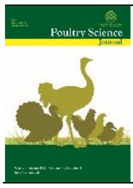

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Effects of Bioherbal and Bioherbal Plus Feed Supplements on Production Performance, Egg Traits, Blood Biochemistry, and Immune Parameters of Laying Hens

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Abstract

The use of medicinal plants represents a new approach for utilizing phytogenic feed additives to improve production, health, and growth performance in the poultry industry. In this study, the effects of different phytogenic diets on egg production, egg quality, and blood parameters in laying hens were investigated. The experimental treatments included the following: (1) control basal diet (CON); (2) basal diet + bioherbal 1.5% + mineral supplement 0.15% (BHM); (3) basal diet + bioherbal 1.5% (BH); (4) basal diet + mineral supplement 0.15% (M); (5) basal diet + bioherbal plus 1.5% + mineral supplement 0.15% (BHPM); and (6) basal diet + bioherbal plus 1.5% (BHP). The results showed that egg quality indicators did not significantly differ in response to the addition of the various phytogenic feed additives, whereas the yolk index significantly differed among treatments only during the first period of the study ($P< 0.05$). The findings of this study revealed that the experimental treatments had no significant effect on the hematological parameters of the blood of the birds, whereas they caused significant changes in the means of the biochemical attributes, including triglyceride (TG), cholesterol (CHO), albumin, total protein (TP) and uric acid. The BHP and M treatments caused the highest and lowest triglyceride contents in the blood, respectively ($P<0.05$). Adding BH to the diet of birds reduced blood cholesterol levels ($P<0.05$). The BHM-containing treatment tended to increase egg production percentage in the whole period of the experiment relative to the control ($P<0.10$). The results revealed that the content of immunoglobulin Y reached the highest value in the BHM treatment group at the second immunization, whereas the lowest value was obtained in the BHP diet group ($P<0.05$). These findings show that the use of Bioherbal supplemented by minerals may have some effect on the performance of laying hens that may be significant in stressful conditions. Therefore, the evaluation of these additives in stress conditions is suggested.

Introduction

The natural products derived from herbs and spices utilized in animal and poultry nutrition for improving health and growth performance are called “phytogenic feed additives” (PFA) (Windisch *et al.*, 2008). The use of PFA, such as plants, essential oils (EO), extracts, and individual or combined active EO ingredients, has recently received much greater attention as an efficient substitute for traditional antibiotics, probiotics, and prebiotics, which maintain desirable characteristics while having minimal adverse impacts on human health and the environment (Abou-Elkhair *et al.*, 2018).

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Antibiotics have unquestionably proven to be of tremendous benefit to both humans and domestic animals (Khattab *et al.,* 2010). The use of antibiotics is a standard practice for illness prevention as well as a vital tool for increasing meat and egg production in poultry systems. Additionally, owing to their ability to promote growth, they are frequently added to animal feed at subtherapeutic levels (Prescott *et al.,* 1993). Thus, these agents are extremely beneficial in preventing producers from suffering significant economic losses as a result of disease outbreaks (Prescott *et al.,* 1988).

Although antibiotics are used less frequently in laying hen husbandry systems compared to meat-type systems, the increasing resistance of pathogenic bacteria to antibiotics in poultry—and its potential transmission to humans—remains a serious concern. Given the wide variety, abundance, and recognized safety of plant-based compounds widely used in the food industry, phytogenic feed additives (PFAs) offer several advantages to conventional antibiotics (Mehdi *et al.,* 2018).

Over the last two decades, studies have shown that phytogenic feed additives exert multiple effects, including antibacterial, antiviral, antifungal, anti-inflammatory, and antioxidant activities (Windisch *et al.,* 2008; Mohammadi Gheisar *et al.,* 2018).

An increasing number of in vivo studies on PFAs and their effects on animal nutrition and gastrointestinal health have been conducted. The enhancement in nutrient digestibility resulting from PFA supplementation may be due to their potential to help nutrient availability and improve feed efficiency through better liver functions (Hernandez *et al.,* 2004; Prakash *et al.,* 2010). However, the mechanisms of action and potential applications of PFAs in poultry nutrition remain poorly understood. Egg production and quality, eggshell strength, and nutrients in eggs rapidly decline with age, and these problems cause economic losses for producers (Liu *et al.,* 2013). At the end of the laying cycle and under the effects of various stress agents, decreases in productive efficiency and egg quality are caused mostly by the oxidative stress accumulated by long-term egg production (Liu *et al.,* 2018). To reduce reliance on synthetic drugs and avoid antibiotic residues in eggs, manufacturers rely on secure substitutes from medicinal plants to enhance egg production persistence, health, and resistance to oxidative stress in aged hens (Liu *et al.,* 2013; Qu *et al.,* 2018).

Many studies have pointed to the role of PFA in increasing the production and health of birds. For example, Yalçin *et al.* (2020) reported that the use of thyme as a supplement was helpful in reducing egg yolk cholesterol and increasing omega-3 fatty acid levels in egg yolks. These findings demonstrate the remarkable potential of thyme as an additive to produce enriched eggs. Supplementing with thyme may be beneficial for treating hyperlipidemic disorders as well as enhancing the antioxidant status and humoral immune response in birds. As a result, nutritionally altered eggs that are high in omega-3 fatty acids and low in cholesterol can be made commercially from dried thyme leaves. Cumin (*Cuminum cyminum* L.) has several medicinal properties, such as antimicrobial, antifungal, anti-inflammatory, antioxidant, antidiabetic, anticarcinogenic, and hypocholesterolemic properties (Saleh *et al.,* 2018; Srinivasan *et al.,* 2018), which may be involved in enhancing broiler chick growth performance (Al-Kassi *et al.,* 2010). Despite limited effects on performance characteristics and the chemical composition of eggs, there have been only a limited number of in vivo studies exploring the inclusion of cumin seeds or their active plant components in the diets of laying hens. (Aydin *et al.,* 2008).

Given several reports highlighting the positive impacts of medicinal plants on broiler chicken performance and some comprehensive investigations on laying poultry, this research was carried out to pinpoint a suitable and safe PFA for developing the parameters related to laying performance, traits of egg production, quality indicators of eggs, blood parameters, and the immune response in laying hens.

Materials and methods

Birds, design and feeding treatments

This study was carried out at the poultry research center of Ferdowsi University of Mashhad. The bioherbal and bioherbal plus (BHP) were purchased from Koohrang Nature's Gold (KNG) Company, Mashhad, Iran. The mineral supplement was also provided by Aryana Company, Mashhad, Iran. The Bioherbal included the powder of dried aerial part of cumin (25%), peppermint (25%), mint (22%), nepeta (25%), and thyme (3%). The bioherbal plus was as follows: fennel (10%), cumin (10%), peppermint (20%), mint (20%), citrus blossom (20%), lavender (10%), sesame (5%), and thyme (5%). The mineral supplement used in this study contained selenium, zinc, chromium and manganese. Five hundred seventy-six 2.5-year-old laying hens of the Hy-line w-36 strain with an average body weight of 1.5 kg and an egg production percentage of 85% were selected and subjected to different treatments for 8 weeks. The experiment was done with six treatment groups, eight replications and 12 laying hens in each replicate, in a randomized complete block design (due to vertical lighting variation). The treatments were as follows: (1) control basal diet (CON); (2) basal diet + bioherbal 1.5% + mineral supplement 0.15% (BHM); (3) basal diet + bioherbal 1.5% (BH); (4) basal diet + mineral supplement 0.15% (M); (5) basal diet + bioherbal plus 1.5% + mineral supplement 0.15% (BHPM); and (6) basal diet + bioherbal plus 1.5%

(BHP). The control diet was based on a corn–soybean meal and was formulated according to the nutritional recommendation guide for Hy-line w-36 laying hens with UFFDA software (Table 1). The birds were raised under a 16 L:8D photoperiod at 25°C with free access to water and feed.

Table 1: Ingredients and composition of the basal diet

Feed ingredients	**Content, %**
Corn	44.55
Wheat	15.00
Soybean meal (44% crude protein)	23.46
Vegetable oil	3.87
Dicalcium phosphate	1.70
Salt	0.35
D, L-Methionine	0.22
L-Lysine HCl	0.03
Calcium carbonate	10.32
Vitamin and mineral premix	0.50
Calculated chemical composition	
Metabolizable energy (kcal/kg)	2813
Crude protein (%)	16
Methionine (%)	0.37
Methionine + cystine (%)	0.67
Lysine (%)	0.74
Threonine (%)	0.52
Calcium (%)	4.74
Available phosphorus (%)	0.38
Arginine (%)	0.79
Tryptophan (%)	0.16

[1]Supplying per kg of feed: vitamin A (all-trans retinol acetate) - 10000 IU; vitamin D3 (Cholecalciferol) - 2500 IU; vitamin E (α-tocopheryl acetate) - 10 IU; vitamin K3 (menadione sodium bisulphite) - 3 mg; vitamin B1 (thiamine hydrochloride) - 2 mg; vitamin B2 (riboflavin) – 5 mg; vitamin B3 (niacin) – 8 mg; vitamin B5 (pantothenic acid) - 12 mg; vitamin B6 (pyridoxine hydrochloride) – 2 mg; vitamin B9 (folic acid) – 0.75mg; vitamin B12 (cyanocobalamin) – 0.015 mg; Biotin (vitamin H) – 0.05 mg; vitamin C (ascorbic acid) – 0.05 mg; choline chloride - 250 mg; Antioxidant – 25 mg. [2] Supplying per kg diet: Zn (Zinc) - 60 mg; Mn (Manganese) – 70 mg; Fe (Iron) – 60 mg; Cu (Copper) – 8 mg; Se (Selenium) – 0.25 mg; I (Iodine) – 1 mg; Co (Cobalt) – 0.25 mg.

Feed intake

The feed intake (FI) for each replicate was calculated weekly via the feed difference at the beginning of the week compared with the remaining feed at the end of the week. The mean of daily feed intake of each chicken was calculated via the following formula.

FI (g/bird) $= \frac{\sum(\mathrm{fw}i) - (\mathrm{Fwb})}{\mathrm{HD}}$, where $\sum$ (fw_i) represents the weight of feed at the beginning of the week; fw_b represents the weight of residual feed at the end of the week; and hen-day (HD) represents the number of hens at the beginning of the week. The feed conversion ratio (FCR) was calculated by dividing the feed consumed each week by the weight of eggs produced in the same week.

Egg quality parameter measurements

The egg quality parameters were measured monthly. The quality characteristics of the eggs, including weight (using an ultrasensitive laboratory scale), shape index (via a Vernier caliper), density, yolk index, yolk color (Roche scale), Haugh unit, and eggshell thickness, were calculated according to previous reports (Guo *et al.,* 2022; Muhammad *et al.,* 2021).

Blood biochemical analysis

At the end of the trial period, blood samples (2 mL) were taken from the wing vein of two randomly selected birds of each replication and then transferred to the laboratory in a tube containing anticoagulant. The blood plasma was separated by centrifugation at 1800 g for 12 min and stored at -20°C until analysis. Biochemical features such as total protein (TP), triglyceride (TG), cholesterol (CHO), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), glucose, calcium (Ca), and phosphorus (P) levels were measured via analytical kits (Pars Azmoon Company; Tehran, Iran) in accordance with the manufacturer's guidelines (Nahavandinejad *et al.,* 2014). Hematological features, including red blood cells (RBCs), white blood cells (WBCs), lymphocytes, heterophils, monocytes, and basophils, and the heterophil/ lymphocyte ratio were evaluated as previously reported (Attia *et al.,* 2017).

Intestinal histomorphology and microflora analysis

A small part of the jejunum from the intestine (approximately 0.5 cm) was separated through an autopsy, washed with physiological saline, and then placed in containers containing 20 mL of 10% formalin for staining. The samples were sent to the histology laboratory to measure their tissue characteristics (Muhammad *et al.,* 2021).

To investigate the microflora of the ileum and cecum, three birds from each treatment group were randomly selected. One gram of each ileum and cecum content was mixed with 9 mL of phosphate-buffered saline (PBS), and serial dilutions were prepared. An aliquot (25 µL) of each diluted solution was then taken and cultured in special culture media. De Man, Rogosa and Sharpe (MRS) (containing cysteine-HCl) and MacConkey (MAC) agar media were used to count the lactobacilli and gram-negative bacteria, respectively.

Immune Responses

On the 16th and 23rd days of the rearing period, 0.5 mL of sheep red blood cell suspension (0.5%) was injected into three birds of each experimental replication through the breast muscle. One milliliter of blood was then taken from the same bird through the wing vein on days 23 and 30. The blood samples were kept overnight at ambient temperature to separate the serum from the blood clot. The obtained serum was centrifuged at 1800 g for 10 minutes and immediately stored at -20°C until further analysis. The microtiter hemagglutination method was used to determine the titer of antibodies produced against sheep red blood cells (Wegmann *et al.,* 1966).

Tibia quality characteristics and mineral content

Two birds whose weights were close to the average weight of the experimental unit were selected, and the left tibias were carefully separated to determine their quality characteristics, such as volume, length, relative weight, density and ash amount. The contents of calcium (Ca), phosphorus (P), chromium (Cr), selenium (Se) and zinc (Zn) in the tibia bone samples were measured via inductively coupled plasma (ICP) via the addition of 6 N HCl to the ash according to the method described by Catala-Gregori *et al*. (2006).

Statistical analysis

All data were analyzed based on a randomized complete block design. The block was designed based on vertical lighting variation. One-way analysis of variance (ANOVA) and the least squares mean procedure set for Tukey's test ($P< 0.05$, and $0.10 \leq p >0.05$ as a tendency) were used to determine the significance of the effects of the treatments and compare their means, respectively, via SAS software (version 9.4).

Results

Feed intake and egg production

The results of this study revealed no significant differences in feed intake or egg production among the treatments during the eight weeks of the experiment. The highest and lowest feed intakes were recorded in the second week of the experiment, as the highest feed intake was recorded for the BHM treatment (95.30 g), which was significantly greater ($P<0.10$) than that of the BH treatment (90.45 g) (Table S1). The highest egg production was obtained in the seventh week in all the treatment groups (Table 2). In the seventh week, the BH treatment resulted in the highest egg production percentage, but the difference was not significant. As shown in Table S3, the average egg mass production in the first and sixth weeks significantly differed from that in the other treatments. Compared with the control, adding BHP alone or in combination with a mineral supplement (BHPM) to the diet of the birds reduced the egg mass production in the first week. The minimum and maximum egg mass rates in the sixth week were obtained in the M (38.29%) and BHM (48.81%) treatments, respectively, and did not significantly differ from those of the control (Table S3). The obtained FCR did not differ among the treatments, with the minimum FCR recorded in BH and BHPM (Table S4).

Egg quality parameters

The effects of different diet treatments on egg quality characteristics were measured every four-week laying period and are presented in Tables S5 and S6. The egg quality indicators, including density, shape index, yolk weight, shell weight, albumen weight, yolk index, shell thickness, and yolk color index, did not significantly differ among the various phytogenic feed additives employed in the first period of laying (Table S5). During the second 4 weeks of laying, egg density and eggshell thickness decreased with the consumption of BHPM, whereas BH resulted in the greatest shell thickness and shell weight in the eggs ($P< 0.05$) (Table S6). During the whole trial laying period, the application of various feed additives to the basal diet of the birds did not cause significant changes in egg quality characteristics, except for albumen weight, which was the lowest in the BH and BHM treatments (Table 2).

Table 2: Effect of treatments on the egg quality traits of laying hens during the whole trial period

Item	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Density (g/cm^3)	1.081	1.081	1.084	1.081	1.081	1.083	0	0.07
Shape index[1]	78.182	77.427	77.729	77.558	77.527	76.889	0.27	0.05
Yolk weight (g)	16.935	16.580	17.024	16.850	16.824	16.670	0.25	0.84
Shell weight (g)	6.912	6.884	6.813	6.811	6.817	6.783	0.09	0.91
Albumen weight (g)	42.499^a	40.647^b	40.387^b	42.434^a	41.263^{ab}	41.213^{ab}	0.55	0.04
Shell thickness (mm)	0.405	0.402	0.403	0.401	0.403	0.399	0	0.88
Yolk color index[2]	5.625	5.575	5.775	5.675	5.650	5.600	0.11	0.84
Yolk index[3]	31.214	31.402	31.953	31.796	31.128	31.826	0.50	0.78

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean. Mean values followed by different letters in the same row are significantly different (p<.05). 1- (Small diameter/large diameter) ×100. 2- Roche index 3- (yolk height/yolk diameter) ×100

Blood parameters

The findings of this study revealed that the experimental treatments had no significant effect on the hematological parameters of the blood of the birds, whereas they caused significant changes in the means of the biochemical attributes, including triglyceride (TG), cholesterol (CHO), albumin, total protein (TP) and uric acid, in the blood serum of the laying hens (Table 3). The BHP and M treatments caused the highest and lowest triglyceride contents in the blood, respectively. Adding BH to the diet of the birds reduced blood cholesterol levels (189 mg/dL). Blood serum ALB had the lowest value in the control treatment, and the highest value was observed in the BHM treatment. The lowest and highest total protein contents were also obtained in the BHM and BHP treatments, respectively. Compared with the control diet, the BHM diet significantly increased the uric acid level in the bird's blood. The HDL-c levels did not significantly change with the use of bioherbal or bioherbal plus alone or in mixtures with mineral.

Humoral immune system

Although the antibody response after the first injection of SRBC did not significantly differ among the experimental treatments (P>0.05), the second SRBC injection stimulated antibody secretion ($P<$ 0.05). Compared with the control (4.12), the BHPM (4.25) and BHP (4.75) treatments increased the level of total anti-SRBC antibody without significant differences. The content of immunoglobulin Y reached the highest value in the BHM treatment group, whereas the lowest value was obtained in the BHP diet group (Table 4).

Table 3: Effect of treatments on blood hematological and biochemical attributes of laying hens

Item	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
RBC ($\times 10^6$/mL)	2.425	2.207	2.46	2.172	2.147	2.027	0.11	0.084
WBC ($\times 10^3$/mL)	11.122	10.345	11.377	10.042	10.007	9.37	0.531	0.127
Hgb (g/dl)	12.5	11.5	12.525	11.275	11.025	10.6	0.571	0.144
Hematocrit (%)	32	29.25	32.75	29	29.25	26.75	1.476	0.098
Heterophil (%)	28.25	28.25	28.25	29	28.5	29.5	0.606	0.66
Lymphocyte (%)	59	57.5	56	56.75	55.25	58.25	1.162	0.252
H/L ratio	0.477	0.495	0.507	0.517	0.512	0.502	0.013	0.361
TG (mg/dL)	5038^{ab}	5676^{a}	4148^{ab}	3175^{b}	3307^{b}	5763^{a}	349	0.032
CHO (mg/dL)	244^{ab}	302^{a}	189^{b}	249^{ab}	207^{b}	303^{a}	13.98	<0.001
Albumin (g/dL)	2.16^{c}	3.76^{a}	2.63^{bc}	2.6^{bc}	2.2^{c}	3.36^{ab}	0.191	<0.001
TP (g/dL)	7.03^{ab}	7.7^{a}	6.1^{ab}	5.13^{b}	6.7^{ab}	8.13^{a}	0.457	<0.001
Uric acid (mg/dL)	6.3^{bc}	9.23^{a}	4.83^{bc}	6.93^{ab}	4.3^{c}	7.1^{ab}	0.524	<0.001
HDL-c (mg/dL)	71	68.66	71.33	69.33	69.33	73	3.327	0.937

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean. Mean values followed by different letters in the same row are significantly different ($P<$ 0.05).

Table 4: Effect of treatments on Anti-SRBC immunoglobulin titer (log_2) of laying hens at the end of days 23 and 30

Item	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Total SRBC 1	3.625	4.375	3.75	3.75	4.125	4.857	0.304	0.68
IgY 1	4.5	4.875	4.375	4.5	4.5	4	0.296	0.527
Total SRBC 2	4.125^{ab}	3.75^{ab}	3.285^{b}	4^{ab}	4.25^{ab}	4.75^{a}	0.285	0.029
IgY 2	4.5^{ab}	5.0^{a}	4.375^{ab}	3.875^{b}	3.625^{b}	3.5^{b}	0.247	0.0006

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean. Mean values followed by different letters in the same row are significantly different ($P<$ 0.05).

Intestinal histomorphology and microflora

The results of this study revealed that when diets based on herbs alone or combined with minerals were used, no significant changes in the morphometric indices of laying hens were detected during the trial period (Table 5).

The effects of the experimental treatments on the microbial flora of the cecum and ileum revealed that the BHPM treatment had the greatest number of ileum gram-negative bacteria. The lowest number of gram-negative bacteria was also recorded in the control treatment. The experimental treatments did not have a significant effect on the populations of ileum and cecum lactobacilli or cecum gram-negative bacteria (Table 6).

Table 5: Effect of treatments on intestinal morphometry of laying hens

Item	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Villus width (µm)	56.6	52.87	49.14	45.28	39.82	40.62	4.483	0.129
Crypt (µm)	117.06	126.73	126.17	105.98	94.52	123.34	11.173	0.313
Intestinal wall thickness (µm)	166.69	141.86	155.09	120.13	114.17	155.65	12.03	0.051
Villus height (µm)	815.49	680.58	701.27	837.62	696.14	787.45	76.433	0.569

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean

Table 6: Effect of treatments on Lactobacilli and gram-negative bacteria of the ileum and cecum of laying hens ($\log_{10}$CFU/g)

Item	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Ileum								
Gram-negative bacteria	3.92	4.03	4.35	4.43	4.50	4.18	0.157	0.12
Lactobacilli	3.58^{ab}	3.31^{b}	3.81^{ab}	3.79^{ab}	3.95^{ab}	3.98^{a}	0.134	0.034
Cecum								
Gram-negative bacteria	5.55	5.44	5.45	5.46	5.46	5.59	0.25	0.99
Lactobacilli	6.10	6.30	5.97	6.36	6.14	5.96	0.23	0.78

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean. Mean values followed by different letters in the same row are significantly different ($P < 0.05$).

Tibial properties and mineral content

The results of the present study revealed that the experimental treatments had no significant effect on tibia characteristics (Table 7). The consumption of the additives significantly affected the Ca and Cr contents of the tibia ash of laying hens, with the lowest contents of Ca and Cr observed in BHPM (31.391 mg/dL) and BH (7.991 mg/dL), respectively, which were significantly different from those of the control (Table 8).

Table 7: The effect of treatments on the tibia properties of laying hens

Item	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Dry weight (g)	4.006	4.01	4.334	4.311	4.161	4.325	0.203	0.711
Dry matter (%)	69.22	67.956	69.61	71.246	70.138	69.031	2.232	0.933
Dry matter without fat (%)	68.09	64.065	62.614	65.881	66.165	64.061	2.602	0.728
Diaphysis diameter (mm)	6.633	6.963	6.913	6.643	6.683	6.656	0.212	0.778
Large epiphysis diameter (mm)	15.55	15.573	16.5	15.796	16.223	16.03	0.218	0.189
Small epiphysis diameter (mm)	10.9	11.09	11.276	11.03	11.526	11.316	0.195	0.308
Length (mm)	80.296	82.913	79.74	79.133	84.103	80.72	1.257	0.415
Ash (%)	50.054	54.694	49.187	50.611	50.59	54.444	1.727	0.174
Splitting force (N)	105.16	111.26	119.83	128.73	116.7	133.26	17.466	0.863
Hardness (N/mm)	296.45	229.1	288.9	273.56	282.36	309	26.197	0.428

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM=The standard error of the mean.

Table 8: The effect of treatments on the mineral content of tibia ash of laying hens

Item	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Ca (mg/g)	38.754^{a}	33.367^{ab}	32.651^{ab}	32.269^{ab}	31.391^{b}	34.638^{ab}	1.4	0.033
P (mg/g)	18.825	18.266	18.365	18.063	18.326	18.392	0.23	0.438
Cr (ppm)	16.149^{a}	11.446^{ab}	7.991^{b}	8.721^{ab}	9.597^{ab}	10.098^{ab}	1.422	0.023
Se (ppm)	3.117	1.474	3.079	2.675	2.275	3.638	0.622	0.281
Zn (ppm)	491	557	486	453	459	516	28.154	0.151

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean. Mean values followed by different letters in the same row are significantly different ($P < 0.05$).

Discussion

The findings of the present study revealed that there was no significant difference between the treatments in terms of feed intake or egg production. Botsoglou *et al.* (2005) reported that supplementation of layer diet with saffron, oregano, or rosemary showed no significant effect on intake, FCR, or egg production. Corresponding to the results of the present study, Florou-Paneri *et al*. (2005) observed that the inclusion of oregano oil did not significantly influence egg weight, feed consumption, or feed conversion efficiency. In another study, the influences of probiotic, thyme, garlic, and caraway herbal extracts were examined on the amount and quality of eggs produced by laying hens, and no significant effects were reported on the quality or quantity of eggs produced in comparison with the control treatment (Behnamifar *et al.*, 2015). Black cumin (*Bunium persicum*) supplementation at different doses did not show any effects on the average of daily body weight change of broiler chickens; however, corresponding to research by Shirzadegan *et al*. (2015), it did alter certain blood parameters.

Behnamifar and colleagues (2018) reported that chamomile, wild mint, and oregano plant extracts have no significant effect on production, egg mass, FCR, egg weight, feed intake, and egg quality indices of Japanese quail. It was reported that the use of cumin seed oil (*Cuminum cyminum* L.) showed no effect on egg production, egg mass and feed intake but increased egg weight, whereas they recorded high FCR when 500 g/ton cumin seed oil was fed to laying hen (Saleh *et al.,* 2019). Çabuk *et al.* (2006) found that incorporating 24 mg of an essential oil mixture (consisting of six different essential oils derived from selected herbs) per kg of diet notably enhanced egg production and feed efficiency while decreasing the rate of broken eggs. Furthermore, the essential oil mixture had a beneficial impact on the rate of egg production and FCR in laying hens. The incorporation of 200 mg/kg of essential oils from thyme, sage, or rosemary into the diet positively impacted egg weight, but did not affect the rate of egg production (Prakash & Srinivasan, 2010). These results provide partial evidence that herbs and essential oils positively influence hen performance. The occurrence of positive effects of essential oils may differ depending on the management conditions. The chronic nutritional deficiencies, health and genetic conditions of birds in different studies may explain the different results. For example, the use of essential oils in birds that are in stressful conditions is more likely to improve their performance (Jang *et al.*, 2007; Çabuk *et al*., 2006). Additionally, EO mixtures had positive effects on the egg production rate and FCR in laying quail, according to Çabuk *et al*. (2006). The inclusion of 200 mg/kg essential oils from thyme, sage, or rosemary in the diet positively impacted egg weight, although it did not affect the rate of egg production. (Bölükbaşı *et al*., 2008).

In the present study, the effect of treatments containing herbal additives was varied on the egg quality during the weeks of recording. According to Saleh *et al.* (2019), dietary cumin seed oil considerably enhanced the quality attributes of egg yolks and shells, whereas other egg quality parameters were unaffected in the present study (Saleh *et al.*, 2019). The phytoestrogens in cumin, which have estrogenic and anti-osteoporotic effects, may be responsible for the improvements in eggshell quality attributes (Patil *et al.,* 2017). Furthermore, Shirke *et al.* (2008) reported that adding cumin extract to rats' food reduced the amount of calcium that was excreted in the urine while increasing the calcium content and mechanical strength of bones (Shirke *et al.,* 2008). As a result, dietary cumin seed oil supplementation may play a role in increasing serum calcium levels, which in turn increases shell calcification, which subsequently results in an improvement in shell quality.

The results of this study revealed that while the experimental treatments had no significant effect on the hematological characteristics of the birds, they significantly altered the mean levels of biochemical attributes in the blood serum of laying hens.

When thyme powder was added to the diets of laying hens, the level of blood cholesterol decreased compared with control diets, according to Cimrin (2019) and Mohammed *et al.* (2022). According to Behnamifar *et al.* (2015), thyme and garlic extracts decreased serum and yolk cholesterol in comparison with the control. The improvement in the serum lipid profile can be attributed to thymol, which is found at high concentrations in thyme plants and improves fat digestion by increasing the production of lipase and bile (Hajiazizi *et al.,* 2016).

This study revealed that BHM was most effective in improving the immune system. An anticipated increase in response to SRBC among the EO groups was due to this herb's ability to enhance the activation of the nonspecific immune system. High-flavonoid herbs, like cumin, support the effects of vitamin C, function as antioxidants, and may thus improve immune performance (Manach *et al*., 1996). Nevertheless, Sadeghi *et al*. (2012) found that infusions (5 grams per liter) of cinnamon, thyme, and turmeric in the diet did not influence antibody titers against Newcastle disease virus vaccine. Furthermore, Aami-Azghadi *et al*. (2010) noted that the inclusion of 0.2 to 0.8 g/kg of cumin essential oil in a broiler diet had no impact on total anti-SRBC, IgG, or IgM titers. According to previous results (Olgun, 2016), the biomechanical properties and mineral content of the tibia are affected by the consumption of a mixture of essential oils, including thyme essential oils. According to several reports, the addition of plant feed additives or essential oils to the diet leads to an increase in serum phosphorus and plasma zinc and a decrease in plasma calcium (Olgun, 2016). Additionally, Olgun and Yildiz (2014) noted that the addition of an essential oil mixture to the diet reduces mineral excretion.

Conclusion

Results of the present study showed that the additive of Bioherbal along with the mineral supplement used in this study tended to increase egg mass, while having no significant effect on egg quality. Also, the treatment contained this additive tended to increase egg production percentage in the whole period of the experiment relative to the control. These findings show that the use of Bioherbal supplemented by minerals may have some effect on the performance of laying hens that may be significant in stressful conditions. Therefore, the evaluation of these additives in stress conditions is suggested.

Ethics approval and consent to participate

All methods in the study were in accordance with the ethics guidelines on animal experiments at Ferdowsi University of Mashhad. The committee on the ethics of animal experiments approved the protocols by certificate number IR.UM.REC.1401.286

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Supplementary data

Table S1. Effect of experimental treatments on average feed intake (g) of laying hens.

Trial period	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Week 1	92.34	92.25	94.25	91.07	91.51	92.33	1.46	0.804
Week 2	92.96	91.94	90.46	92.58	95.39	92.94	1.08	0.086
Week 3	95.22	94.12	95.87	92.87	94.57	93.29	1.47	0.854
Week 4	94.36	93.65	93.62	93.60	93.16	93.11	1.30	0.988
Week 5	92.90	92.20	91.98	92.11	93.91	91.98	1.90	0.974
Week 6	96.27	96.90	95.67	94.43	95.87	96.73	1.00	0.547
Week 7	93.48	95.97	95.19	94.78	93.52	94.31	1.18	0.639
Week 8	88.34	90.48	89.81	91.45	88.93	88.72	1.68	0.775
Means	92.71	93.44	92.40	92.30	92.80	92.38	0.73	0.89

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean. Mean values followed by different letters in the same row are significantly different ($P < 0.05$).

Table S2. Effect of experimental treatments on egg production (%) of laying hens.

Trial period	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Week 2	74.25	77.38	76.78	77.08	77.83	72.77	1.72	0.246
Week 3	76.34	75.15	76.02	74.55	77.83	76.04	1.79	0.851
Week 4	76.04	79.61	76.78	76.78	76.34	77.98	1.89	0.777
Week 5	74.40	69.49	68.60	70.24	67.86	69.64	2.75	0.626
Week 6	59.00	64.28	66.67	57.34	64.28	62.05	2.31	0.118
Week 7	75.89	80.13	81.99	78.72	78.99	80.78	2.18	0.476
Week 8	57.89	66.52	60.12	59.22	62.20	56.10	2.61	0.105
Means	69.07	71.82	70.70	69.36	70.16	68.91	0.76	0.08

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean. Mean values followed by different letters in the same row are significantly different ($P < 0.05$).

Table S3. Effect of different diet treatments on egg mass production (kg) of laying hens.

Trial period	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Week 1	44.29[ab]	43.44[ab]	45.81[ab]	49.63[a]	41.49[b]	41.49[b]	1.70	0.014
Week 2	47.58	49.67	49.00	49.20	51.93	47.21	1.48	0.28
Week 3	51.86	50.32	50.54	51.00	53.12	49.76	1.29	0.496
Week 4	48.09	49.54	48.74	50.21	50.25	50.55	1.61	0.871
Week 5	48.20	44.81	48.10	48.53	46.41	46.99	1.72	0.517
Week 6	41.81[ab]	48.81[a]	45.48[ab]	38.29[b]	43.98[ab]	43.15[ab]	1.91	0.011
Week 7	49.72	52.16	52.11	50.00	50.41	50.82	1.56	0.796
Week 8	42.24	46.55	44.74	46.21	46.30	44.23	1.99	0.606
Means	393	417	412	409	409	392	981	0.38

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean. Mean values followed by different letters in the same row are significantly different ($P < 0.05$).

Table S4. Effect of different diet treatments on the feed conversion ratio (FCR) of laying hens.

Trial period	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Week 1	2.10	2.14	1.99	1.86	2.20	2.25	0.101	0.092
Week 2	1.96	1.86	1.82	1.89	1.84	1.94	0.056	0.429
Week 3	1.81	1.87	1.90	1.79	1.79	1.86	0.054	0.620
Week 4	1.93	1.91	1.91	1.84	1.93	1.85	0.074	0.882
Week 5	1.90	1.99	1.94	1.92	2.03	1.99	0.088	0.871
Week 6	2.25	2.00	2.14	2.27	2.17	2.18	0.964	0.449
Week 7	1.90	1.88	1.83	1.94	1.86	1.87	0.059	0.871
Week 8	2.14	1.95	2.07	1.98	1.94	2.05	0.086	0.560
Means	2.01	1.96	1.95	1.96	1.95	2.00	0.03	0.71

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean. Mean values followed by different letters in the same row are significantly different ($P < 0.05$).

Table S5. Effect of different diet treatments on the egg quality traits during the first 4 weeks of laying.

Item	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Density	1.081	1.079	1.082	1.082	1.08	1.08	0.001	0.857
Shape index	78.226	77.169	77.243	78.123	77.387	77.582	0.664	0.801
Yolk weight	17.376	16.767	17.204	16.728	16.643	16.224	0.612	0.816
Shell weight	6.996	7.223	6.843	7.085	6.99	6.916	0.225	0.902
Albumen weight	42.185	39.307	40.793	42.594	41.611	42.485	1.813	0.786
Shell thickness	0.402	0.404	0.404	0.401	0.406	0.403	0.003	0.901
Yolk colour index	5.375	5.375	5.75	5.75	5.625	5.5	0.247	0.786
Yolk index	32.062	32.396	32.615	32.262	31.863	33.048	0.545	0.763

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean. Mean values followed by different letters in the same row are significantly different ($P < 0.05$).

Table S6. Effect of different diet treatments on the egg quality traits during the second 4 weeks of laying.

Item	CON	BHM	BH	M	BHPM	BHP	SEM	*P*-value
Density	1.084[ab]	1.083[ab]	1.084[a]	1.079[bc]	1.078[c]	1.084[ab]	0.001	0.001
Shape index	79.466	79.246	78.131	77.927	77.993	78.022	0.65	0.349
Yolk weight	17.603	15.825	17.323	16.522	16.383	17.682	0.484	0.05
Shell weight	7.303[ab]	7.058[ab]	7.698[a]	7.205[ab]	6.578[b]	6.935[ab]	0.177	0.005
Albumen weight	44.505	42.875	42.991	42.772	42.174	42.47	1.37	0.879
Shell thickness	0.409[ab]	0.403[ab]	0.418[a]	0.405[ab]	0.395[b]	0.406[ab]	0.004	0.028
Yolk color index	5.625	5.625	5.75	5.875	5.875	5.75	0.221	0.935
Yolk index	31.44	30.67	32.953	30.914	30.254	33.669	1.161	0.253

CON: Basal diet; BHM: basal diet + bioherbal 1.5% + mineral supplement 0.15%; BH: basal diet + bioherbal 1.5%; M: basal diet + mineral supplement 0.15%; BHPM: basal diet + bioherbal plus 1.5% + mineral supplement 0.15%; BHP: basal diet + bioherbal plus 1.5%. SEM= The standard error of the mean. Mean values followed by different letters in the same row are significantly different ($P < 0.05$).