come from many plants (Nabavi *et al.*, 2012) so the contents of bee pollen can vary significantly (Schreck *et al.*, 2001). Pollen contains remarkable amounts of fatty acids, proteins, carbohydrates, lipids, vitamins, minerals and phenolic and immunological compounds that can be important for human or animal health such as aquatics (Xu *et al*., 2009).

Propolis (honey bee glue), the composition of which is directly dependent on the composition of the vegetation of the region, is a dark-coloured, resinous material produced by mixing their own waxes in hypo pharyngeal secretions with resins collected from plants for protection of hives as sealer (Selamoglu Talas *et al*., 2012). It is used as a phenolic compound capable of preventing human or animal diseases by decreasing oxidative stress. As regards its role on the immune response of mammals, propolis may be capable of activating macrophage and lymphocyte functions as well as resisting few microbes in several species (Cuesta *et al*., 2005). Choobkar *et al.* (2014) showed that propolis compared with the control group enhanced the immune system in addition to wound healing in the skin of the carp.

Different species have different reactions to stress. Species may vary in their physiological response to stressors. For example, handling may change the timing of reproduction as the case in species such as rainbow trout (*Oncorhynchus mykiss*) (Kakoolaki, Shapour *et al*., 2013). The most potent microbicidal component in propolis is *flavanone pinocembrin* (Yin *et al*., 2009).

Rainbow trout is a fast-growing and an eminently commercial coldwater fish species, highly familiar in Iran and is one of the most highly delicate foods. This fish is intensively cultivated in Iran, Norway, USA, Chile, Denmark and France (Shamloofar *et al*., 2015).

The motile *Aeromonas* group, especially *A. hydrophila*, affects a wide

variety of freshwater fish species. *A. hydrophila* is more abundant in waters with a high organic load than in relatively unpolluted water (Yin *et al*., 2009). This pathogen has been responsible for a number of disease outbreaks in Iran and the immunostimulants may be of great promise in the prevention of infectious diseases of rainbow trout such as *Streptococcus iniae*.

Because of the importance of rainbow trout (*O. mykiss*) in fish aquaculture and the proven immunostimulatory effects of propolis and pollen to give early activation to the non-specific immune responses in mammals, we tested the effects of propolis and pollen administered in vivo on the innate immune response of this fish.

Materials and methods

Fish and experimental design

O. mykiss, a common cold water fish in Iran, was used as the experiment species. One hundred and eighty fish weighing 50±5.0 g were used for non-specific cellular assays and disease resistance tests. All the fish were distributed among 15 glass aquaria (250 L) as three treatments (1% pollen, 1% propolis, 0.5% pollen + 0.5% propolis), negative control and positive control in triplicate, each consisting of 30 animals. They were then acclimated to the determined water conditions (temperature: 15.50 ± 1.0 and pH: 6.8±0.1) prefilled with clean aerated well water and did not show any signs of disease during the acclimation period. They were kept and siphoned during the experiment before exposure to *A. hydrophila* in the 5th week in which the condition of disease resistance was revealed. Water flow was maintained at 6 L/h (It was approximately replaced once a day for each aquarium). Fish were fed ad libitum 4 times a day with a commercial pelleted feed containing either pollen (1%), or propolis (1%), or a combination of pollen (0.5%) and propolis (0.5%) for 5 weeks. They were kept at the ambient, uncontrolled temperature of 15±1.0^{o}C under natural photoperiod.

Crude propolis, pollen and ethanolic-extract

Propolis and pollen ethanolic-extract was prepared by adding 30 mL of absolute ethanol to 3 g of each of them in bottles, which were sealed and kept from light and moderately shaken for 1 day at room temperature. The extract was then filtered twice, dried and stored in sealed bottles at 4°C until use (Abd-El-Rhman, 2009). They were used in combination with food as 1% during the experiment.

Blood sampling and total protein plasma

Nine Fish blood samples (three fish/group/replication) were withdrawn from t hecaudal vein 1, 2, 3 and 4 weeks after the start of feeding using 1 mL syringes with 24-gauge needle (Kakoolaki *et al*., 2011). They were pre-filled with heparin as an anticoagulant for Individual fish to be

sampled once to prevent the interference in the experiment due to multiple bleeding in the fish. A part of blood was then transferred into 1.5 mL heparinated tubes (Trittau, Germany) for hematology study and the other part transferred into non-heparinated tube for plasma biochemistry analysis. Heparinated blood samples were placed in a refrigerator at 4°C. Non-heparinated samples were immediately centrifuged at 4°C with 1500 ×g for 4 minutes. Plasma was collected with a micropipette and stored at −80°C until analysis. (Baba *et al.*, 2015). Total protein concentration of plasma (PTP) were analyzed using an autoanalyser (Mindray BS-200, China), with commercial clinical investigation kits (Pars Azmoon Kit, Tehran, Iran).

Separation of leucocytes from the blood

Leucocytes for assay were separated from each blood sample by density-gradient centrifugation. One milliliter of histopaque 1.119 (Sigma) containing 100 µL of bacto hemagglutination buffer, pH 7.3 (Difco, USA) was dispensed into siliconised tubes. One milliliter of a combination of histopaque 1.077 and hemagglutination buffer was layered carefully on the top of the gradient (Ardó *et al*., 2008). Bleeding procedure was completed within 1 min. The sample preparations were centrifuged at 700 g for 15 min at 4°C. Plasma was then collected and stored in sterile eppendorf tubes at -80°C for future application. Separated leucocytes were gently removed and dispensed into siliconised tubes, containing phenol red free Hanks Balanced Salt Solution (HBSS, Sigma). The Cells were then washed twice in HBSS and adjusted to 10^7 viable cells mL^{-1} (Christybapita *et al*., 2007).

Phagocitic assay

Phagocytic activity of blood leucocytes was determined spectrophotometrically (Seeley *et al.*, 1990). This assay involves the measurement of congo red-stained yeast (*Saccharomyces cerevisiae*) cells that have been phagocytised by leucocytes. To perform the assay, 1000 mL of the leucocyte solution was mixed with 2000 mL of the congo red-stained and autoclaved yeast cell suspension (providing a yeast cell: leucocyte ratio of 20:1). The combinations were incubated at room temperature for 60 min. Following incubation, 1 mL ice-cold HBSS was added and 1 mL of histopaque (1.077) was injected into the bottom of each sample tube. The samples were centrifuged at 850 g for 5 min to separate leucocytes from free yeast cells. Leucocytes were harvested and washed twice in HBSS. The cells were then resuspended in 1 mL trypsin–EDTA solution (5.0 g L^{-1} trypsin and 2.0 g L^{-1} EDTA, Sigma) and incubated at 37°C overnight. The absorbance of the samples was measured at 510 nm using trypsin–EDTA as a blank (Yin *et al*., 2009).

Respiratory burst activity

Respiratory burst activity of isolated leukocytes was quantified by the nitroblue tetrazolium (NBT) assay

(Secombes, 1990), which measures the quantity of intracellular oxidative free radicals with slight changes to the concentration of nitro blue tetrazolium (NBT) solution of 2 mg/mL modified by Ardo *et al*. (2008) as follows. The intracellular production of superoxide anion was analysed by the formation of formazan crystals. 100 µL of leukocyte solution was mixed with 100 µL of NBT (0.2% in PBS) containing phorbol 12-myristate 13-acetate PMA (PMA, Sigma, 1 µg/mL), and superoxide dismutase (SOD, Sigma, 300 U/mL). After incubation at room temperature for 50 min with slow movement, plates were centrifuged at 500 × g for 2 min and the supernatants were discarded. Cells were then washed twice with HBSS and fixed in 70% methanol. Formazan crystals were dissolved by adding a 120 µL of 2 M KOH and 140 µL DMSO. After the formation of the blue-colored solutions, absorbance values (OD) were read in a spectrophotometer operating at 620 nm using KOH/DMSO (120 µL of 2 M KOH/140 µL DMSO) as blank.

Disease resistance

A. hydrophila (ATCC 7966), was inoculated in tryptone soya broth (TSB) and incubated at 28^{o}C. The culture was centrifuged at 800 ×g for 15 min. The packed cells were washed and the targeted dose was reached in phosphate buffered saline to 1×10^{8} cells. The treatment groups and untreated control were injected peritoneally with virulent *A. hydrophila* (1×10^{8} cells/fish). The mortality number for each group and replications were recorded daily for 5 days. The cause of mortality was confirmed by chemical test using isolated organism from liver of 5 % of dead fish. Mortality percent was calculated by the formula of Kakoolaki *et al*. (2011) as follows:

$$\text{Mortality percent} = \frac{\text{Number of dead fish}}{\text{Total no. of fish}} \times 100$$

Statistical analysis

The effect of different stimulants in the different weeks (Time × stimulants) on selected innate immune responses was analyzed with the Repeated Measures ANOVA using SPSS 18. The greater and lower values of Pillai's Trace and Wilks' Lambda showed more effectiveness of the independent factors on different values of Phagocytosis, Respiratory Burst, Protein Total Plasma while the data were presented as mean± SEM at the level of α= 0.05.

Table 1: Multivariate test to determine the effectiveness of independent factors on immunological variables (*n*=9).

Criteria	Effect	Multivariate Tests			
		Test	**Value**	**F**	***p***
phagocytosis	Week × stimulants	Pillai's Trace	1.50	10.76	0.000
		Wilks' Lambda	0.04	20.28	0.000
Respiratory Burst		Pillai's Trace	1.29	8.14	0.000
		Wilks' Lambda	0.12	10.78	0.000
Plasma Total Protein		Pillai's Trace	2.23	31.11	0.000
		Wilks' Lambda	0.00	48.43	0.000

Table 2: Estimated Marginal Means of different immunological criteria affected by stimulants and time (*n*=9).

Criteria	Groups	Weeks			
		1	**2**	**3**	**4**
Phagocytosis	1% Pollen	0.06±0.05	1.41±0.05	2.24±0.10	1.27±0.08
	1% propolis	1.71±0.5	2.48±0.05	2.42±0.10	2.03±0.08
	Pol. 0.5% + Pro 0.5%	1.66±0.05	2.42±0.05	2.19±0.10	2.19±0.08
	Control	0.98±0.05	1.21±0.05	1.30±0.10	2.26±0.08
Respiratory burst	1% Pollen	0.17±0.01	0.38±0.02	0.33±0.02	0.33±0.02
	1% propolis	0.18±0.17	0.27±0.02	0.40±0.02	0.40±0.02
	Pol. 0.5% + Pro 0.5%	0.21±0.01	0.52±0.02	0.38±0.02	0.40±0.02
	Control	0.21±0.01	0.22±0.02	0.22±0.02	0.34±0.02
PTP	1% Pollen	31.88±0.51	33.11±0.38	31.22±0.44	31.33±0.61
	1% propolis	32.77±0.51	32.88±0.38	35.22±0.44	25.88±0.61
	Pol. 0.5% + Pro 0.5%	43.66±0.51	33.33±0.38	32.33±0.44	38.22±0.61
	Control	28.77±0.51	27.88±0.38	30.11±0.44	31.77±0.61

Pol.: pollen, Pro.: Propolis, PTP: Plasma Total Protein.

Results

The results of preliminary test, multivariate statistical assess showed a strong and significant effects of independent variables, time (week) and stimulants, 1% pollen, 1% propolis, combination of them and control group on the dependent variables included phagocytosis, Respiratory Burst, Protein Total Plasma given in Table 1.

The effects of two factors, Persian bee propolis and pollen extracts at different sampling times (4 weeks) on enhancing the non-specific immune response of rainbow trout (*O. mykiss*) are listed in Table 2.

As shown in Table 2 and Fig. 1, there were significant differences (p>0.05) based on OD (absorbance) of estimated marginal means of phagocytic activity between groups, 1% propolis and combination of Pollen and Propolis in weeks 1 and 2 after feeding with herbs. These marginal means were significantly different (p>0.05) from the control ones at week first up to week third.

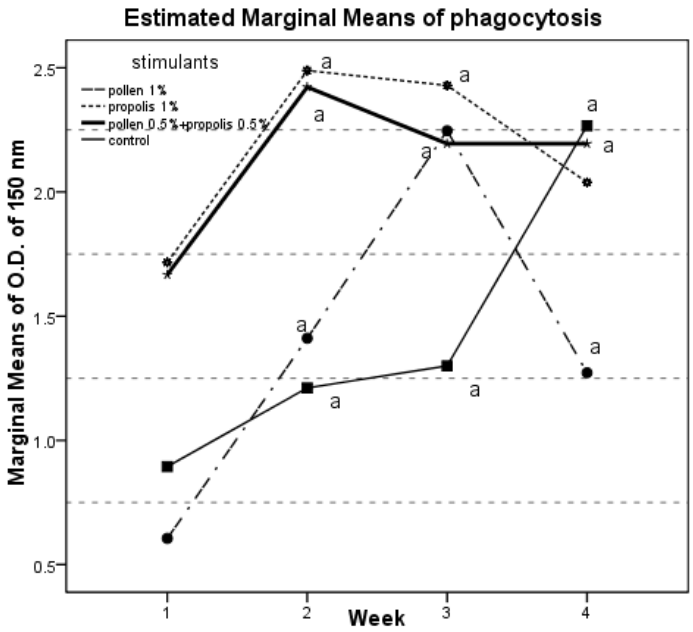

Figure 1: Phagocytic activity of phagocytic cells in the control group and groups fed diets containing different concentration of pollen and propolis. Similar scripts show no significant difference for each line (group).

The production of intracellular oxidative free radicals showed a significant increase particularly in the second week in all treated groups (Fig. 2), in comparison to the untreated group (control).

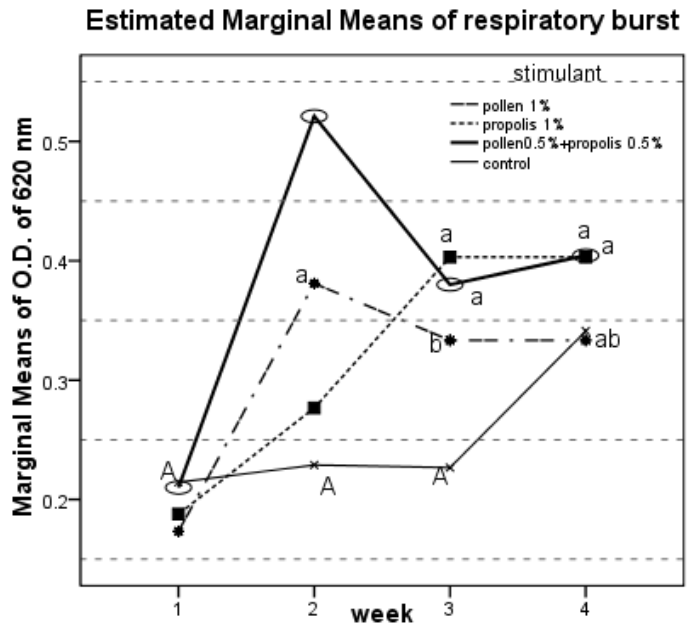

Figure 2: Respiratory burst activity of phagocytic cells withdrawn from blood of fish in control group and in groups fed pollen, propolis and combination of them.

No significant differences (p>0.05) was measured in the first, third and fourth weeks between 0.5% pollen+0.5% propolis and 1% propolis. The maximum marginal means of O.D. of respiratory burst activity for 1% propolis and 0.5% pollen+0.5% propolls was measured respectively, 0.27±0.02 and 0.52±0.02 into the second week and 0.40±0.02 and 0.38±0.02 in the third week.

A significant gradual increase in plasma total protein level was measured only in fish fed the diet containing 1% propolis from the first to third week, while fish fed with 0.5% pollen+ 0.5% propolis showed a significant decrease (p<0.05) in the level of PTP from first to third week which went up at the end of experiment. (Table 2, Fig. 3).

Fish were introduced with *A. hydrophila* after four weeks of the experiment and cumulative mortality was recorded during 5days (Fig. 4). All three treatment groups including 1% pollen, 1% propolis and 0.5% pollen+ 0.5% propolis showed lower mortality compared to the positive control. Diets containing 1% pollen, 1% propolis and 0.5% pollen+ 0.5% propolis reduced the mortality by 40%, 45% and 65%, respectively. With the exception of the negative control which showed mortality close to zero percent, minimum mortality was observed in group fed with 0.5% pollen+0.5% propolis.

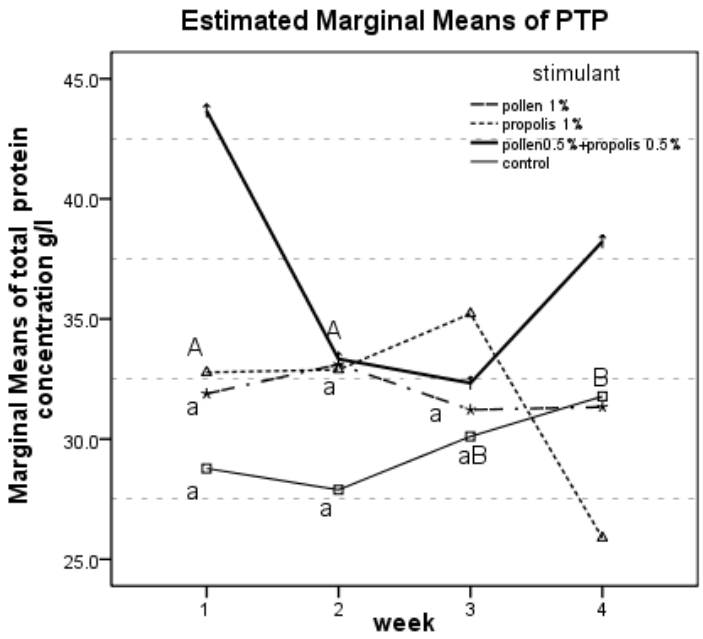

Figure 3: Fluctuation in plasma total protein levels in control group and in groups fed diets containing pollen, propolis and combination of them.

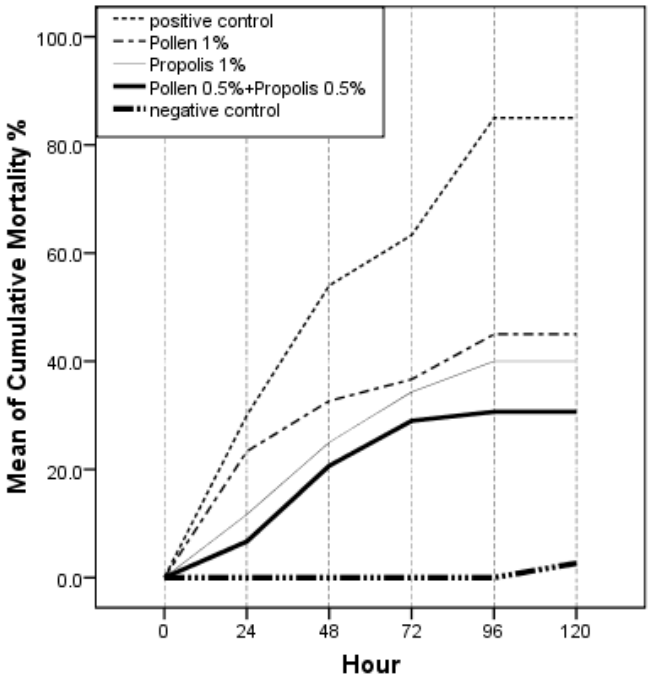

Figure 4: Cumulative mortalities (%) of fish in control group and in groups fed diets containing pollen, propolis and combination of them. The figure shows a Five-day period following an artificial infection with *Aeromonas hydrophila*.

Discussion

During recent decades, the application of potential dietary medicinal herbs has been shown to enhance the innate immune response in fish (Magnadóttir, 2006). This research was conducted to find the possible effect of pollen and propolis extracts, which were added to the commercial feed to immune response and control mortality in rainbow trout exposed to *A. hydrophyla* causing mortality in the warmer areas, and passing the colder condition particularly summer and spring.

The results of this trial showed that feeding rainbow trout with both pollen and propolis extracts or in combination with each other was able to enhance the nonspecific immune response. Meanwhile one percent doses of propolis extract as well as a combination of 0.5% propolis and 0.5% pollen extract (group 3) for three weeks from the first week without remarkable change significantly enhanced the phagocytic response of white blood cells. Several studies have proved that some herb-based immunostimulants could enhance phagocytosis in various fish species (Sakai, 1999; Ardó *et al*., 2008; Bilen *et al*., 2011; Harikrishnan *et al*., 2011; Wang *et al.*, 2015). Phagocytosis has been identified as an important mechanism in the innate immune system of fish (Magnadóttir, 2006). Phagocytosis, which is performed by neutrophils and macrophages shows an important defense against pathogenic bacteria by producing toxic oxygen species during

a process named respiratory burst (Neumann *et al*., 2001). The phagocytic activity of blood leukocytes increased in rainbow trout fed diets with plant extracts containing food, especially 1% ginger (*Zingiber officinale*) root (Dügenci *et al*., 2003). Zeranol has advantageous effects on phagocytic activity of rainbow trout, which increased with the nutritional dose (Keleş, 2002). The phagocytic activity of white blood cells was increased in carp fed with Chinese herbs only in weeks 3 and 4 of exposure to *A. hydrophila* (Yin *et al*., 2009). On the contrary results of the present study showed an increasing trend in phagocytic activity on week 2 up to the end of experiment on week 4 (Fig. 1).

Phagocytes also produce toxic oxygen forms during a process called respiratory burst. Respiratory burst (ROS, reactive oxygen species), the increase of oxidation level in phagocytes enhanced by foreign agents, is considered as an important indicator of the innate immune system in fish, where O^{-2} is the first product to be released (Harikrishnan *et al*., 2011). Secombes (1990) also found that the release of superoxide anion, hydrogen peroxide, into the phagosome during the respiratory burst is considered to be one of the most important mechanisms involved in the bactericidal activity of macrophages. The increase of intracellular activity has been reported in Atlantic salmon (*Salmo salar*) and rainbow trout (*O. mykiss*) fed diet with different doses of glucan (Dügenci *et al.*, 2003). Both phagocytic and respiratory burst activity significantly increased in fish fed with 1.0% and 2.0% *Sytrax* japonica supplementation diets when challenged with *V. harveyi* (Harikrishnan *et al*., 2011). In contrast, Cuesta *et al.* (2005) reported that no significant effects were seen with the use of propolis on either of the humoral parameters assayed, peroxidase content and alternative complement pathway. In the present study, ROS production was provoked after feeding with the combination of 0.5% propolis and 0.5% pollen and 1% propolis or 1% pollen on week 2. The maximum marginal mean of O.D. at 620 nm was recorded for the incorporated extract and was rather flat on weeks 3 and 4 with less value compared to week 2 for both, a combination of 0.5% propolis and 0.5% pollen and 1% propolis, with no significant difference from each other (Fig. 2). In contrast to group 3 (incorporated extract), the curve of 1% propolis slowly went up and was stablized from the third to fourth weeks indicating higher potential for intracellular respiratory burst activity in rainbow trout. The effect of Astragalus and Lonicera extracts in tilapia on the fourth week after feeding (Ardó *et al*., 2008) probiotics (Kim and Austin, 2006) and glycyrrhizine isolated from *Glycyrrhiza glabra* (Christybapita *et al.*, 2007) in rainbow trout were reported to enhance the respiratory burst activity of macrophages. On the other hand vitamins E, C and A, have been shown to have little effect upon macrophage or phagocytic cells-

respiratory burst activity (Yin *et al.*, 2009).

Plasma proteins contain the humoral constituents of the innate immune system, e.g. immunoglobulins, transferrin, agglutinins or precipitins (Magnadóttir, 2006). No significant effect was seen on PTP due to administration of propolis in gilthead seabream (Ardó *et al*., 2008). Our study showed that a pattern of marginal means of PTP (g/L) is in line with the performance of respiratory burst during the sampling weeks after feeding with propolis and pollen in rainbow trout particularly in group 3 (combination of pollen and propolis) that increased, abruptly on week 3. Generally, PTP did not go up to the second week in the fish fed diet with pollen, propolis or incorporated extracts similar to the result of Kolman *et al*. (1998) who found a decrease of PTP level in Russian sturgeon (*Acipenser güldenstadti*) after feeding with glucan, chitosan and finnstim.

The result showed feeds supplemented with the combination of pollen and propolis was the most effective and the mortality of the fish reduced by 65%. The results showed a significant decrease of cumulative mortality compared to either the positive control or 1% pollen or 1% propolis following challenge with *A. hydrophila.* Recently, decreased mortality on challenge with *A. hydrophila* was reported in *Labeo rohita* fed with 0.5% Achyranthes seed incorporated diet (Rao *et al*., 2006). These results are in line with the decrease in cumulative mortality percent of infected fish fed diets administrated with various immunostimulants (Sakai, 1999; Rao *et al*., 2006), chitosan and levamisole (Gopalakannan and Arul, 2006).

Based on the results, it is concluded that enhancing the non-specific immunity of the fish fed a combination of Persian bee pollen and propolis extracts abruptly increased through the first week up to the second. Nevertheless, the immunity of the 1% of propolis extract administrated orally provoked with the slower slope and could be kept more stable even after four weeks. Meanwhile, the mean of Cumulative Mortality due to application of both pollen and propolis remarkably was less than that of Persian honeybee propolis. Finally, it is suggested that diets containing both pollen and propolis were more effective than that of propolis alone.

This work suggests a new perspective for the use of herbs in rainbow trout which can be applied before outbreaks of diseases to prevent high numbers of mortalities from occurring due to provoking the innate immunity.

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