

Impacts of Herbal Extracts and Different Dietary Oil Sources on Performance, Tibia bone characteristics and Mineralization of Broiler Chicken

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Abstract

This study aimed to investigate the effects of the supplementation of *Withania coagulans* extracts (fruit (WCF) and root (WCR)) and two oil types (linseed oil (LO) and fish oil (FO)) on the growth performance, tibia bone characteristics, calcium and phosphorus content in tibia and serum, biochemical indicators of bone metabolism, and some blood parameter in broilers. The experimental materials comprised 720 male Ross 308 broilers (nine treatments, 8 replications, 10 birds per replication). Dietary treatments included: basal diet (control), 200mg/kg WCR, 200 mg WCF /kg, 2% FO, or 2% LO added to basal diet. BW and BWG increased at 1–21 and 22–42 days with an adding FO and WCF compared to the control group. The highest values of stiffness, ultimate load, and BMD were obtained supplemented with a mixture of FO + WCF, while the lowest Stiffness, ultimate load, and BMD were observed basal diet without supplement. Increased serum calcium and tibia calcium levels observed by diets containing fish oil supplemented with WC extracts compared to other groups ($P < 0.05$). LO and FO supplementation reduced PGE2 concentration compared to other groups treatment ($P < 0.05$). No significant difference was observed in serum TRAP levels in all experimental groups ($P > 0.05$). In conclusion, feeding a combination of oil source (FO) and extract (WCF) improved the performance of birds, increased bone characterized and calcium levels in serum and tibia by altering biochemical indicators of bone metabolism in serum.

Introduction

Industrial broiler chicken has been selected to reduce the time required to achieve its desired body weight and feed conversion. However, there have been several indirect effects on selected programs. Numerous studies have shown the negative effect of rapid growth on the skeletal system of broilers [1]. Skeletal problems and poor bone quality in these birds appear to be affected by the relationship between genetic potential, environment, and feed [2]. 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 [3].

Linseed and fish oil are sources of n-3 PUFA. Linseed contains protein (22%), oil (34%), and rich source ALA [4]. Linseed oil can be beneficial to improve the omega-3 content of the broiler carcass without adversely influencing broiler performance. Fish oil and seafood contain long-chain unsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). N-3 and n-6 fatty acids are the two main polyunsaturated fatty acids (PUFAs) whose equivalent dietary intake has gained considerable attention in global nutrition because of their feasible health advantages [5]. Zhong et al. [6] reported that linseed oil supplementation or blending with palm oil increased apparent calcium digestibility, reduced serum calcium, and boosted calcium levels in the tibia. N-3 PUFA may improve bone health by increasing calcium absorption in the gut and increasing osteoblast differentiation and activity, reducing osteoclast activity, and promoting mineral deposition in developing bones [7]. They are favorable for preventing and treating osteoporosis [8]. PUFAs can improve bone health through calcium absorption, hormonal changes, gene expression, lipid peroxidation, and the production of eicosanoid. The bone mineral changes may be the consequence of the formation of intestinal soaps with calcium that decreases its absorption [9].

Diets containing alpha-linoleic acid (ALA) reduce N-terminal telopeptide of type 1 collagen levels and maintain phosphatase activity, thereby adjusting bone modeling and mineralization [10]. Mineralization induces bone health, which permits the skeleton to tolerate gravitation and extra loading. Bone health is specified not only by the mass of bone tissue and the microarchitectural structure but also by the rate of mineralization of the bone matrix [11]. Evidence proves the interconnection of PUFAs and bone health and growth. Feeding diets enriched with n-3 ALA significantly developed skeletal health in laying hens and biochemical proof recommends that improved bone turnover has enhanced the bone mechanical characteristics [12]. Administering omega-3 to poultry diets improved tibia and femur strength. Moreover, it had positively affected on joint disease and bone metabolism [12–15]. The growth and production performance of poultry are enhanced the supplementation of fatty acids or their sources.

Several studies have suggested vitamin D might play an essential role in regulating intestinal calcium metabolism [16, 17]. Estradiol applied to chicks has been announced to stimulate the creation of 1,25-dihydroxy vitamin D [1,25-(OH)₂D], the physiologically active form of vitamin D [18]. Secondary metabolites are a wide range of chemical substances in the form of natural products that do not directly participate in plant growth and development. Plant secondary metabolites like phenylpropanoids, terpenoids, and alkaloids play significant functions in plant durability under specific biological situations. In opposition with primary metabolites, secondary metabolites are often limited in distribution, and in many examples, a particular secondary metabolite is combined with specific taxonomic groups or a plant species. Medicinal plants have long been the basis of herbal drugs for the inhibition and remedy of different diseases and secondary metabolites are associated with the medicinal characteristics [19].

Some plant-derived natural, comprehensive quantities of estradiol are practiced in poultry diets. *Withania coagulans* relating to the Solanaceae family have considerable estradiol compounds. Steroidal compounds, alkaloids, saponins, terpenoids, saponins, glycosides, phenols, tannins, and Flavonoids are some of the secondary metabolites reported in WC [20]. Withanolides are steroidal lactones having C-26 and C-22 stereochemistry. WC fruits were previously identified as containing various compounds withanolide compounds [21]. An alcoholic root extract of the WS was added to the diet of aged laying hens for 130 mg/kg to improve calcium and phosphorus absorption in the tibia [22].

Therefore, the purpose of this experiment was to investigate the effects of the supplementation of *Withania coagulans* extracts (fruit and root) and type of oils (fish and linseed oil) on the growth performance, tibia bone characteristics, calcium and phosphorus content of tibia and serum, biochemical indicators of bone metabolism, and some blood parameter in broilers.

Material And Methods

Animals and experimental design

The Local Ethics Committee approved the experiment for Animal Experimentation at the Higher Education Complex of Saravan (Saravan, Sistan and Baluchestan, Iran). A total of 720 male Ross 308 broilers were randomly allocated into nine groups. Each treatment was replicated eight times with 10 chicks in a completely randomized design. The birds were provided a basal diet containing corn and soybean meal with two different supplements, oils and extracts. The dietary treatments were control, basal diet without supplementation; WCR, basal diet containing 200 mg/kg *Withania coagulans* root extract; WCF, basal diet containing 200 mg/kg *Withania coagulans* fruit extract ; FO, basal diet containing 2% of fish oil; LO, basal diet containing 2% linseed oil; WCR + FO, basal diet containing 200 mg/kg *Withania coagulans* root extract + 2% fish oil; WCF + FO, basal diet containing 200 mg/kg *Withania coagulans* fruit extract + 2% fish oil; WCR + LO, 200 mg/kg *Withania coagulans* fruit extract + 2% linseed oil; WCR + LO, 200 mg/kg *Withania coagulans* root extract + 2% linseed oil. All diets were formulated based on the NRC (1994). The ingredient of the basal, FO, LO diet, and nutrient level for the starter phase (1–21 d) and finisher phase (22–42 d) are exhibited in Table 1. The fatty acid combinations of the diets are shown in Tables 1. Chicks were kept in floor pens (1.2 × 1.6 m) with wood shavings in an environmentally managed room held at 34–36°C during d 1; after d 14, the temperature was gradually reduced to 26°C and was then maintained at room temperature through to the end of the trial. Water and experimental diets were provided *ad libitum* to the birds.

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

	Starter			Finisher		
Ingredients (%)	Control ^a	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	2.00	0.00	0.00	2.00	0.00
Fish Oil	0.00	0.00	2.10	0.00	0.00	2.00
Dical. Phos.	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 (MJ/kg)	12.55	12.55	12.55	13.43	13.43	13.43
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						

^aBroiler dietary treatments include: basal diet, basal diet supplemented with 2% fish oil (FO), 2% linseed oil (LO) and 200 mg of *Withania coagulans* root or fruit extract.

^bMineral premix contains iron 120 mg, Zn 100 mg, Mn 150 mg, Cu 20 mg, Mg 12 mg, Co 0.6 mg and Se 0.20 mg

^cVitamin premix contains 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.

^dn-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

	Starter			Finisher		
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
^a Broiler dietary treatments include: basal diet, basal diet supplemented with 2% fish oil (FO), 2% linseed oil (LO) and 200 mg of <i>Withania coagulans</i> root or fruit extract.						
^b Mineral premix contains iron 120 mg, Zn 100 mg, Mn 150 mg, Cu 20 mg, Mg 12 mg, Co 0.6 mg and Se 0.20 mg						
^c Vitamin premix contains 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*

The fruit and root of *Withania coagulans* were purchased from the herbal medicine store. Then, 300 g of powdered fruit and root were mixed with 50% ethanol in a container and placed soaking for 72 h. A rotary evaporator under vacuum (Laborota 4000, Heidolph, Germany) was used to extract, filter, and concentrate the soluble materials, and finally the residues were collected and used for the analysis and storage. Total Phenolic contents (TPC) and flavonoids contents were measured by Spectrometry method. Folin-Ciocalteu Reagent determined total phenols. The extracts of root and fruit were mixed in Gallic acid, sodium carbonate and Folin-Ciocalteu reagent. The spectrophotometer readings at 765nm after 15 min and at 725nm after 30 min were recorded. Total phenol values are expressed as Gallic acid equivalent (mg/g of dry mass), which is a standard reference compound. To determine total flavonoids content, the aluminium chloride colorimetric method was used for flavonoids determination. Root and fruit extracts in aqueous were separately mixed with methanol, aluminium chloride, potassium acetate, and distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a UV-Visible spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at concentrations of 12.5 to 100 g/ml in an aqueous (Mirakzahi et al. 2017; Azhar et al. 2020). The compounds of WC and WR are shown in Table 2.

Table 2
Total Phenolic and Flavonoids Contents of *Withania coagulans* fruit and roots extracts.

	TPC (mgGAE/ml)	TFC (mgQE/ml)
<i>Withania Coagulans</i> fruit	17.9 $\pm$ 0.85	1.48 $\pm$ 1.3
<i>Withania Coagulans</i> root	10.02 $\pm$ 0.49	1.02 $\pm$ 1.2
(n = 5) Values are expressed as means $\pm$ SD, TPC: Total Phenol Content, TFC: Total Flavonoids Content		

Sample collections

Bodyweight was recorded for separately replicate at 1, 21, and 42 d of age, and feed intake was determined over these periods to assess feed conversion. At 42 d of age, two chicks per replicate were randomly chosen and weighed after feed deprivation for 12 h. Blood samples were collected from broiler chickens on day 42. The serum was separated by centrifugation at $1500 \times g$ for 15 minutes and stored at -20°C until analyses. After the collection of blood samples, birds were sacrificed by cervical dislocation followed quickly by exsanguination. Then, the tibia bones were removed, cleared of surrounding soft tissues, and measured 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 [23].

Bone Density and Bone Mineral Content

Tibias were randomly selected for a three-point bending test using an Instron Universal Testing Machine (Model H5KS, Tinius Olsen Company). Ultimate bending strength values, which indicate the stiffness of callus, were calculated. Bone mineral content, bone density, and geometric parameters of the harvested tibia were measured by DXA. BMD was measured by dual-energy X-ray absorptiometry using a Hologic Discovery-W machine (Hologic, Inc. Waltham, MA, USA). Bone density measurement by DXA is based on projection bone density and is described as grams per squared centimeters. Tibia ash content was determined by ashing the bones in the oven for 18 h at 600°C [24]

Bone Strength: Breaking Bones

The meat was removed before the right tibia was tested for bone-breaking strength. According to [25], the bone-breaking strength was measured with a universal testing machine (model 441, Instron, Ltd. England). Right tibiae were measured for weight, diameter, and length. Averages of tibia diameters were taken at the narrowest and widest points. The rate of deformation was 5mm/min. The tracing of force was recorded at a steady rate. The graphs displayed plateau curves of maximal force (kg) reached to measure the energy stored in the bone.

Blood metabolites

Serum tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) were determined following methods administered by the diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum PGE2 was measured by an enzyme-linked immunosorbent assay using a commercial kit (Blue Gene Biotechnology Co. Ltd, Shanghai, China). It was 5.0% for the coefficient of variation between tests. Metabolites of blood samples were analyzed using an automatic blood chemical analyzer (Random Access Analyser A15, Biosystem Corp, Spain) for calcium and phosphorus total protein, glucose, albumin, and triglyceride and globulin levels according to the procedures of the manufacturer. The levels of cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides in serum were measured via reagent kits (Wako Pure Chemical Industries Ltd. Tokyo, Japan), following the manufacturer's instructions [26].

Statistical Analysis

All data were analysed using the one-way GLM procedure of SAS (2002) for the analysis of variance. It was used in a completely randomized design using male broiler chickens (Ross 308 strain). Significant differences among treatments were identified at a 5% level by Duncan's multiple range test.

Results

Broiler Performance

The impact of feeding different sources of extract and oil on the average daily gain, feed intake, and feed conversion ratio at 1–21 days and 22–42 days are presented in Table 3. Dietary treatments had no significant differences in feed intake. Bodyweight and body weight gain (BWG) increased at 1– 21 and 22–42 days with an adding level of PUFA sources of oil (FO) and *Withania coagulans* extracts fruit compared to the control group. Significantly higher ($P < 0.05$) superior feed

efficiency (1.92) were found in broilers fed dietary treatment WCF + FO at 22–42 days; However, no differences were observed between diets supplemented with oil and extracts.

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

	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	
WCF	38.49	940.40 ^{ab}	901.91 ^{ab}	1080.11	1.20	2780.80 ^{ab}	1840.40 ^a	3568	1.94 ^{ab}	
WCR	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	
WCF + FO	39.24	956.68 ^a	917.44 ^a	1096.25	1.19	2799.10 ^a	1842.42 ^a	3540	1.92 ^b	
WCR + FO	38.27	929.54 ^{ab}	891.27 ^b	1081.89	1.21	2764.20 ^b	1834.66 ^{ab}	3562	1.94 ^{ab}	
WCF + LO	38.98	933.62 ^b	894.63 ^b	1089.89	1.22	2756.70 ^{bc}	1823.08 ^b	3560	1.95 ^{ab}	
WCR + LO	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-c Within a column, means with different superscripts are significantly different (P < 0.05)										
WCF: <i>Withania coagulans</i> extracts fruit; WCR: <i>Withania coagulans</i> extracts root; FO: Fish oil, LO: Linseed oil; SEM: Standard error mean; BW: Body Weight; FI: Feed Intake; FCR: Feed Conversion Ratio										

Bone characteristics

Results regarding tibia growth and bone characteristics are shown in Table 4. The tibia weight of birds supplemented with FO and WCF 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 noted in the tibia of chickens fed with an unsupplemented diet, and the highest value of ultimate load (249.70 N) was observed in birds receiving WCF + FO diet. Similarly, the highest values of Stiffness (14.29 N × 104/m) and BMD (0.701 g/cm²) were obtained supplemented with a mixture of FO + WCF, while the lowest Stiffness (15.08 N × 104/m) and BMD (0.729g/cm²) were observed basal diet without supplement. The inclusion of *Withania coagulans* extracts fruit in diets led to an increase (Weight, Ultimate load, Stiffness) in tibia bone in comparison with diet containing *Withania coagulans* extracts root. 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 4

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

Dietary treatment	Weight (g)	Diameter (mm)		Length (cm)	Ultimate load (N)	Stiffness (N × 10 ⁴ /m)	BMD (g/cm ²)
Control	15.12 ^d	9.06		11.08	204.30 ^d	14.29 ^d	0.701 ^c
WCF	15.90 ^b	9.10	11.09	228.25 ^b	14.84 ^b	0.715 ^b	
WCR	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}	
WCF + FO	16.41 ^a	9.11	11.12	249.70 ^a	15.08 ^a	0.729 ^a	
WCR + FO	16.26 ^{ab}	9.08	11.20	236.80 ^{ab}	14.76 ^b	0.721 ^{ab}	
WCF + LO	15.97 ^b	9.06	11.14	234.25 ^{ab}	14.89 ^b	0.726 ^a	
WCR + LO	15.90 ^{bc}	9.09	11.13	230.50 ^b	14.65 ^c	0.722 ^{ab}	
SEM	0.087	1.3574	0.0942	0.7364	0.0323	0.0007	
P-Values	< .0001	0.09	1.20	< .0001	< .0001	< .0001	
a-c Within a column, means with different superscripts are significantly different (P < 0.05)							
WCF: <i>Withania coagulans</i> extracts fruit; WCR: <i>Withania coagulans</i> extracts root; FO: Fish oil, LO: Linseed oil; SEM: Standard error mean							

Biochemical indicators, Calcium, and phosphorus

The effects of different oil sources and extracts on calcium and phosphorus in serum and tibia are presented in Table 5. Diets containing fish oil supplemented with *Withania coagulans* root and fruit extract showed the highest amount of calcium in serum and tibia compared to other groups. No significant differences were observed in phosphorus concentration in serum or tibia at 42 days of age. The dietary treatments did not effect on the tibia ash of birds.

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

Dietary treatment	Serum		Tibia		Ash (%)
	calcium (mmol/l)	phosphorus (mmol/l)	calcium (mg/g)	phosphorus (mg/g)	
Control	2.80 ^c	2.49	38.84 ^c	18.92	43.12
WCF	3.12 ^a	2.52	39.17 ^b	19.20	43.33
WCR	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
WCF + FO	3.13 ^a	2.53	39.32 ^a	19.20	43.59
WCR + FO	3.12 ^a	2.50	39.28 ^a	19.12	43.48
WCF + LO	3.10 ^a	2.49	39.16 ^b	19.10	43.34
WCR + LO	3.06 ^a	2.45	39.14 ^b	19.08	43.29
SEM	1.9536	0.0026	0.0893	2.4527	1.4729
P-Values	< .001	2.14	< .001	0.08	1.12
a-c Within a column, means with different superscripts are significantly different (P < 0.05)					
WCF: <i>Withania coagulans</i> extracts fruit; WCR: <i>Withania coagulans</i> extracts root; FO: Fish oil, LO: Linseed oil; SEM: Standard error mean					

Effects of *Withania coagulans* extracts (fruit and root) and type of oils (fish and linseed oil) on biochemical indicators of bone metabolism in serum summarized in Table 6. The inclusion of fish and linseed oil in the bird's diet showed a decrease in serum PGE2 concentration compared to other groups (P < 0.05). The addition of the extract did not show a significant effect on the serum concentration of PGE2 (P > 0.05). Birds fed with linseed oil or fish oil, as well as 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 in all experimental groups (P > 0.05).

Table 6

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

Dietary treatment	Item		
	PGE2 (mg/ml)	ALP (U/L)	TRAP (U/I)
Control	5.32 ^a	112.24 ^b	5.51
WCF	5.21 ^a	113.67 ^b	5.40
WCR	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
WCF + FO	4.80 ^b	133.87 ^a	5.42
WCR + FO	4.81 ^b	129.50 ^a	5.43
WCF + LO	4.90 ^b	133.87 ^a	5.25
WCR + LO	4.87 ^b	124.75 ^a	5.37
SEM	0.052	0.254	0.654
P-Values	< .0001	1.70	0.09
a-c Within a column, means with different superscripts are significantly different (P < 0.05)			
WCF: <i>Withania coagulans</i> extracts fruit; WCR: <i>Withania coagulans</i> extracts root; FO: Fish oil, LO: Linseed oil; SEM: Standard error mean			

Blood parameters

Data presented in Table 7 shows the effects of diet supplementation *Withania coagulans* extracts and type of oils on blood parameters at 42 days of age. Blood amounts of cholesterol were decreased ($P < 0.05$) in the diet containing *extracts and oils* treatments compared to control. The lowest level of serum glucose was observed in birds receiving *Withania coagulans extracts*. No significant differences were observed in the total protein, Albumin, and LDL serum of birds at 42 days of age. The content of HDL content in broilers whose diet was supplemented only with oil tended to increase compared to the control group, although this difference was observed only numerically. Also, lower numerical triglycerides are observed in the diet containing oils and extracts than in the control diet. an the control group ($P < 0.05$). No significant difference was observed in serum TRAP levels in all experimental groups ($P > 0.05$).

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

Dietary treatment	Item							
	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 ^c	0.32	1.90	0.80	12.20 ^b
WCF	34.20	16.01	11.90	3.12 ^b	0.28	1.89	0.82	11.300 ^a
WCR	34.12	16.06	11.95	3.11 ^b	0.28	1.91	0.81	11.28 ^a
FO	34.04	15.95	11.98	2.80 ^a	0.26	1.87	0.88	12.10 ^b
LO	34.00	16.07	12.00	2.88 ^a	0.27	1.91	0.85	12.11 ^b
WCF + FO	34.18	16.08	11.89	2.73 ^a	0.25	1.92	0.91	11.32 ^a
WCR + FO	34.15	16.03	11.93	2.81 ^a	0.25	1.90	0.92	11.31 ^a
WCF + LO	34.16	16.04	12.02	2.86 ^a	0.29	1.89	0.94	11.40 ^a
WCR + LO	34.15	15.97	11.96	2.84 ^a	0.30	1.88	0.92	11.29 ^a
SEM	2.2541	0.7351	1.2967	0.0624	1.4261	0.0651	0.079	0.0235
P-Values	0.075	1.54	0.29	< .0001	0.19	0.85	0.09	< .0001
a-c Within a column, means with different superscripts are significantly different (P < 0.05)								
WCF: <i>Withania coagulans</i> extracts fruit; WCR: <i>Withania coagulans</i> extracts root; FO: Fish oil, LO: Linseed oil; SEM: Standard error mean; HDL: high density lipoprotein; LDL: Low Density Lipoprotein								

Discussion

Higher BW and BWG were observed in birds fed different combinations of n-3 PUFA diet with *Withania coagulans* extracts. Also, chickens fed with oils and extracts alone showed better performance than the control. Better performance was reported in chickens fed diets containing n-3 PUFA sources than in chickens fed a saturated fat diet [27, 28]. Wang et al. [29] illustrated that the effect of n-3 PUFA in broiler diet was associated with their dietary level since low levels of dietary fish oil are more useful than high levels in increasing performance and feed gain. This study contradicted the results of Kalakuntla et al. [30] whose investigation showed that dietary supplementation with soy oil, linseed oil, and fat oil did not affect the performance of broilers.

So far, numerous studies have been performed using herbs, spices, and extracts and have shown conflicting results in chicken performance. However, some studies have shown that plant compounds have positive effects on body weight gain and FCR in chickens [6, 31]. Others reported either an improved chicken body weight gain without affecting FCR, which is in line with our findings. Previous chemical studies of this plant have identified several withanolides in the fruit and roots of the WC [32, 33]. Azhar et al. [34] revealed the presence of secondary metabolites such as phenols and flavonoids in all parts of WC and stated that its extract may be an important source of vital natural antioxidants. Both parts of root and fruit

extracts had potent antioxidant activity, but the study data showed that fruit extract has higher bioactive substances than the root (Table 2).

Polyunsaturated fatty acids are prone to oxidation because they are the first target for a free radical attack at the commencement of peroxidation [35]. On the other hand, the inclusion of n-3-containing compounds (such as fish and flaxseed oil) in chicken diets increases the content of the long-chain n-3 PUFA and the susceptibility of meat lipids to oxidation [36]. El-Samee et al [35] reported that the addition of 2 g sweet chestnut tannins/kg in the diet containing fish and linseed oil as antioxidant sources inhibited lipid oxidation and enhanced antioxidant activity value in a broiler. 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 [37]. 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, tibia growth and bone characteristics such as weight, ultimate load, stiffness, and bone mineral density (BMD) were significantly increased in birds fed with FO, LO, and WC extract compared to the control group. In several animal investigations, n-3 fatty acids or a low ratio of n-6 to n-3 fatty acids has revealed a positive influence on bone characteristics [38]. Studies have demonstrated that various rates of n-6 to n-3 fatty acids was altered the synthesis of prostaglandin and insulin-like growth factor I [39]. High levels of n-6 fatty acids increased prostaglandin E2 (PGE2) production [40, 41] and supplementation with n-3 fatty acids or lower ratios of n-6 to n-3 fatty acids improve calcium transportation [42], and calcium retention [43]. The results of this study on PGE2 obtained by supplementing fish and linseed oil in poultry diets confirm the findings (Table 1).

Bone mineral density (BMD) exhibits the bone mineral content per unit mass of bone material significantly affecting the bone strength. This biomarker is of importance as improved bone strength could contribute more support to the rapid growth of broilers. Rats fed diets with 40 g/kg n-3 PUFA illustrated a 5.2% improvement in femur BMD compared to control [44]. Subsequently, the bone biomechanical response is likewise an indicator of bone strength. In line with the current findings, Tarlton et al. [45] 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 additionally announced stronger, tougher, and harder bones as measured through bone-breaking strength in the tibia from n-3 PUFA group. According to earlier findings, in growing Japanese quails fed supplements containing 2% fish oil and linolenic acid, their tibia breaks broke stronger than those fed unsupplemented fish oil and linolenic acid.

The current study showed that the fruit and roots of the WC extract improved bone parameters. These results are consistent with the results of Hosseini et al. [46] that in their studies it has been stated that increasing 100 g of WC fruit extract developed some characteristics of the tibia bone in broilers. WC has several therapeutic effects including anti-inflammatory, anticancer, chemoprotective, immunomodulatory, hepatoprotective, antifungal, and antibacterial, as well as cardiovascular and central nervous system activities [47, 48]. The chemistry of WC has been completely studied and different groups of chemical ingredients such as steroidal lactones, alkaloids, flavonoids, and tannin have been recognized, extracted, and isolated [48]. The main chemical constituents of the plant, withanolide, and withaferin A (WFA) are largely restricted in fruit and roots [49]. Oral administration of WFA to osteopenic ovariectomized mice increased osteoprogenitor cells within the bone marrow and developed the expression of osteogenic genes. 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 TNF α), and expression of skeletal osteoclastogenic genes. It also increased new bone formation and expression of osteogenic genes in the femur bone [50, 51]. 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 [52]. Several other feeding studies reported the same effective association between bone mineral content and n-3 PUFA in growing animals [53, 54]. Japanese quail supplemented with n-3 PUFA showed higher levels of bone mineral in the tibia than controls [55]. However, some studies

report no effect of n-3 PUFAs on bone minerals in mice or birds, regardless of whether they are included in diets [56]. According to a study feeding pullet breeders either DHA, ALA, or a standard control diet, the offspring produced by the mothers fed 1% DHA increased in tibia ash weight and percentage [57]. It may be possible to apply these conclusions in broiler nutrition to support bone strength in broiler chicks. No significant difference in the amount of ash of different chickens fed with n-3 dietary sources was found in this experiment, which contradicts these results. Hosseini et al. [46] 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 following the report of Mirakzehi et al. [33] 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 [58]. Improvement in calcification tibia (Ca) may be due to the presence of these compounds in the extract.

In the present study, it was 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 [59]. It has been derived from AA 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 [60, 61]. Supplementing the diet with n-3 fatty acids through fish oil and chemical compounds in the extract may have improved Bone characterized and calcium levels in serum and tibia by altering biochemical indicators of bone metabolism in serum.

Hosseini et al. [46] Showed that adding *Withania* coagulans extracts to broiler diets did not affect serum ALP activity. These results are consistent with our results, as well as the study of Tahmasbi et al. [62] who used different levels of *Withania* coagulans in laying poultry diets. Among the markers of bone regeneration, ALP is considered one of the markers of bone formation that indicates osteoblastic activity [63]. While TRAP is considered as one of the markers of bone resorption that reflects osteoclastic activity [64]. Increasing the amount of ALP indicate a beneficial effect of PUFA on hepatic cell membrane integrity that might be due to enriched phospholipids as an essential part of cell membrane integrity containing two hydrophobic LCFA [65]. Reports of *Withania* coagulans effects on TRAP have not yet been found in broiler.

The herbal plant used in this study is known to have antioxidant properties. It has been reported that adding to the diet increases insulin secretion and decreases glucose levels [32, 66]. Gorelick et al. [67] reported the withaferin-A from *W. somnifera* that significantly changes the serum glucose level. Various studies exhibited direct influences of glucose on the bone cells: osteoblasts, osteoclasts, and osteocytes [68]. 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 [69, 70] Compared to control treatments and extracts, fish oil and linseed oil decreased total cholesterol. It was reported that high cholesterol levels increase osteoporosis by inhibiting osteoblasts [71].

Conclusions

In conclusion, feeding a combination of fish oil and fruit extracts of *Withania coagulans* were enhanced bone characteristics (Weight, Ultimate load, Stiffness), increased calcium levels in serum and tibia, reduced total cholesterol, and had a positive influence on growth performance in broiler. Therefore, the inclusion of mixed *Withania coagulans* fruit extract and fish oil in the diet may have positive effects on the tibia and bone quality of broilers.

Declarations

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Author Contribution

All the authors contributed to the experimental design and performed experiments and also contributed to data analysis, interpretation of the results, and drafting of the manuscript. The main responsible for the drafting of the Manuscript was Hassan Saleh.

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Data Availability Statement

The data that support this study will be shared upon reasonable request to the corresponding author.

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