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EFFECTS OF TWO PLANT EXTRACTS AND VITAMIN D_3 ON BONE MECHANICAL AND HISTOLOGICAL PROPERTIES OF BROILER CHICKENS

M. T. Mirakzehi[1*], S. J. Hosseini[2] and H. Saleh[1]

[1]Department of Animal Science, Higher Educational Complex of Saravan. P.O. Box:99516-34145, Saravan, Sistan and Baluchestan, Iran; [2]Centre of Excellence in the Animal Science Department, Ferdowsi University of Mashhad, PO Box 91775-1163, Mashhad, Iran.
[*]Correspondence Author E-mail:mt_mirakzehi@yahoo.com

ABSTRACT

This study was performed to evaluate the effects of hydroalcoholic extracts of *Withania somnifera* (WS) root, *Withania coagulans* (WC) fruit and 1, 25-dihydroxycholecalciferol [1, 25-$(OH)_2$ D_3] on bone mineralization, mechanical ,and histological properties of broiler chickens. A total of 550 male one-day-old Ross 308 broiler chickens were randomly allotted to 55 pens, with 10 birds per pen (replicate), and reared for 42 days. A completely randomized design was used with 11 dietary treatments including a positive control diet with adequate Ca; a negative control diet (Ca concentration reduced by 30%); and a negative control diet supplemented with either WS or WC extracts at three levels (0, 75 and 150 mg/kg diet) or 1, 25-$(OH)_2$-D_3 at two different levels (0 and 0.5 µg/kg diet). Diets were given *ad libitum* from one to 42 day of age. Dietary treatments did not affect feed intake and feed conversion efficiency. On days 21 and 42, one bird per replicate was killed and tibiae were removed. Among the birds that were given negative control diet, the highest Ca retention values were noted in those that received 75 mg/kg WC (84.67%) or 150 mg/kg WS (83.09%) with 0.5 µg/kg 1, 25-$(OH)_2$ D_3. At 21 days of age, the highest tibia Ca values were obtained in birds fed negative control diet supplemented with 150 mg/kg WS or WC with no dietary supplementation of 1, 25-$(OH)_2$ D_3 (38.652 and 38.433 % respectively). No significant effects on tibia Ca were noted at 42 days. There were no significant differences ($P>0.05$) between experimental treatments for all tibia bone biomechanical properties. The addition of 75 mg/kg WS and 1, 25-$(OH)_2$ D_3 to negative control diets resulted in significant increase ($P<0.05$) in tibia mineralized zone width. The present study showed that dietary supplementation of WS and WC at 150 and 75 mg/kg, respectively improved Ca retention and the effects were more pronounced in the presence of 1, 25 $(OH)_2$ D_3. Administration of 150 mg/kg WS and WC had beneficial effects on bone calcification. Also, synergistic effects of WS and 1, 25-$(OH)_2$ D_3 resulted in significant increase in tibia mineralized zone width.

Key words: *Withania somnifera, Withania coagulans*, 1, 25-dihydroxycholecalciferol, Bone, Broiler chicken.

INTRODUCTION

The active form of vitamin D_3 in bird is produced by two sequential hydroxylation reactions. The first hydroxylation of vitamin D_3 molecule occurred at position of 25 in the liver to produce 25-OH-cholecalciferol (25-OH-D_3), the major circulating form of vitamin D_3 in the blood. The circulating 25-OH-cholecalciferol is then transported to the kidneys and hydroxylated at position 1 by the enzyme 1-alpha-hydroxylase (VD3 1α hydroxylase) (E.C.1.14.13.13) forming 1, 25-dihydroxycholecalciferol [1, 25-$(OH)_2$ D_3], the most biologically active, hormonal metabolite of the vitamin D_3 (Norman, 2008). 1, 25-$(OH)_2$ D_3 plays a well-recognized role in the regulation of mineral metabolism. Previous findings suggest that supplementation of practical diets containing low Ca and high or adequate in P with 1, 25-$(OH)_2$ D_3 may improve the bone mineralization and bone breaking strength in birds (Ledwaba and Roberson, 2003; Han *et al*. 2012; Hosseini *et al*., 2016). Also, beneficial effects of 1, 25 $(OH)_2$ D_3 on the development of bone mineralized zone was reported by Mirakzehi *et al.* (2013). Edwards (1990) suggested that the synthesis of 1, 25 $(OH)_2$ D_3 by the kidney is not sufficient to stimulate maximum Ca absorption and bone formation in fast-growing broiler chickens fed diets with low Ca contents and adequate amounts of cholecalciferol. It is well documented that oestradiol hormones are powerful modulators of Ca metabolism by activating renal enzyme VD3 1α hydroxylase in the kidney (Dick *et al.,* 2005; Bansal *et al.,* 2013). Panwar and Tarafdar (2006) reported that *Withania somnifera* (WS) and *Withania coagulans* (WC) are known as economically important species of *Withania* and are widely cultivated. *Withania somnifera* L. (Solanaceae) is an annual herb and a rich source of bioactive compounds (Khan *et al.,* 2009). *Withania coagulans* L. Dunal is a small evergreen shrub that has been shown to possess varied medicinal properties as a remedy for dyspepsia, flatulent colic and other intestinal diseases (AbouZid *et al.,* 2010). Several pharmacological activities of the plants are attributed to a group of steroidal lactones called withanolides, consisting

of a group of C-22 and C-26 patterns which are oestrogenic compounds (Dewir *et al.,* 2010). Tahmasbi *et al.* (2012) reported that dietary supplementation of root alcoholic extract of WS (65 and 130 mg/kg diet) increased bone mineralization in aged laying hens. Also, previous report by Mirakzehi *et al.* (2013), showed better Ca retention at supplementary levels of WS at 150 mg/kg in birds given negative control diet. They suggested that oestrogen-like withanolides may stimulate the activity of renal 25-hydroxyvitamin D_3 1α-hydroxylase and subsequent elevated production of 1 α, 25-$(OH)_2$ D_3. Because of the involvement of 1, 25-$(OH)_2$ D_3 in bone mineralization and the possible role of withanolides on Ca and P metabolism, it was therefore of interest to evaluate the effects of 1, 25 $(OH)_2$ D_3 and hydroalcoholic extracts of WS root and WC fruit in low and adequate Ca diets on bone mineralization and bone mechanical and histological characteristics of male broiler chickens.

MATERIALS AND METHODS

Preparation of plant extracts: Dried fruits of WC were purchased from the local market and the roots of WS grown in natural habitat were collected during the month of October from Saravan, Sistan and Baluchestan, Iran. The roots and fruits were accurately identified and authenticated at the Herbarium of Botany Directorate in University of Sistan and Baluchestan, Iran. The fruits were coarsely powdered and soaked in 50% ethanol with occasional shaking at room temperature. Roots were thoroughly washed with sterile water, air-dried, and ground into a fine powder form. After 3 days, the ethanol soluble materials were filtered and concentrated using a rotary evaporator (Laborota 4000, Heidolph Germany), and then freeze-dried for 24 h to yield extracts. Dried extracts were stored at -20ºC until used for experimental work.

Birds, diets and experimental design: A total of 550 male one-d-old Ross 308 broilers were obtained from a commercial hatchery and reared in 55 floor pens with wood shavings litter at a stocking rate of 10 birds per pen (1 × 1 m). Feed and water were provided *ad libitum* throughout the 6-week experimental period. The temperature and lighting regime were controlled according to Ross broiler management manual (2009). A completely randomized design was used with eleven dietary treatments replicated in five pens each. The dietary treatments were as follows: basal diets (positive and negative control) and the negative control diet supplemented with either WS or WC extracts at three levels (0, 75 and 150 mg/kg diet) or 1, 25-$(OH)_2$ D_3 at two different levels (0 and 0.5 µg/kg diet). The compound 1, 25-$(OH)_2$ D_3 (Sigma Aldrich, St. Louis, MO, USA) was used in liquid form using corn oil as a carrier (10 µg/ml). Both control diets (Table 1) were based on corn and soybean meal and were formulated to meet the requirements suggested by the Ross 308 broiler nutrient specifications (Ross 2007) for all nutrients except Ca, which was reduced by 30% (obtained by reducing limestone and adding fine sand) in negative control diet. Feed intake and body weights were recorded at 1, 11, 24, and 42 days of age, and body weight gain (BWG) and feed conversion efficiency (kg feed per kg gain, FCE) were calculated. All the research procedures were approved by the Animal Use and Care Committee of the Higher Educational Complex of Saravan.

Experimental procedures: Feed intake (FI) and body weights were recorded at 11, 24 and 42 days of age to calculate body weight gain (BWG) and feed conversion efficiency (FCE). The BWG and FCE were calculated using the formulas shown below:

BWG= finish weight – start weight FCE= feed intake/weight gain

At 15 days of age, birds were exposed to fasting for 16 h, and experimental diets containing 0.3% chromium oxide were assigned to measure mineral retention (ash, Ca and P). After 24 h adaptation period, excreta from each pen were collected for 72 h to determine ash, Ca and P retention. Feed and Excreta samples were oven-dried at 105°C for 24 h. At 21 and 42 days of age, one bird was removed from each pen and blood samples were taken from the brachial vein. The sera of blood samples were separated by centrifugation at 1500 × g for 15 minutes, stored at -20°C until analysis for Ca, P and alkaline phosphatase (ALP). After the birds were slaughtered by cervical dislocation, both tibia were dissected from the carcass and adherent tissues were removed. The left tibia was stored at -20°C until subsequent measurement of mechanical properties and mineral content. The right tibia bones were fixed in 10% phosphate buffer formalin for 12 h at 4℃ and then proximal epiphysis and mid-diaphysis were slit to collect 0.5 cm thickness sections. The both longitudinal epiphyseal and latitudinal diaphyseal sections were decalcified in 10% formic acid embedded in paraffin and cut into 5-µm sections by microtome. The sections were stained with hematoxylin and eosin (H and E). Growth plate histological parameters (widths of the proliferative zone, hyperthrophic zone, mineralized zone) and cortical thickness were measured with an optical microscope (Olympus BX41TF, Tokyo, Japan), photographed with a digital camera system (Olympus DP12 U-TV0.5 XC-2, Japan) and the images were analyzed using Soft Imaging System (Olysia Soft Imaging System, Germany). The 3-point bending test was used to determine the mechanical properties of the left tibial bones. At the time of mechanical testing the frozen left tibia bones were thawed in room temperature for 4 h, oven-dried at 105°C for 24 h and defatted with diethyl ether for 48 h. The dried fat free bone weight, bone diameter at the center of

diaphysis biomechanical properties and bone Ca and P were measured. The mechanical properties of left tibia were determined using Instron Universal Testing Machine (Model H5KS, Tinius Olsen Company). The bones were held in identical positions. The distance between the two supporting ends was 5 cm and the 10 mm diameter crosshead probe (50 kg) approached the bone at 5 mm/min until fracture occurred. During the experiment, care was taken to ensure that the impact of crosshead was at the midpoint. Using the software (Q Mat), mechanical parameters such as ultimate shear force, maximal deflection before fracture, shear fracture energy and stiffness (tangent to the angle α) were calculated. Broken tibia pieces were collected, dried over night at 105 °C in an oven, and ashed at 600 °C for 16 h to determine mineral content.

Chemical analysis: The concentrations of Ca, inorganic P and ALP in serum samples were measured using an automatic blood chemical analyser (Random Access Analyser A15, Biosystem Corp, Spain). Feed, excreta, and bone analyzed for ash, Ca and total P. The concentration of Ca in samples was determined by atomic absorption spectrophotometry (Varian SpectrAA 50B Atomic Absorption Spectrometer: Varian Ltd, USA) according to AOAC (2005) procedures (method 927.02) and total P was measured colorometrically using the molybdovanadate method (AOAC, 2005 method 965.17). The concentration of Chromium in dried feed and excreta samples was measured according to the procedure described by Williams *et al.* (1962) using an atomic absorption spectrophotometry (Varian SpectrAA 50B Atomic Absorption Spectrometer: Varian Ltd, USA).

Statistical analysis: Data were analyzed using the general linear model analysis of variance (ANOVA) (SAS Institute, 2004) in a completely randomized design. Means were compared using Duncan's multiple range test. All differences were considered significant at $P \leq 0.05$. The statistical model of experiment is shown below.

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where:
Y_{ij}: being any observation
μ: is the general mean
T_i: effect of the i-th treatment
e_{ij}: effect of random error

RESULTS

Growth performance: Table 2 presents the effects of dietary treatments on body weight gain (BWG), feed intake (FI), feed conversion efficiency (FCE) at 42 days of age and mineral retention of broilers at 19-21 days of age. Dietary supplementation of WS, WC and 1, 25-$(OH)_2$ D_3 did not alter ($P>0.05$) the growth performance of chickens. Also, ash (%) and P (%) retention were not affected ($P>0.05$) by dietary treatments. Experimental treatments significantly influenced Ca retention. Among the birds that were given negative control diet, the highest Ca retention values were noted in those that received 75 mg/kg WC (84.67%) or 150 mg/kg WS (83.09%) with 0.5 µg/kg 1, 25-$(OH)_2$ D_3. Birds fed on these diets had significantly ($P<0.05$) higher dietary Ca retention values compared with those receiving a positive control diet with no dietary supplementation of WS, WC and 1, 25-$(OH)_2$ D_3. Feeding broilers with WS, WC and 1, 25-$(OH)_2$ D_3 did not significantly affect growth performance including BWG, FI and FCE.

Bone physical characteristics and mineralization: The dietary treatments had no significant impact on tibia physical characteristics (weight, length and diameter) at 21 and 42 days of age ($P>0.05$) (Table 3 and Table 4). Except for tibia bone Ca (21 day), birds given experimental treatments had similar tibia ash (%), Ca (%) and P (%) ($P>0.05$). At 21 days of age, the highest tibia Ca values were obtained in birds fed negative control diet supplemented with 150 mg/kg WS or WC with no dietary supplementation of 1, 25-$(OH)_2$ D_3 (38.652 and 38.433 % respectively). In these birds tibia Ca was significantly higher compared to that in birds fed on positive or negative control basal diets with no dietary supplement (33.876 and 33.175 % respectively) ($P<0.05$).

Bone mechanical properties: The effects of dietary treatments on bone mechanical properties at 21 and 42 days of age are given in Table 5. Overall, there were no significant differences ($P>0.05$) between experimental treatments for all tibia bone biomechanical properties. Feeding diets containing low dietary Ca level did not show any significant effect on bone biomechanical strength.

Bone histology: The tibia bone histological measurements at 21 and 42 days of age are presented in Table 6. Tibia bone histological measurements were not significantly affected by inclusion of plant extract or 1, 25-$(OH)_2$ D_3, except for mineralized zone width at 42 days of age. The addition of 75 mg/kg WS and 1, 25-$(OH)_2$ D_3 to negative control diets resulted in significant increase ($P<0.05$) in tibia mineralized zone width compared with negative control with no dietary supplement or diet that supplemented with only 150 mg/kg WC (2377 vs. 1775 and 1595 µm, respectively). Also, results showed that birds fed 0.5 µg/kg 1, 25-$(OH)_2$ D_3 in negative control diets with no dietary plant extract significantly had higher bone mineralized zone width compared to those given negative control diet with no dietary supplement (2228 vs. 1775 µm, respectively). The birds given 75 mg/kg WS alone had significantly lower mineralized zone width compared to those offered similar diet with 0.5 µg/kg 1, 25-$(OH)_2$ D_3 (2377 vs. 1901 µm, respectively).

Serum Ca, P and ALP: The effects of dietary treatments on serum Ca and P concentration and ALP activity are shown in Table 7. Dietary supplementation with WS and WC extracts or 1, 25-$(OH)_2$ D_3 did not have any significant effects on blood characteristics.

Table 1. Composition of the basal diets (g/kg).

Ingredient	Starter (1-10d)		Grower (11-23d)		Finisher (24-42d)	
	-	+	-	+	-	+
Corn	520	520	532.0	532.0	530.0	530.0
Soybean meal	350	350	370.0	370.0	369.0	369.0
Gluten	50	50	-	-	-	-
Vegetable oil	32.7	32.7	58.0	58.0	65.6	65.6
Limestone	5.3	13.1	4.0	10.7	3.7	10.3
Dicalcium phosphate	17.5	17.5	15.5	15.5	14.0	14.0
Salt	3.5	3.5	4.7	4.7	4.1	4.1
Methionin	3.2	3.2	2.8	2.8	2.0	2.0
Lysine	4.0	4.0	1.3	1.3	-	-
Threonine	1.0	1.0	-	-	-	-
Vitamin premix[1]	2.5	2.5	2.5	2.5	2.5	2.5
Mineral premix[2]	2.5	2.5	2.5	2.5	2.5	2.5
Sand	7.8	-	6.7	-	6.6	-
Calculated nutrients and energy						
AME, (MJ/kg)	12.6	12.6	13.1	13.1	13.3	13.3
Crude protein (g/kg)	235.2	235.2	211.5	211.5	209.1	209.1
Lysine (g/kg)	14.4	14.4	12.4	12.4	11.3	11.3
Methionine (g/kg)	7.0	7.0	6.1	6.1	5.2	5.2
TSAA (g/kg)	10.7	10.7	9.5	9.5	8.6	8.6
Calcium (g/kg)	7.3	10.4	6.3	9.0	5.9	8.5
Nonphytate P (g/kg)	5.0	5.0	4.5	4.5	4.2	4.2
Total P (g/kg)	7.0	7.0	6.8	6.8	6.5	6.5
Analyzed Ca and total P concentration						
Calcium (g/kg)	7.5	10.7	6.5	9.3	6.1	8.8
Total P (g/kg)	7.4	7.4	7.1	7.1	6.7	6.7

[1]Vitamin premix provided per kilogram of diet: retinyl acetate, 11,000 IU; cholecalciferol, 1,800 IU; DL-α-tocopheryl acetate, 11 mg; menadione sodium bisulphate, 2 mg; riboflavin, 5.7 mg; pyridoxine hydrochloride, 2 mg; cyanocobalamin, 0.024 mg; nicotinic acid, 28 mg; folic acid, 0.5 mg; pantothenic acid, 12 mg; choline chloride, 250 mg.
[2]Mineral premix provided per kilogram of diet: Mn, 100 mg; Zn, 65 mg; Cu, 5 mg; Se, 0.22 mg; I, 0.5 mg; and Co, 0.5 mg.

Table 2. Effect of *Withania somnifera (WS)*, *Withania coagulans (WC)* and 1, 25 $(OH)_2$ D_3 in negative control diets on body weight gain (BWG), feed intake (FI),feed conversion efficiency(FCE) at 42 day of age and mineral retention of broilers at 19-21 days of age.

Treatment			BWG (g)	FI (g)	FCE (g/g)	Mineral retention		
Control	Plant extract(mg/kg)	1, 25 $(OH)_2$ D_3(μg/kg)				Ash (%)	Ca (%)	P (%)
+	0	0	2339	3927	1.679	58.332	69.308^{c}	72.400
-	0	0	2235	3883	1.741	64.948	82.450^{a}	79.380
-	0	0.5	2219	3868	1.742	63.102	81.799^{a}	77.333
-	75 WS	0	2368	3880	1.639	63.581	73.362^{bc}	74.223
-	75 WS	0.5	2396	3939	1.648	57.240	79.500^{ab}	75.907
-	150 WS	0	2299	3888	1.693	60.977	81.993^{a}	77.478
-	150 WS	0.5	2441	4006	1.642	60.664	83.098^{a}	76.138
-	75 WC	0	2357	4019	1.706	64.404	82.645^{a}	78.160
-	75 WC	0.5	2291	3812	1.666	57.900	84.676^{a}	76.175
-	150 WC	0	2284	3828	1.676	62.467	81.394^{a}	76.877
-	150 WC	0.5	2428	3923	1.614	63.031	82.307^{a}	77.656

SEM	56.273	90.822	0.037	2.220	1.823	1.718
P-value	0.108	0.869	0.298	0.182	0.0001	0.333

Table 3. Effect of *Withania somnifera (WS)*, *Withania coagulans (WC)* and 1, 25 $(OH)_2$ D_3 in negative control diets on bone characteristics of broilers at 21 day of age.

Treatment			Tibia weight (g)	Tibia length (mm)	Tibia diameter (mm)	Tibia ash (%)	Tibia Ca (%)	Tibia P (%)
Control	Plant extract(mg/kg)	1, 25 $(OH)_2$ D_3(µg/kg)						
+	0	0	1.592	64.990	5.302	51.580	33.876[b]	24.222
-	0	0	1.666	65.978	5.348	50.803	33.175[b]	24.348
-	0	0.5	1.663	65.878	5.148	51.709	35.335[ab]	24.162
-	75 WS	0	1.845	66.302	5.752	51.400	36.086[ab]	24.596
-	75 WS	0.5	1.589	66.248	5.286	52.119	37.745[a]	24.287
-	150 WS	0	1.733	67.824	5.564	52.825	38.652[a]	24.332
-	150 WS	0.5	1.647	66.066	5.048	52.620	35.804[ab]	24.126
-	75 WC	0	1.533	64.770	5.190	52.669	34.978[ab]	24.089
-	75 WC	0.5	1.637	66.296	4.982	51.507	36.681[ab]	24.196
-	150 WC	0	1.486	64.390	5.204	52.058	38.433[a]	23.983
-	150 WC	0.5	1.748	65.792	5.360	52.142	36.605[ab]	24.360
SEM			0.106	0.848	0.185	1.071	0.996	0.171
P-value			0.532	0.324	0.204	0.905	0.024	0.600

Table 4. Effect of *Withania somnifera (WS)*, *Withania coagulans (WC)* and 1, 25 $(OH)_2$ D_3 in negative control diets on bone characteristics of broilers at 42 day of age.

Treatment			Tibia weight (g)	Tibia length (mm)	Tibia diameter (mm)	Tibia ash (%)	Tibia Ca (%)	Tibia P (%)
Control	Plant extract(mg/kg)	1, 25 $(OH)_2$ D_3(µg/kg)						
+	0	0	5.487	95.882	7.930	48.509	38.108	24.544
-	0	0	5.597	96.220	7.958	49.693	38.402	24.344
-	0	0.5	5.300	97.670	7.564	49.725	38.621	24.427
-	75 WS	0	5.696	96.340	7.816	48.345	38.008	23.878
-	75 WS	0.5	5.966	97.134	7.926	49.333	39.028	24.377
-	150 WS	0	5.817	98.658	7.742	49.999	38.759	24.427
-	150 WS	0.5	5.497	98.478	7.540	47.832	38.583	24.111
-	75 WC	0	5.484	95.582	7.594	47.762	38.033	24.261
-	75 WC	0.5	5.616	97.166	7.866	49.477	38.659	24.227
-	150 WC	0	5.471	90.654	7.530	49.296	38.433	24.011
-	150 WC	0.5	5.162	95.210	7.776	49.316	37.532	24.610
SEM			0.248	2.337	0.235	0.754	0.432	0.204
P-value			0.619	0.652	0.784	0.416	0.164	0.318

Table 5. Effect of *Withania somnifera (WS)*, *Withania coagulans (WC)* and 1, 25 $(OH)_2$ D_3 in negative control diets on bone mechanical properties of broilers at 21 and 42 days of age.

Treatment			21 day				42 day			
Control	Plant extract(mg/kg)	1, 25 $(OH)_2$ D_3(µg/kg)	SF[1] (N)	FD[2] (mm)	FE[3] (N-mm)	Stiffness (N/mm)	SF (N)	FD (mm)	FE (N-mm)	Stiffness (N/mm)
+	0	0	108.78	0.600	0.031	187.54	172.60	0.724	0.056	280.77
-	0	0	104.40	0.595	0.031	178.04	188.06	0.631	0.059	304.98
-	0	0.5	95.68	0.567	0.029	191.48	184.37	0.728	0.070	276.52
-	75 WS	0	129.02	0.579	0.037	224.18	219.82	0.625	0.042	322.58
-	75 WS	0.5	109.12	0.595	0.034	186.54	211.66	0.597	0.066	359.34

-	150 WS	0	89.48	0.429	0.024	185.32	204.80	0.642	0.066	323.80
-	150 WS	0.5	100.52	0.528	0.026	193.04	202.44	0.702	0.075	287.58
-	75 WC	0	92.00	0.525	0.026	174.86	208.70	0.704	0.065	296.98
-	75 WC	0.5	91.76	0.497	0.023	184.84	199.98	0.707	0.075	286.60
-	150 WC	0	99.32	0.594	0.029	169.64	173.85	0.564	0.051	314.42
-	150 WC	0.5	112.18	0.713	0.038	166.26	180.87	0.605	0.058	290.25
SEM			12.079	0.059	0.006	16.905	18.901	0.046	0.009	21.860
P-value			0.508	0.306	0.839	0.598	0.761	0.211	0.371	0.296

Table 6. Effect of *Withania somnifera (WS)*, *Withania coagulans (WC)* and 1, 25 $(OH)_2$ D_3 in negative control diets on bone histological measurements of broilers at 21 and 42 days of age.

Treatment			21 day				42 day			
Control	Plant extract(mg/kg)	1, 25 $(OH)_2$ D_3(µg/kg)	PZ[1] (µm)	HZ[2] (µm)	MZ[3] (µm)	CT[4] (µm)	PZ (µm)	HZ (µm)	MZ (µm)	CT (µm)
+	0	0	921	324.45	2375	1532	938	272	2029^{abc}	1560
-	0	0	846	306.76	1762	1286	990	265	1775^{cd}	1657
-	0	0.5	711	309.05	1981	1334	1033	251	2228^{ab}	1416
-	75 WS	0	753	279.23	2073	1372	991	288	1901^{bcd}	1620
-	75 WS	0.5	785	272.44	1926	1266	1020	263	2377^{a}	1569
-	150 WS	0	890	286.15	2086	1292	961	304	2046^{abc}	1517
-	150 WS	0.5	713	297.18	2037	1187	1091	303	1891^{bcd}	1589
-	75 WC	0	725	279.31	1880	1323	1011	276	1762^{cd}	1703
-	75 WC	0.5	740	320.87	2094	1190	947	289	2032^{abc}	1657
-	150 WC	0	799	344.91	2031	1329	1007	257	1595^{d}	1515
-	150 WC	0.5	775	272.72	2226	1542	891	261	1963^{abcd}	1632
SEM			47.41	31.64	110.60	89.92	61.73	19.60	130.35	86.73
P-value			0.083	0.591	0.072	0.197	0.671	0.600	0.009	0.568

[a, d] Means within each column with no common superscript differ significantly (P<0.05).
[1]PZ= Proliferative zone
[2]HZ= Hypertrophic zone
[3]MZ= Mineralized zone
[4]CT=Cortical thickness

Table 7. Effect of *Withania somnifera (WS)*, *Withania coagulans (WC)* and 1, 25 $(OH)_2$ D_3 in negative control diets on blood characteristics of broilers at 21 and 42 days of age.

Treatment			21 day			42 day		
Control	Plant extract(mg/kg)	1, 25 $(OH)_2$ D_3(µg/kg)	Ca (mg/dL)	P (mg/dL)	ALP (U/L)	Ca (mg/dL)	P (mg/dL)	ALP (U/L)
+	0	0	9.74	5.95	8627	9.28	5.75	2686
-	0	0	8.58	6.58	12049	8.36	6.80	3047
-	0	0.5	9.92	5.78	12879	8.48	6.60	2298
-	75 WS	0	8.52	6.26	13996	9.73	5.25	2329
-	75 WS	0.5	8.84	6.62	9673	10.15	7.00	1906
-	150 WS	0	9.15	5.66	14231	10.17	7.20	2729
-	150 WS	0.5	8.48	6.78	14312	9.64	6.50	2210
-	75 WC	0	8.79	6.21	13481	8.19	5.00	2476
-	75 WC	0.5	9.61	5.94	13726	9.96	5.75	2929
-	150 WC	0	8.19	5.60	12037	9.56	5.66	2260
-	150 WC	0.5	9.89	7.04	10199	10.33	7.60	2045
SEM			0.594	0.657	1861.09	0.838	0.682	259.65
P-value			0.537	0.788	0.474	0.607	0.164	0.291

DISCUSSION

The results of growth performance were consistent with the findings of Mirakzehi *et al.* (2013) and Tahmasbi *et al.* (2012) with broiler and laying hens experiments, respectively. The results of our work showed that dietary supplementation of WS and WC alone or with 1, 25-$(OH)_2$ D_3 in negative control diets were not effective on improving broiler growth performance under normal dietary Ca level or when dietary Ca decreased to 70% of the recommended concentration. The observed increase of Ca retention in birds given 150 mg/kg WS or 75 mg/kg WC in the current study was consistent with the results of Mirakzehi *et al.* (2013) who reported that administration of 150 mg/kg WS significantly improved dietary Ca retention in birds receiving a negative control compared to those that consumed a positive control diet. The results of the current experiment are in accordance with the report of Hosseini *et al.* (2016) who showed that supplementation of fruit hydroalcoholic extract of WC improved dietary Ca retention in birds receiving a negative control compared to those that consumed a positive control diet. The reports reveal that the whole WC extract contains various chemical components such as steroidal lactones (withanolides), alkaloids, tannins, and flavonoids (Shabbir *et al.* 1999). Liel *et al.* (1999) reported that the beneficial effects of phytosterols could be mediated through oestrogenic nature by simultaneous increase of vitamin D receptor expression and specific 1, 25 $(OH)_2$ D-related activities in the intestinal mucosa leading to stimulation of Ca absorption. Furthermore, the observed results are in agreement with hypothesis that estradiol acts on the kidney to increase renal tubular reabsorption of calcium, extending previous concepts of the importance of estrogen effects on calcium metabolism (Devine *et al.* 2005). Devine *et al.* (2005) reported that association between free estradiol concentration and renal calcium handling is an important factor in the mobilization of Ca. Similar effects of WS on bone calcification were found by others (Nagareddy and Lakshmana, 2006; Tahmasbi *et al.* 2012; Mirakzehi *et al.* 2013). They hypothesized that the improvements in bone calcification were associated with the presence of a large number of withanolides, oestrogen-like compounds. Rege *et al.* (1989) reported that the effects depend to a great extent on the chemistry of the phytoestrogenic compounds. The presence of a diphenolic ring in chemical structure of these compounds makes them act in a similar manner to endogenous oestrogens, oestradiol and, diethylstilbestrol (Rege *et al.* 1989). Oestrogen is known to increase 1, 25-$(OH)_2$ D_3, which in turn causes stimulation of Ca absorption from the intestine (Mishra *et al.* 2000). The results from this experiment combined with earlier studies (Nagareddy and Lakshmana, 2006; Tahmasbi *et al.* 2012; Mirakzehi *et al.* 2013) indicate that the extracts prevented bone loss and improved bone calcification in conditions of severe Ca deficiency.

Non significant effects of negative control diets on biomechanical strength of bone suggested that the lowest amount of Ca fed in this experiment (70% of Ca requirement) was not low enough to cause negative effect on bone biomechanical properties. Sharp *et al.* (2000) reported that bone mechanical strength depends on the amount of bone (density and mineralization) and its architectural spatial organization. Bonser and Casinos (2003) reported that the usefulness of ash content would be a sufficient predictor of differences in mechanical competence between-bone comparisons. Our results showed that Ca deficiency did not result to decreased tibia ash (%) at 21 and 42 days of age. These results are reflected on bone biomechanical properties. Treatment with WS and WC only improved tibia bone Ca at 21 days of age. In spite of significant effects of dietary plant extracts on tibia bone Ca, no significant effects were found on bone biomechanical properties.

Results of bone histology suggested that synergistic effects of 1, 25-$(OH)_2$ D_3 and WS can beneficially influence mineralized zone width. This was in accordance with Mirakzehi *et al.* (2013), who reported that supplementation of 1, 25 $(OH)_2$ D_3 in negative control diets containing 75 mg/kg WS significantly increased mineralized zone width compare to those fed higher level of WS with 1, 25 $(OH)_2$ D_3 or fed no dietary supplementation of 1, 25 $(OH)_2$ D_3. The results of this study are consistent with a report by Hosseini *et al.* (2016) who found that dietary supplementation with 1, 25-$(OH)_2$ D_3 improved mineralized zone width. It has been shown that 1, 25 $(OH)_2$ D_3 modulate C-myc proteins. C-myc protein is a potent inducer of apoptosis, and increases matrix vesicle synthesis which mediates mineralization of the growth plate matrix (Farquharson and Jefferies, 2000). Also, Nagareddy and Lakshmana (2006) hypothesized that WS exerts its beneficial effects through endocrine system and may increase bone formation and reduce resorption of bone minerals into the systemic circulation and consequent excretion.

These data of blood characteristics are in agreement with the findings of Roberson and Edwards (1994) and Edwards (1993). They reported no differences in serum Ca concentration of broilers fed 5 or 10 μg/kg 1, 25-$(OH)_2$ D_3. The present results agree with the results of Mirakzehi *et al.* (2013) and Hosseini *et al.* (2016), who observed no significant effects of dietary plant extracts of WS and WC on blood characteristics of broilers at 21 and 42 days of age.

Conclusions: In conclusion, the results of our experiment show that dietary supplementation of WS and WC at 150 and 75 mg/kg, respectively improved Ca retention and the effects were more pronounced in the presence of 1, 25 $(OH)_2$ D_3 in broiler chickens. It is observed that

administration of 150 mg/kg WS and WC had beneficial effects on bone calcification at 21 days of age. Also, synergistic effects of WS and 1, 25-$(OH)_2$ D_3 resulted in significant increase in tibia mineralized zone width. Further studies are required to evaluate the exact mechanism of action of active constituents of WS and WC extracts.

REFERENCES

Abouzid, S. F., A. A. El-Bassuony, A. Nasib, S. Khan, J. Qureshi and M. I. Choudhary. (2010). Withaferin A production by root cultures of *Withania coagulans*. Int. J. Appl. Res. Nat. Prod. 3 (1): 23-27.

AOAC International. (2005). Official Methods of Analysis of AOAC International, 18th ed (Gaithersburg, MD, AOAC Int.).

Bansal, N., R. Katz, I. H. de Boer, B. Kestenbaum, D. S. Siscovick, A. N. Hoofnagle, R. Tracy, G. A. Laughlin, M. H. Criqui, M. J. Budoff, D. Li and J. H. Ix. (2013). Influence of estrogen therapy on calcium, phosphorus, and other regulatory hormones in postmenopausal women: the MESA study. J. Clin. Endocrinol. Metab. 98 (12): 4890-4898.

Bonser, R. H. C., and A. Casinos. (2003). Regional variation in cortical bone properties from broiler fowl--a first look. Br. Poult. Sci. 44 (3): 350-354.

Devine, A., I. M. Dick, S. S. Dhaliwal, R. Naheed, J. Beilby and R. L. Prince. (2005). Prediction of incident osteoporotic fractures in elderly women using the free estradiol index. Osteoporos. Int. 16(2): 216-221.

Dewir, Y. H., D. Chakrabarty, S-H. Lee, E-J. Hahn and K-Y. Paek. (2010). Indirect regeneration of *Withania somnifera* and comparative analysis of withanolides in in vitro and greenhouse grown plants. Biol. Plantarum. 54 (2): 357-360.

Dick, I.M., A. Devine, J. Beilby and R. L. Prince. (2005). Effects of endogenous estrogen on renal calcium and phosphate handling in elderly women. Am. J. Physiol. Endocrinol. Metab. 288 (2): E430-E435.

Edwards, HM. JR. (1990). Efficacy of several vitamin D compounds in the prevention of tibial dyschondroplasia in broiler chickens. J. Nutr. 120 (9): 1054-1061.

Edwards, HM. JR. (1993). Dietary 1, 25-Dihydroxycholecalciferol Supplementation Increases Natural Phytate Phosphorus Utilization in Chickens. J. Nutr. 123 (3): 567-577.

Farquharson, C., and D. Jefferies. (2000). Chondrocytes and longitudinal bone growth: the development of tibial dyschondroplasia. Poult. Sci. 79 (7): 994-1004.

Han, J. C., Y. L. Wang, H. X. Qu, F. Liang, J. L. Zhang, C. X. Shi, X. L. Zhang, L. Li, Q. Xie, C. L. Wang and Y. Y. Yan. (2012). "One alpha-hydroxycholecalciferol improves growth performance, tibia quality, and meat color of broilers fed calcium-and phosphorus-deficient diets." Asian-Australas. J. Anim. Sci. 25 (2): 267-271.

Hosseini, S. J., H. Kermanshahi, H. Nassirimoghaddam, A. Nabipour, M. T. Mirakzeh, H. Saleh and M. Kazemifard. (2016). Effects of 1.25-Dihydroxycholecalciferol and Hydroalcoholic Extract of *Withania Coagulans* Fruit on Bone Mineralization and Mechanical and Histological Properties of Male Broiler Chickens. Braz. J. Poultry Sci. 18 (1): 73-86.

Khan, S., F. Malik, K. A. Suri and J. Singh. (2009). Molecular insight into the immune up-regulatory properties of the leaf extract of Ashwagandha and identification of the Th1 immunostimulatory chemical entity. Vaccine. 27 (43): 6080-6087.

Ledwaba, M. F., and K. D. Roberson, 2003. Effectiveness of twenty-five-hydroxycholecalciferol in the prevention of tibial dyschondroplasia in Ross cockerels depends on dietary calcium level. Poult. Sci. 82 (11): 1769-1777.

Liel, Y., S. Shraga, P. Smirnoff and B. Schwartz. (1999). Estrogen increases 1, 25-dihydroxyvitamin D receptors expression and bioresponse in the rat duodenal mucosa. Endocrinology. 140 (1): 280-285.

Mirakzehi, M. T., H. Kermanshahi, A. Golian and A. R. Raji. (2013). The effects of dietary 1, 25-dihydroxycholecalciferol and hydroalcoholic extract of *Withania somnifera* root on bone mineralisation, strength and histological characteristics in broiler chickens. Br. Poult. Sci. 54 (6): 789-800.

Mishra, LC., BB. Singh and S. Dagenais. (2000). Scientific basis for the therapeutic use of *Withania somnifera* (ashwagandha): a review. Altern. Med. Rev. 5 (4): 334-346.

Nagareddy, PR., and M. Lakshmana. (2006). *Withania somnifera* improves bone calcification in calcium-deficient ovariectomized rats. J. Pharm. Pharmacol. 58 (4): 513-519.

Norman, AW. (2008). From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. Am. J. Clin. Nutr. 88 (2): 491S-499S.

Panwar, J., and J. C. Tarafdar. (2006). Distribution of three endangered medicinal plant species and their colonization with arbuscular mycorrhizal fungi. J. Arid. Environ. 65 (3): 337-350

Rege, N. N., H. M. Nazareth, A. A. Isaac, S. M. Karandikar and S. A. Dahanukar. (1989). Immunotherapeutic modulation of intraperitoneal adhesions by Asparagus racemosus. J. Postgrad. Med. 35 (4): 199-203.

Roberson, K. D., and H. M. Edwards. (1994). Effects of ascorbic acid and 1, 25-dihydroxycholecalciferol on alkaline phosphatase and tibial dyschondroplasia in broiler chickens. Br. Poult. Sci. 35 (5): 763-773.

Ross. (2007). Ross 308 Broiler: Nutrition Specification (Aviagen, Scotland, UK). Available: http://www.aviagen.com/. Accessed 25 May 2009.

Ross. (2009). Broiler Management Manual, Aviagen Incorporated Bradford Drive, Huntsville, AL, USA

SAS. (2003). SAS 9.1 (Cary, NC, SAS Institute Inc.).

Shabbir, M., M. Yousaf, S. Qureshi, A. Naz and M. I. Choudhary. (1999). Three withanolides from *Withania coagulans*. Phytochemistry. 52 (7): 1361-1364.

Sharp, J. C., J. C. Copps, Q. Liu, L. N. Ryner, R. A. Sebastian, G. Q. Zeng, S. Smith, J. O. Niere, B. Tomanek and M. Sato. (2000). Analysis of Ovariectomy and Estrogen Effects on Body Composition in Rats by X-Ray and Magnetic Resonance Imaging Techniques. J. Bone Miner. Res. 15 (1): 138-146.

Tahmasbi, A. M., M. T. Mirakzehi, S. J. Hosseini, M. J. Agah and M. Kazemi Fard. (2012). The effects of phytase and root hydroalcoholic extract of *Withania somnifera* on productive performance and bone mineralisation of laying hens in the late phase of production. Br. Poult. Sci. 53 (2): 204-214.

Williams, C. H., D. J. David and O. Iismaa. (1962). The determination of chromic oxide in faeces samples by atomic absorption spectrophotometry. J. Agric. Sci. 59 (3): 381-385.