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Effects of Alcoholic and Aqueous Extract of Propolis on Growth Performance, Hemato-Immunological Parameters and Disease Resistance of Common Carp (*Cyprinus Carpio*)

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Abstract

In this study, the effects of oral administration of an alcoholic and aqueous extract of propolis on growth performance indices, hematological parameters and immune responses of *Cyprinus carpio* were investigated. Six hundred and thirty juvenile *Cyprinus carpio* (weighing 58.5 ±4.2 g, Mean±SD) were randomly divided into seven equal groups in triplicates. Three groups (G1 to G3) were fed diets containing 0.1%, 0.25% and 0.5% of Propolis-ethanolic Extract (PEE) respectively, whereas groups 4 to 6 were fed a diet containing the same level of Propolis Aqueous Extract (PAE), group seven was received free extract normal food. Growth indices and hemato-immunological parameters in treated fish were evaluated and compared among the groups. The results showed a significant increase in serum lysozyme activity, total serum protein and globulin in fish fed diets containing 0.5% PEE compared to the control group (p<0.05). But no significant differences were observed in growth-performance indices and other immunological and hematological parameters compared to the control group (P>0.05). Meanwhile, cumulative mortality after the bacterial challenge of fish fed on a diet containing 0.5% PPE significantly decreased compared to control group (p<0.05). According to the results, supplementation of food with 0.5% PEE stimulated some innate immune responses and resistance against *A.hydrophila* infection. Then 0.5% PEE in diet can be a good candidate for immunostimulant against bacterial infection in common carp.

Keywords: Cyprinus carpio, Propolis, Growth indices, Hematological parameters, Immune responses.

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Introduction

Several immunostimulants such as vitamins (Anderson, 1992), substances with microbial origin (Dalmo, & Bogwald, 2008), extracts from animals and plants (Baba, Acar, Ontas, Kesbic, & Yilmaz, 2016a and 2016b), synthetic compounds like levamisole (Sakai, 1999) and sub-products of other industries such as chitosan and propolis (Sforcin, 2007) have been reported that play a promising role in aquaculture by enhancing the disease resistance in fish species (Abdy, Alishahi, Tollabi, Ghorbanpour, 2017).

Propolis is a complex resinous sticky substance that its color varies from green, red to dark brown. Honeybees collect it from buds and exudates of various plants, mix it with their own salivary secretions and waxes, and thought to be used as a protective barrier and sterilant in beehives (Beyraghdar Kashkooli, Ebrahimi Dorcheh, Mahboobi-Soofiani, & Samie, 2011). Propolis in nature is composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollen, and other substances (Burdock, 1998). Propolis has several biological and pharmacological properties, such as immunomodulatory, antitumor, antimicrobial, anti-inflammatory, and antioxidant (Kanbur, Eraslan, & Silici, 2009). It is used as a sealant for small open spaces in the hive. Propolis has been used as a medicine in local and popular medicine in many parts of the world like Egyptians, Greeks and Romans since ancient times, at least to 300 BC, Because of its biological Properties as an antimicrobial, antifungal, antiprotozoal and antiviral agent (Sforcin, 2007). Propolis also shows immunomodulatory effect, which modulates the non-specific immunity via macrophage activation, action on lymphocytes and antibody production. Propolis was able to activate macrophages and enhance its fungicidal action and bactericidal activity (Sforcin, 2007). As regards its role in the immune system, propolis has been shown to have both immunomodulatory and anti-inflammatory effects in mammals (Dimov, Ivanovska, Bankova, & Popov, 1992; Ansorge, Reinhold, & Lendeckel, 2003). Besides, long-term administration of propolis extracts presents very low toxicity to rainbow trout (*Oncorhynchus mykiss*) (Beyraghdar Kashkooli *et al.*, 2011) and experimental animals (Arvouet-Grand, Lejeune, Bastide, Pourrat, Privat, & Legret, 1993).

In recent years, many studies have been done on Propolis, with various medical effects (Orsolic & Basic, 2003; Hu, Hepburn, Li, Chen, Radloff, & Daya, 2005; Kanbur *et al.*, 2009), but few studies have been done on the effect of propolis on fish species (Zhang, Gong, Yu, & Yuan, 2009; Abd-El-Rhman 2009; Yonar, Yonar, & Silici, 2011). The aim of this study was to compare the effects of an alcoholic and aqueous extract of propolis, a honeybee product, on growth performance, hematological parameters and immune responses of common carp (*Cyprinus carpio*).

Materials and Methods

Fish

A total of 630 Juvenile artificial reproduced and pond reared, *Cyprinus carpio* (58.5±4.2 g, Mean±SD) was obtained from a cyprinid fish farm in Ahvaz, Khuzestan province, Iran. Fish were kept in 300 L tanks, with running aerated and dechlorinated water at 25±1°C and kept one week to acclimate. Aquarium equipped with external biofilters and thermostatic heaters.

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Fish were fed with commercial pellets (Behparvar Company, Iran) twice a day. Water quality factors were recorded during the experiment as temperature, 25±1C; Dissolved oxygen, 8-10 ppm; pH, 7.8±0.2; NO2 <0.01ppm and NH3 <0.1ppm. The water exchange rate was 20% of water volume daily.

Crude Propolis and It's Ethanolic and Aqueous Extract

The crude Propolis sample was collected in summer from north of Khuzestan province using propolis traps and kept in a dark and dry place until used. Propolis analyzed before extraction and its composition was: Resins 49%, Waxes and fatty acids 33%, Essential oils 9%, Protein 4% and Minerals and other organics 5%. Propolis-ethanolic-extract (PEE) was prepared by adding 30 ml of absolute ethanol to 3 g minced propolis in bottles, which were sealed and shaken in darkness for 1 day at room temperature. The extract was then filtered twice and put in 80 ºC water bath for 3 hours to evaporation of alcohol, stored in sealed bottles at 4 °C until used (Cuesta, Rodriguez, Esteban, & Meseguer, 2005). Also, aqueous extract of propolis was prepared. One hundred grams of solid propolis resolved in 1 liter of distilled water and put it in the lab for 3 days at room temperature. Once per day mixed it with an electric mixer. Then, it filtered two times with filter paper. Next boiled it for 2 hours and then filtered again. Prepared extract keeps in the dark and closed container at 4 ^{0}C temperature in the refrigerator before use (Cuesta *et al.*, 2005).

Experimental Settings

Six hundred and thirty fish were randomly divided into 7 equal groups, each group with three replicates (30 fish in each replicate). Fish were fed for 60 days under following treatments: groups 1 to 3 (G1 to G3) received diet containing 0.1%, 0.25 % and 0.5% PEE respectively whereas groups 4 to 6 (G4 and G6) fed with diet supplemented with 0.1%, 0.25 % and 0.5% PEE the control group (G7) received free extract normal food. Fish were hand-fed ad libitum twice a day. The fish biometrical assay for growth performance indices was conducted just before the start and at the end of the study.

Fish were anesthetized with 100 mg l^{-1} MS222 and blood samples were collected from the caudal peduncle vein of 4 fish from each replicate in 0, 20th, 40th and 60th days of the experiment, by using needles previously rinsed in heparin for the evaluation of hematological parameters (Schaperclaus, Kulow, & Schreckenbach, 1991). For serum separation, another 0.5 ml blood samples were withdrawn into blood Eppendorf tubes without anticoagulant in the syringe. The Eppendorf tubes, containing the blood samples were centrifuged at 3000 g for 15 min and the supernatant serum was collected. The serum was stored at -20ºC until used for serum immunological assays.

Experimental Feed Preparation

Commercial basal diet (crude protein 32%, crude fat 8%, ash 10%, crude fibre 6%, moisture 10%, nitrogen-free extract 32%, and gross energy 3963 kcal/kg) was crushed, for each extract (PEE and PAE) food divided into four parts. The first part was mixed with 0.1% extract (G1 and G4), the second part was mixed with 0.5% extract (G2 and G5) the third part was mixed with 1% extract (G3 and G6) and the fourth part used as control food. The

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diet was reformed into pellets, spread to dry and stored at 4 ºC for the feeding experiment (Cuesta *et al.*, 2005). All groups were fed with their experimental diet for 60 days.

Growth Performance Indices

Feed conversion ratio (FCR), specific growth rate (SGR), the average weight-gain (AWG), feed efficiency ratio (FER) and Condition factor (CF) were calculated according to the following equations:

CF= weight (g)*100 / (length, cm)^3

FCR= Feed intake (g) / weight gain (g).

SGR (%/day) = 100 (In final body weight (g) - ln initial body weight (g) / experimental period (day).

W Daily Gain (g/day) = Average final weight (g) - Average initial weight (g) / experimental period (day).

FER = Body weight gain (g) / Feed intake (g).

Immunological Parameters

Serum Lysozyme Activity

Serum lysozyme activity was measured following Ellis, (1990) and based on turbidometric method, The lyophilized *Micrococcus lysodeikticus* (Sigma, USA) at a concentration of 0.3 mg mL^{-1} (in 0.05 M sodium citrate buffer pH=5.1) were added to sera ratio of 1:10 v/v in the same buffer. Immediately after adding *M. lysodeikticus*, the first OD was read at 450 nm. The second OD was read 6 minutes later. Lysozyme activity was expressed as unit mL^{-1} min^{-1}, where one unit is defined as the decrease in absorbance of 0.001 min^{-1}.

Serum Bactericidal Activity

Serum bactericidal activity was measured according to Sunyer & Tort (1995) with slight modification. Sera samples from each group were diluted three times with 0.1% gelatin-veronal buffer (GVBC2) (pH 7.5, containing 0.5 mM ml-1 Mg^{2+} and 0.15 mM ml-1 Ca^{2+}). *A. hydrophila* (live, washed cells) was suspended in the same buffer to make a concentration of 1 ×10^5 cfu ml-1. The diluted sera and bacteria were mixed at 1:1, incubated for 90 min at 25 °C and shaken. One control group containing bacterial suspension in the same buffer was also incubated for 90 min at 25 °C. The numbers of viable bacteria were then calculated by counting the colonies from the resultant incubated mixture on TSA plates in triplicate (three plates per sample) after 24 h incubation.

Total Serum Protein, Albumin and Globulin

The total serum protein level was estimated by the method of Bradford (Bradford, 1976) using the standard protein estimation kit (Zist shimi co, Iran). For globulin estimation 50 ml saturated ammonium sulfate solution was added dropwise to 50 ml serum followed by vortexing. Centrifugation was done at 10,000 gr for 5 min. Then 20 ml of this sample dissolved with 80 ml carbonate-bicarbonate buffer (pH 9.3) and the protein content was estimated by the method of Bradford using the standard protein estimation kit (Zist shimi co, Iran). Albumin content was measured using a standard albumin estimation kit (Zistchem Diagnostics, Iran)

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Hematological Parameters

Blood samples immediately analyzed for the estimation of numbers of erythrocytes and, hemoglobin (Hb), hematocrit (Hct), the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH) and the mean corpuscular hemoglobin concentration (MCHC). Numbers of erythrocytes count were determined by the hemocytometer method (Ellis, 1990), hematocrit was determined by the microhematocrit method (Fox, White, Koa, & Fernald, 1997) and hemoglobin measurement was determined by the cianometa-hemoglobin method (Paul, Goldenfarb, Frank, Hall, & Brosious, 1971). MCV, MCH and MCHC were calculated by using the formulas as follow (Hu *et al.*, 2005):

MCV (µm3 cell-1) = (Packed cell volume as percentage/RBC in millions cell mm3) ×10

MCH (pg cell-1) = (Hb in g 100 ml-1/ RBC in millions cell mm3) ×10

MCHC (g 100 ml-1 Hct) = (Hb in g100 mL-1/ packed cell volume as percentage) ×100

White Blood Cell Count (WBC), Differential Count

White blood cell count was made from 6 animals of each group in a Neubauer counting chamber as described by Schaperclaus *et al.* (1991). For the Differential count of leukocytes whole blood on glass microscope slides, dried in air, and stained with May-Grunwald/Giemsa. Leucogram was assessed for each fish under an oil immersion lens. One hundred white blood cells from each smear were assessed and the percentages of different types of leukocytes were calculated following the method of Schaperclaus *et al.* (1991).

Challenge with *Aeromonas hydrophila*

After the administration of food supplemented with a different dose of PEE or PAE, for 60 days, 15 fish from each aquarium (45 fish from each group) were transferred to new aquariums. For challenging assay *A.hydrophila* (AH04: originally isolated from cyprinid farms in Iran) were used. Bacteria from a frozen stock (-70 ºC) were inoculated into tryptone soy broth (TSB) media, grown overnight at 25 ºC in a shaker, The broth was centrifuged at 2000 × g for 15 min. Packed cells were washed and demand concentration was prepared in phosphate buffered saline (PBS).The fish in each new aquarium were injected intraperitoneal with 0.1 ml of LD_{50} suspension of *A. hydrophila* (1.6×10^7cfu per fish) in PBS. Mortality of challenged fish was recorded daily for 10 days. The cause of death was ascertained by re-isolating the infecting organism from kidney and liver of dead fish according to Misra *et al.* (2006).

Statistical Analysis

All statistical analyses were performed using SPSS 16 software. Data were tested for normal distribution with Shapiroe-Wilk's test and for a homogeneous variance with Levene's test. Differences between means of data in groups were tested with one-way analysis of variance (ANOVA) and Tukey's comparison of means, which the significance level was defined as $P<0.05$.

Results

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Growth Performance

The results of growth indices have been presented in table 1. Results obtained indicate that administration of a different dose of PEE had no significant difference in all growth parameters (alcoholic and aqueous extract of propolis) include: SGR, FCR, FER, PWG, and CF. (P>0.05).

Lysozyme activity

The serum lysozyme activity in PEE 0.5% treatment in 20, 40, 60 days increased significantly in comparison with the control group (table 2). The serum lysozyme activity in PEE 0.1% and 0.25% treatment had no significant difference in comparison with control treatment.

Serum bactericidal activity

The Serum bactericidal activity had no significant difference in comparison with control treatment (table 2).

Total serum protein

The results of total serum protein values have been presented in table 2. The serum total protein in PEE 0.5% treatment in 20, 40, 60 days increased significantly in comparison with the control group (P<0.05). The serum total protein in PEE 0.25% treatment in 60 days increased significantly in comparison with the control group (P<0.05). The serum total protein in PEE 0.1% treatment had no significant difference in comparison with control treatment

Serum globulin

The results of Serum globulin values have been presented in table 2. The Serum globulin in PEE 0.5% treatment in 20, 40, 60 days increased significantly in comparison with the control group (P<0.05). The Serum globulin in PEE 0.25% treatment in 60 days increased significantly in comparison with the control group (P<0.05).

Serum albumin

The results of Serum albumin values have been presented in table 2. The Serum albumin in PEE 0.1%, 25% and 0.5% treatment had no significant difference in comparison with control treatment.

Hematological parameters

The results of hematological parameters and White blood cell count (WBC), Differential count of treatments have been presented in Table 3 and 4. Our results indicated that PCV, RBC, Hb, MCV, MCH, MHCH, WBC, Lym, Het, Mono in treatments fed with different concentrations of PEE and PAE (experimental groups) had no significant difference in comparison with control group ($p < 0.05$).

Challenge test

The percent of fish mortality in 10th-day post-challenge have been presented in Fig 1. The percent of fish mortality after challenge with *A. hydrophila* in G3 treatment (fish fed with food supplemented with 0.5%PEE) decreased significantly in comparison with other groups ($p < 0.05$). Post-challenge mortality in G1 and G2 treatments had no significant difference in comparison with control group.

Discussion

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Among the different immunostimulants, materials with animal origin showed comparative advantages over chemical ones in aquaculture. In the past decade, many studies have been conducted on propolis and its medicinal properties (Kanbur *et al.*, 2009; Hu *et al.*, 2005; Orsolic & Basic, 2003). However, few studies have been focused on the propolis administration in aquaculture (Zhang *et al.*, 2009). In the present study effect of different level of an ethanolic and aqueous extract of propolis on growth indices, hematological and immune parameters of *Cyprinus carpio* were reported. Growth performance indices (FCR, SGR, PWG, FER, and CF) were not affected by oral administration of PEE and PAE in *Cyprinus carpio*. Results obtained in the growth performance of present work are consistent with a previous study in rainbow trout (Beyraghdar *et al.*, 2011) and sea bream (*Sparus aurata* L.). Rainbow trout fed with propolis supplemented food for 8 weeks showed no significant change in growth indices and physiological parameters. Similarly, the growth indices of sea bream were not affected by the dietary intake of propolis (0.1% in food) for six weeks (Cuesta *et al.*, 2005). But there was a controversially report by Abd- El-Rahman, (2009) and Tukmechi, Karimi Rad, Farrokhi, Agh, & Jalili, (2014) in tilapia and rainbow trout respectively. Which showed a significant increase in growth performance indices in tilapia and rainbow trout fed with propolis ethanolic extract enriched diet (Abd-El-Rhman, 2009; Tukmechi *et al.*, 2014).they claim that propolis properties such flavonoids (flavones and flavanones) vitamins (B1, B, C, E) and essential minerals (iron, aluminum, manganese) and silicon can improve the digestive cofactors and enzymatic activity which cause growth stimulation. The difference between the later reports and our finding can be referred to the variation of propolis source and level, fish species, duration of the study and environmental factors. We believe although propolis contains growth stimulating properties, it's oral administration with the concentration of 0.1%, 0.25% and 0.5% for 60 days didn't induce significant growth indices of common carp. The probability role of antinutritional factors of propolis can't be ignorable.

In this work, some immune responses of propolis-treated fish were affected significantly compared to control group ($P<0.05$). The serum lysozyme activity in PEE 0.5% group in 20, 40, 60 days increased significantly in comparison with the control group ($P<0.05$). In other groups, a mild increase in lysozyme activity was seen in comparison with the control group but, not insignificant extent. The propolis induced effects that were noted were at the cellular level, namely, phagocytosis and cytotoxicity (Cuesta *et al.*, 2005). One of the components of the innate immune system is humoral elements, including lysozyme or complement system. The serum lysozyme level is one of the humoral elements that are mostly used to measure the innate immune response in fish (Zhang *et al.*, 2009). It seems that PEE includes more effective ingredients of propolis and it can induce more immunostimulatory effects to compare to PAE. Increase in lysozyme activity has been reported after administration of propolis in some fish species. Cuesta *et al.* (2005) reported that propolis in intraperitoneal and oral routes has immunostimulatory effects in gilthead sea bream (*Sparus aurata* L.) although intraperitoneal administration was more effective than dietary intake. They showed a notable increase in lysozyme activity in both routes. Also, a significant increase in serum lysozyme activities was found in PEE treated *O. niloticus* and rainbow trout (Abd-El-Rhman, 2009; Tukmechi *et al.*, 2014). Elevated lysozyme activity was reported after 4 and 6 weeks administration of 0.1 and 1% propolis in Chinese sucker, *Myxocyprinus asiaticus* (Zhang *et al.*, 2009). Similarly, increase in lysozyme activity was noted following oral administration of propolis in Barbus barbules

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(Alishahi & Jangrannejad, 2012) and other biological immunostimulants such as algal extract (Alishahi, Karamifar, & Mesbah, 2015).

The bacteriolytic activity of serum constitutes an important part of natural humoral immunity of fish has an effective role towards a range of microorganisms. Although the serum bactericidal activity was enhanced by oral administration of 0.25% and 0.5% of PEE in food, this enhancement was not insignificant extent. Similar to the present results Dotta, Mouriño, Mouriño, & Martins, (2011) reported that supplementation of food with 0.5 and 1% propolis for 20 days didn't cause a change in antimicrobial potency (evaluated *against Aeromonas hydrophila*, *Enterococcus durans* and *Escherichia coli*) in tilapia. Also Alishahi & Jangeran Nejad, (2012) did not found a difference in the serum bactericidal activity of *barbus barbulus* supplemented with 0.5 and 1% Propolis in the diet for 60 days. Tukmechi *et al.* (2014) found a notable increase in serum antibacterial activity of propolis-treated rainbow trout. Abd-El-Rhman, (2009) reported a significant increase in serum bactericidal activity following the administration of propolis in tilapia. They suggest that antibacterial activity can be attributed to the effect of propolis on liver and leukocyte production, the important sites for the synthesis of antibacterial proteins. 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 antibacterial activity in gilthead seabream (*Sparus aurata*). Zhang *et al.* (2009) fed Chinese sucker fish (*Myxocyprinus asiaticus*) with different dosage of traditional Chinese medicine formulated from propolis and herbal drug. They showed higher respiratory burst activity of phagocytes and serum lysozyme and complement activity (Zhang *et al*., 2009). The apparent discrepancy among these studies may be attributed to the propolis source, dose, and fish species.

It is important to estimate the bacterial resistance of treated fish to determine the efficiency of an immunostimulant. In the present study, the lowest mortality after challenge with *A.hydrophila* was recorded in *0.5%* EEP group which was significantly lower than the control group. The higher survival rate in *0.5%* EEP group may be related to the presence of some bactericidal and immunostimulating component in EEP. Similarly, the results of the study Wei-Hua Chu in 2006, was shown the propolis can stimulate the immune response in *C. auratus gibelio* against *A. hydrophila* and leukocyte activity and antibody titer in vaccinated fish and increased the survival rate following challenge. It may have a potential as an adjuvant or immunostimulant in fish (Chu, 2006). Abd-El-Rahman investigated antagonism of *A. hydrophila* by Propolis and its effect on the performance of Nile tilapia, *Oreochromis niloticus.*

Besides the results of this work showed that total protein and globulin in PEE group at 20, 40 and 60 days of study were significantly higher than the control group. Total plasma protein concentration is one of the most important factors in the blood and their clinical significance has been considered as an indicator of health, stress, and welfare in both terrestrial and aquatic organisms (Dotta, de Andrade, Tavares Gonçalves, Brum, Mattos, Maraschin, & Martins, 2014). Their values usually change in different physiological and pathological condition. The increase in serum protein content might be correlated with an increase of proteins like serum lysozyme, complement component, acute phase proteins, cytokines, lectins and bactericidal peptides. Probably, the moderate increase in the leukocyte count and their functions might have resulted in the enhancement of the serum protein and globulin level. Among total serum proteins, globulins correspond to proteins present in the blood

responsible for the organism's immune defense system, such as immunoglobulins (Maqsood, Samoon, & Singh 2009). Certain herbal immunostimulants have been reported to increase total protein as well as total globulin in fish (Vasudeva Rao, Romesh, Singh, & Chakrabarti, 2004).

Hematological parameters are used as a clinical indicator of health diagnosis (Dotta *et al.,* 2011). When herbal medicines are used as an immunostimulant, the blood parameters levels generally have not been influenced without a stimulus or challenge by some invading pathogen (Dotta *et al.,* 2014). In the present study, the hematological parameters have not been influenced by oral administration of PEE and PAE ($P>0.05$). Our results indicated that PCV, RBC, Hb, MCV, MCH, MCHC, WBC and differential count of leukocytes: Lymphocytes, Neutrophils, eosinophil and Monocytes in the fish fed with different concentrations of PEE and PAE (experimental groups) had no significant difference in comparison with control group ($p<0.05$). No change in hematological parameters showed that these doses of propolis are not toxic in *Cyprinus carpio*. Inconsistent with our study Beyraghdar *et al.* (2011) showed that long-term exposure to high concentration of propolis in rainbow trout didn't change hematological parameters such as PCV, Hb, RBC and total protein, globulin and cholesterol, when compared to the control group, But Talas & Gulhan (2009) in rainbow trout suggested that the WBC, MCV, MCH values and granulocytes rates increased ($p<0.05$) in 0.02 and 0.03 g/L propolis treated groups. They reported a decrease in agranulocytes, erythrocytes, hemoglobin and hematocrit values in fish exposed to 0.02 and 0.03 g/L propolis ($p<0.05$).The contradictory reports may be based on the difference between the physiology of fish species, the origins of propolis, which influence its quality, and water quality can be reasons for the incoherence among the different works.

Our results indicated that diet supplemented with 0.5% ethanolic extract of propolis enhanced some none specific immune responses and resistance against bacterial infection in common carp in a dose-dependent manner. Meanwhile, supplementation of food with a different level of aqueous extract of propolis didn't induce a significant change in immune responses of common carp. Growth indices and hematological parameters were not influenced in carp treated with ethanol or aqueous extract of propolis. According to the results of this study, it can be concluded that supplementation of food with 0.5% ethanolic extract of propolis in common carp can be used as an immunostimulant against bacterial infection in common carp.

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Table 1. Effect of oral administration of different concentration of PEE and PAE on SGR, FCR, FER, PWG and CF of *Cyprinus carpio* fed for 60 days. G1: groups fed with a commercial basal diet containing 0.1% PEE, G2: groups fed with a commercial basal diet containing 0.25% PEE, G3: group fed with a commercial basal diet containing 0.5% PEE and Control: groups fed with a commercial basal diet free from PEE.

groups	SGR	FCR	FER	PWG	CF
G1	1.31 0.19[a]	3±0.44 [a]	0.33±0.045 [a]	108±21.5 [a]	1.38±0.17 [a]
G2	1.41±0.22 [a]	2.85±0.49 [a]	0.36±0.1 [a]	131±39.6 [a]	1.41±0.21 [a]
G3	1.28±0.18 [a]	2.9±0.51 [a]	0.34±0.085 [a]	120±27.5 [a]	1.51±0.27 [a]
G4	1.21±0.2 [a]	3.2±0.43 [a]	0.32±0.05 [a]	110±23.4 [a]	1.34±0.23 [a]
G5	1.33±023 [a]	3.1±0.49 [a]	0.34±0.06 [a]	120±32.4 [a]	1.39±0.3 [a]
G6	1.2±0.21 [a]	3±0.48 [a]	0.33±0.076 [a]	118±24.5 [a]	1.43±0.32 [a]
G7	1.19±0.12 [a]	3.1±0.39 [a]	0.32±0.26 [a]	104±21.3 [a]	1.41±0.16 [a]

Table 2. The effect of administration of food supplemented with a different dose of PEE and PAE on various immunological parameters (serum lysozyme, total serum protein and globulin, Serum albumin and bactericidal activity) in *Cyprinus carpio*.

day	Extra acts	groups	Lysozyme	bactericidal activity	Total protein	Total globulin	Albumin
Day 0	PEE	Control	124.64±22.48 a	127.09±18.43 a	3.15±0.57 a	1.56±0.49 a	1.62±0.28 a
		0.1%	125.6±22.4 a	124.9±19.67 a	3.21±0.64 a	1.51±0.41 a	1.59±0.34 a
		0.2%	118.4±12.5 a	125.67±13.46 a	3.14±0.62 a	1.49±0.39 a	1.6±0.31 a
		0.5%	121.4±23.8 a	127.09±25.43 a	3.16±0.55 a	1.55±0.46 a	1.69±0.3 a
	PAE	Control	116.64±22.34 a	105.09±18.42 a	3.07±0.57 a	2.80±0.49 a	1.43±0.28 a
		0.1%	114.64±24.21 a	110.9±21.72 a	3.17±0.61 a	2.76±0.49 a	1.31±0.31 a
		0.2%	112.45±26.45 a	109.12±23.21 a	3.11±0.56 a	2.83±0.50 a	1.53±0.26 a
		0.5%	117.79±24.4 a	105.29±17.2 a	3.12±0.53 a	2.81±0.51 a	1.38±0.29 a
Day 20	PEE	Control	128.64±22.48 b	127.09±18.43 a	3.05±0.57 b	1.46±0.49 b	1.62±0.28 a
		0.1%	138.33±29.44 ab	137.33±23.83 a	3.02±0.65 b	1.56±0.87 b	1.48±0.32 a
		0.2%	143.33±19.41 ab	142.50±33.04 a	3.18±0.85 ab	1.98±1.06 ab	1.20±0.35 a
		0.5%	170±40.37 a	148.83±63.73 a	3.99±0.67 a	2.63±0.74 a	1.26±0.31 a
	PAE	Control	116.64±22.48 a	100.09±18.42 a	3.07±0.57 a	1.70±0.49 a	1.24±0.28 a
		0.1%	126.33±29.44 a	110.33±23.83 a	2.94±0.65 a	1.81±0.87 a	1.19±0.32 a
		0.2%	128.00±19.41 a	105.50±21.04 a	3.10±0.85 a	1.75±0.81 a	1.20±0.35 a
		0.5%	140.56±20.37 a	101.83±23.73 a	3.16±0.66 a	1.95±0.74 a	1.15±0.31 a
Day 40	PEE	Control	129.29±20.99 c	129.88±21.07 a	2.93±0.55 b	1.40±0.39 b	1.51±0.25 a
		0.1%	144.17±18.00 b	111.67±8.87 a	3.11±0.47 ab	1.83±0.27 ab	1.32±0.19 a
		0.2%	142.00±17.61 b	119.17±2.93 a	3.41±0.56 ab	2.03±0.72 ab	1.45±0.41 a
		0.5%	153.33±7.53 a	118.33±25.34 a	3.88±0.24 a	2.51±0.29 a	1.29±0.20 a
	PAE	Control	124.45±20.99 a	102.87±21.07 a	3.11±0.55 a	1.71±0.39 a	1.34±0.25 a
		0.1%	132.17±18..00 a	92.67±18.87 a	3.12±0.47 a	1.65±0.27 a	1.52±0.18 a
		0.2%	131.00±17.61 a	92.17±16.93 a	3.29±0.56 a	1.53±0.7[3] a	1.48±0.41 a
		0.5%	139.33±7.52 a	91.33±25.33 a	3.17±0.24 a	1.68±0.43 a	1.43±0.20 a
Day 60	PEE	Control	126.25±12.46 b	124.13±10.08 a	3.21±0.62 b	1.72±0.57 b	1.56±0.45 a
		0.1%	143.33±16.02 ab	126.50±9.61 a	3.15±0.54 b	1.88±0.65 b	1.25±0.38 a
		0.2%	136.67±11.69 ab	116.20±9.04 a	3.93±1.63 a	2.61±1.52 a	1.28±0.62 a
		0.5%	215.00±47.62 a	123.17±16.96 a	3.97±0.60 a	2.59±1.12 a	1.30±0.36 a
	PAE	Control	119.78±12.46 b	97.12±10.08 a	3.17±0.62 a	3.03±0.57 a	1.20±0.29 a
		0.1%	131.33±16.02 ab	99.50±14.61 a	3.07±0.54 a	3.03±0.65 a	1.20±0.38 a
		0.2%	134.00±11.69 ab	89.20±12.04 a	3.28±0.76 a	3.18±0.62 a	1.17±0.62 a
		0.5%	145.00±27.62 a	90.17±16.96 a	3.19±0.60 a	3.08±0.81 a	1.08±0.36 a

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Table 3. Hematological parameters (PCV, RBC, Hb, MCV, MCH, and MCHC) in the blood of *Cyprinus carpio* administration of food supplemented with different concentrations of PEE and PAE.

day	extracts	groups	PCV	RBC	Hb	MCV	MCH	MCHC
Day 0	PEE	Control	32.86±5.42 [a]	1.64±0.31 [a]	3.77±0.97 [a]	212.20±59.5 [a]	23.68±7.8 [a]	11.06±3.3 [a]
		0.1%	33.86±5.2 [a]	1.41±0.33 [a]	3.45±0.76 [a]	223.23±56.5 [a]	21.56±6.44 [a]	12.11±3.4 [a]
		0.2%	34.6±3.4	1.34±0.23 [a]	3.34±0.56 [a]	202.34±32.4 [a]	24.56±5.26 [a]	11.06±3.2 [a]
		0.5%	31.23±3.89 [a]	1.64±0.43 [a]	3.23±0.67 [a]	220.21±34.2 [a]	22.59±7.23 [a]	11.06±3.21 [a]
	PAE	Control	32.23±4.57 [a]	1.76±0.26 [a]	3.92±0.86 [a]	180.12±75.5 [a]	22.30±6.83 [a]	11.91±3.04 [a]
		0.1%	31.66±4.46 [a]	1.54±0.56 [a]	3.36±0.67 [a]	178.23±46.5 [a]	23.30±4.8 [a]	12.11±3.11 [a]
		0.2%	31.89±4.23 [a]	1.43±0.22 [a]	3.79±0.45	180.46±44.6 [a]	24.30±6.25 [a]	11.56±3.43 [a]
		0.5%	33.03±4.24 [a]	1.49±0.49 [a]	3.98±0.89 [a]	178.34±46.57 [a]	21.30±5.83 [a]	11.91±3.81 [a]
Day 20	PEE	Control	31.15±4.25 [a]	1.54±0.35 [a]	3.51±1.09 [a]	219.48±62.03 [a]	22.49±10.65 [a]	10.77±3.71 [a]
		0.1%	27.89±4.59 [a]	1.43±0.37 [a]	3.22±1.20 [a]	211.26±54.70 [a]	25.91±12.30 [a]	11.37±2.71 [a]
		0.2%	33.25±2.38 [a]	1.61±0.26 [a]	3.77±0.97 [a]	212.81±17.83 [a]	23.91±8.22 [a]	10.93±2.84 [a]
		0.5%	32.56±3.43 [a]	1.57±0.45 [a]	3.56±1.14 [a]	232.14±89.74 [a]	17.65±11.06 [a]	10.02±5.25 [a]
	PAE	Control	32.46±4.07 [a]	1.66±0.30 [a]	3.66±0.98 [a]	195.88±49.03 [a]	22.11±9.60 [a]	10.83±4.19 [a]
		0.1%	31.89±3.98 [a]	1.55±0.32 [a]	3.37±1.09 [a]	205.71±41.70 [a]	21.72±11.25 [a]	10.09±3.19 [a]
		0.2%	34.22±3.20 [a]	1.73±0.21 [a]	3.92±0.86 [a]	197.77±40.83 [a]	22.68±7.17 [a]	11.03±3.32 [a]
		0.5%	32.26±3.25 [a]	1.69±0.40 [a]	3.71±1.03 [a]	190.67±76.74 [a]	21.91±10.01 [a]	11.02±5.73 [a]
Day 40	PEE	Control	37.43±3.41 [a]	1.71±0.26 [a]	3.95±0.92 [a]	201.98±64.21 [a]	23.65±4.91 [a]	11.07±3.19 [a]
		0.1%	35.80±3.35 [a]	1.58±0.20 [a]	3.77±1.00 [a]	228.37±46.3 [a]	25.57±4.91 [a]	11.24±3.80 [a]
		0.2%	38.20±2.49 [a]	1.78±0.34 [a]	4.01±0.96 [a]	206.29±30.9 [a]	22.47±2.87 [a]	11.04±2.71 [a]
		0.5%	38.50±4.51 [a]	1.74±0.21 [a]	4.08±0.92 [a]	171.26±96.22 [a]	23.09±6.78 [a]	10.89±3.85 [a]
	PAE	Control	36.43±3.23 [a]	1.83±0.21 [a]	4.10±0.81 [a]	199.52±51.21 [a]	22.46±3.86 [a]	10.85±3.67 [a]
		0.1%	34.80±3.17 [a]	1.70±0.15 [a]	3.92±0.89 [a]	204.31±33.3 [a]	23.00±3.86 [a]	10.83±4.28 [a]
		0.2%	34.20±3.31 [a]	1.90±0.29 [a]	4.16±0.85 [a]	180.00±17.9 [a]	21.88±1.82 [a]	11.72±3.19 [a]
		0.5%	34.50±4.33 [a]	1.86±0.16 [a]	4.23±0.81 [a]	185.32±83.22 [a]	22.72±5.73 [a]	11.82±4.33 [a]
Day 60	PEE	Control	34.65±6.08 [a]	1.66±0.30 [a]	3.89±0.85 [a]	213.73±56.61 [a]	24.69±7.53 [a]	11.40±3.06 [a]
		0.1%	31.67±4.95 [a]	1.68±0.36 [a]	3.62±1.09 [a]	201.51±73.2 [a]	23.65±11.56 [a]	11.61±3.68 [a]
		0.2%	37.22±6.69 [a]	1.74±0.24 [a]	3.90±0.54 [a]	215.21±49.32 [a]	22.89±2.93 [a]	10.16±1.72 [a]
		0.5%	35.13±5.72 [a]	1.57±0.31 [a]	4.18±0.75 [a]	224.48±49.32 [a]	27.08±4.45 [a]	12.19±3.14 [a]
	PAE	Control	34.65±5.90 [a]	1.78±0.25 [a]	4.04±0.74 [a]	194.82±43.61 [a]	22.71±6.48 [a]	11.23±3.54 [a]
		0.1%	31.67±4.77 [a]	1.80±0.31 [a]	3.77±0.98 [a]	175.93±60.2 [a]	20.95±10.51 [a]	11.43±4.16 [a]
		0.2%	35.22±5.51 [a]	1.86±0.19 [a]	4.05±0.43 [a]	189.75±36.32 [a]	21.84±1.88 [a]	11.09±2.20 [a]
		0.5%	33.13±5.09 [a]	1.69±0.26 [a]	4.33±0.64 [a]	195.8±36.32 [a]	25.61±3.40 [a]	12.62±3.62 [a]

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Table 4. White blood cell count (WBC), Differential count in the blood of *Cyprinus carpio* administration of food supplemented with different concentrations of PEE and PAE.

day	extracts	groups	WBC	Lym (%)	Neut (%)	Mono (%)	Baso	Eos
Day 0	PEE	Control	7.02±1.79 [a]	69.97±7.6 [a]	29.22±7.85 [a]	0.66±0.52 [a]	0.33±0.52 [a]	0.0±0.0 [a]
		0.1%	6.98±1.23 [a]	70.56±7.2 [a]	29.27±5.56 [a]	0.66±0.52 [a]	0.5±0.55 [a]	0.17±0.41 [a]
		0.2%	6.72±1.36 [a]	69.12±7.3 [a]	29.67±7.23 [a]	0.5±0.55 [a]	0.5±0.55 [a]	0.33±0.52 [a]
		0.5%	7.12±1.04 [a]	68.76±4.2 [a]	29.5±7.44 [a]	0.17±0.41 [a]	0.0±0.0 [a]	0.17±0.41 [a]
	PAE	Control	6.27±1.64 [a]	67.47±6.80 [a]	32.22±5.75 [a]	0.5±0.55 [a]	0.33±0.52 [a]	0.0±0.0 [a]
		0.1%	6.56±1.78 [a]	65.4±4.21 [a]	33.89±5.56 [a]	0.17±0.41 [a]	0.33±0.52 [a]	0.0±0.0 [a]
		0.2%	5.98±1.05 [a]	68.67±5.56 [a]	31.77±4.90 [a]	0.5±0.55 [a]	0.84±0.68 [a]	0.17±0.41 [a]
		0.5%	6.33±1.33 [a]	67.89±6.26 [a]	31.67±4.75 [a]	0.17±0.41 [a]	0.5±0.55 [a]	0.0±0.0 [a]
Day 20	PEE	Control	6.74±1.88 [a]	67.11±7.28 [a]	32.37±7.50 [a]	0.5±0.55 [a]	0.33±0.52 [a]	0.17±0.41 [a]
		0.1%	6.67±2.01 [a]	68.22±6.20 [a]	31.11±6.79 [a]	0.0±0.0 [a]	0.17±0.41 [a]	0.33±0.52 [a]
		0.2%	6.75±1.86 [a]	66.00±8.19 [a]	33.33±8.37 [a]	0.0±0.0 [a]	0.33±0.52 [a]	0.17±0.41 [a]
		0.5%	7.22±2.09 [a]	67.11±8.01 [a]	32.67±8.11 [a]	0.17±0.41 [a]	0.5±0.55 [a]	0.0±0.0 [a]
	PAE	Control	5.99±1.73 [a]	66.30±6.48 [a]	33.00±5.40 [a]	0.5±0.55 [a]	0.33±0.52 [a]	0.17±0.41 [a]
		0.1%	5.92±1.86 [a]	65.72±5.40 [a]	33.11±4.69 [a]	0.17±0.41 [a]	0.0±0.0 [a]	0.0±0.0 [a]
		0.2%	6.00±1.71 [a]	64.00±7.39 [a]	35.33±6.27 [a]	0.17±0.41 [a]	0.33±0.52 [a]	0.0±0.0 [a]
		0.5%	7.00±1.94 [a]	64.61±7.21 [a]	34.67±5.90 [a]	0.66±0.52 [a]	0.17±0.41 [a]	0.17±0.41 [a]
Day 40	PEE	Control	7.08±1.36 [a]	69.11±7.43 [a]	30.22±7.19 [a]	0.5±0.55 [a]	0.5±0.55 [a]	0.0±0.0 [a]
		0.1%	6.62±1.33 [a]	68.67±6.53 [a]	31.67±6.65 [a]	0.17±0.41 [a]	0.33±0.52 [a]	0.0±0.0 [a]
		0.2%	7.23±0.87 [a]	69.00±9.19 [a]	30.33±8.62 [a]	0.66±0.52 [a]	0.17±0.41 [a]	0.0±0.0 [a]
		0.5%	7.33±1.74 [a]	69.67±7.74 [a]	29.67±7.53 [a]	0.66±0.52 [a]	0.0±0.0 [a]	0.17±0.41 [a]
	PAE	Control	6.33±1.21 [a]	67.61±6.63 [a]	32.22±5.09 [a]	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0 [a]
		0.1%	5.87±1.18 [a]	66.17±5.73 [a]	33.67±4.55 [a]	0.5±0.55 [a]	0.17±0.41 [a]	0.0±0.0 [a]
		0.2%	6.78±0.72 [a]	66.50±8.39 [a]	32.33±6.52 [a]	0.66±0.52 [a]	0.0±0.0 [a]	0.17±0.41 [a]
		0.5%	6.35±1.59 [a]	67.17±6.94 [a]	32.67±5.43 [a]	0.0±0.0 [a]	0.33±0.52 [a]	0.0±0.0 [a]
Day 60	PEE	Control	7.10±1.96 [b]	70.41±6.88 [a]	28.41±7.23 [a]	0.5±0.55 [a]	0.17±0.41 [a]	0.0±0.0 [a]
		0.1%	6.84±0.98 [b]	71.00±8.66 [a]	26.89±8.43 [a]	0.0±0.0 [a]	0.33±0.52 [a]	0.0±0.0 [a]
		0.2%	7.29±1.6 [ab]	73.78±7.84 [a]	25.89±8.78 [a]	0.66±0.52 [a]	0.17±0.41 [a]	0.33±0.52 [a]
		0.5%	8.51±1.83 [a]	74.44±3.71 [a]	24.44±4.22 [a]	0.0±0.0 [a]	0.5±0.55 [a]	0.17±0.41 [a]
	PAE	Control	6.80±1.81 [a]	68.00±6.08 [a]	28.00±5.13 [a]	0.5±0.55 [a]	0.17±0.41 [a]	0.0±0.0 [a]
		0.1%	6.09±0.83 [a]	69.50±7.86 [a]	28.89±6.33 [a]	0.17±0.41 [a]	0.5±0.55 [a]	0.0±0.0 [a]
		0.2%	7.11±1.39 [a]	70.00±7.04 [a]	29.00±6.68 [a]	0.5±0.55 [a]	0.17±0.41 [a]	0.0±0.0 [a]
		0.5%	7.12±2.68 [a]	70.00±2.91 [a]	28.80±2.12 [a]	0.66±0.52 [a]	0.33±0.52 [a]	0.17±0.41 [a]

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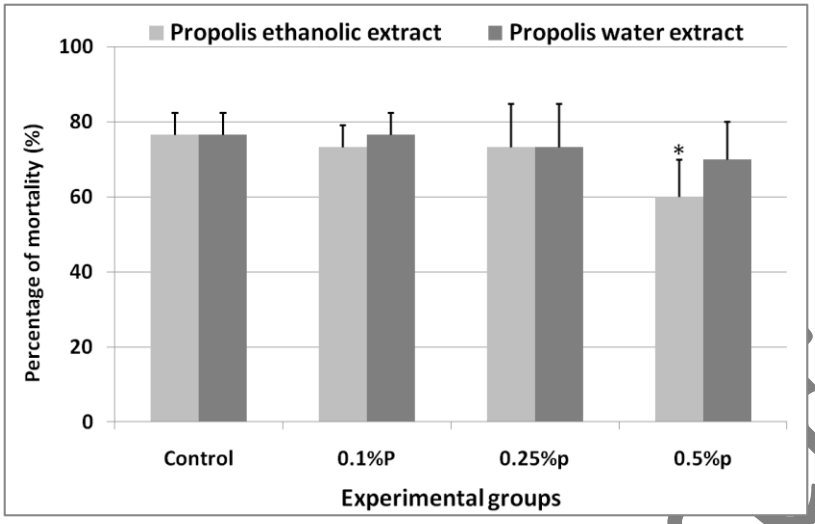

Figure 1. The cumulative mortality of *Cyprinus carpio* after bacterial challenge in experimental groups treated with a different level of PEE or PAE.