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The effects of phytase and root hydroalcoholic extract of *Withania somnifera* on productive performance and bone mineralisation of laying hens in the late phase of production

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Abstract 1. A 6-week study was conducted to investigate the effects of phytase and hydroalcoholic extract of *Withania somnifera* root (WS) on productive performance and bone mineralisation of laying hens in the late phase of production.

2. Diets were arranged factorially ($3 \times 2 \times 2$) and consisted of a positive control with adequate Ca (4·37%) and nonphytate P (NPP; 0·39%) and a negative control diet with Ca (4·06%) and NPP (0·36 %); three concentrations of *Withania somnifera* (0, 65 and 130 mg/kg diet); and two concentrations of microbial phytase (0 and 300 U/kg diet).

3. A total of 144 72-week-old Hy-Line W36 laying hens were randomly assigned to the 12 treatment groups. Each treatment was replicated 4 times (4 x 3 hens). Egg production and egg weight were recorded daily, while feed intake and egg quality traits were recorded every two weeks. Bone quality traits were evaluated at the end of experiment.

4. *Withania somnifera* supplementation increased egg production and lowered egg weight only in the second two weeks of the experiment. Addition of phytase significantly depressed specific gravity of the eggs for the entire experiment period. No dietary treatment effects were observed on egg shell thickness and yolk weight.

5. *Withania somnifera* at 130 mg/kg did not affect feed intake. The hens fed on the positive control diet had higher albumen weight than the negative control diet in the second two-week period. Supplementation of the positive control diet with 65 mg/kg *Withania somnifera* in the absence of phytase significantly improved shell weight compared with the negative control (5·779 vs. 5·273 g respectively).

6. Supplementing *Withania somnifera* significantly improved Ca and P retention in tibia bone. In addition, an increase in tibia bone P was observed with phytase supplementation. There were significant interactions between *Withania somnifera* content and phytase for tibia bone Ca and P.

7. The results of this experiment indicated that dietary *Withania somnifera* has beneficial effects on tibia bone Ca and P content, and phytase improved tibia bone P retention without adverse effects on productive performance.

INTRODUCTION

Most of the phosphorus in feedstuffs of plant origin is in the form of phytate P (Carlos and Edwards, 1998). Bound P in phytate is poorly available to birds because of the lack of endogenous phytases (Van der Klis *et al*., 1997). On the other hand, phytic acid can form insoluble salts with Ca^{2+}, potentially rendering Ca unavailable for intestinal absorption. Phytase has the ability to release Ca^{2+} from the insoluble salts and make it available for absorption (Kornegay *et al*., 1996). Studies have demonstrated that addition of phytase improves performance, and Ca and P

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digestibility, in layers fed on a maize and soyabean-based diet (Lim *et al*., 2003; Panda *et al*., 2005; Wu *et al*., 2006). Gordon and Roland (1998) reported that phytase supplementation of Ca-deficient diets significantly increases Ca utilisation. Similarly, Lim *et al*. (2003) found that supplementation of microbial phytase (300 U/kg) in the diet of laying hens can improve egg production and decrease the number of broken and soft eggs. In addition, the effects of phytase supplementation are significantly modified by the concentrations of Ca and nonphytate P (NPP). By contrast, Snow *et al*. (2003) reported that phytase supplementation of diets based on maize and soyabean with 0·25% NPP and 3·55% Ca, did not affect hen performance. Several investigators reported that aged hens were less efficient in absorbing Ca than younger ones (Grobas *et al*., 1999*a*; Al-Batshan *et al*., 1994). Also, Scheideler and Sell (1987) presented evidence that utilisation of phytate P was reduced with the increasing age of the hens. However, aged birds were capable, when stimulated, of producing 1α,25-dihydroxycholecalciferol [1α,25$(OH)_2D_3$] or absorbing Ca at rates similar to those of young birds (Bar and Hurwitz, 1987). Additionally, they reported that the decline in kidney 25-Hydroxyvitamin D_3 1-alpha-hydroxylase (VD3 1A hydroxylase) (E.C.1.14.13.13) and plasma 1,25$(OH)_2D_3$ with age, could have been due to the lower egg production rate of the older hens, because a decline in egg shell production is associated with decreased production of 1,25$(OH)_2D_3$. Osteoporosis in laying hens is defined as a decrease in the amount of fully mineralised structural bone, leading to increased fragility and susceptibility to fracture (Whitehead and Fleming, 2000). In the old laying hen, the phenomenon probably develops because of a high demand for calcium for eggshell formation. With the loss or qualitative changes of the medullary bone as an effective calcium reserve, there is considerable impairment in eggshell quality in laying hens more than 500 days old (Abe *et al*., 1982).

Withania somnifera L., a member of the *Solanaceae* family, is well known for its medicinal uses. It is an annual herb, and several pharmacological activities of the plant have been attributed in general to substances in the roots. The medicinal properties of this plant are associated with certain steroidal alkaloids and steroidal lactones in a class of constituents called withanolides (Jayapraksam *et al*., 2004; Khan *et al*., 2009). Withanolides are oestrogenic compounds and it is possible that their oestrogenic nature, similar to that of phytosterols, accounts for their anti-osteoporotic activity (Nagareddy and Lakshmana, 2006). Nagareddy and Lakshmana (2006) investigated the potential beneficial effects of an alcoholic extract of WS root (65 mg/kg body weight) on osteoporosis caused by ovariectomy and concurrent calcium deficiency in rats. They found that WS treatment improved the tibial bone strength, prevented bone loss and markedly increased the ash weight, ash calcium and phosphorus concentrations. Therefore, the aim of this study was to determine: (1) the efficacy of *Escherichia coli*-derived phytase, and potential effects of WS using either low-Ca, low-NPP or conventional diets on the productive performance, egg quality, and bone mineralisation of aged laying hens; and (2) The interaction between Ca and NPP, phytase and WS concentrations in the diet on the productive performance, egg quality, and bone mineralisation of aged laying hens.

MATERIALS AND METHODS

Preparation of *withania somnifera* extract

The roots of WS were collected during November from Saravan, Sistan and Baluchestan, Iran. The root was identified and authenticated at the Herbarium of Botany Directorate in Ferdowsi University of Mashhad. The root was washed, air-dried and ground to a fine powder. The finely ground root powder was extracted with 50% ethanol in a rotary evaporator (Laborota 4000, Heidolph, Germany) at 50°C to obtain a semi dry extract. The aqueous extract was then freeze-dried for 24 h. Dried extracts were placed in a bottle, stoppered and then stored at −20°C until used.

Husbandry and experimental diets

All experimental procedures used in this experiment were approved by the Animal Care Committee of the Ferdowsi University of Mashhad. A total of 144 72-week-old Hy-Line W36 layers were allocated randomly into 12 treatments, with 4 replicates per treatment and three layers per replicate. All birds were housed in three-tiered cages and offered feed and water *ad libitum*. Room temperature was kept at 21°C, and the light programme consisted of 16 h light daily throughout the experiment. Based on two weeks of pre-experimental egg production, three days pre-experimental egg weight, and three days pre-experimental egg specific gravity and body weight, the treatment means for these traits were kept similar ($P > 0{\cdot}05$) at the start of the experiment. Birds were randomly assigned to experimental diets and fed to 78 weeks of age. The experimental diets were formulated to have similar AME_N and crude protein (Table 1). Nutrient concentrations of the positive control diet met the requirements suggested in the

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

Ingredient	Negative control (g/kg)		Positive control (g/kg)	
Maize	620·6	620·6	601·9	601·9
Soyabean meal	198·7	198·7	202·4	202·4
Barley	40·0	40·0	40·0	40·0
Methionine	1·2	1·2	1·1	1·1
Sodium chloride	3·8	3·8	3·8	3·8
Limestone	97·4	97·4	104·7	104·7
Dicalcium phosphate	13·5	13·5	14·8	14·8
Vitamin premix[2]	2·5	2·5	2·5	2·5
Mineral premix[3]	2·5	2·5	2·5	2·5
Vegetable oil	19·5	19·5	26·0	26·0
Phytase (U/kg)[4]	0	300	0	300
Calculated nutrients and energy				
AME, (MJ/kg)	11·71	11·71	11·71	11·71
Crude protein (g/kg)	145·6	145·6	145·6	145·6
Lysine (g/kg)	7·4	7·4	7·4	7·4
Methionine (g/kg)	3·6	3·6	3·5	3·5
TSAA (g/kg)	6·1	6·1	6·0	6·0
Calcium (g/kg)	40·6	40·6	43·7	43·7
Nonphytate P (g/kg)	3·6	3·6	3·9	3·9
Total P (g/kg)	5·6	5·6	5·8	5·8
Analysed nutrient concentration	41·3	41·3	44·5	44·5
Calcium (g/kg)	5·9	5·9	6·3	6·3
Total P (g/kg)				

[1]Each diet was supplemented with 0, 65 and 130 mg/kg root hydroalcoholic extract of *Withania somnifera* (WS).
[2]Vitamin premix provided per kilogram of diet: vitamin A (retinyl acetate), 8800 IU; cholecalciferol, 2200 IU; DL-*α*-tocopheryl acetate, 11 IU; menadione sodium bisulphate, 2·2 mg; riboflavin, 4·4 mg; D-calcium pantothenate, 8·8 mg; nicotinic acid, 44 mg; pyridoxine hydrochloride, 2·2 mg; d-biotin, 0·11 mg; thiamine hydrochloride, 2·5 mg; ethoxyquin, 125 mg.
[3]Mineral premix provided per kilogram of diet: $MnSO_4$: H_2O, 185 mg; ZnO, 62 mg; $FeSO_47H_2O$, 149 mg; $CuSO_4$: $5H_2O$, 19·6 mg; KI, 1·4 mg; Na_2SeO_3, 0·22 mg.
[4]Phyzyme XP 5000, Danisco Animal Nutrition.

Hy-Line W-36 Commercial Management Guide (Hy-Line International, 2009–2011). The negative control diet was formulated as for the positive control diet, but with Ca and NPP concentrations reduced by 7·09 and 7·69%, respectively. The 12 experimental diets were factorially arranged as three concentrations of WS extract (0, 65 and 130 mg/kg diet), two concentrations of phytase (0 and 300 U/kg diet) and two types of diet, negative and positive control. The phytase product (Phyzyme XP) contained a minimum phytase activity of 5,000 phytase units (FTU)/g (EC 3·1·3·26) and was derived from *E. coli* and expressed in *Schizosaccharomyces pombe*.

Productive performance and egg quality

Egg production, egg weight, and number of broken and soft-shell eggs were recorded daily, whereas feed intake was recorded every two weeks. Egg weight and specific gravity were determined on all eggs produced during a 4-d period at the end of each two weeks. The eggs from d 1 and 2 were used for measurement of egg weights and egg components, whereas eggs from d 3 and 4 were used for measurement of specific gravity. The specific gravity was determined according to the Archimedes method (Hempe *et al.*, 1988).

Three eggs from each replication in each treatment were randomly used to determine the mass of the main egg components (shell, yolk, and albumen). The eggs were broken on a flat surface. The yolks were separated from the albumen, rolled on a paper towel to remove adhering albumen and weighed. The shells were carefully washed with warm water and dried at 60°C overnight in a drying oven and then weighed. All records were summarised bi-weekly. The shell thickness was measured at three locations on the egg (air cell, equator, and sharp end) using a digital micrometer with an accuracy of $\pm 1\,\mu m$ (series 500, Mitutoyo, Tokyo, Japan) and was averaged. The shell weight per unit of surface area (SWUSA) was calculated by dividing the dried shell weight by the surface area of each egg. The surface area of the egg was calculated as indicated by Ousterhout (1980).

Bone quality

On the last day of the experiment, one reproductively active hen from each replicate was killed by cervical dislocation, and the left tibia removed for subsequent analysis. The bones were

Table 2. *Effect of* Withania somnifera *(WS), Phytase and type of Control diet on egg production, and egg weight*

Treatment			Hen-day egg production (%)[1]				Egg weight(g)			
WS (mg/kg)	phytase (U/kg diet)	Control	72 to 74 weeks	74 to 76 weeks	76 to 78 weeks	72 to 78 weeks	72 to 74 weeks	74 to 76 weeks	76 to 78 weeks	72 to 78 weeks
0	0	−	67·85^{ab}	76·78	75·926	73·523	66·11	66·69	66·78	66·53^{ab}
0	0	+	75·00^{ab}	79·16	73·14	75·77	64·90	64·87	67·36	65·71^{ab}
0	300	−	70·83^{ab}	73·81	63·88	69·51	67·16	67·31	70·45	68·30^{ab}
0	300	+	75·00^{ab}	82·14	75·92	77·69	67·56	67·19	71·36	68·70^{a}
65	0	−	72·61^{ab}	76·19	75·92	74·91	66·68	67·96	67·01	67·22^{ab}
65	0	+	64·88^{b}	69·64	66·66	67·06	67·82	69·24	68·38	68·45^{a}
65	300	−	74·40^{ab}	71·42	72·22	72·68	68·45	67·86	67·93	68·08^{ab}
65	300	+	67·85^{ab}	76·19	72·22	72·09	66·91	67·08	67·46	67·15^{ab}
130	0	−	76·19^{a}	80·35	75·00	77·18	65·20	64·08	65·74	65·01^{b}
130	0	+	70·83^{ab}	79·76	75·00	75·19	67·72	67·63	68·66	68·00^{a}
130	300	−	69·64^{ab}	79·76	72·22	73·87	66·91	67·08	71·86	68·62^{a}
130	300	+	73·21^{ab}	80·95	75·00	76·38	66·16	65·22	67·53	66·30^{ab}
SEM			3·245	3·669	5·466	3·567	1·301	1·428	2·184	1·224
Main effect										
WS (mg/kg)	0		72·17	77·97^{ab}	72·22	74·12	66·43	66·51^{a}	68·99	67·31
	65		69·94	73·36^{b}	71·75	71·68	67·46	66·03^{ab}	67·70	67·73
	130		72·48	80·20^{a}	74·30	75·66	66·50	66·00^{b}	68·45	66·98
Phytase(U/kg diet)	0		71·23	76·98	73·61	73·94	66·41	66·74	67·32	66·82
	300		71·82	77·38	71·91	73·70	67·19	66·96	69·43	67·86
Control	−		71·92	76·38	72·53	73·61	66·75	66·83	68·30	67·29
	+		71·13	77·97	72·99	74·03	66·85	66·87	68·46	67·39
P										
WS			0·295	0·028	0·298	0·336	0·587	0·043	0·949	0·454
Phytase			0·842	0·611	0·584	0·959	0·329	0·747	0·262	0·193
Control			0·939	0·293	0·884	0·569	0·585	0·947	0·581	0·337
WS × Phytase			0·561	0·973	0·856	0·718	0·355	0·285	0·718	0·511
WS × Control			0·046	0·438	0·614	0·267	0·704	0·524	0·955	0·803
Phytase × Control			0·694	0·145	0·336	0·209	0·396	0·153	0·146	0·187
WS × Phytase × Control			0·520	0·582	0·713	0·908	0·227	0·102	0·262	0·038

[1]Hen-day egg production = (100 × number of eggs laid)/(number of hens × days).
[a-b]Means within each column with no common superscript differ ($P < 0·05$).

cleaned of attached tissue. Tibia bones were oven-dried at 103°C for 24 h, and then fat was extracted for 48 h in diethyl ether, the bones dried again, and the dry-defatted weight was calculated (Cheng and Coon, 1990). Tibias were ashed at 600°C for 36 h and the weight of ash, and percentage of Ca and P, of the defatted tibia were determined.

Chemical analysis

After ashing, the concentrations of Ca and P in samples were determined by atomic absorption spectrophotometry (Varian SpectrAA 50B Atomic Absorption Spectrometer: Varian Ltd, USA) according to AOAC (2005) procedures (method 927·02). P concentrations were determined colorometrically using the molybdovanadate method (AOAC, 2005 method 965·17).

Statistical analysis

All data were analysed according to a completely randomised design with a $3 \times 2 \times 2$ factorial arrangement. The General Linear Models of SAS (SAS Institute, 2003) were used to analyse all the data. Differences among treatment means were measured by Duncan's multiple range and considered significant at $P < 0·05$.

RESULTS AND DISCUSSION

Productive performance

The effects of different dietary treatments on egg production and egg weight are presented in Table 2. In the first two weeks of the experiment, a WS concentration x control interaction ($P < 0·05$) was observed. The effect of WS on egg production was more evident in hens fed on 130 mg/kg WS and the negative control diet. There was a significant difference ($P < 0·05$) between birds given this treatment, and those fed on the positive control diet supplemented with 65 mg/kg WS. However, other dietary treatments had no effect on egg production during this period. From 74 to 76 weeks of age (second two weeks), hens fed on 130 mg/kg WS

Table 3. *Effect of* Withania somnifera *(WS), phytase and type of Control diet on feed intake and feed conversion*

Treatment		Feed intake(g/day)				Feed conversion(g/100 g egg mass)			
Main effect		72 to 74 weeks	74 to 76 weeks	76 to 78 weeks	72 to 78 weeks	72 to 74 weeks	74 to 76 weeks	76 to 78 weeks	72 to 78 weeks
WS (mg/kg)	0	138·72	117·60	114·34	123·55	2·91	2·27	2·33	2·51
	65	138·86	115·98	113·53	122·79	2·97	2·34	2·36	2·56
	130	141·54	120·89	120·90	127·78	2·94	2·29	2·43	2·55
SEM		2·978	2·590	3·133	206·62	0·082	0·069	0·101	0·072
Phytase(U/kg diet)	0	138·09	116·18	113·24	122·50	2·93	2·27	2·32	2·51
	300	141·337	120·13	119·28	126·91	2·95	2·34	2·43	2·57
SEM		2·432	2·115	2·558	2·174	0·067	0·056	0·083	0·059
Control	−	138·43	116·80	115·48	123·57	2·89	2·30	2·38	2·53
	+	140·99	119·51	117·03	125·84	2·99	2·31	2·37	2·55
SEM		2·432	2·115	2·558	2·174	0·067	0·056	0·083	0·059
P									
WS		0·985	0·402	0·203	0·528	0·937	0·809	0·908	0·859
Phytase		0·563	0·195	0·103	0·219	0·763	0·360	0·100	0·429
Control		0·662	0·370	0·671	0·486	0·683	0·814	0·984	0·938

had significantly ($P < 0·05$) higher egg production than those fed on 65 mg/kg WS (80·20 vs. 73·36%, respectively). Supplementation of phytase did not affect egg production. Egg production during the third period and during the entire experiment was not influenced by dietary concentrations of WS, regardless of type of control diet. Some investigators observed that egg production and egg mass were significantly improved when Leghorn pullets and female quails were treated with oestradiol (El-Afifi and Abu Taleb, 2002; and Hamdy *et al*., 2002), but no effect was found in this study. Hens fed on a diet supplemented with the highest WS concentration (130 mg/kg) had significantly ($P < 0·05$) lower egg weight than those fed on the diet without WS during 74 to 76 weeks of age. However, hens fed on diets supplemented with WS or phytase did not show significant differences from those fed on non supplemented diets, whether on Ca and NPP deficient diets, or diets with adequate concentrations of them, throughout the period of the experiment. Interaction of the WS, phytase and control was also significant ($P < 0·05$) over the entire period of the experiment. It is assumed that decreased egg weight from feeding 130 mg/kg WS from 74 to 76 weeks of age is related to a simultaneous increase in egg production. Birds that are laying regularly will have continuously high oestrogen, whereas birds laying very few eggs are likely to have long periods with low levels of oestrogen (Whitehead, 2004). These findings are in accordance with those of Bar and Hurwitz (1987), who reported that 20-month-old birds produced significantly fewer eggs with higher weight.

The interaction between dietary treatments was not significant for feed intake and feed conversion; therefore only the main effects are presented (Table 3). Feed intake and feed conversion ratio were not significantly different among birds fed on different concentrations of WS and phytase, in both deficient and adequate Ca and NPP diets.

Throughout the production experiment, phytase supplementation at 300 U/kg significantly decreased specific gravity of the eggs (Table 4). There were interactions of WS × phytase × control in the 72 to 74 week and 74 to 76 week periods. The highest value of specific gravity was obtained in hens fed on the positive control diet supplemented with 65 mg/kg WS. The effects of the experimental treatments were not significant on egg shell thickness. Percentage of broken and soft eggs was not influenced by dietary treatments (data not shown). Determination of specific gravity is an appropriate index because it is related to shell thickness and $CaCO_3$ deposition. Keshavarz (2000) also observed that the main effect of phytase on specific gravity was significant, as it was lower in the presence of phytase in the diet during the 30 to 42, and 54 to 66 week periods, respectively. In contrast, Gordon and Roland (1998) reported that phytase supplementation in diets with low NPP (0·1%) improved specific gravity. Lim *et al*. (2003) found that supplementation of phytase in hens at 21 to 41 weeks of age did not affect specific gravity. Also, no phytase supplementation effect on specific gravity was observed by Boling *et al*. (2000) during the 20 to 70 week period, when maximum concentrations of NPP in diets were 0·2%. In this experiment, the minimum concentration of NPP was 0·36% and probably adequate for support of performance. Specific gravity of the eggs of the birds fed on both diets regardless of Ca and NPP concentrations was inversely affected by phytase

Table 4. *Effect of* Withania somnifera *(WS), phytase and type of Control diet on specific gravity and egg shell thickness*

Treatment			Specific gravity				Egg shell thickness(mm)			
WS (mg/kg)	Phytase (U/kg diet)	Control	72 to 74 weeks	74 to 76 weeks	76 to 78 weeks	72 to 78 weeks	72 to 74 weeks	74 to 76 weeks	76 to 78 weeks	72 to 78 weeks
0	0	−	1·073[a]	1·071[ab]	1·054	1·066	0·403	0·392	0·371	0·389
0	0	+	1·069[ab]	1·068[ab]	1·051	1·063	0·398	0·373	0·379	0·383
0	300	−	1·068[ab]	1·065[b]	1·051	1·062	0·391	0·386	0·373	0·383
0	300	+	1·068[ab]	1·068[ab]	1·049	1·062	0·392	0·380	0·378	0·383
65	0	−	1·067[ab]	1·064[b]	1·051	1·061	0·381	0·376	0·348	0·368
65	0	+	1·074[a]	1·074[a]	1·048	1·066	0·404	0·402	0·373	0·393
65	300	−	1·068[ab]	1·067[ab]	1·048	1·061	0·407	0·384	0·375	0·389
65	300	+	1·066[b]	1·066[ab]	1·049	1·060	0·396	0·368	0·385	0·383
130	0	−	1·069[ab]	1·069[b]	1·053	1··064	0·392	0·403	0·370	0·389
130	0	+	1·070[ab]	1·068[ab]	1·049	1·062	0·414	0·386	0·367	0·389
130	300	−	1·063[b]	1·065[b]	1·042	1·057	0·388	0·390	0·340	0·373
130	300	+	1·069[ab]	1·065[b]	1·047	1·060	0·389	0·367	0·359	0·372
SEM			0·0015	0·0014	0·0016	0·0011	0·0099	0·0087	0·0079	0·0063
Main effect										
WS (mg/kg)	0		1·070	1·068	1·051	1·063	0·396	0·383	0·375	0·385
	65		1·069	1·068	1·049	1·062	0·397	0·382	0·370	0·383
	130		1·068	1·067	1·048	1·061	0·396	0·387	0·359	0·381
Phytase(U/kg diet)	0		1·071[a]	1·069[a]	1·051[a]	1·064[a]	0·399	0·389	0·368	0·385
	300		1·067[b]	1·066[b]	1·048[b]	1·060[b]	0·394	0·379	0·368	0·380
Control	−		1·068	1·067	1·050	1·062	0·394	0·389	0·363	0·382
	+		1·069	1·068	1·049	1·062	0·399	0·379	0·373	0·384
P										
WS			0·450	0·755	0·229	0·190	0·988	0·865	0·133	0·809
Phytase			0·008	0·040	0·025	0·002	0·546	0·194	0·966	0·374
Control			0·420	0·388	0·355	0·668	0·518	0·207	0·119	0·681
WS × Phytase			0·968	0·881	0·583	0·756	0·457	0·619	0·067	0·237
WS × Control			0·214	0·403	0·846	0·386	0·787	0·361	0·745	0·600
Phytase × Control			0·999	0·706	0·101	0·589	0·328	0·410	0·935	0·397
WS × Phytase × Control			0·030	0·034	0·401	0·058	0·569	0·295	0·489	0·337

[a-b]Means within each column with no common superscript differ ($P < 0·05$).

supplementation. Hughes *et al*. (2009) reported that nutrient utilisation, such as protein and amino acids, decreased with phytase supplementation to the P-adequate diet. Metabolic interaction exists between Ca and P in the laying hen. Egg shell calcification normally improves as the supply of dietary Ca increases, whereas it declines with increasing dietary P (Hartel, 1990). Too high or low P has also been known to prevent adequate egg shell calcification (Carlos and Edward, 1998). Also, Keshavarz (2003) observed that supplementation of phytase in diets with 0·25 or 0·20% NPP in four strains of laying hens did not affect the egg specific gravity for the entire experiment (20 to 63 weeks of age). This author postulated that increasing the NPP content of the diets by phytase overcame the expression of the beneficial effect of the low-P diets on specific gravity. Whereas, Boling *et al*. (2000) found that the effects of supplementation of phytase in layer diets at 70 to 76 weeks of age became more pronounced at 0·10% of NPP.

At 74 to 76 weeks of age, hens consuming adequate concentrations of Ca and NPP had significantly ($P < 0·05$) higher albumen weight compared with hens fed on deficient concentrations of Ca and NPP (Table 5). The differences between dietary treatments were not significant over the entire period of the experiment. However, there were WS × phytase × control interactions at the 74 to 76 week period. The birds fed on 130 mg/kg WS, or those fed on the positive control diet supplemented with 65 mg/kg WS plus 300 U/kg of phytase, had significantly ($P < 0·05$) higher albumen weight. Yolk weight was not influenced by supplementation of WS and phytase, regardless of Ca and NPP concentrations in the diet. Jalal and Scheideler (2001) reported that concentration of NPP can significantly affect egg composition irrespective of phytase supplementation. These authors reported that hens fed on the diet with 0·35% NPP had significantly higher albumen weight than those fed on lower NPP concentrations. As shown with the NPP concentrations reported here, it seems that these significant differences are not related to NPP concentration. It is possible that these changes of albumen weight

Table 5. *Effect of* Withania somnifera *(WS), phytase and type of Control diet on albumen weight and yolk weight*

Treatment			Albumen weight(g)				Yolk weight(g)			
WS (mg/kg)	Phytase (U/kg diet)	Control	72 to 74 weeks	74 to 76 weeks	76 to 78 weeks	72 to 78 weeks	72 to 74 weeks	74 to 76 weeks	76 to 78 weeks	72 to 78 weeks
0	0	−	41·06	41·92 ^{abc}	42·11	41·70	18·94	19·49	20·48	19·64
0	0	+	40·64	40·36^{bc}	41·54	40·85	19·20	18·77	20·07	19·35
0	300	−	42·14	40·79 ^{bc}	41·28	41·41	19·77	19·07	19·58	19·47
0	300	+	43·12	44·75 ^{ab}	43·12	43·66	18·97	19·13	20·99	19·36
65	0	−	41·47	39·69 ^{bc}	39·56	40·24	19·44	19·07	20·98	19·83
65	0	+	41·22	45·59^{a}	42·68	43·17	18·48	19·91	20·00	19·47
65	300	−	42·10	41·73 ^{abc}	40·32	41·39	19·02	19·11	19·87	19·67
65	300	+	40·70	43·09 ^{abc}	43·73	42·51	19·26	19·49	19·99	19·58
130	0	−	43·14	43·14 ^{abc}	43·15	43·14	20·00	19·37	20·64	20·00
130	0	+	42·25	43·06 ^{abc}	41·87	42·39	18·83	19·28	20·44	19·52
130	300	−	40·90	44·31^{ab}	43·83	43·01	19·56	18·70	20·31	19·52
130	300	+	41·27	45·37^{a}	42·98	43·20	19·33	18·07	20·22	19·20
SEM			1·531	1·345	1·457	1·029	0·578	0·517	0·521	0·364
Main effect										
WS (mg/kg)	0		41·74	41·96	42·01	41·90	19·22	19·11	20·03	19·46
	65		41·38	42·53	41·57	41·83	19·05	19·40	20·46	19·64
	130		41·89	43·97	42·95	42·94	19·43	18·85	20·40	19·56
Phytase(U/kg diet)	0		41·63	42·29	41·82	41·91	19·15	19·32	20·43	19·63
	300		41·71	43·34	42·54	42·53	19·32	18·93	20·16	19·47
Control	−		41·80	41·93^{b}	41·71	41·81	19·46	19·13	20·48	19·69
	+		41·54	43·70^{a}	42·65	42·63	19·01	19·11	20·12	19·41
P										
WS			0·887	0·107	0·401	0·247	0·905	0·345	0·556	0·782
Phytase			0·933	0·186	0·395	0·307	0·523	0·200	0·448	0·436
Control			0·764	0·028	0·270	0·178	0·276	0·935	0·353	0·198
WS × Phytase			0·304	0·514	0·957	0·744	0·989	0·421	0·626	0·735
WS × Control			0·878	0·237	0·120	0·295	0·975	0·333	0·498	0·913
Phytase × Control			0·779	0·653	0·537	0·532	0·515	0·898	0·420	0·623
WS × Phytase × Control			0·805	0·040	0·849	0·252	0·276	0·609	0·638	0·993

[a-c]Means within each column with no common superscript differ ($P < 0·05$).

might be attributed to a higher concentration of fat in the positive control diet compared with the negative control. The increasing fat content led to an increase in both yolk and albumen weight (Safaa *et al*., 2008), but in some research the improvement was proportionally greater for the albumen than for the yolk (Grobas *et al*., 1999*b*). Whitehead (1995) suggested that the effect of fat on albumen weight was due to the influence of certain unsaturated fatty acids on the production of oestrogen, which is mainly responsible for albumen secretion. Ewan (1991) hypothesised that increasing fat content may lead to increased digestibility of nutrients such as protein and amino acids through slowing passage rate.

Significant interaction was observed between WS × phytase × control in shell weight over the total period of experiment (Table 6). Supplementation of the positive control diet with 65 mg/kg WS in the absence of phytase significantly improved shell weight compared with the negative control diet (5·779 vs. 5·273 g respectively). The effects of dietary treatments on SWUSA were not significantly different ($P > 0·05$).

Bone quality

There were no significant effects of dietary treatments on tibia bone weight. A significant WS × phytase interaction was observed for weight of tibia ash. Supplementation of 65 and 130 mg/kg WS in dietary treatments resulted in a significant ($P < 0·05$) increase in tibia P and Ca (% of ash) respectively. Also, P retention in tibia bone was significantly ($P < 0·05$) improved by phytase supplementation. The birds fed on the positive control diet had significantly ($P < 0·05$) higher Ca retention in tibia bone than those fed on negative control diet. Significant interactions between dietary WS with phytase or control were observed for tibia Ca and P. The birds fed on the positive control diet supplemented with 130 mg/kg WS and phytase had the highest value of tibia Ca. Significant WS × phytase × control interaction was also observed for tibia P. The highest value of tibia P was obtained in hens fed on the negative control diet with supplementation of phytase and 65 mg/kg WS. As mentioned earlier, WS root extract has oestrogen-like compounds. Nagareddy and Lakshmana (2006)

Table 6. *Effect of* Withania somnifera *(WS), phytase and type of Control diet on shell weight and shell weight per unit surface area (SWUSA)*

Treatment			Shell weight (g)				SWUSA (mg/cm^2)			
WS (mg/kg)	Phytase (U/kg diet)	Control	72 to 74 weeks	74 to 76 weeks	76 to 78 weeks	72 to 78 weeks	72 to 74 weeks	74 to 76 weeks	76 to 78 weeks	72 to 78 weeks
0	0	−	5·634	5·778	5·557	5·656^{ab}	73·54	75·00	72·05	73·54
0	0	+	5·561	5·188	5·756	5·502^{ab}	73·54	68·68	74·17	72·15
0	300	−	5·349	5·503	5·487	5·446^{ab}	69·06	70·96	68·92	69·59
0	300	+	5·624	5·537	5·810	5·657^{ab}	72·42	71·50	72·29	72·06
65	0	−	5·154	5·390	5·275	5·273^{b}	66·87	69·02	68·21	68·04
65	0	+	5·571	5·822	5·545	5·779^{a}	71·46	73·79	75·77	73·65
65	300	−	5·704	5·497	5·761	5·654^{ab}	72·79	70·62	73·87	72·42
65	300	+	5·264	5·270	5·878	5·471^{ab}	68·18	68·16	75·63	70·69
130	0	−	5·626	5·633	5·689	5·649^{ab}	74·23	75·22	74·64	74·65
130	0	+	5·570	5·472	5·525	5·523^{ab}	71·48	70·29	70·20	70·66
130	300	−	5·422	5·720	5·505	5·549^{ab}	70·23	73·93	67·86	70·58
130	300	+	5·360	5·131	5·404	5·298^{ab}	70·03	67·68	69·53	69·07
SEM			0·217	0·191	0·206	0·146	2·706	2·601	2·637	1·933
Main effect										
WS (mg/kg)	0		5·542	5·501	5·652	5·565	72·14	71·54	71·86	71·83
	65		5·424	5·495	5·715	5·544	69·83	70·40	73·37	71·20
	130		5·495	5·489	5·531	5·505	71·49	71·78	70·56	71·24
Phytase(U/kg diet)	0		5·519	5·547	5·624	5·564	71·85	72·00	72·51	72·12
	300		5·454	5·443	5·641	5·512	70·45	70·48	71·35	70·73
Control	−		5·482	5·587	5·545	5·538	71·12	72·46	70·93	71·47
	+		5·492	5·403	5·720	5·538	71·19	70·02	72·93	71·38
P										
WS			0·740	0·995	0·448	0·838	0·465	0·727	0·330	0·874
Phytase			0·604	0·350	0·891	0·548	0·376	0·317	0·452	0·223
Control			0·934	0·104	0·153	0·997	0·965	0·112	0·195	0·935
WS × Phytase			0·550	0·626	0·467	0·622	0·475	0·911	0·195	0·408
WS × Control			0·867	0·188	0·189	0·245	0·714	0·196	0·266	0·226
Phytase × Control			0·498	0·487	0·612	0·381	0·727	0·851	0·863	0·884
WS × Phytase × Control			0·145	0·052	0·448	0·049	0·199	0·171	0·288	0·096

[a-b]Means within each column with no common superscript differ ($P<0·05$).

found similar results of significantly improved Ca and P retention in femur bone and tibial bone strength of calcium-deficient ovarectomised rats by supplementation of WS. They reported that the mechanism behind these changes is driven by oestrogen-like withanolides. Oestrogen mediates the increased intestinal absorption of Ca and P in egg laying birds (Bar *et al*., 1978). As hens age and their egg size increases, the shell quality decreases and this is closely associated with calcium availability at the site of either its source (primarily diet, secondarily bone) or its delivery (shell gland) (Hansen *et al*., 2003). Oestrogen treatment of egg laying birds leads to an increase in renal 25(OH)D_3-1-hydroxylase activity and plasma 1,25 $(OH)_2D_3$ concentrations in egg laying birds (Tanaka *et al*., 1976; Baksi and Kenny, 1977; Pike *et al*., 1978). In laying birds, 1,25$(OH)_2D_3$ is thought to be essential not only for intestinal calcium absorption and mobilisation of calcium from medullary bone, but also for calcium transport in the shell gland. It is suggested that renal biosynthesis of 1,25$(OH)_2D_3$, probably controlled by gonadal activity, may be impaired in old laying hens (Abe *et al*., 1982). It is generally accepted that oestrogen declines over the production year (Johnson, 1986). Similarly, Hansen *et al*. (2003) found a dramatic decrease in oestrogen in hens at 70 weeks of age compared with those in peak production (29 weeks). However, results of this experiment showed that supplementation of WS improved Ca and P retention in tibia bone, but these improvements were not reflected in egg shell quality parameters. Abe *et al*. (1982) showed that older hens had decreased renal 25(OH)D_3-1-hydroxylase concentrations, and smaller pool sizes of 25(OH)D_3 and 1,25 $(OH)_2D_3$ than young ones. As shown in Table 7, supplementation of 65 mg/kg WS improved P concentration in tibia ash. Oestrogen is required for activation of the 25$(OH)_2$-1-α-hydroxylase, and thus the activation of vitamin D_3 (Tanaka *et al*., 1978). It up-regulates gut mucosal 1,25D_3 receptors in the chicken (Wu *et al*., 1994) and in the rat (Schwartz *et al*., 2000), and it is required, with 1,25 D3, for synthesis of CaBP-D28K in gut mucosa (Nys *et al*., 1992). Similar to the findings of this experiment, Hughes *et al*. (2009)

Table 7. *Effect of* Withania somnifera *(WS), phytase and type of Control diet on tibia bone quality*

Treatment WS (mg/kg)	Phytase (U/kg diet)	Control	Tibia weight(g)	Tibia ash(g)	Tibia Ca (% ash)	Tibia P (% ash)
0	0	−	3·963	2·313^{d}	38·26^{bcd}	21·21^{cd}
0	0	+	4·490	2·675^{abcd}	39·42^{abc}	21·46^{bc}
0	300	−	4·400	2·660^{abcd}	37·34^{cde}	20·84^{d}
0	300	+	4·210	2·623^{abcd}	34·50^{e}	22·17^{a}
65	0	−	4·190	2·606^{abcd}	35·31de	21·44^{bcd}
65	0	+	4·593	2·863^{ab}	40·16^{abc}	21·31^{bcd}
65	300	−	4·170	2·460^{dc}	37·77^{bcde}	22·31^{a}
65	300	+	4·310	2·710^{abc}	38·99^{abc}	21·92^{ab}
130	0	−	4·276	2·520^{bcd}	36·79^{cde}	21·52^{bc}
130	0	+	3·940	2·405^{dc}	39·42^{abc}	21·37^{bcd}
130	300	−	4·670	2·930^{a}	40·94^{ab}	21·37^{bcd}
130	300	+	4·473	2·760^{abc}	41·80^{a}	21·32^{bcd}
SEM			0·175	0·111	0·979	0·172
Main effect						
WS (mg/kg)	0		4·283	2·660	37·52^{b}	21·42^{b}
	65		4·315	2·651	38·06^{b}	21·74^{a}
	130		4·347	2·576	39·70^{a}	21·39^{b}
Phytase(U/kg diet)	0		4·272	2·676	38·37	21·38^{b}
	300		4·354	2·578	38·15	21·67^{a}
Control	−		4·255	2·687	37·58^{b}	21·44
	+		4·366	2·561	38·92^{a}	21·62
P						
WS			0·839	0·436	0·013	0·019
Phytase			0·226	0·069	0·582	0·019
Control			0·586	0·183	0·035	0·193
WS × Phytase			0·085	0·014	0·001	0·011
WS × Control			0·124	0·070	0·024	0·001
Phytase × Control			0·193	0·260	0·013	0·171
WS × Phytase × Control			0·272	0·408	0·737	0·034

[a-e]Means within each column with no common superscript differ ($P < 0·05$).

showed that phytase supplementation of diets adequate in P (0·25% NPP) had no effect on bone ash at 42 or 61 weeks of age. An increase in phytate P retention greater than 60% with the addition of 5 µg/kg of 1,25-$(OH)_2D_3$ alone was shown earlier by Edwards (1993) and Mitchell and Edwards (1996*a*, *b*). Signs of a P deficiency became evident when the dietary P concentration fell to 3·2 g/kg (Hartel, 1990). Improved tibia P retention by phytase supplementation agrees with the hypothesis that the amount of P required to prevent losses as a consequence of osteoporosis is higher than that for optimal egg production (Hartel, 1990).

The results of this experiment indicate that the performance and egg quality parameters of aged laying hens were not improved with supplementation of WS or exogenous *Escherichia coli*-derived phytase. Addition of phytase causes a negative effect on the specific gravity of the eggs. It is observed that administration of 130 mg/kg WS had beneficial effects on bone Ca and P retention. Furthermore, concentration of tibia bone P improved significantly with addition of 65 mg/kg WS or phytase. Further research is required to confirm the mechanisms by which WS improved Ca and P retention in tibia bone. In addition, it is necessary to carry out further phytochemical investigations to identify the active constituents of WS.

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