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The effects of Hydroalcoholic Extract of *Withania Somnifera* Leaf and Fish Oil on Growth Performance, Bone Calcification, Morphological and Mechanical Characteristics and Gene Expression in Broiler Chickens

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Abstract

This experiment was conducted to study the effects of hydroalcoholic extract from *Withania somnifera* (*WS*) leaf and fish oil on performance, mineral retention, bone morphological and mechanical characteristics, and Calbindin-D 28K (CALB1) gene expression in broiler chickens. Treatments were arranged in a CRD with a 2 × 3 × 2 factorial arrangement consisted of two dietary Ca levels (low: 30% less than normal Ca level, and adequate: normal Ca level), three concentrations of *WS* (0, 100 and 200 mg/kg diet) and two concentrations of fish oil (0 and 2 %). A total of 600 one-day-old Ross 308 male broilers were divided into 12 treatments with 5 replicates and 10 chickens in each. At 24 d of age, one bird per replicate was randomly killed, and tibiae were removed. Results showed that dietary supplementation of *WS* significantly improved feed conversion ratio (FCR) ($P < 0.05$). Birds fed diets supplemented with *WS* had significantly higher Ca content in the tibia ($P < 0.05$). Dietary inclusion of fish oil significantly increased the width of the bone proliferative zone ($P < 0.05$). In biomechanical properties, dietary supplementation of *WS* and fish oil significantly increased the shear force ($P < 0.05$). Synergistic effects of *WS* and fish oil showed that the addition of *WS* at 200 mg/kg in birds fed diets containing fish oil led to a significant increase in tibial stiffness ($P < 0.05$). Low-Ca diet up-regulated duodenal CALB1 mRNA expression ($P < 0.05$). Supplementation of *WS* also resulted in a significant up-regulation in the gene expression of CALB1in both duodenum and jejunum ($P < 0.001$). In conclusion, dietary supplementation of *WS* may have beneficial effects on bone calcification and strength by increasing the CALB1 gene expression and Ca retention. Also, synergistic effects of *WS* and fish oil may improve the mechanical properties of the tibia.

Introduction

Over recent years, significant advances have been made in genetic selection to improve growth performance in broilers without simultaneous selection for the skeletal system that keeps up with increased body mass (Dibner *et al.*, 2007). Leg disorders, especially deformity and lameness, are the most important factors affecting the health and welfare status of broiler chickens nowadays (Güz *et al.*, 2019). Bone development is affected by several factors including genetics, management, growth rate, nutrition, and locomotion (González-Cerón *et al.*, 2015; Reiter and Bessei, 2009; Saunders-Blades *et al.*, 2009). Bone strength depends on the organic and inorganic composition of the bone tissue. Traditionally, vitamins D_3, Ca, and P have often been used in the diet to improve the mineral matrix, but higher-than-normal Ca intake does not stimulate the ossification response (Watkins *et al.*, 1997). Ca deficiency is usually rare in the commercial poultry

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industry. However, there may be malabsorption problems that can impair intestinal Ca absorption (Zanu *et al.,* 2020). Ca deficiency can lead to osteoporosis, and its homeostatic imbalance impairs the mineralized bone mass and leads to increased bone fragility and susceptibility to fracture (Whitehead, 2004).

Medicinal plants produce a vast variety of natural compounds, which have been used as therapeutic drugs since ancient times and are the natural sources of most new drugs (Mushtaq *et al.,* 2018). *Withania somnifera* L., *(WS) (*family Solanaceae) is an annual plant known as winter cherry that is widely used to treat various diseases such as tumors, rheumatism, asthma, diabetes, ulcers, arthritis, and vitiligo (Ariel and Serhan, 2007). Previous studies have revealed the beneficial effects of *WS* root and leaf extract on bone mineralization, chondroprotective, and osteoclastogenesis inhibition (Ichikawa *et al.,* 2006; Mirakzehi *et al.,* 2013; Nagareddy and Lakshmana, 2006; Rasool and Varalakshmi, 2006). The plant extract contains numerous active compounds, namely, withanolides, alkaloids, reducing sugars, and a few flavonoids (Mishra *et al.,* 2000). The estrogenic nature of withanolides, similar to phytosterols, may contribute to the anabolic response by promoting bone mineralization, osteoblast cell proliferation, and differentiation (Setchell and Lydeking-Olsen, 2003).

Dietary lipids play a significant role in the growth, development, and modeling of long bones. Fish oil is a good source of n-6 polyunsaturated fatty acids (PUFA), which differently affect bone mineralization and osteoblast function than the common fat sources of broiler diets containing ω-6 PUFA, such as maize oil, palm oil, and soybean oil (Watkins *et al.,* 2003). It was suggested that ω-3 PUFA is involved in maintaining bone health (Lau *et al.,* 2009). In this regard, several mechanisms have been discovered to mediate the effects of dietary fats on bone, including changes in Ca absorption and Ca excretion, osteoblast formation, prostaglandin synthesis, and lipid oxidation (Salari *et al.,* 2008). In addition, ω-3 PUFA exerts inhibitory effects on bone resorption and stimulatory effects on mineral deposition and bone formation through synergy with estrogen compounds. It was found that ω-3 fatty acids may be involved in the up-regulation of osteoblastogenesis or a down-regulation of osteoclastogenesis (Watkins *et al.,* 2001). Dietary PUFAs may also modulate bone strength. Liu *et al.,* (2003b) showed that feeding diets containing fish oil compared with chicken fat and soybean oil increased bone shear stress and shear force in quails . In general, various mechanisms such as intestinal absorption, storage and exchange with bones, and renal reabsorption or excretion lead to calcium and phosphorus homeostasis (Alexander *et al*., 2014). Absorption of dietary Ca can occur via active, transcellular, or passive paracellular transport mechanisms (Dimke *et al.,* 2011). Calbindin-D $_{28K}$ (CALB1), vitamin D_3-induced Ca-binding protein, is the major player involved in transcellular Ca transport in the intestine (Gloux *et al.,* 2019). It has been reported that the mRNA expression of calbindin-D $_{9K}$ increases in response to a low-Ca^{2+} diet in mice models (Woudenberg-Vrenken *et al.,* 2012). Also, it has been previously reported that estrogenic compounds increase the mRNA expression of uterine calbindin-D $_{9K}$ in rats (An *et al.,* 2002). Therefore, the objective of the present study was to investigate the efficacy of fish oil and the potential effects of *WS* and their interactions using either adequate or low-Ca diets on growth performance, bone calcification, morphological and mechanical traits, and mRNA expression of CALB1 in broiler chickens at 24 days of age.

Materials and Methods

Extraction of *WS* leaf

For extraction, fresh two-year-old *WS* plants were collected from an agricultural farm in Saravan, Baluchestan in September. The collected plants were identified and authenticated by the herbarium of the Department of plant production, Faculty of Agriculture of Saravan Higher Education Complex. The leaves were slowly separated from the plant shade dried, and ground into a powder. They were then mixed with 50% ethanol at room temperature for 72 hours and shaken frequently. Finally, the liquid-soluble material was separated from the remaining solids by vacuum filtration and concentrated by a rotary evaporator (Laborota 4000, Heidolph, Germany). The concentrated extract was then freeze-dried for 24 h. and stored at -20°C until further use (Tahmasbi *et al.,* 2012).

Quantitative Phytochemical Analysis

Total phenol content in *WS* hydroalcoholic extract was measured by spectrophotometry (Barreira *et al.,* 2008) and the results were expressed as micrograms of gallic acid equivalent (GAEs) per mg of extract. Total flavonoid content was determined by spectrophotometry (Kathirvel and Sujatha, 2016) and the results were expressed in micrograms equivalent to catechins (CEs) per mg of extract. The total amount of tannin was determined by the Folin-Dennis method (Oyaizu, 1986) and the results were expressed in micrograms equivalent of tannic acid per mg of extract (Table 1).

Birds, husbandry, and dietary treatments

A total of 600 one-day-old Ross 308 commercial male broiler chickens were purchased from a commercial hatchery. The birds were randomly allocated to 60-floor pens (10 birds per pen, 1 × 1 m^2). During the 24-day trial period, feed and water were provided *ad libitum*. The standard temperature, lighting regime, and ventilation were controlled in accordance with the operating procedures of Ross broiler. The trial was conducted as a completely randomized design in a 2 × 3 × 2 factorial arrangement comprising low and adequate concentrations of dietary Ca, three concentrations of *WS* leaf extract (0, 100, and 200 mg/kg diet), and two concentrations of fish oil (0 and 2 %). The corn-soybean meal basal diets were formulated to meet the nutrient requirement recommendation manual for breeding the Ross 308 broiler except for dietary Ca level (Aviagen, 2007). The experimental diets were isocaloric and isonitrogenous in each growing phase. The low Ca diet was formulated similar to the adequate Ca diet with 30% less Ca (Table 2). This experiment was approved by the Animal Use and Care Committee of Saravan Higher Education Complex.

Table 1. Total phenolic, flavonoids, and tannin contents of *WS* hydroalcoholic extract

Phytochemical constituents	Content (µg/mg)
Total phenolic	11.95
Total flavonoid	39.98
Total tannins	154.77

Table 2. Composition of the basal diets (g/kg as fed)

Ingredient (g/kg)	Starter (1-10d)				Grower (11-23d)			
	Low Ca		Adequate Ca		Low Ca		Adequate Ca	
	Fish oil (0%)	Fish oil (2%)	Fish oil (0%)	Fish oil (2%)	Fish oil (0%)	Fish oil (2%)	Fish oil (0%)	Fish oil (2%)
Corn	536.9	536.9	536.9	536.9	560	560	560	560
Soybean meal (44%)	352.1	352.1	352.1	352.1	330	330	330	330
Corn gluten (60%)	40	40	40	40	29.2	29.2	29.2	29.2
Soybean oil	30	10	30	10	44	24	44	24
Fish oil	-	20	-	20	-	20	-	20
Dicalcium phosphate	14	14	14	14	12	12	12	12
Limestone	5.9	5.9	13	13	4.8	4.8	12	12
Salt	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
DL-Methionine	1.8	1.8	1.8	1.8	1.6	1.6	1.6	1.6
L-Lysine	3.4	3.4	3.4	3.4	2.9	2.9	2.9	2.9
Vitamin premix[1]	2.5	2.5	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	2.5	2.5
L-Threonine	0.8	0.8	0.8	0.8	0.3	0.3	0.3	0.3
Sand	7.1	7.1	-	-	7.2	7.2	-	-
Calculated value								
AME (kcal/kg)	2999	2999	2999	2999	3099	3099	3099	3099
Crude protein (g/kg)	230	230	230	230	215	215	215	215
L-Lysine (g/kg)	13.9	13.9	13.9	13.9	12.9	12.9	12.9	12.9
DL-Methionine (g/kg)	5.5	5.5	5.5	5.5	5.1	5.1	5.1	5.1
TSAA (g/kg)	8.4	8.4	8.4	8.4	7.9	7.9	7.9	7.9
Calcium (g/kg)	6.7	6.7	9.6	9.6	5.8	5.8	8.7	8.7
Non-phytate P (g/kg)	4.5	4.5	4.5	4.5	4.3	4.3	4.3	4.3
Total P (g/kg)	6.4	6.4	6.4	6.4	6.0	6.0	6.0	6.0
Analyzed value								
Calcium (g/kg)	6.7	6.8	9.7	9.7	5.10	5.09	8.9	8.9
Total P (g/kg)	6.5	6.5	6.5	6.5	6.2	6.2	6.2	6.2

[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.

Data Collection, Sampling, and Measurements

All chickens were individually weighed on days 0, 11, and 24. Feed intake (FI) was measured per pen on 11 and 24 days of age. The performance was determined per replicate on 11 and 24 days of age as average feed intake (FI), average body weight gain (BWG), and FI/BWG. Mortality was recorded and all the data were corrected as they occurred. On day 15,

all the birds were deprived of feed for 16 h and subsequently were offered 12 experimental diets containing chromium oxide as an indigestible marker at 0.3 % until 21 days of age. During the period of 19 to 21 days of age, excreta were collected to determine Ca, P, and ash retention. The collected excreta and feed samples were dried in a 105 ºC oven for 24 h. At 24 days of age, the birds were deprived of feed and water, and after 12-h fasting, one bird per replicate was randomly selected and bled via the brachial vein to measure the blood parameters. The blood samples were centrifuged for 15 min at 1500 g at room temperature, and sera were stored at -20 ºC for the determination of alkaline phosphatase (ALP), Ca and, P. Birds were then weighed individually and humanly killed using cervical dislocation. Tissue samples from the midpoint of the jejunum and duodenum (150-200 mg) were taken to evaluate the gene expression of CALB1. The samples were rinsed with ice-cold PBS twice, minced, and placed into RNA later (Qiagen, Hilden, Germany), and stored at -80°C for subsequent mRNA analysis. Both tibiae were removed from the carcass and cleaned of all attached tissues. The left tibia was immediately frozen and kept at -40°C until further analysis. The right tibia was fixed for 24 hours at 4°C in 10% phosphate-buffered formalin solution and then split longitudinally in half on the epiphyseal plate. 0.5-cm mid-diaphyseal cross-sections were also excised from the same bones. The longitudinal slices and cross-sectional segments were decalcified using a 10% formic acid solution. Then, after embedding in paraffin, the samples were cut into 5 µm sections by microtome. All sections were stained using hematoxylin and eosin (H and E) to measure tibia histomorphometric parameters including cortical thickness, the width of the proliferative zone, hypertrophic zone, and mineralized zone. The width of the mentioned parameters was measured using a light microscope (Olympus BX41TF, Tokyo, Japan) with a digital camera (Olympus DP12 U-TV0.5 XC-2, Japan) which was connected to the image analysis system (Olysia Soft Imaging System, Germany). The left tibia bones were thawed before mechanical testing. The diameter and length at the center of the diaphysis were determined using calipers. The bones were subsequently oven-dried at 105 ºC for 24 h. After drying, bones were defatted in diethyl ether for 48 h to measure the fat-free dried bone weight, bone length, mechanical properties, and bone mineral content (Ca and P). The bones were subjected to a three-point bending test using an Instron Universal Testing Machine (Instron, Model H5KS, Tinius Olsen Company). In brief, the bones were mounted across supporting steel bars with a gap of 50 mm. The 10 mm diameter cross head probe (50 kg) was applied to the shaft's midpoint at 5 mm/min until a fracture occurred. Ultimate shear force, shear fracture energy, stiffness (tangent to the angle α), and maximal deflection before fracture were determined using the software (Q Mat). The sheared tibia pieces were collected oven-dried overnight at 105ºC. The dried bone samples were kept at 600 ºC for 16 h to determine ash, Ca, and P contents.

Laboratory measurements of Ca, P, Cr, and ALP

Serum Ca, P, and ALP was determined using an automatic blood chemical analyzer (SELECTRA-ProM, ELITech Group VITAL, Scientific, Dieren-The Netherland). Excreta, diet and, bone samples were analyzed for ash, total P and, Ca. Ca concentration in all samples was determined by atomic absorption (Varian SpectrAA 50B Atomic Absorption Spectrometer: Varian Ltd, USA) according to AOAC method 927.02 (AOAC, 2005). P concentrations were determined colorimetrically using a molybdovanadate reagent (AOAC, 2005, method 96517). The chromium concentration in dried excreta and feed samples was measured by atomic absorption (Varian SpectrAA 50B Atomic Absorption Spectrometer, Varian Ltd, USA) according to Williams *et al.* (1962).

Total RNA extraction and reverse transcription

The extractions of total RNA from duodenum and jejunum tissue samples were conducted using a RNA extraction kit (RNX-plus Kit, SinaClon Iran). Then, using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), purity and concentration of isolated RNA were determined at the absorbance ratio of 260-280 nm. All RNA samples were stored at -80 °C until cDNA synthesis. Briefly, for cDNA synthesis, total RNA at 1-10 µg and a 2-step RT-PCR kit (Cat. No. RTPL12, Vivantis Technologies) were used according to the manufacturer's protocol.

Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in a 48-well plate format of a Real-Time PCR System (Applied Biosystems). A total of 20 µL of PCR reaction mixture for every sample was prepared using the SYBR Green Master Mix (Applied Biosystems), containing 10 µL of SYBR Green, 1 µL of sample cDNA, 1 µL of each of the respective forward and reverse primers, and 7 µL of RNase-free water. All reactions were performed in triplicate, and no-template controls were included in each run. The sequence of forward and reverse primers of the target (CALB1), and housekeeping (β-actin) genes are shown in Table 3. The primer of CALB1 used for real-time PCR was previously reported in Li *et al.*, (2012) study. The real-time PCR cycling conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, 59 °C for 30 sec, 72 °C for 40 sec, followed by one cycle of final extension at 72 °C for 10 min; the melting curve was obtained

over the range 60-95 °C. The relative gene expression based on the housekeeping gene (β-actin) as endogenous control was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analysis

All data were statistically analyzed using the GLM procedure of SAS (SAS Institute, 2003) as a three-factorial arrangement, including dietary Ca concentration (low and adequate), *WS* leaf extract (0, 100, and 200 mg/kg diet), and fish oil (0 and 2 %). Significant differences among treatments were tested using Duncan's multiple range and considered significant at $P < 0.05$.

Table 3. Primer sequences used for the quantitative real-time PCR

Gene[a]	Primer sequence[b] (5`-3`)	Annealing temp (˚C)	Amplicon (bp)	Reference
CALB1	F:AATCTGCGTTGCTTCCATACA R: CATTTAGTTGCCTGAGTTCACCT	59 ºC	218	Li *et al.,* (2012)
β-actin	F: AACACCCACACCCCTGTGAT R: TGAGTCAAGCGCCAAAAGAA	60 ºC	100	

[a] CALB1: Calbindin 1

[b]F: Forward primer; R: Reverse primer

Results

Growth performance

The effects of dietary treatments on the birds' performance from day 0 to 24 are shown in Table 4. There were no significant differences in FI and BWG among birds during the experimental period ($P > 0.05$). The significant main effects of *WS* on FCR were observed ($P < 0.01$). Supplementation of *WS* at the levels of 100 or 200 mg/kg significantly improved FCR throughout the experiment (1.30 and 1.29 vs 1.34; $P < 0.01$).

Table 4. Effect of dietary Ca, *Withaania somnifera* (*WS*), and fish oil on feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR) of broiler chickens at 24 d of age

Treatment			FI (g)	BWG (g)	FCR (g/g)
Ca (%)	*WS* (mg/kg)	Fish oil (%)			
Low	0	0	1618.31	1184.67	1.36
Low	0	2	1610.00	1224.52	1.31
Low	100	0	1612.74	1248.84	1.29
Low	100	2	1536.88	1195.93	1.28
Low	200	0	1593.66	1232.60	1.29
Low	200	2	1533.88	1198.71	1.27
Adequate	0	0	1549.44	1165.65	1.37
Adequate	0	2	1549.53	1180.36	1.31
Adequate	100	0	1613.34	1219.49	1.32
Adequate	100	2	1615.10	1219.16	1.32
Adequate	200	0	1596.32	1238.82	1.29
Adequate	200	2	1608.56	1240.12	1.29
SEM			35.911	26.989	0.019
Main effects					
Ca		-	1584.41	1214.22	1.30
		+	1596.22	1210.60	1.32
WS		0	1593.31	1188.80	1.34[a]
		100	1594.52	1220.86	1.30[b]
		200	1583.11	1227.57	1.29[b]
Fish oil		0	1604.81	1215.02	1.32
		2	1575.82	1209.80	1.30
P-value					
Ca			0.571	0.817	0.188
WS			0.885	0.105	0.001
Fish oil			0.168	0.739	0.078
Ca × *WS*			0.189	0.356	0.406
Ca × Fish oil			0.372	0.506	0.729
WS × Fish oil			0.961	0.333	0.118
Ca × *WS* × Fish oil			0.447	0.568	0.866

[a-b]Means within each column with no common superscript differ significantly ($P < 0.05$).

Mineral retention

As shown in Table 5, the experimental treatments did not show any significant effect on ash and phosphorus retention ($P > 0.05$). Regarding Ca, significant main effects of dietary Ca and *WS* levels on Ca retention were observed ($P < 0.05$). The results showed that birds fed low-Ca diets had significantly higher Ca retention than those fed adequate-Ca diets (79.32 vs. 76.93%; $P < 0.05$). The results indicate that birds fed *WS* (at both levels of 100 and 200 mg/kg) had significantly higher values of Ca retention than those on non-supplemented diets (79.40 and 79.02 vs 75.96%; $P < 0.01$). Also, a significant interaction was observed between dietary Ca and *WS* on Ca retention. This interaction showed that regardless of fish oil level when birds are fed diets with adequate Ca, the addition of *WS* at 100 or 200 mg/kg leads to a significant increase in Ca retention (79.60 and 79.06 vs 73.06%; $P < 0.05$).

Table 5. Effect of dietary Ca, *Withaania somnifera* (*WS*), and fish oil on mineral retention of broiler chickens at 19-21 days of age

Treatment			Mineral retention		
Ca (%)	*WS* (mg/kg)	Fish oil (%)	Ash (%)	Ca (%)	P (%)
Low	0	0	62.28	78.71[a]	75.33
Low	0	2	66.70	78.90[a]	73.89
Low	100	0	61.25	80.23[a]	75.70
Low	100	2	61.27	79.30[a]	74.43
Low	200	0	60.31	80.49[a]	75.58
Low	200	2	61.54	78.27[a]	75.21
Adequate	0	0	65.54	73.06[b]	74.22
Adequate	0	2	62.56	73.18[b]	75.66
Adequate	100	0	60.94	79.60[a]	74.25
Adequate	100	2	56.82	78.46[a]	75.61
Adequate	200	0	61.70	78.26[a]	75.09
Adequate	200	2	65.54	79.06[a]	75.58
SEM			2.887	1.605	1.180
Main effect					
Ca		-	62.22	79.32[a]	75.02
		+	62.18	76.93[b]	75.07
WS		0	64.27	75.96[b]	74.78
		100	60.07	79.40[a]	75.00
		200	62.27	79.02[a]	75.36
Fish oil		0	62.00	78.39	75.03
		2	62.40	77.86	75.06
P-value					
Ca			0.980	0.013	0.944
WS			0.131	0.007	0.777
Fish oil			0.811	0.568	0.957
Ca × *WS*			0.461	0.049	0.956
Ca × Fish oil			0.376	0.625	0.125
WS × Fish oil			0.531	0.863	0.999
Ca × *WS* × Fish oil			0.464	0.724	0.804

[a-b]Means within each column with no common superscript differ significantly ($P < 0.05$).

Bone physical characteristics and mineral content

As shown in Table 6, the experimental treatments had no significant effect on physical characteristics, including tibia weight, length, and diameter ($P > 0.05$). The results showed that experimental treatments did not have a significant effect on the tibia ash and P content ($P > 0.05$). Regarding the Ca content of the tibia, significant main effects of *WS* were recorded ($P < 0.05$). Birds fed diets supplemented with both 100 and 200 mg/kg *WS* had significantly higher Ca content in the tibia (37.04 and 37.34 vs 36.02 %; $P < 0.05$).

Bone morphology

As shown in Table 7, the significant main effects of fish oil on the width of the proliferative zone were recorded ($P < 0.05$). Birds fed a diet containing fish oil had a significantly higher width of the proliferative zone than those without fish oil (794.31 vs 754.66 μm; $P < 0.05$). Two-way interaction was also observed between *WS* and fish oil on the width of the proliferative zone. The interaction showed that

regardless of dietary Ca level, the addition of fish oil to diets without *WS* leads to a significant increase in the width of the proliferative zone (872.45 vs 702.55 μm; $P < 0.001$). Experimental treatments did not show a significant effect on hypertrophic zone width and cortical thickness ($P > 0.05$). A significant two-way interaction was also observed between dietary Ca level and *WS* for mineralized zone width. This interaction showed that regardless of fish oil level, dietary addition of *WS* when birds are fed low-Ca diets leads to a significant increase in the width of the mineralized zone (2263.20 vs 1783.70 μm; $P < 0.05$).

Table 6. Effect of dietary calcium, *Withaania somnifera* (*WS*), and fish oil on Tibia bone physical characteristics and mineral content of broiler chickens at 24 days of age.

Treatment			Physical characteristics			Mineral content		
Ca (%)	*WS* (mg/kg)	Fish oil(%)	weight (g)	length (mm)	diameter (mm)	ash (%)	Ca (%)	P (%)
Low	0	0	1.63	66.91	5.33	51.66	35.80	24.59
Low	0	2	1.93	66.17	5.30	52.22	35.85	24.04
Low	100	0	1.66	66.17	5.20	52.46	37.10	24.34
Low	100	2	1.76	66.40	5.13	52.41	37.02	24.23
Low	200	0	1.72	64.51	5.01	51.27	37.30	24.03
Low	200	2	1.66	66.31	5.08	52.39	36.95	24.08
Adequate	0	0	1.70	64.05	5.34	53.67	36.23	24.10
Adequate	0	2	1.60	66.27	5.47	51.15	36.20	24.01
Adequate	100	0	1.70	65.86	5.74	52.00	36.84	24.00
Adequate	100	2	1.72	64.16	5.20	53.39	36.84	24.24
Adequate	200	0	1.78	64.72	5.24	53.64	37.18	24.35
Adequate	200	2	1.64	65.40	5.08	51.79	37.24	24.34
SEM			0.103	0.853	0.184	0.862	0.681	0.172
Main effect								
Ca		-	1.72	65.91	5.17	52.07	36.67	24.22
		+	1.69	65.08	5.34	52.61	36.90	24.17
WS		0	1.71	65.85	5.36	52.18	36.02[b]	24.18
		100	1.71	65.40	5.320	52.57	37.04[a]	24.20
		200	1.70	65.23	5.10	52.27	37.34[a]	24.20
Fish oil		0	1.70	65.20	5.31	52.45	36.83	24.23
		2	1.71	65.78	5.21	52.22	36.72	24.15
P-value								
Ca			0.595	0.096	0.118	0.284	0.508	0.660
WS			0.969	0.576	0.114	0.801	0.024	0.989
Fish oil			0.756	0.243	0.341	0.652	0.747	0.425
Ca × *WS*			0.533	0.693	0.676	0.871	0.848	0.060
Ca × Fish oil			0.127	0.709	0.400	0.129	0.989	0.216
WS × Fish oil			0.348	0.467	0.391	0.401	0.780	0.241
Ca × *WS* × Fish oil			0.444	0.052	0.478	0.118	0.923	0.533

[a-b]Means within each column with no common superscript differ significantly ($P < 0.05$).

Also, the birds fed diets containing fish oil had significantly higher shear force values (112.54 vs 104.95 N; $P < 0.05$). No significant effects of experimental treatments on fracture deflection and fracture energy were recorded ($P > 0.05$). On the other hand, a significant main effect on stiffness was observed for *WS*. Higher stiffness values were recorded for birds fed diets containing *WS* at both 100 and 200 mg/kg (194.69 and 199.09 vs 172.98 N/mm, $P < 0.05$). The results also showed a two-way interaction between *WS* and fish oil on tibial bone stiffness. This interaction showed that regardless of the dietary Ca level when birds are fed diets containing fish oil, the addition of *WS* at 200 mg/kg leads to a significant increase in tibial stiffness (221.36 vs 164.52 N/mm; $P < 0.05$).

Blood serum parameters

The results of blood serum parameters at the end of the experiment are shown in Table 9. No significant effect of experimental treatments on serum Ca, P, and ALP was observed ($P > 0.05$).

CALB1 gene expression

As shown in Table 10, a decrease in dietary Ca levels resulted in a significant increase in CALB1 gene expression in the duodenum (1.84 vs 1.62; $P < 0.05$). On the other hand, no significant effect was observed

in the jejunum ($P > 0.05$). The data showed that *WS* supplementation also led to a significant increase in CALB1 gene expression. The highest expression values were obtained at 200 mg/kg, which shows a significant difference with both 0 and 100 mg/kg (2.29 vs 1.69 and 1.21; $P < 0.001$). The significant main effects of *WS* on CALB1 gene expression were observed with a similar trend in the jejunum. The group fed at the level of 200 mg/kg showed a significant difference compared to the groups that received other levels (2.08 vs 1.64 and 1.32; $P < 0.001$).

Table 7. Effect of dietary calcium, *Withaania somnifera* (*WS*), and fish oil on bone morphological characteristics of broiler chickens at 24 days of age.

Treatment			Morphological characteristics[1]			
Ca (%)	*WS* (mg/kg)	Fish oil (%)	PZ (μm)	HZ (μm)	MZ (μm)	CT (μm)
Low	0	0	702.55^{d}	285.30	1783.70^{b}	1316.30
Low	0	2	872.45^{a}	317.06	1979.70^{ab}	1327.10
Low	100	0	816.56^{abc}	338.27	1958.6^{ab}	1345.60
Low	100	2	779.94^{bcd}	279.15	2049.30^{ab}	1296.70
Low	200	0	776.33^{bcd}	279.88	2068.20^{ab}	1232.00
Low	200	2	773.28^{cd}	321.69	2263.20^{a}	1424.40
Adequate	0	0	718.93^{cd}	334.93	2046.40^{ab}	1348.20
Adequate	0	2	866.89^{ab}	279.74	2075.3^{ab}	1408.40
Adequate	100	0	755.36^{cd}	301.88	2223.20^{ab}	1409.20
Adequate	100	2	730.31^{cd}	280.26	2203.30^{ab}	1565.00
Adequate	200	0	758.23^{cd}	278.50	1903.60^{ab}	1324.70
Adequate	200	2	743.00^{cd}	327.90	1938.50^{ab}	1412.30
SEM			29.472	28.367	141.050	94.431
Main effect						
Ca		-	786.85	303.56	2019.11	1322.92
		+	762.12	300.53	2065.02	1411.29
WS		0	790.20	304.26	1974.27	1349.98
		100	770.54	299.89	2108.58	1407.21
		200	762.71	301.99	2043.35	1348.34
Fish oil		0	754.66^{b}	303.13	1999.27	1328.75
		2	794.31^{a}	300.97	2084.86	1405.66
P-value						
Ca			0.152	0.854	0.559	0.118
WS			0.403	0.976	0.394	0.650
Fish oil			0.024	0.895	0.287	0.172
Ca × *WS*			0.352	0.816	0.047	0.608
Ca× Fish oil			0.826	0.672	0.374	0.653
WS × Fish oil			<0.0001	0.103	0.902	0.707
Ca × *WS* × Fish oil			0.918	0.279	0.988	0.526

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

Table 8. Effect of dietary calcium, *Withaania somnifera* (*WS*), and fish oil on bone mechanical characteristics of broiler chickens at 24 days of age.

Treatment			Mechanical characteristics [1]			
Ca (%)	*WS* (mg/kg)	Fish oil (%)	SF (N)	FD (mm)	FE (N-mm)	S (N/mm)
Low	0	0	96.40	0.535	32.360	151.08^{c}
Low	0	2	107.92	0.555	30.920	180.38^{abc}
Low	100	0	107.44	0.570	30.380	194.10^{abc}
Low	100	2	116.70	0.604	24.860	182.14^{abc}
Low	200	0	109.92	0.553	27.660	171.90^{abc}
Low	200	2	113.15	0.590	31.720	221.36^{a}
Adequate	0	0	96.66	0.535	27.100	195.92^{abc}
Adequate	0	2	108.80	0.652	32.380	164.52^{bc}
Adequate	100	0	107.86	0.587	22.620	202.10^{ab}
Adequate	100	2	112.77	0.598	35.920	200.40^{abc}
Adequate	200	0	111.46	0.649	36.660	181.82^{abc}
Adequate	200	2	115.90	0.557	31.960	221.28^{a}
SEM			5.720	0.045	5.483	15.212
Main effect						
Ca		-	108.58	0.568	29.650	183.49
		+	108.90	0.596	31.107	194.34
WS		0	102.44^{b}	0.569	30.690	172.98^{b}
		100	111.19^{a}	0.590	28.445	194.69^{a}
		200	112.60^{a}	0.587	32.00	199.09^{a}
Fish oil		0	104.95^{b}	0.571	29.463	182.82
		2	112.54^{a}	0.593	31.293	195.01
P-value						
Ca			0.923	0.284	0.647	0.222
WS			0.031	0.788	0.653	0.042
Fish oil			0.026	0.426	0.566	0.171
Ca × *WS*			0.889	0.795	0.703	0.890
Ca× Fish oil			0.899	0.728	0.381	0.257
WS × Fish oil			0.613	0.341	0.863	0.041
Ca × *WS* × Fish oil			0.931	0.224	0.214	0.246

[a-c]Means within each column with no common superscript differ significantly ($P < 0.05$).
[1] SF= Shear force; FD= Fracture deflection; FE= Fracture energy; S= Stiffness.

Table 9. Effect of dietary Ca, *Withaania somnifera* (*WS*), and fish oil on blood parameters of broiler chickens at 24 days of age.

Treatment			Ca (mg/dl)	P (mg/dl)	ALP (U/l)
Ca (%)	*WS* (mg/kg)	Fish oil (%)			
Low	0	0	8.71	6.02	13881
Low	0	2	8.74	5.64	10901
Low	100	0	8.89	6.18	14322
Low	100	2	8.97	6.91	11383
Low	200	0	8.73	6.19	13961
Low	200	2	9.15	6.00	11881
Adequate	0	0	8.79	6.88	11092
Adequate	0	2	8.84	6.41	11307
Adequate	100	0	9.44	6.05	12133
Adequate	100	2	9.29	6.03	11786
Adequate	200	0	9.13	5.96	11355
Adequate	200	2	9.49	5.89	10950
SEM			0.326	0.511	1493.4
Main effect					
Ca		-	8.87	6.15	12722
		+	9.16	6.17	11437
WS		0	8.77	6.19	11796
		100	9.15	6.29	12406
		200	9.11	6.01	12037
Fish oil		0	8.95	6.19	12791
		2	9.07	6.14	11368
P-value					
Ca			0.127	0.872	0.142
WS			0.208	0.717	0.844
Fish oil			0.497	0.826	0.105
Ca × *WS*			0.736	0.199	0.915
Ca × Fish oil			0.811	0.700	0.155
WS × Fish oil			0.626	0.575	0.981
Ca × *WS* × Fish oil			0.960	0.819	0.936

Table 10. Effect of dietary calcium, *Withaania somnifera* (*WS*), and fish oil on the relative mRNA expression of CALB1 in the intestine of broiler chickens (n=60) at 24 days of age.

Treatment			Duodenum	Jejunum
Ca (%)	*WS* (mg/kg)	Fish oil (%)	CALB1[1]	CALB1
Low	0	0	1.35	1.51
Low	0	2	1.33	1.52
Low	100	0	1.77	1.66
Low	100	2	1.99	1.77
Low	200	0	2.27	2.07
Low	200	2	2.33	2.12
Adequate	0	0	1.06	1.14
Adequate	0	2	1.08	1.12
Adequate	100	0	1.10	1.35
Adequate	100	2	1.91	1.76
Adequate	200	0	2.29	2.07
Adequate	200	2	2.28	2.08
SEM			0.185	0.182
Main effect				
Ca		Low	1.84[a]	1.78
		Adequate	1.62[b]	1.59
WS		0	1.21[c]	1.32[c]
		100	1.69[b]	1.64[b]
		200	2.29[a]	2.08[a]
Fish oil		0	1.64	1.63
		2	1.82	1.73
P-value				
Ca			0.045	0.077
WS			<0.001	<0.001
Fish oil			0.102	0.370
Ca × *WS*			0.377	0.376
Ca× Fish oil			0.387	0.725
WS × Fish oil			0.102	0.543
Ca × *WS* × Fish oil			0.420	0.753

[a-c]Means within each column with no common superscript differ significantly ($P < 0.05$).
The results are expressed as $2^{-\Delta\Delta Ct}$

Discussion

In this experiment, the effects of supplementation of hydroalcoholic extract of *WS* leaf and fish oil in diets with adequate or low Ca levels on performance, Ca and P retention, bone characteristics, and CALB1 gene expression were investigated. The results showed that *WS* supplementation improved FCR throughout the experiment. These results are consistent with the previous findings indicating that supplementation of *WS* root powder in the diet of broiler chickens resulted in improved FCR (Dwivedi *et al.,* 2015; Vasanthakumar *et al.,* 2015). Vasanthakumar *et al.,* (2015) reported that improved FCR because of *WS* extract supplementation might be attributed to the free radical scavenging activity of the secondary metabolites. Different parts of a plant, including roots, fruits, and, leaves contain flavonoids, withanolides, glycosides, alkaloids, phenolics, tannins, and saponin (Dhuley *et al.,* 1993). The plant contains major active ingredients such as withanolide 1, withanolide 2, withanine, and withaferin A, which not only act as antioxidants and antibacterial agents but also do as stimulants of digestive enzymes, improve digestion of dietary nutrients and FCR (Abou-Douh, 2002). Also, the flavonoids in the plant, which are the main group of phenolic compounds, act as an effective donor of hydrogen and have antimicrobial activity against intestinal pathogens, thus, preventing the physical attachment of pathogenic microorganisms to the intestinal epithelium, which in turn improving digestion and absorption of nutrients and, intestinal health (Liang *et al.,* 2010).

In this experiment, reducing dietary Ca level by 30% resulted in a significant increase in Ca retention. Birds seem to compensate for the Ca deficiency through an adaptation mechanism. This result is consistent with a previous report by Plumstead *et al.* (2008), who showed that the reduction of dietary Ca level from 1.16 % to 0.47 % with a constant dietary P level leads to a linear increase in apparent Ca digestibility. This result is supported by the findings obtained from a study by Tancharoenrat and Ravindran (2014), who reported that reducing the Ca level in the broilers' diet from 13 to 7 g/kg leads to a significant increase in Ca retention in the gastrointestinal tract. Increased Ca retention in the present experiment may be due to the activation of adaptive mechanisms, including increased expression of the CALB1 gene, synthesis of 1, 25 $(OH)_2$ D_3, sodium-Ca exchanger, plasma membrane Ca-transporting, Ca pumps, plasma membrane Ca^{2+} ATPase (PMCA), and sodium-dependent phosphate transport protein 2B which increase mineral retention (Brini and Carafoli, 2011). Regarding the beneficial effects of *WS* on Ca retention, the results of this experiment are in line with observations from Nagareddy and Lakshmana (2006), who found that oral injection of *WS* root hydroalcoholic extract at 65 mg/kg body weight twice a day for 16 weeks in ovariectomized rats leads to a decrease in excreted Ca or in other words, an increase in its retention. However, we will discuss the stimulatory effects of *WS* on the expression of the CALB1 gene as one of the possible factors. In this regard, the presence of a large number of withanolides, particularly withaferin A, estrogen-like compounds, has been previously mentioned as one of the possible mechanisms involved in Ca retention (Mishra *et al.,* 2000).

The results of this experiment in terms of the effect of experimental treatments on the physical properties of the tibia are in accordance with the findings of Mirakzehi *et al.,* (2013, 2018), who reported that there is no significant difference between experimental treatments due to supplementation of *WS* hydroalcoholic extract in broiler chickens at 21 and 42 days of age. Regarding the lack of significant effect of fish oil on the physical properties of the tibia, similar results were recorded by other researchers in broilers and Japanese quails (Güz *et al.,* 2019; Liu *et al.,* 2003a).

The findings of this experiment showed that the inclusion of *WS* extract in the diet improved the calcification of the tibia, which is consistent with the results of other researchers in broilers, Ca-deficient ovariectomized rats, and laying hens respectively (Mirakzehi *et al.,* 2013; Nagareddy and Lakshmana 2006; Tahmasbi *et al.,* 2012). The results of this experiment indicate that the improvement of Ca retention through the intestine by supplementation of *WS* extract has led to increased calcification of the tibia. As mentioned earlier, the beneficial effects of *WS* extract are mainly related to a large number of estrogen-like compounds called withanolides. Phytoestrogenic compounds have a diphenolic ring in their chemical structure that makes them structurally similar to endogenous estrogen, diethylstilbestrol, and estradiol. Phytoestrogens in the gut are affected by metabolic processes and converted to heterocyclic phenols, which have a structure similar to estrogen (Lorand *et al.,* 2010). In birds, as in mammals, estrogen plays an important role in the process of osteogenesis. Estrogen has been shown to increase the activity of the enzyme 25(OH) D_3-1α-hydroxylase, and the expression of 1, 25 $(OH)_2$ D_3 receptors in the intestine and bone, not only increase the absorption of Ca in the intestine but also increase the mobilization of Ca into the bones (Nie *et al.,* 2020). Morphological findings of this study showed that the width of the proliferative zone increased in birds that consumed diets containing fish oil. The interaction between *WS* and fish oil also illustrates this well. Our observations are in line with the findings of Liu *et al.,* (2003a), who reported that the width of the cartilaginous proliferative zone in progeny from quails fed fish oil supplemented diet is

significantly greater compared to those fed diets containing soybean oil. Also, this result is supported by Watkins *et al.,* (1996), who demonstrated that feeding broilers with fish oil (high in ω-3 PUFAs) resulted in a higher bone formation rate compared to the soybean oil group (high in ω-6 PUFAs). PUFAs may exert their effects by modulating PGE synthesis and reducing inflammation. Also, ω-3 fatty acids have been shown to increase the proliferation and differentiation of osteoblasts by increasing the expression of core-binding factor alpha 1 (Cbfa1). Cbfa1 is a transcription factor that plays an important role in the initiation and regulation of cell proliferation, matrix maturation, and mineralization phases (Watkins *et al.,* 2003). Regarding the beneficial effects of *WS* on increasing the width of the mineralized zone of birds fed low-Ca diets, similar results have been obtained by Kuang *et al.,* (2020), who reported that withanolides positively affect osteogenic differentiation and inhibit osteoclastogenesis by inhibiting NF-κB signaling pathways.

The results of bone mechanical properties showed that *WS* supplementation has positive effects on increasing shear force. The bones of birds in this group need more energy to break. Similar results have been reported by other researchers (Mirakzehi *et al.,* 2013; Nagareddy and Lakshmana 2006; Reddy *et al.,* 2004). The results also indicate the beneficial effects of dietary *WS* on bone stiffness. Our findings confirm the results of Mirakzehi *et al.* (2013), who reported that *WS* supplementation increased the bone stiffness of broilers at 21 and 42 days of age. Polyphenols, flavonoids, phytosterols, and vitamin C in the *WS* have been reported to prevent the degradation of bone connective tissues by inhibiting collagenase and prostaglandin E_2 (Rasool and Varalakshmi, 2007). Also, the estrogenic nature of phytosterols in the leaf and root of *WS* leads to the positive effects of the plant on Ca retention, bone calcification, and its mechanical properties. They also stimulate protein synthesis in the bone matrix by increasing the expression of vitamin D receptors in osteoblasts (Liel *et al.,* 1999). Stiffness is an important factor for effective locomotion in birds, and stiffer bones improve leg movements (Kim *et al.,* 2011). Bone mineralization contributes to its stiffness and strength (Liu *et al.,* 2012). It seems that supplementation of *WS* improves bone stiffness by increasing its mineralization. Our findings agree with those of Liu *et al.* (2003a), who reported that progeny from hens consuming diets containing fish oil had higher tibial shear force and stiffness at 7 and 14 d of age. Fish oil contains ω-3 and ω-6 PUFAs, which differently affect osteoblast function and bone strength than the common fat sources, such as soybean oil, corn oil, and palm oil (Watkins *et al.,* 2003). Supplementation of the diet with fish oil elevates 20:6 (ω-3) and 20:5 (ω-3) PUFAs in phospholipids of bone. Consequently, growing birds fed diets containing ω-3 PUFAs showed a significantly greater bone formation rate compared to those given soybean oil (Watkins *et al.,* 1997). Approximately 90% of the organic matrix of bone is type I collagen. Mature type I collagen in bone is bound together by special molecules called pyridinoline and deoxypyridinoline, which have a strong positive correlation with bone mechanical strength in normal and osteoporotic bones (Rath *et al.,* 1999). It has been shown that manipulating dietary essential fatty acids leads to changes in pyridinium cross-links in the organic bone matrix (Kruger *et al.,* 1997).

Our findings showed that serum levels of Ca, P, and ALP were similar in all groups. Our results are consistent with the findings of other researchers who reported that *WS* supplementation did not affect serum biochemical markers in broilers (Mirakzehi *et al.,* 2018) and rats (Nagareddy and Lakshmana, 2006). Homeostatic mechanisms appear to be involved in maintaining normal levels of serum biochemical markers despite dietary Ca deficiency. In case of poor Ca consumption or absorption 1, 25 $(OH)_2$ D_3 will mobilize Ca from the bone and even inhibit mineral deposition in the bone matrix to maintain normal serum Ca at the expense of bone strength (Carmeliet *et al.,* 2015).

In the present experiment, low dietary Ca levels up-regulated CALB1 gene expression in the duodenum. Similarly, as in this experiment, Li *et al.* (2012) also found that feeding diets containing low levels of Ca and normal P to broilers up-regulated duodenal CALB1 mRNA expression. Low plasma Ca levels lead to increased parathyroid secretion, which in turn releases Ca from the bones by activating the enzyme 1α-hydroxylase in the kidney. A gradual increase in the production of 1, 25 $(OH)_2$ D_3 in the kidney can increase Ca absorption in the small intestine and its reabsorption in the kidney. Therefore, vitamin D plays its role as a transcription factor and leads to increased Ca transporter expression (Proszkowiec-Weglarz and Angel, 2013). Regarding the effects of *WS* on the up-regulation of CALB1 mRNA in both duodenum and jejunum, as mentioned earlier, the mechanism associated with these changes seems to be related to estrogen-like withanolides. Estrogen increases renal 25 (OH) D_3-1-hydroxylase activity and plasma levels of 1, 25 $(OH)_2$ D_3. Furthermore, 1, 25 $(OH)_2$ D_3 in turn increases the absorption of Ca from the intestine and the mobilization of Ca from bone (Nie *et al.,* 2020). It has been reported that 1, 25 $(OH)_2$ D_3 can increase Ca absorption and reabsorption by increasing the expression of calcium-binding proteins including, CALB1 in the intestine and calbindin-D_{28K} (CaBP28K) in the kidney (Yang *et al.,* 2018).

Conclusion

In conclusion, the results of the present study suggest that dietary supplementation of *WS* exert beneficial biological effects on CALB1 gene expression, intestinal Ca absorption, bone calcification, and strength. Also, the results suggest that dietary *WS* and fish oil may have synergistic effects on bone strength. Further experiments are needed to investigate the mechanism of action of active constituents of *WS* on bone quality and strength.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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