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Impacts of *Withania coagul*ans extracts, linseed, and fish oil on performance, tibia bone characteristics, and mineralization in broiler chicken

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Abstract This study was designed to evaluate the impact of *Withania coagulans* extracts [fruit (WFE) and root (WRE)], linseed oil (LO), and fish oil (FO) on growth performance, tibia bone characteristics, calcium and phosphorus content in tibia and serum, biochemical indicators of bone metabolism, and certain blood parameters in broilers. The study involved 720 male Ross 308 broilers, which were allotted to a completely random design containing 9 treatments with 8 replications of 10 birds each. The experimental treatments included a basal diet (Control), basal diet + 200 mg/kg WRE or WFE, basal diet + 2% fish oil or flaxseed oil, a diet containing 2% fish oil + 200 mg/kg WRE or WFE, and a diet containing 2% flaxseed oil + 200 mg/kg WRE or WFE. Body weight and body weight gain increased from 1–21 and 22–42 days with the addition of FO and WFE compared to the control group. The highest values of bone stiffness, ultimate load, and mineral density (BMD) were observed in birds supplemented with a mixture of FO + WFE, while the lowest values were observed in birds fed the basal diet without supplements. Diets containing fish oil supplemented with WC extracts led to increased serum calcium and tibia calcium levels compared to other groups ($P \leq 0.05$). Supplementation with LO and FO reduced PGE2 concentration compared to other treatment groups ($P \leq 0.05$). No significant difference was observed in serum serum tartrate-resistant acid phosphatase (TRAP) levels across all experimental groups ($P>0.05$). In conclusion, the combination of oil source (FO) and extract (WFE) in the diet improved the performance of birds, increased bone characteristics and calcium levels in serum and tibia, and altered biochemical indicators of bone metabolism in serum, suggesting that this combination could be beneficial to the health and performance of broilers.

Keywords: broiler, fish oil, linseed oil, tibia, *Withania coagulans*

Introduction

Polyunsaturated fatty acids are the primary target for free radical attack at the onset of peroxidation (El-Samee et al., 2019). However, inclusion of n-3-containing compounds (such as fish and flaxseed oil) in chicken diets increases the content of long-chain n-3 polyunsaturated fatty acid (PUFA) and the susceptibility of meat lipids to oxidation (Koreleski et al., 2006).

Linseed is rich in protein (22%), oil (34%), and alpha-linolenic acid (ALA) (Jia and Slominski, 2010). Linseed oil can enhance the omega-3 content of the broiler carcass without negatively affecting broiler performance. Fish oil and seafood are rich in long-chain unsaturated fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid

(DHA). The N-3 and n-6 fatty acids are the two main polyunsaturated fatty acids (PUFAs) and a balanced diet containing the fatty acid is beneficial for health (Simopoulos, 2010).

It has been reported that feeding chickens with diets containing n-3 fatty acids (fish and flax oil) improves performance (Saleh et al., 2009, 2018; Wang et al., 2011). It has been reported that feeding chickens with diets containing n-3 fatty acids (fish and flax oil) improves performance (Saleh et al., 2009, 2018; Wang et al., 2011). Zhang et al. (2009) reported that supplementing linseed oil or blending it with palm oil increased the apparent calcium digestibility, reduced serum calcium, and boosted calcium levels in the tibia. The N-3 PUFA may enhance bone health by increasing calcium absorption in the gut, promoting osteoblast differentiation and activity, reducing osteoclast activity, and encouraging mineral deposition in developing bones (Mailhot et al., 2010). Also, PUFAs improve gene expression, lipid peroxidation, and eicosanoid production through hormonal changes. They are beneficial for preventing and treating osteoporosis (El-Sayed and Ibrahim, 2017).

Diets rich in alpha-linolenic acid (ALA) have been found to reduce the level of N-terminal telopeptide of type 1 collagen and maintain phosphatase activity, thereby regulating bone modeling and mineralization (Griel et al., 2007). Mineralization is crucial for bone health as it enables the skeleton to withstand gravitational and additional loads. Bone health is determined not only by the quantity of bone tissue and its microarchitectural structure but also by the rate of mineralization of the bone matrix (Boivin and Meunier, 2002). There is evidence to suggest a link between PUFAs and bone health and growth. Diets enriched with n-3 ALA have been shown to significantly improve skeletal health in laying hens (Baird et al., 2008). Biochemical evidence suggests that enhanced bone turnover has improved the mechanical characteristics of the bone (Tarlton et al., 2013a). The addition of omega-3 to poultry diets has been found to strengthen the tibia and femur. It also had a positive effect on joint disease and bone metabolism (Baird et al., 2008; Ebeid et al., 2008; Lau et al., 2013; Tarlton et al., 2013). The growth and production performance of poultry can be enhanced by supplementing their diets with fatty acids or their sources (El-Sayed and Ibrahim, 2017). This suggests that dietary interventions can play a significant role in promoting the health and productivity of poultry.

Medicinal plants have long been the source of herbal drugs for the prevention and treatment of various diseases, and secondary metabolites are linked to their medicinal properties (Croteau et al., 2000). Several studies reported that diets containing medicinal plants could improve performance, immune system, antioxidant effects, and reduce skeletal problems (Saleh et al., 2016, 2018; Ali Tavakkoli et al., 2022). El-Samee et al. (2019) reported that adding 2 g sweet chestnut tannins/kg in a diet containing fish and linseed oil as antioxidant sources inhibited lipid oxidation and enhanced antioxidant activity value in broilers.

Certain plant-derived natural compounds, containing substantial quantities of estrogens, are used in poultry diets. *Withania coagulans* (WC), a member of the Solanaceae family, is known to contain significant amounts of estrogenic compounds. This plant has been reported to contain various secondary metabolites such as steroidal compounds, alkaloids, saponins, terpenoids, glycosides, phenols, tannins, and flavonoids (Ali Tavakkoli et al., 2021). Withanolides, which are steroidal lactones with C-26 and C-22 stereochemistry, are among these compounds. Various withanolides have been identified in the fruits of *Withania coagulans* (Prasad et al., 2010a). It has been reported that the application of estradiol to chicks stimulated the production of 1,25-dihydroxy vitamin D_3 [1,25-(OH)2D_3], which is the physiologically active form of vitamin D (Beck and Hansen, 2004). Numerous studies have indicated that vitamin D may play a crucial role in the regulation of intestinal calcium metabolism (Hosseini et al., 2016; Mirakzehi et al., 2017). An alcoholic extract of *Withania somnifera* (WS) root, added to the diet of aged laying hens at 130 mg/kg, enhanced the absorption of calcium and phosphorus in the tibia (Tahmasbi et al., 2012).

Industrial broiler chickens have been selectively bred to achieve their desired body weight and feed conversion in a shorter time. However, this selective breeding has had several indirect side-effects, such as the adverse impact of rapid growth on the broiler's skeletal system (Williams et al., 2000). The skeletal problems and poor bone quality in these birds seem to be influenced by the interplay between genetic potential, environment, and diet (Huang et al., 2019). In addition to calcium and vitamin D, the quantity and quality of dietary polyunsaturated omega-3 fatty acids (n-3 PUFAs) may also affect skeletal system health (Shang et al., 2004).

Therefore, the aim of this experiment was to explore the effects of supplementing broiler diets with *Withania coagulans* extracts (fruit and root) and two types of oils (fish and linseed oils). The study focused on their impact on growth performance, tibia bone characteristics, calcium and phosphorus content in tibia and serum, biochemical indicators of bone metabolism, and certain blood parameters in broilers.

Materials and methods

Animals and experimental design

The experiment was approved by the Local Ethics Committee for Animal Experimentation at the Higher Education Complex of Saravan (Saravan, Sistan and Baluchestan, Iran). The study involved 720 male Ross 308 broilers, which were randomly divided into nine groups. Each treatment was replicated eight times with 10 chicks per replication in a completely randomized design. The birds were fed a basal diet of corn and

soybean meal, supplemented with fish oil, linseed oil (LO), *Withania coagulans* root extract (WRE), and *Withania coagulans* fruit (WFE) extract. The experimental treatments included a basal diet (control), a basal diet + 200 mg/kg WRE or WFE, a basal diet + 2% fish oil or flaxseed oil, a diet containing 2% fish oil + 200 mg/kg WRE or WFE, and a diet containing 2% flaxseed oil + 200 mg/kg WRE or WFE. All diets were formulated based on the NRC (1994). The ingredients of the basal, FO, and LO diets, and nutrient levels for the starter phase (1–21 days) and finisher phase (22–42 days) are presented in Table 1. The fatty acid compositions of the diets are shown in Table 1. Chicks were housed in floor pens (1.2 × 1.6 m) with wood shavings in a temperature-controlled room. The temperature was maintained at 34–36°C during the first day and was gradually reduced to 26°C after day 14. It was then kept at room temperature until the end of the trial. Water and diets were provided *ad libitum*.

Table 1. Composition and calculated nutrient content of starter and finisher diet of broilers

	Starter			Finisher		
Ingredients (%)	Control	Fish oil	Linseed oil	Control	Fish oil	Linseed oil
Corn	56.90	56.90	56.90	64.70	64.70	64.70
Soybean meal 48%	35.68	35.68	35.68	26.87	26.87	26.87
Gluten meal	1.00	1.00	1.00	1.25	1.25	1.25
Canola oil	2.10	0.10	0.10	3.80	1.80	1.80
Linseed oil	0.00	0.00	2.00	0.00	2.00	0.00
Fish oil	0.00	2.00	0.00	0.00	0.00	2.00
Dicalcium phosphate.	1.89	1.89	1.89	1.60	1.60	1.60
Limestone	1.30	1.30	1.30	1.00	1.00	1.00
Common salt	0.40	0.40	0.40	0.25	0.25	0.25
dl-Methionine	0.16	0.16	0.16	0.03	0.03	0.03
l-Lysine HCl	0.00	0.00	0.00	0.00	0.00	0.00
Choline Cl -70%	0.07	0.07	0.07	0.00	0.00	0.00
Mineral premix[b]	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin premix[c]	0.25	0.25	0.25	0.25	0.25	0.25
Calculated composition						
CP (%)	23.00	23.00	23.00	19.51	19.51	19.51
AME (kcal/kg)	3000.00	3000.00	3000.00	3200.00	3200.00	3200.00
Fat (%	4.64	4.64	4.64	6.56	6.56	6.56
Calcium (%)	1.00	1.00	1.00	0.80	0.80	0.80
Available phosphorus (%)	0.45	0.45	0.45	0.30	0.30	0.30
Methionine (%)	0.51	0.51	0.51	0.32	0.32	0.32
Lysine (%)	1.12	1.12	1.12	0.85	0.85	0.85
Fatty acid composition of experimental diets (as % of total fatty) [d]						
n-3 PUFA	5.03	43.65	37.05	4.93	36.35	31.22
n-6 PUFA	50.09	15.25	22.29	52.26	22.15	28.86
SFA	16.55	14.59	12.76	16.25	13.08	13.06
MUFA	26.15	25.06	21.85	25.35	27.75	25.14
PUFA	55.95	59.08	64.35	56.95	58.02	59.82

Dietary treatments were a basal diet, basal diet supplemented with 2% fish oil (FO), 2% linseed oil (LO), and 200 mg of *Withania coagulans* root or fruit extract.

Mineral premix contained iron120 mg, Zn 100 mg, Mn 150 mg, Cu 20 mg, Mg 12 mg, Co 0.6 mg, and Se 0.20 mg

Vitamin premix contained retinol 2 mg, α-tocopherol 0.02 mg, cholecalciferol 0.03 mg, menadione 1.33 mg, thiamine 0.83 mg, cobalamin 0.03 mg, riboflavin 2.0 mg, biotin 0.03 mg, folic acid 0.33 mg, niacin 23.30 mg, pantothenic acid 3.75 mg and pyridoxine 1.33 mg.

[d]n-3 = ω-3 fatty acids; n-6 = ω-6 fatty acids; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; FA composition expressed as percentage of total FA

Extraction of fruits and roots of Withania coagulans

Fruits and roots of *Withania coagulans* were procured from an herbal medicine store. Separately, 300 g of the powdered fruit and root were combined with 50% ethanol in a container and soaked for 72 hours. A rotary evaporator under vacuum (Laborota 4000, Heidolph, Germany) was utilized to extract, filter, and concentrate the soluble materials. The residues were then collected and stored at -20 ºC for analysis. Total phenolic contents (TPC) and flavonoid contents were measured by spectrophotometer. The Folin-Ciocalteu reagent was used to determine total phenols. The extracts of the root and fruit were mixed with gallic acid, sodium carbonate, and Folin-Ciocalteu reagent. Spectrophotometer readings at 765nm and 725nm were taken after 15 and 30 minutes, respectively. Total phenol values are expressed as gallic acid equivalent (mg/g of dry mass), which serves as a standard reference compound. To determine the total flavonoid content, the aluminum chloride colorimetric method was employed. Root and fruit extracts in aqueous form were separately mixed with methanol, aluminum chloride, potassium acetate, and distilled water. After remaining at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm with a UV-visible spectrophotometer (Gesan Chem 200 autoanalyzer, Italy). A calibration curve was prepared by creating quercetin solutions at concentrations ranging from 12.5 to 100 g/mL in an aqueous solution (Mirakzehi et al.,

2017; Azhar et al., 2020). The compounds in *Withania coagulans* fruit and root extracts are detailed in Table 2.

Table 2. Total phenolic (TPC) and flavonoid (TFC) contents in *Withania coagulans* fruit and root extracts (means±SD, n=5)

	TPC (mgGAE/mL)	TFC (mgQE/mL)
Withania coagulans fruit	17.9±0.85	1.48±1.3
Withania coagulans root	10.02 ± 0.49	1.02±1.2

Sample collection

Bodyweight was recorded individually for each replicate at 1, 21, and 42 days of age, and feed intake was measured over these periods to calculate the feed conversion. At 42 days of age, two chicks per replicate were randomly selected and weighed after 4 hours of feed deprivation. Blood samples were collected from the brachial vein on day 42. Blood serum was separated by centrifugation at 1500 × g for 15 minutes and stored at -20°C for later analysis. Following the collection of blood samples, the birds were humanely euthanized by cervical dislocation, followed quickly by exsanguination. Subsequently, the tibia bones were removed, cleared of surrounding soft tissues and prepared for subsequent measurement of mechanical properties and mineral content. The left tibia bones were stored at -20°C for later measurement of ash, calcium, and phosphorus content. This methodical approach ensured accurate and reliable data collection for the study (Abraham et al., 2005).

Bone density and bone mineral content

The tibiae were randomly selected and tested for three-point bending, ultimate load, and stiffness, which indicate the structural characteristics of the bone, using an Instron universal testing machine (Model H5KS, Tinius Olsen Company). The bone mineral content, bone density, and geometric parameters of the tibia were measured using dual-energy X-ray absorptiometry (DXA). Bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry using a Hologic Discovery-W machine (Hologic, Inc. Waltham, MA, USA). The measurement of bone density by DXA is based on projection bone density and is expressed as grams per square centimeter. The ash content of the tibia was determined by the ash of the bones in an oven for 18 hours at 600°C (Jämsä et al., 1998). These methods ensured a comprehensive analysis of the tibia's physical properties and mineral content.

Bone breaking strength

The meat was removed before testing the right tibia for bone-breaking strength. The bone-breaking strength was measured using a universal testing machine (Model 441, Instron, Ltd. England) as described by Incharoen et al. (2016), The right tibiae were measured for weight, diameter, and length. The average of the tibia diameters was taken at the narrowest and widest points. The rate of deformation was set at 5mm/min. The tracing of force was recorded at a steady rate. The graphs displayed plateau curves of maximal force (kg) reached, which were used to measure the energy stored in the bone. This comprehensive analysis provided valuable insights into the physical properties of the tibia.

Blood metabolites

Serum tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) were measured using methods outlined by the diagnostic kits from the Nanjing Jiancheng Bioengineering Institute, Nanjing, China. Serum PGE2 was quantified using an enzyme-linked immunosorbent assay with a commercial kit from Blue Gene Biotechnology Co. Ltd, Shanghai, China. The coefficient of variation between tests was 5.0%. Blood metabolites were analyzed using an automatic blood chemical analyzer (Random Access Analyzer A15, Biosystem Corp., Spain) to determine levels of calcium, phosphorus, total protein, glucose, albumin, triglycerides, and globulin. This was done according to the manufacturer's procedures. Serum levels of cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides were measured using the reagent kits from Wako Pure Chemical Industries Ltd., Tokyo, Japan, following the manufacturer's instructions (Sousa et al., 2011). These comprehensive analyses provided valuable insights into the biochemical indicators of bone metabolism in broilers.

Statistical analysis

The data were analyzed using the one-way GLM procedure of SAS (2002) in a completely randomized design. Significant differences among treatment means were identified at a 5% level by the Duncan's multiple range test.

Results

The effects of oil and *Withania coagulans* extract supplementation on the average daily gain, feed intake, and feed conversion ratio at 1–21 days and 22–42 days are presented in Table 3. There were no significant differences in feed intake across dietary treatments. Body weight and body weight gain (BWG) increased at 1–21 and 22–42 days with the addition of fish oil (FO) and *Withania coagulans* fruit extract compared to the control group. Significantly higher ($P \leq 0.05$) superior feed efficiency (1.92) was found in broilers fed dietary treatment WFE+ FO at 22–42 days; however, no differences were observed between diets supplemented with oil and extracts.

Regarding the tibia growth and bone characteristics (Table 4), the tibia weight of birds supplemented with FO

and WFE was significantly higher than other birds. There was no difference in tibia diameter and length of chickens fed different experimental diets (P>0.05). The lowest value of ultimate load (204.30 N) was recorded in the tibia of chickens fed with an unsupplemented diet, and the highest value of ultimate load (249.70 N) was the WFE+FO diet. Similarly, the highest values for stiffness (15.08 N × 10^4/m) and BMD (0.729 g/cm2) were obtained in broilers supplemented with a mixture of FO + WFE, while the lowest stiffness (14.29 N × 10^4/m) and BMD (0.701g/cm2) were observed for the basal diet without supplement. The inclusion of *Withania coagulans* fruit extract in the diets led to an increase (weight, ultimate load, stiffness) in tibia bone in comparison with diet containing *Withania coagulans* root extract. In addition, feeding chickens with fish oil, compared to linseed oil showed similarly significant results (P<0.05). The diameter and length of the tibia were not affected by oil and extract supplementation.

Table 3. Effects of *Withania coagulans* extract (fruit and root) and type of oil (fish and linseed oils) on broiler growth performance

Diet	1-21 days					22-42 days			
	Initial body weight (g)	Body weight (g)	BWG (g)	Feed intake (g)	FCR	Body weight (g)	BWG (g)	Feed intake (g)	FCR
Control	38.29	909.19^{c}	870.90^{c}	1072.29	1.23	2710.50^{d}	1801.31^{c}	3550	1.97^{a}
WFE	38.49	940.40^{ab}	901.91^{ab}	1080.11	1.20	2780.80^{ab}	1840.40^{a}	3568	1.94^{ab}
WRE	39.02	929.54^{b}	890.52^{b}	1073.75	1.21	2752.65^{bc}	1823.11^{b}	3562	1.95^{ab}
FO	38.91	934.97^{b}	896.06^{b}	1074.26	1.20	2761.10^{b}	1826.13^{b}	3536	1.94^{ab}
LO	38.26	922.76^{bc}	884.51^{b}	1090.52	1.23	2742.65^{c}	1819.89^{b}	3560	1.96^{a}
FO+WFE	39.24	956.68^{a}	917.44^{a}	1096.25	1.19	2799.10^{a}	1842.42^{a}	3540	1.92^{b}
FO+WRE	38.27	929.54^{ab}	891.27^{b}	1081.89	1.21	2764.20^{b}	1834.66^{ab}	3562	1.94^{ab}
LO+WFE	38.98	933.62^{b}	894.63^{b}	1089.89	1.22	2756.70^{bc}	1823.08^{b}	3560	1.95^{ab}
LO+WRE	39.01	924.12^{bc}	885.10^{b}	1089.25	1.23	2752.65^{bc}	1828.53^{b}	3590	1.96^{ab}
SEM	0.32	10.28	11.08	11.40	0.022	23.22	21.25	35.67	0.029
P-value	0.46	0.04	0.03	0.23	0.18	0.01	0.01	0.12	0.04

a,b: Within columns, mean values with common superscript (s) are not different (P<0.05; Duncan's multiple range test)
WFE: *Withania coagulans* fruit extracts; WRE: *Withania coagulans* root extracts; FO: Fish oil, LO: Linseed oil; SEM: Standard error of the mean; BW: Body Weight; FI: Feed Intake; FCR: Feed Conversion Ratio

Table 4. Effects of *Withania coagulans* extract (fruit and root) and type of oil (fish and linseed oils) on bone characteristics in 42-d-old broilers

Diet	Weight (g)	Diameter (mm)	Length (cm)	Ultimate load (N)	Stiffness (N × 10^4/m)	BMD (g/cm2)
Control	15.12^{d}	9.06	11.08	204.30^{d}	14.29^{d}	0.701^{c}
WFE	15.90^{b}	9.10	11.09	228.25^{b}	14.84^{b}	0.715^{b}
WRE	15.32^{d}	9.09	11.08	218.75^{c}	14.58^{c}	0.711^{b}
FO	16.04^{b}	9.08	11.10	235.50^{ab}	14.57^{c}	0.719^{ab}
LO	15.88^{bc}	9.09	11.16	222.35^{bc}	14.45^{cd}	0.719^{ab}
FO+WFE	16.41^{a}	9.11	11.12	249.70^{a}	15.08^{a}	0.729^{a}
FO+WRE	16.26^{ab}	9.08	11.20	236.80^{ab}	14.76^{b}	0.721^{ab}
LO+WFE	15.97^{b}	9.06	11.14	234.25^{ab}	14.89^{b}	0.726^{a}
LO+WRE	15. 90^{bc}	9.09	11.13	230.50^{b}	14.65^{c}	0.722^{ab}
SEM	0.087	1.357	0.094	0.736	0.032	0.001
P-values	0<.0001	0.09	1.20	0<.0001	0<.0001	<.0001

a,b: Within columns, mean values with common superscript (s) are not different (P<0.05; Duncan's multiple range test)
WFE: *Withania coagulans* fruit extracts; WRE: *Withania coagulans* root extracts; FO: Fish oil, LO: Linseed oil; SEM: Standard error of the mean

The effects of treatments on calcium and phosphorus in serum and tibia are presented in Table 5. Diets containing fish oil supplemented with *Withania coagulans* root and fruit extracts showed the highest amount of calcium in the serum and tibia compared to other groups. No significant differences were observed in the phosphorus concentration in serum or tibia at 42 days of age. The dietary treatments did not affect the tibia ash. Biochemical indicators of bone metabolism in serum are summarized in Table 6. Inclusion of fish and linseed oil in the diet caused a decrease in serum PGE2 concentration compared to other groups (P≤0.05) but was not affected by extract addition (P>0.05). Birds fed with linseed oil, fish oil, or a combination of oils and extracts, had higher levels of ALP than the control group (P≤0.05). No significant difference was observed in serum TRAP levels between experimental groups (P>0.05).

Table 7 shows the effects of dietary supplementation with *Withania coagulans* extracts and oils on blood parameters at 42 days of age. Serum levels of cholesterol were decreased (P≤0.05) due to diet containing oils compared to the control and diet without oil-containing extract. The lowest level of serum glucose was observed in birds receiving *Withania coagulans* extracts. No significant differences were observed in serum total protein, albumin, and LDL at 42 days of age. The HDL content in broilers whose diet was supplemented with oil and *Withania* extract tended to increase compared to the control group, although this difference was observed only numerically. Also, lower numerical triglycerides were observed in the diet contain-

ing oils and extracts than in the control diet.

Table 5. Effects of *Withania coagulans* extract (fruit and root) and type of oil (fish and linseed oils) on serum and tibia calcium and phosphorus in 42-d-old broilers

	Serum		Tibia		
Diet	calcium (mmol/L)	phosphorus (mmol/L)	calcium (mg/g)	phosphorus (mg/g)	Ash (%)
Control	2.80^{c}	2.49	38.84^{c}	18.92	43.12
WFE	3.12^{a}	2.52	39.17^{b}	19.20	43.33
WRE	3.02^{b}	2.50	39.08^{b}	19.00	43.13
FO	3.11^{a}	2.44	39.24^{ab}	19.13	43.46
LO	3.05^{b}	2.54	39.13^{b}	19.08	43.25
FO+WFE	3.13^{a}	2.53	39.32^{a}	19.20	43.59
FO+WRE	3.12^{a}	2.50	39.28^{a}	19.12	43.48
LO+WFE	3.10^{a}	2.49	39.16^{b}	19.10	43.34
LO+WRE	3.06^{a}	2.45	39.14^{b}	19.08	43.29
SEM	0.953	0.003	0.089	2.452	1.472
P-Values	0<.001	2.14	0<.001	0.08	1.12

a,b: Within columns, mean values with common superscript (s) are not different (P<0.05; Duncan's multiple range test)
WFE: *Withania coagulans* fruit extracts; WRE: *Withania coagulans* root extracts; FO: Fish oil, LO: Linseed oil; SEM: Standard error of the mean

Table 6 Effects of *Withania coagulans* extracts (fruit and root) and type of oil (fish and linseed oils) on biochemical indicators of bone metabolism in serum in 42-d-old broilers

Diet	Item		
	PGE2 (mg/mL)	ALP (U/L)	TRAP (U/L)
Control	5.32^{a}	112.24^{b}	5.51
WFE	5.21^{a}	113.67^{b}	5.40
WRE	5.25^{a}	114.52^{b}	5.23
FO	4.85^{b}	126.10^{a}	5.45
LO	4.90^{b}	128.54^{a}	5.34
FO+WFE	4.80^{b}	133.87^{a}	5.42
FO+WRE	4.81^{b}	129.50^{a}	5.43
LO+WFE	4.90^{b}	133.87^{a}	5.25
LO+WRE	4.87^{b}	124.75^{a}	5.37
SEM	0.052	0.254	0.654
P-Values	<.0001	1.70	0.09

a,b: Within columns, mean values with common superscript (s) are not different (P<0.05; Duncan's multiple range test)
WFE: *Withania coagulans* fruit extracts; WRE: *Withania coagulans* root extracts; FO: Fish oil, LO: Linseed oil; SEM: Standard error of the mean

Table 7. Effects of *Withania coagulans* extract (fruit and root) and type of oil (fish and linseed oils) on blood parameters in 42-d-old broilers

	Item							
Diet	Total protein (mg/mL)	Albumin (mg/mL)	Globulin (mg/mL)	Total cholesterol (µmol/mL)	Triglyceride µmol/mL	LDL (µmol/mL)	HDL (µmol/mL)	Glucose (µmol/mL)
Control	34.13	15.95	12.05	3.36^{a}	0.32	1.90	0.80	12.20^{a}
WFE	34.20	16.01	11.90	3.12^{b}	0.28	1.89	0.82	11.30^{b}
WRE	34.12	16.06	11.95	3.11^{b}	0.28	1.91	0.81	11.28^{b}
FO	34.04	15.95	11.98	2.80^{c}	0.26	1.87	0.88	12.10^{a}
LO	34.00	16.07	12.00	2.88^{c}	0.27	1.91	0.85	12.11^{a}
FO+WFE	34.18	16.08	11.89	2.73^{c}	0.25	1.92	0.91	11.32^{b}
FO+WRE	34.15	16.03	11.93	2.81^{c}	0.25	1.90	0.92	11.31^{b}
LO+WFE	34.16	16.04	12.02	2.86^{c}	0.29	1.89	0.94	11.40^{b}
LO+WRE	34.15	15.97	11.96	2.84^{c}	0.30	1.88	0.92	11.29^{b}
SEM	2.254	0.735	1.297	0.062	1.426	0.065	0.079	0.023
P-Values	0.075	1.54	0.29	<.0001	0.19	0.85	0.09	<.0001

a,b: Within columns, mean values with common superscript (s) are not different (P<0.05; Duncan's multiple range test)
WFE: *Withania coagulans* fruit extracts; WRE: *Withania coagulans* root extracts; FO: Fish oil, LO: Linseed oil; SEM: Standard error mean; HDL: high-density lipoprotein; LDL: Low-Density Lipoprotein

Discussion

Birds fed with various combinations of n-3 PUFA diet and *Withania coagulans* extracts exhibited higher body weight (BW) and body weight gain (BWG). Additionally, chickens fed with oils and extracts alone demonstrated better performance than the control group. Improved performance was noted in chickens fed diets containing n-3 PUFA sources compared to those fed a saturated fat diet (Saleh et al., 2009, 2018). Wang et al. (2011) suggested that the effect of n-3 PUFA in the broiler diet

was associated with their dietary level, as low levels of dietary fish oil are more beneficial than high levels in enhancing performance and feed gain. However, this study contradicts the findings of Kalakuntla et al. (2017) who reported that dietary supplementation with soy oil, linseed oil, and fish oil did not affect the performance of broilers. This highlights the complexity of dietary impacts on broiler performance and the need for further research Kalakuntla et al. (2017).

Numerous studies have been conducted using herbs, spices, and extracts, yielding varying results on chicken performance. Some studies have shown that plant compounds positively affected the body weight gain and feed conversion ratio (FCR) in chickens (Zhang et al., 2009; Khattak et al., 2014), aligning with our findings. Previous chemical studies have identified several withanolides in the fruits and roots of *Withania coagulans* (Prasad et al., 2010; Mirakzehi et al., 2017). Azhar et al. (2020) revealed the presence of secondary metabolites such as phenols and flavonoids in all parts of WC, suggesting that its extract may be a significant source of essential natural antioxidants. Both root and fruit extracts exhibited potent antioxidant activity, but our data showed that the fruit extract contained higher bioactive substances than the root (Table 2).

Improved feed efficiency of broiler chicks was reported by supplementation of vitamin E and pomegranate pomace extract as an antioxidant that affected the utilization of dietary nutrients (Saleh et al., 2016). Therefore, the dietary supplementation of WC extracts, which contain phenolic and flavonoid compounds as natural antioxidants, may improve BW, BWG, and FCR by reducing oxidative damage in broilers fed the diet supplemented with LO and FO.

In this study, significant improvements in tibia growth and bone characteristics such as weight, ultimate load, stiffness, and BMD were observed in birds fed with FO, LO, and WC extract compared to the control group. This aligns with several animal studies reporting a positive influence of n-3 fatty acids or a low ratio of n-6 to n-3 fatty acids on bone characteristics (Watkins et al., 2003). Studies have shown that varying rates of n-6 to n-3 fatty acids can alter the synthesis of prostaglandins and insulin-like growth factor I (Albertazzi and Coupland, 2002). High levels of n-6 fatty acids increased prostaglandin E2 (PGE2) production (Marshall and Johnston, 1982; Watkins et al., 1996), while supplementation with n-3 fatty acids or lower ratios of n-6 to n-3 fatty acids improved calcium transportation (Coetzer et al., 1994), and calcium retention. The results of this study on PGE2 obtained by supplementing fish and linseed oil in poultry diets confirm these findings (Table 1).

Bone mineral density significantly affects bone strength and is an important biomarker in improving bone strength which could provide more support to the rapid growth of broilers. Rats fed diets with 40 g/kg n-3 PUFA showed a 5.2% improvement in femur BMD compared to control (Green et al., 2004). Similarly, the bone biomechanical response is also an indicator of bone strength. In line with the current findings, Tarlton et al. (2013) reported that the humerus of laying hens fed 10% linseed oil diets (supplying ALA) revealed a higher BMD compared to those from n-6 FA-fed birds. The researchers also reported stronger, tougher, and harder bones as measured through bone-breaking strength in the tibia from the n-3 PUFA group. In growing Japanese quail fed a supplement containing 2% fish oil and linolenic acid, the tibial fractures were higher than those fed with a fish oil and linolenic acid diet.

The extract of WC fruit or root improved the bone parameters. These results are consistent with the results of Hosseini et al. (2016) who reported that increasing 100 g of WC fruit extract developed some characteristics of the tibia bone in broilers. *Withania coagulans* has several therapeutic effects including anti-inflammatory, anticancer, chemoprotective, immunomodulatory, hepatoprotective, antifungal, and antibacterial, as well as cardiovascular and improving central nervous system activities (Gupta and Rana, 2007; Jain et al., 2012). The main chemical constituents of the plant, withanolide, and withaferin A (WFA), are largely concentrated in fruits and roots (Maurya and pharmacology, 2010). Oral administration of WFA to osteogenic ovariectomized mice increased the osteoprogenitor cells within the bone marrow and developed the expression of osteogenic genes. The WFA supplementation increased trabecular micro-architecture of the long bones, enhanced biomechanical strength factors of the vertebra and femur, reduced bone turnover markers (osteocalcin and TNFα), and expression of skeletal osteoclastogenic genes. It also increased new bone formation and expression of osteogenic genes in the femur (Hie and Tsukamoto, 2010). Withanolide, being a proteasome inhibitor, can bind to the specific catalytic β subunit of 20S proteasome to promote the proliferation and differentiation of osteoblasts, and can effectively inhibit bone resorption and promote new bone formation (Khedgikar et al., 2015). Several other feeding studies reported the same effective association between bone mineral content and n-3 PUFA in growing animals (Lau et al., 2010; Lukas et al., 2011). Japanese quails supplemented with n-3 PUFA showed higher levels of bone minerals in the tibia than control (Liu et al., 2003). Indeed, while some studies have reported no effect of n-3 PUFAs on bone minerals in mice or birds, regardless of their inclusion in diets (Mollard et al., 2005), others have shown positive effects. For example, a study feeding pullet breeders either DHA, ALA, or a standard control diet found that the offspring produced by the mothers fed 1% DHA showed an increase in tibia ash weight and percentage (Akbari Moghaddam Kakhki et al., 2020). This suggests that these conclusions could potentially be applied in broiler nutrition to support bone strength in chicks.

In this study, no significant difference in the content of ash of different chickens fed with n-3 dietary sources was found, which contradicts these results. However, Hosseini et al. (2016) reported that, despite the lack of

significant effect of dietary WC on tibia mineral retention, the tibia cortical thickness of broilers fed the WC-supplemented diets increased. The results of the current experiment are in line with the report of Mirakzehi et al. (2017) who showed that supplementation of fruit hydroalcoholic extract of WC improved dietary Ca retention in birds. The reports reveal that the whole WC extract contains various chemical components such as steroidal lactones (withanolides), alkaloids, tannins, and flavonoids (Uddin et al., 2012). Improvement in tibia calcification may be due to the presence of these compounds in the extract.

The current study observed that fish oil and linseed oil reduced PGE2 levels compared to the control group. There is evidence that PGE2 production is reduced by supplementation with n-3 PUFA (Bautista-Ortega et al., 2009). Prostaglandin E2 is synthesized from arachidonic acid and is a modulator of bone remodeling interacting in both bone resorption and formation. Enhanced synthesis of PGE2 influences negatively on bone remodeling. Several studies have revealed an improvement in osteoblastic markers while PGE2 production was reduced or restricted (Evans et al., 1990; Kajii et al., 1999). Supplementing the diet with n-3 fatty acids through fish oil and chemical compounds in the extract may have improved bone characteristics and calcium levels in serum and tibia by modulating the biochemical indicators of bone metabolism.

The addition of WC extracts to broiler diets did not affect serum ALP activity, as shown by Hosseini et al. (2016) and Tahmasbi et al. (2012). ALP is considered a marker of bone formation that indicates osteoblastic activity (van Straalen et al., 1991), while TRAP is a marker of bone resorption reflecting osteoclastic activity (Minkin, 1982). Increased secretion of ALP indicates a beneficial effect of PUFA on hepatic cell membrane integrity, possibly due to enriched phospholipids, an essential part of cell membrane integrity containing two hydrophobic long-chain fatty acids (Attia et al., 2020).

The herbal plant used in this study, known for its antioxidant properties, has been reported to increase insulin secretion and decrease glucose levels when added to the diet (Prasad et al., 2010; Ali Tavakkoli et al., 2021). Gorelick et al. (2015) reported that withaferin-A isolated from *Withania somnifera* significantly reduced the serum glucose level. Various studies have shown direct influences of glucose on bone cells, including osteoblasts, osteoclasts, and steocytes. Hyperglycemia had detrimental effects on osteoblasts and osteoclasts and increased the sclerostin production in osteocytes; thus, both bone resorption and formation seemed to decrease during hyperglycemia (Hothersall et al., 2014; Cipriani et al., 2020). Compared to control treatments and extracts, fish oil and linseed oils decreased total cholesterol. It has been reported that high cholesterol levels increase osteoporosis by inhibiting osteoblasts (You et al., 2011). These findings highlight the complex interplay between diet, metabolic factors, and bone health.

Conclusions

The findings of this study suggested that a combination of fish oil and fruit extracts of *Withania coagulans* in the diet of broilers can enhance bone characteristics such as weight, ultimate load, and stiffness. It also increased calcium levels in serum and tibia, reduced total cholesterol, and positively influenced t growth performance. Therefore, including a mixture of *Withania coagulans* fruit extract and fish oil in the diet may have beneficial effects on the tibia and overall bone quality of in broilers. This could potentially lead to healthier and more robust broilers, which is beneficial for both the poultry industry and the consumers. However, further research is needed to confirm these findings and to explore the potential mechanisms behind these effects.

Conflict of interests

There is no conflict of interest.

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