THE EFFECT OF DIET SUPPLEMENTATION WITH PROPOLIS ON IMMUNOLOGICAL AND PHYSICOCHEMICAL PROPERTIES OF TIBIA BONES IN BROILER CHICKENS

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SUMMARY

A trial was carried out to determine the effects of diet supplementation with propolis on immunological status and bone physicochemical properties of broiler at 6 weeks of age. Two hundred and forty one-day-old unsexed Cobb broilers chicks were randomly distributed into four equal experimental groups; with three replicates of 20 chicks each. They were fed on one of the following propolis levels; 0, 125, 250 and 500 mg/kg diet. The tibia length, percentage relative to live body weight, dry matter, ash of tibia bone, phosphorus and calcium concentrations were determined at the end of the experiment. The results showed that, propolis supplementation at 125, 250 and 500 mg/kg diet increased ($p<0.05$) the tibia length by 3, 2.4 and 3.4%, respectively, when compared to the control group. The higher value of tibia percentage relative to live body weight was recorded in the group fed propolis supplementation at 125 mg/kg diet, followed by groups fed 250, 500 mg propolis/kg diet and group that fed control diet. Propolis supplementation at 125, 250 and 500 mg/kg increased phosphorus concentrations by 4.6, 1.1 and 1.8 %, respectively. Also, calcium concentrations in ash were increased by 0.6, 2.9 and 3.1 %, respectively, when compared to the control group. Although, all groups had haemagglutination inhibiting antibodies against NDV, the highest haemagglutination inhibiting antibody titer was obtained from birds fed diets supplemented with 125 and 250 mg propolis/ kg diet at 35 days of age. In addition, data showed that the concentration of 500 mg propolis/kg diet did not significantly influence the humoral immune response of broiler against NDV.

Keywords: *Broilers, propolis, Ca, P and immune response.*

INTRODUCON

Broiler chickens are characterized by a very fast growth rate; therefore broiler diets should contain high concentrations of available nutrients (Zimnoch *et al.,* 2000). The adverse effect of broiler selection programs that focus on increasing growth rate and improve the immune-response against diseases, a higher incidence of leg problems, in particular bone deformities and abnormalities (Leterrier and Nys 1992, Horn 2000, Williams *et al.,* 2004).

Recently, natural materials have been investigated in order to prove its effectiveness as a growth promoter such as Propolis. It is a resinous substance collected by worker bees (*Apis mellifera*) from the bark of trees and leaves of plants (Nada *et al.,* 2011).

Propolis plays an important role in the elimination of acute and chronic pain and mortality resulting from osteoporotic fractures which pose serious animal welfare concerns (Danbury *et al.,* 2000) and increase economic loss (Cook, 2000) by increased mortality resulting from skeletal diseases (Sullivan, 1994; Thorp, 1994).

Furthermore, several researchers (Sforcin, 2007; Galal *et al.,* 2008; Attia *et al.,* 2011b,c; Popiela-Pleban *et al.,* 2012) demonstrated that Propolis could be used as growth promoters and immune enhancers as an alternative to antibiotics. In addition, Propolis has been suggested as a suitable health supplement for consumers in developed countries (Bankova, 2005).Also, the oxidative stress that can be caused by environmental factors, disease, infection, inflammation, aging, reactive oxygen species (ROS) production include free radicals and other oxygenated molecules resulting from these factors may be minimized by antioxidant defense mechanisms that protect the cell against cellular oxidants and repair systems that prevent the accumulation of oxidative damaged molecules on 14, 21 and 28 days respectively.

Ihsan *et al.* (2013) reported that, propolis has a positive role against Newcastle disease virus when they studied the effect of propolis in diet supplementation on the histopathological changes in some organs and challenge tests against Newcastle disease in broiler chicks.

Therefore, the aim of this study is to investigate the effect of propolis supplementation in broiler diets on immunological and bone physiological status.

MATERIALS AND METHODS

This study was carried out at two parts; the first one was the extraction procedure of the propolis alcoholic extract in the laboratory using ethanol alcohol 96%, the second was a field study carried out at a private commercial farm under supervision of Animal Production Department, Faculty of Agriculture, Tanta University, during the period from October to December 2014 to investigate the effect of feeding diets with 0, 125, 250, 500 mg Ethanolic Extracts of Propolis/kg diet on broiler physiological and immunological status.

Preparation of ethanolic extracts of propolis (EEP):

Propolis samples were purchased from Elzahaby phytopharm,in powder form. 35gm of propolis powder were mixed with 70 ml of 96% ethanol alcohol and left for 14 days, in a dark cool place (not in refrigerator) and was shaken also 3 times per day. After two weeks the solution was filtered; the liquid portion was stored in a dark green bottle in a cool, dry and dark place. The ethanolic extract solution was then filtered through a Whattman No.1 filter paper and restored to the original volume with 96% ethanol (Khojasteh and Shivazad 2006), then different concentrations of EEP were prepared at a rate of 125, 250 and 500 mg and stored in a dark green bottle in a cool, dry and dark place.

Experimental design:

Two hundred and forty one-day-old unsexed Cobb broilers chicks were used in this experiment, randomly distributed into four equal experimental groups; with three replicates of 20 chicks each. The average initial body weights of the treatments groups were nearly similar with no observed significant differences. Chicks were grown in floor pens and subjected to 23 hrs. lighting along the experimental period which extended to 6 weeks of age. The house temperature was kept at about 34ºC during the first 3 days, 32ºC during next 4 days and thereafter, gradually decreased by 3ºC weekly down to 24ºC. Pellets feed form and water were available *ad libitum* throughout the experimental period. All experimental groups were reared under similar managerial and hygienic conditions.

Experimental Diets:

The basal diet was a commercial corn-soybean meal diet formulated to meet or exceed the nutritional requirement of growing chicks as recommended by NRC (1994) as shown in Table 1.

Dietary treatments evaluated included:

Control (Basal diet without any addition), T2 (Basal diet supplemented with 125 mg/kg EEP), T3 (Basal diet supplemented with 250 mg/kg EEP) and T4 (Basal diet supplemented with 500 mg/kg EEP).

Measurements:

Tibia bones were separated, removing cartilage. Their weight and length of the tibia were determined. The measurements were taken with an electronic caliper, based on the geometric characteristics of the tibia.

The content of crude ash and minerals (calcium and phosphorus) in tibia bones was determined after dry mineralization at 650 °C. Ca content was measured by spectrophotometer, and P content was estimated calorimetrically. Bone samples were mineralized in a mixture of nitric acid and perchloric acid (3:1). Mineralization was carried out in an aluminum heating block with temperature control. Reference samples were prepared together with test samples. The Ca content of mineralizates was determined by flame atomic absorption spectrometry (acetylene-air flame), with the use of Unicom 939 atomic absorption spectrometer, equipped with an Optimus data station, a background correction source (deuterium lamp) and cathode lamps (Whiteside and Miner 1984). In order to determine Ca content, a 10 % aqueous solution of lanthanum chloride was added to all experimental solutions, in the amount ensuring the final La+3 concentration of 1 %. Phosphorus concentration was determined in mineralizates by colorimeter with ammonium molybdate and with sodium sulfate and hydroquinone. Absorbance was

measured using a VIS 6000 spectrophotometer (A. KRÜSS Optronic GmbH, Hamburg, Germany), at a wavelength of λ=610 nm (Fiske and Subbarow1925). The content of Ca and P was determined using standards at a concentration of 1 mg/cm3, diluted with a 0.1 M solution of HNO3 (Kleczeket *et al.*, 2012).

Haemagglutination Inhibition (HI) Test:

After 10 days of Lasota vaccination, blood samples were collected. Blood serum was separated by centrifugation at 3000 rpm for 20 min. The collected serum was kept frozen at - 20ºC until assay. The beta-procedure of the HI test was employed as a micro-test in plastic plates as outlined in "Methods for Examining Poultry Biologics and for Identifying and Quantifying Avian Pathology" (Anon, 1990).

Table (1). The composition and calculated analysis of experimental starter, grower and finisher diets.

Ingredients	Experimental diets		
	Starter	Grower	Finisher
Yellow corn	54	61	63.5
Soybean meal (44%)	29	26.5	24
Wheat brain	2.3	0.8	-
Corn gluten meal (62%)	8.5	4.8	4.8
Soybean oil	2.5	3.2	4
Dl-Methionine	0.1	0.1	0.1
L. Lysine	0.1	0.1	0.1
Limestone	1.4	1.4	1.4
Dicalcium phosphate	1.5	1.5	1.5
Salt	0.3	0.3	0.3
Premix**	0.3	0.3	0.3
Total	100.00	100.00	100.00
Calculated analysis			
Crude protein (%)	23.01	20.05	19.03
ME (Kcal/Kg)	3025	3109	3196
Ether extract (%)	3.40	3.8	4.0
Crude fiber (%)	3.50	3.60	3.20
Calcium (%)	0.95	0.94	0.94
Available phosphorus (%)	0.41	0.40	0.39
Methionine (%)	0.5	0.43	0.44
Lysine (%)	1.1	1.00	0.93
Mt cysteine	0.94	0.90	0.88

** *Each 3kg of premix contained: Vit. A 12000IU, Vit.D 2200IU, Vit.E 10mg, Vit.K_3 2000mg, Vit.B_1 1000mg, Vit. B_2 5000mg, Vit. B_6 1500mg, Vit. B_{12} 10mg, Pantothenic acid 10mg, Niacin 30mg, Folic acid 1000mg, Biotin 50mg, Choline chloride 300mg, Manganese 60mg, Zinc 50mg, Copper 10mg, Iron 30mg, Iodine 1000mg, Selenium 100mg, Cobalt 100mg and $CaCo_3$ to 3g.*

Statistical analysis:

Data were statistically analyzed by one-way ANOVA, using the general linear model procedure (SAS, 1996). Tests of significance for differences among treatments were done according to Duncan (1955). The statistical model was used for the analysis of variance to estimate the effect of dietary propolis supplementation as follows:

$Y_{ijk} = U + T_i + R_j + e_{ijk}$

Where, Y_{ijk} is the observations; U is overall mean; T_i is effect of i treatment (i = 0, 1, 2 and 3); R_j is effect of j replicate (j = 1, 2 and3) and e_{ijk} is residual (random error).

RESULTS AND DISCUSSION

Bone physiological status:

Table (2) shows the effect of propolis supplementation on tibia weight, length and tibia percentage of live body weight. It is obvious that, propolis supplementation increased significantly ($p<0.01$) tibia

weights. The highest value of tibia weights was in the group fed propolis supplementation at 125 mg/kg diet. While, the least tibia weights were in the group fed control diet.

Propolis supplementation at 125, 250 and 500 mg/kg diet increased significantly ($p<0.05$) the tibia length by 3, 2.4 and 3.4 % respectively, when compared to the control group.

The highest value of tibia percentage relative to live body weight was recorded in the group fed propolis supplementation at 125 mg/kg diet, followed by groups fed 250, 500 mg propolis/kg diet and group that fed control diet.

Table (2). Effect of dietary propolis supplementation on physical properties (Mean±SE) of the tibia bone.

Traits \ Treatments	Control(T1)	125 (T2)	250 (T3)	500 (T4)	Sign,
Tibia weight g	20.07±0.75^{b} (100%)	23.47±0.58^{a} (116.9%)	21.86±0.76^{ab} (108.91%)	22.16±0.52^{a} (110.4%)	**
Tibia length mm	92.63±0.98^{b} (100%)	95.37±0.59^{a} (102.95%)	94.83±0.46^{a} (102.37%)	95.77±0.75^{a} (103.38%)	*
Tibia percentage in the total body weight %	1.02±0.003^{b} (100%)	1.05±0.009^{a} (102.94%)	1.04±0.009^{ab} (101.96%)	1.04±0.007^{ab} (101.96%)	*

-Means of each column followed by the same letter are not significantly different at the 5% level according to Duncan's Multiple Range Test.
- indicate P<0.05 -** indicate P<0.01*
-T1=Control (C) Basel diet, T2= (125 mg pro/kg), T3= (250 mg pro/kg), T4= (500 mg pro/kg).

Bone chemical properties:

Table (3) shows the effect of propolis supplementation on mineral composition of the tibia bone. The dry matter and Ash of tibia bone for broiler groups were not statistically affected by propolis containing diets, when compared to control group. The highest content of dry matter and ash were noted in the tibia bones of broilers fed a diet supplemented with propolis at 250 mg/kg. followed by 500mg/kg diet; 250 mg/kg diet and the control respectively.

Phosphorus and calcium concentrations in ash were highest in the tibia bones of broilers fed supplemented diet with propolis when, compared to the control group. Propolis supplementation at 125, 250 and 500 mg/kg increased Phosphorus concentrations by 4.6, 1.1 and 1.8 % respectively, when compared to the control group. Also, calcium concentrations in ash were increased by 0.6, 2.9 and 3.1 % respectively, when compared to the control group.

Table (3). Effect of dietary propolis supplementation on minerals composition (Mean±SE) of the tibia bone.

Traits \ Treatments	Control(T1)	125 (T2)	250 (T3)	500 (T4)	Sign,
Tibia dry matter % (1mg/cm3)	96.75±0.16 (100%)	96.67±0.21 (99.91%)	97.02±0.05 (100.28%)	97.00±0.08 (100.26%)	NS
Ash in tibia dry matter% (1mg/cm3)	55.72±0.42 (100%)	55.78±0.4 (100.1%)	56.57±0.2 (101.52%)	55.88±0.37 (100.29%)	NS
Phosphorus in ash % (1mg/cm3)	17.58±0.23^{b} (100%)	18.38±0.25^{a} (104.55%)	17.78±0.31^{ab} (101.14%)	17.9±0.1^{ab} (101.82%)	*
Calcium in ash % (1mg/cm3)	40.23±0.22^{b} (100%)	40.47±0.15^{b} (100.60%)	41.4±0.17^{a} (102.90%)	41.47±0.19^{a} (103.08%)	***

-Means of each column followed by the same letter are not significantly different at the 5% level according to Duncan's Multiple Range Test.
-NS indicate not significant. - indicate P<0.05 -*** indicate P<0.001*
-T1=Control (C) Basel diet, T2= (125 mg pro/kg), T3= (250 mg pro/kg), T4= (500 mg pro/kg)

Similar results were found by Haro *et al.,* (2000) who demonstrated that addition of propolis caused increase of phosphorus level in bones. Probably propolis inclusion in the diet increases absorption of phosphorus from the blood to the bone and thus decreased the level of these elements in the blood.

These results are Confirmed by the discussion of Ang *et al.,* (2009), who demonstrated that propolis has been shown to have anti-tumor and anti-inflammatory properties, Low concentrations of caffic acid phenyl ester(cape) <1microM dose dependently inhibited RANKL-induced osteoclastogenesis in RAW264.7 cell and bone marrow macrophage (BMM) cultures, as well as decreasing the capacity of human osteoclasts to resorb bone. CAPE inhibited both constitutive and RANKL-induced NF-kappaB and NFAT activation, concomitant with delayed IkappaBalpha degradation and inhibition of p65 nuclear translocation. At higher concentrations, CAPE induced apoptosis and caspase 3 activities of RAW264.7 and disrupts the microtubule network in osteoclast like (OCL) cells, which results in the attenuation of osteoclastogenesis and bone resorption, implying that CAPE is a potential treatment for osteolytic bone diseases.

The haemagglutination inhibition of broilers chicks affected by propolis supplementation is listed in Table (4). Propolis supplemented group at 125 and 250 mg/kg were (5, 3.5) higher than the control group and supplemented group of 500 mg/kg (3, 2.67) in the (NDV) antibodies titer, 10 days after vaccination.

A similar results were found by *Taheri et al.,* (2005) who found that, antibody titer against avian Influenze, newcastle disease virus and BD were significantly ($P<0.05$) increased with oil extract of propolis supplementation, without any effect on IB.

In addition, Hegazi *et al.* (2012) reported that, propolis extract treated group was the highest in the (NDV) antibodies titer (4.9, 6.4 and 7.7) when compared with control group (2.7, 2.2 and 1.9) on 14, 21 and 28 days.

Table (4). Effect of dietary propolis supplementation on HI (Mean±SE) against NDV of broiler chickens after 10 days of vaccination.

Treatments	Propolis levels				significance
	T1	T2	T3	T4	
HI (%)	3 ± 0.52^{b}	5 ± 0.87^{a}	3.5 ± 0.43^{ab}	2.67 ± 0.21^{b}	*

-Means of each column followed by the same letter are not significantly different at the 5% level according to Duncan's Multiple Range Test.
-NS indicate not significant.
- indicate P<0.05*
-T1=Control (C) Basel diet, T2= (125 mg pro/kg), T3= (250 mg pro/kg), T4= (500 mg pro/kg).

CONCLUSIONS

In conclusion, this study demonstrated that propolis supplementation may be a good enhancer to the tibia length, tibia percentage of the total body weight, phosphorus and calcium concentrations as well as immunological status against NDV.

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تاثير إضافة البروبوليس على الحالة المناعية والخواص الفزيائية والكيميائية لعظام الساق فى دجاج اللحم.

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أجريت هذه الدراسة لتحديد تاثير إضافة البروبوليس فى العلائق على الحالة المناعية والخواص الفزيائية والكميائية لعظام الساق فى دجاج اللحم. تم توزيع 240 كتكوت من سلالة الكوب عمر يوم واحد في أربع معاملات قسمت داخليا إلي 4 مكررات كل واحدة منها تضم 20 كتكوت. وتضم المستويات المختلفة من البروبوليس (0، 125، 250، و 500 ملجم / كجم عليقة) لمدة 6 أسابيع. وكانت أهم النتائج المتحصل عليها هي حدوث زيادة معنوية في طول الساق بمعدل 3, 2.4 و 3.4 % عند اضافة البروبوليس بمعدل 125, 250 و 500 ملجم/كجم عليقة عند مقارنتها بالكنترول. سجلت المعاملة الثانية (125 ملجم /كجم) أعلى وزن نسبى للساق ثم يليها المعاملة الثالثة (250 ملجم/كجم) ثم المعاملة الرابعة (500 ملجم/كجم)ثم معاملة الكنترول. أيضا حدث زيادة معنوية فى تركيز كلا من الفسفور والكالسيوم فى المعاملات الثانية والثالثة والرابعه على التوالى بمعدل 4.6, 1.1و 1.8 : 0.6, 2.9 و3.1 على التوالى عند مقارنتها بالكنترول. وعلي الرغم من ان كلا الطيور بالمستويات المختلفة من البروبوليس كانت تمتلك اجسام مضادة لفيروس النيوكاسل. وكان هناك فروق معنوية بين المعاملات في مستوي الاجسام المضادة وكانت الطيور المغذاة علي علائق محتوية علي 125و250 ملجم/كجم بروبوليس أظهرت ارتفاعا في مستوي الاجسام المضادة لمرض النيوكاسل مقارنتا بالكنترول. ومن هنا نوصى باستخدام البروبوليس بمعدل 125 ملجم /كجم فى علائق الدواجن .