Effects of diets containing different concentrations of propolis on hematological and immunological variables in laying hens

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ABSTRACT To evaluate the effect of 4 different levels of propolis supplementation on the hematological and immunological parameters of laying hens, a trial was conducted with 60 White Leghorn layer hens. The experiment was conducted by using a randomized design with 5 treatments, 4 replicates, and 3 hens in each replicate. Treatments included basal diet (control) and basal diet plus 0.5, 1, 3, and 6 g of propolis/kg of diet, respectively. At the end of the 12-wk treatment period, samples of blood were collected to determine hematological and immunological values. The results showed that the addition of propolis at 3 g/kg in the diet resulted in significant increases ($P < 0.05$) in the serum IgG and IgM levels and significant decreases ($P < 0.05$) in the peripheral blood T-lymphocyte percentage compared with those of the control and other treatment groups. In addition, the level of 3 g/kg of propolis supplementation significantly increased ($P < 0.05$) erythrocyte count (red blood cells) compared with the other treatments. On the other hand, hemoglobin and hematocrit values and total leucocyte (white blood cells) and differential leucocytes counts were not influenced by propolis supplementation. These results indicate that the inclusion of propolis at the level of 3 g/kg of diet may have a positive effect on humoral immunity of laying hens.

Key words: propolis, hematological variable, immunological variable, laying hen

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INTRODUCTION

Plant-derived substances receive considerable interest because of their biological activities as antioxidants and antimicrobials. Consumer concern over the possible adverse effect of certain chemical preservatives and the ban of several antibiotics caused a resurgence of infectious diseases and economical losses in the poultry industry. This prompted many researchers to search for natural alternatives to antibiotics. In a previous study, we found that probiotic and prebiotic supplementation to diets of turkey elevated immunoglobulin levels (Çetin et al., 2005). Several medicinal herbs have been shown to have beneficial pharmacological and physiological effects. In recent years, attention has been focused on the use of propolis as a health supplement suited to consumers in developed countries (Banskota et al., 2001; Bankova, 2005).

Propolis (bee glue) is a resinous material collected by honey bees (*Apis mellifera* L.) from different plant exudates, which is used to inhibit microorganism growth in their nests and to repair the hives. More than 200 constituents have been identified in different propolis samples. Flavonoids, aromatic acids, caffeic acid, terpenes, and phenolic constituents appear to be the principal components responsible for the biological and pharmacological activities of propolis samples (Bankova et al., 2000; Orsolić et al., 2004). The ethanolic extract of propolis has been reported to posses various biological activities such as antibacterial (Kujumgiev et al., 1999; Sforcin et al., 2005; Silici and Kutluca, 2005), antiviral (Vynograd et al., 2000), antiinflammatory, analgesic and tissue regenerative (De Castro, 2001), antioxidant (Orhan et al., 1999; Banskota et al., 2000), and cytostatic and hepatoprotective activities (Banskota et al., 2000).

Immunostimulation through natural substances may be considered an alternative for the prevention and cure of infectious diseases. Stimulation of the immune system by natural products has already been reported (Orsi et al., 2000; Kong et al., 2004). It has also been suggested that dietary bioactive food components that interact with the immune response have considerable potential to reduce susceptibility to infectious diseases (Kogut, 2009). Several reports on the immunomodula-

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Table 1. Chemical composition of ethanol extract of propolis sample [% of total ion current (TIC),[1] gas chromatography-mass spectrometry]

Compound	RT[2] (min)	TIC (%)
Flavonoids		
Chrysin	52.48	6.06
Acacetin	53.57	2.76
Aliphatic acids		
9-Octadecanoic acid	38.95	0.48
Hexadecanoic acid	33.58	0.87
Aromatic acids		
Caffeic acid	29.19	0.52
Ferulic acid	41.35	0.42
2-Propenoic acid	29.40	0.55
3-Hydroxy-4-methoxycinnamic acid	30.34	0.32
3,4-Dimethoxycinnamic acid	30.56	1.04
Esters		
Benzylcinnamate	36.99	1.49
Alcohol, terpene, and kinons		
2-Methoxy-4-vinylphenol	12.97	0.48
β-Eudesmol	24.07	0.86
2-Naphthalenemethanol	24.19	0.93
α-Bisabolol	24.93	4.09
Others		
2,3-Dihydroxybenzofuran	10.33	0.35
Benzene	47.35	15.58
2-Nonadecanone	38.18	0.32
Heneicosane	38.6	0.98
Tricosane	44.34	0.98
2-Propene-1-one	45.39	12.19
4H-1-Benzopyran-4-one	50.58	2.35
2,4-Cycloheptadien-1-one	53.93	7.09

[1]The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.

[2]RT = retention time.

tory activities of water-soluble derivatives of propolis (**WSDP**) have been published (Orsi et al., 2000; Takagi et al., 2005; Fischer et al., 2007), but little is known about the effect of ethanolic extracts of propolis on the immunological variables in hens. Also, the long-term effect of propolis administration on the biochemical and physiological parameters of hens has not yet been studied. Considering the possibility of future antibiotic restrictions and potential benefits of propolis, the effect of propolis on the immune system should be researched in laying hens. In addition, the effect of different doses of propolis on immunological and hematological parameters has not been compared previously. Therefore, the purpose of the study was to determine the effect of different levels of dietary propolis intake on the hematological and immunological parameters in laying hens.

MATERIALS AND METHODS

Origin of Propolis

Propolis sample was collected from Kayseri (Central Anatolia) in Turkey. Hand-collected propolis was kept desiccated in the dark until the processing. Thirty grams of propolis was extracted for a week with 100 mL of 70% ethanol at room temperature to obtain the extract. After filtration, the extract was evaporated by vacuum evaporator at 50°C.

Chemical Analysis of Propolis

Gas chromatography-mass spectrometry was carried out to detect the main components of propolis by using an Agilent GC 6890 gas chromatograph coupled to an Agilent MSD 5973 mass detector (Agilent Technologies, Santa Clara, CA) under electron impact ionization. The chromatographic column for the analysis was a Zebron (ZB-1) methyl polysiloxane column (30 m × 0.25 mm i.d., df: 0.25 μm; Phenomenex, Aschaffenburg, Germany). Helium was used as a propulsive gas at a flow rate of 10 mL/min. Propolis sample was analyzed with the column held initially at 100°C for 5 min and then increased to 150°C and then kept at 150°C for 2 min. Finally, the temperature was raised to 280°C with a 2°C/min heating ramp, and the column was maintained at 280°C for 60 min. The injection was performed in split mode at 250°C, and the peaks were identified by computer searches in commercial reference libraries. The main components of propolis samples were determined by considering their areas as a percentage of the total ion current. The main compounds of propolis sample were presented in Table 1.

Birds, Experimental Design, and Diet

A total of sixty 40-wk-old White Leghorn laying hens were used in this study. The experiment was conducted by using a completely randomized design, and 5 dietary treatments (4 replicates and 3 hens in each replicate) were used. Treatments included basal diet (control) and basal diet plus 0.5, 1, 3, and 6 g of propolis/kg of diet, respectively. Layers were fed with experimental diets for 12 wk. Feed and water were provided ad libitum throughout the experiment. The hens were housed in cages, maintained at room temperature, and provided with 16 h of light daily. Chemical composition of the basal diet fed to the laying hens was shown in Table 2.

Sample Collection and Analyses

At the end of the 12 wk, blood samples from birds were taken for hematological and immunological analysis by puncturing the brachial vein. The blood samples were collected into heparinized tubes for hematological analyses and into nonheparinized tubes for immunological analyses. For serum isolation, blood samples in nonheparinized tubes were centrifuged at 1,400 × *g* for 15 min. Isolated sera were stored at −20°C. Hematological analyses were performed on the same day that the blood samples were taken.

Erythrocyte and total leucocyte counts were determined using Natt-Herrick's solution. Hemoglobin concentration was measured spectrophotometrically (Shimadzu UV 1700 V, Shimadzu, Kyoto, Japan). Hematocrit was measured using a microhematocrit capillary tube reader after centrifugation on a hematocrit centrifuge at 2,240 × *g* for 5 min. Differential white

Table 2. Ingredients and chemical composition of basal (control) diet[1]

Item	40 to 52 wk
Ingredient	
Corn, yellow	54.84
Soybean meal 44%	12.30
Wheat	10.00
Limestone	9.22
Sunflower meal	5.8
Wheat bran	2.00
Meat-bone meal	1.97
Full-fat soybean	1.48
Corn gluten	1.21
Dicalcium phosphate	0.50
Salt	0.30
Vitamin-mineral mix[2]	0.25
DL-Methionine	0.08
Lysine	0.05
Calculated values	
DM	89.03
CP	16.00
Crude ash	12.40
Crude fiber	3.54
Lysine	0.75
Choline	1.71
Methionine	0.37
Calcium	4.00
Phosphorus-T	0.62
Phosphorus-K	0.40
Linoleic acid	1.50
ME, kcal/kg	2,750

[1]Values are expressed as percentages.

[2]Provided per 2.5 kg of vitamin-mineral mix: vitamin A, 12,000.000 IU; vitamin D_3, 2,400.000 IU; vitamin E, 30,000 mg; vitamin K_3, 2,500 mg; vitamin B_1, 3,000 mg; vitamin B_2, 7,000 mg; vitamin B_6, 4,000 mg; vitamin B_{12}, 15 mg; nicotinamide, 40,000 mg; calcium D-pantothenate, 8,000 mg; folic acid, 1,000 mg; D-biotin, 45 mg; vitamin C, 50,000 mg; choline chloride, 125,000 mg; Mn, 80,000 mg; Fe, 40,000 mg; Zn, 60,000 mg; Cu, 5,000 mg; I, 400 mg; Co, 100 mg; Se, 150 mg; and antioxidant, 10,000 mg.

blood cell (**WBC**) counts were performed using blood smears stained with May-Grunwald-Giemsa stain. One hundred WBC were examined per laying hen using a microscope, and the percentage of each of 5 basic leucocytes (heterophils, lymphocytes, monocytes, eosinophils, and basophils) was calculated (Konuk, 1981).

Peripheral blood T-lymphocyte percentages were determined according to Mueller et al. (1975). Briefly, for α-naphthyl acetate esterase (**ANAE**) demonstration, blood smears were prepared from blood samples and dried in air. They were then fixed in a mixture of glutaraldehyde and acetone solution for 3 min at −10°C, rinsed in distilled water, and dried in air. They were then incubated in the incubation solution (pH 5.8) for 3 h, rinsed with distilled water, and counterstained with methylene blue for 10 min. After application of ANAE enzyme staining, they were examined under a light microscope. The lymphocytes with reddish-brown reaction products were regarded as ANAE-positive T lymphocytes. The percentage of ANAE-positive T lymphocytes in every smear was determined by counting 200 lymphocytes totally under a light microscope.

Total IgG and IgM concentrations in individual serum samples were measured automatically by the BNII nephelometric immunoassay of Beckman Coulter (Brea, CA).

Statistical Analysis

Data were analyzed using the SPSS for Windows software, Version 12.0 (SPSS Inc., Chicago, IL). Statistically significant differences between groups were determined by ANOVA. When the differences were significant, Tukey's honestly significant difference multiple range test was performed. Mean values were considered significantly different at $P < 0.05$. Data are expressed as mean values ± SD.

RESULTS

The effect of dietary supplementation of propolis on some hematological parameters of the laying hens is presented in Table 3. There was significant increase ($P < 0.05$) in erythrocyte count of birds given 3 g/kg of propolis compared with control and other treatment groups. However, interestingly, the erythrocyte count was not significantly altered by doses of 0.5, 1, and 6 g/kg of propolis. Although a slight increase was determined in hemoglobin and hematocrit values in the group receiving 3 g/kg of propolis in the diet, these increases were found not to be significant statistically ($P > 0.05$) in comparison to the control and other treatment groups.

The WBC count and the percentages of differential WBC were not significantly influenced ($P > 0.05$) by dietary supplementation with propolis. On the other

Table 3. Effect of diets containing different levels of propolis on some hematological parameters of the laying hens[1]

Dose (g/kg of diet)	Erythrocyte ($10^6/\mu L$)	Hemoglobin (g/dL)	Hematocrit (%)
Control			
0	2.34 ± 0.32[a]	11.75 ± 1.0	27.19 ± 1.4
Propolis			
0.5	2.40 ± 0.27[a]	12.00 ± 1.1	26.32 ± 3.5
1	2.71 ± 0.35[a]	12.19 ± 0.8	27.34 ± 1.8
3	3.30 ± 0.29[b]	13.21 ± 0.6	29.78 ± 3.7
6	2.45 ± 0.28[a]	12.21 ± 1.26	26.47 ± 3.6

[a,b]Mean values with different superscripts in the column are significantly different ($P < 0.05$).

[1]Data are expressed as mean ± SD (n = 12).

hand, serum IgG and IgM levels were significantly higher ($P < 0.05$) in the group receiving 3 g/kg of propolis in the diets when compared with the control and other treatment groups. The level of 3 g/kg of propolis supplementation significantly decreased ($P < 0.05$) the percentage of ANAE-positive T lymphocytes.

The effects of dietary supplementation of propolis on some immunological parameters of the laying hens are presented in Table 4. The best response to the propolis supplementation as regards humoral immunity occurred in birds receiving 3 g/kg of propolis. Interestingly, compared with 3 g/kg, the 6 g/kg dose of propolis did not produce a significant elevation in the serum IgG and IgM level. The level of 1 g/kg of propolis supplementation increased serum IgG and IgM concentrations compared with the control group, but the difference was not statistically significant ($P > 0.05$).

DISCUSSION

In this study, the effects of different levels of propolis on some hematological and immunological parameters were investigated. In the present study, erythrocyte counts had significantly increased ($P < 0.05$) in the group receiving 3 g/kg of propolis in the diets when compared with the control and other treatment groups. On the other hand, we found that propolis at doses of 0.5, 1, and 6 mg/kg fed to hens did not have a significant effect on erythrocyte count. In this experiment, a significant elevation of the number of erythrocytes in birds of groups given 3 g/kg of propolis suggests that propolis might have a stimulatory effect on the synthesis of these cells in bone marrow. Our results are consistent with those previously reported by Orsolić and Basic (2005), who observed increased erythrocyte counts in mice treated with WSDP and suggested that propolis acts directly on hematopoietic bone marrow cells and enhances their growth and differentiation into colony-forming cells. The slightly increased hemoglobin level, in response to 3 g/kg of propolis supplementation observed in this study, could be explained by assuming that propolis improves the digestive utilization of iron and the regeneration efficiency of hemoglobin (Haro et al., 2000). The above findings suggest that dietary propolis supplementation at 3 g/kg of diet may help prevent anemia.

The present data showed insignificant difference among treated groups for WBC counts, which agree with those of Orsolić et al. (2006), who found that WSDP have no effect on WBC count in mice. Contrary to our findings, Orsolić and Basic (2003, 2005) reported that WSDP given to mice caused a significant elevation of leucocytes in peripheral blood. Also, we found that differential WBC counts were not influenced by propolis supplementation. These results agree with the findings of Orsolić and Basic (2005), who reported that WSDP did not change the ratios of leukocyte types in peripheral blood of mice.

Serum antibody level is the indicator of humoral immunity. Our results showed that serum IgG and IgM levels in the group receiving 3 g/kg of propolis were significantly higher than those of control and other groups, suggesting that 3 g/kg of propolis could promote humoral immunity in laying hens. A similar, but not significant, trend was observed for 1 and 6 mg/kg of propolis. Several studies have showed that propolis activates the immune system in different animal species, increasing macrophage activity (Dimov et al., 1991; Park et al., 2004) and increasing interleukin-1, interleukin-2, and interleukin-4 levels (Orsolić and Basic, 2003; Park et al., 2004). These cytokines stimulate B lymphocytes and then they are changed to plasma cells, which would be able to produce immunoglobulins (Diker, 1998). Therefore, in our study, the increased levels of IgG and IgM in birds of groups given 3 g/kg of propolis may be related to the stimulation of B lymphocytes by these cytokines.

Our findings were supported by Ziaran et al. (2005), who reported that humoral immunity was modulated by different levels of propolis in the diet of broilers. In that study, they observed that low levels of dietary propolis increased antibody titer, whereas high levels of propolis decreased antibody titer, thereby exhibiting a bell-shaped dose-response relation. Similarly, Taheri et al. (2005) have observed a relatively negative effect of a higher concentration of propolis on humoral immunity of broilers and concluded that the broiler's

Table 4. Effects of diets containing different levels of propolis on IgG and IgM levels and α-naphthyl acetate esterase-positive [ANAE (+)] T-lymphocyte percentage in laying hens[1]

Dose (g/kg of diet)	IgM (mg/dL)	IgG (mg/dL)	ANAE (+) T lymphocytes (%)
Control			
0	172.65 ± 29.2[a]	79.70 ± 16.4[a]	50.23 ± 1.32[a]
Propolis			
0.5	180.50 ± 34.8[a]	84.87 ± 14.6[a]	48.14 ± 1.32[a]
1	208.50 ± 38.5[a]	104.50 ± 31.38[a]	50.76 ± 1.32[a]
3	261.00 ± 44.4[b]	141.77 ± 35.71[b]	40.32 ± 1.32[b]
6	211.00 ± 39.7[a]	98.50 ± 28.27[a]	51.34 ± 1.32[a]

[a,b]Mean values with different superscripts in the column are significantly different ($P < 0.05$).

[1]Data are expressed as mean ± SD (n = 12).

immune system may respond to propolis on a crucial dosage. Furthermore, Scheller et al. (1988) indicated that further increase in the propolis dose or in the frequency of its administration has an inhibitory effect on the antibody production in immunized mouse spleen cells. On the other hand, 6 g/kg of propolis did not produce a significant elevation in the serum IgG and IgM level, and this effect could be attributed to the main constituents of this propolis preparation such as benzene and flavonoids. It is also possible that benzene can have negative effects on immune function. Lange et al. (1973) have reported decreased levels of serum IgA and IgG with no decrease in the IgM level in persons exposed to benzene and its homologs and suggested that this observation reflects a suppressive action of benzene on immunoglobulin-producing cells, resulting in the inhibition of DNA synthesis. Similarly, Irons and Moore (1980) concluded that benzene has a relatively selective cytotoxic effect on circulating B lymphocytes in the rabbit, depending on the dose. Meanwhile, it was reported that ethanol (propolis solvent) did not influence its activities in immunological assays (Sforcin, 2007).

We observed that 3 g/kg of propolis supplementation decreased the peripheral blood T-lymphocyte ratio of laying hens. Similarly, Sforcin (2007) reported that propolis could act directly on the T cells, inhibiting their differentiation. Furthermore, Park et al. (2004) reported that the treatment of caffeic acid phenethyl ester, the active component of propolis, directly or indirectly causes the immune cells to decrease in cell number, especially T cells. In this study, the suppressive effect of 3 g/kg of propolis on T lymphocytes could be attributed to caffeic acid phenethyl ester.

Because of the high chemical complexity of propolis, it is difficult to identify which components are responsible for its biological activities. With respect to hematological and immunological parameters, discrepancies between our results and those reported by others may be related to the type and dosage of propolis and its constituents in the diets, duration of feeding with experimental diets, study population (i.e., in age, weight, or breed), and animal species.

Based on the result of the present work, we conclude that the inclusion of propolis at the level of 3 g/kg of diet may stimulate IgG and IgM production in laying hens and may be more effective than low- or high-dose propolis, and dosage could be an important factor in using propolis for immune stimulation in laying hens. Moreover, fowls are subjected to frequent stress factors such as environmental, nutritional, physical, social, and pathological stress. These stressful conditions may suppress immune function and lead to birds' starvation and infectious disease. Taken together, we can say that the inclusion of propolis at the level of 3 g/kg of diet may protect the birds from the harmful effect of stress and reduce susceptibility to infectious diseases.

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