



Effect of In-ovo Injection of Herbal Extracts on Post-hatch Performance, Immunological, and Physiological Responses of Broiler Chickens

K.H. El-Kholy*, Doaa M.A. Sarhan, and Eman A. El-Said

Poultry Production Department., Faculty of Agriculture, Damietta University, Damietta, 34518, Egypt

*Corresponding author's Email: khelkholy@du.edu.eg; ORCID: 0000-0002-2562-2311

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ABSTRACT

In-ovo injection with exogenous materials, such as natural antioxidants, throughout incubation could be a technique to boost hatchlings' performance. The objective of the present study was to determine the effect of in-ovo injection of cinnamon, thyme, and clove extracts on the subsequent growth performances, immunity, and physiological responses of newly-hatched chickens. A total of 450 fertile eggs used in the current experiment were obtained from avian broiler breeder flocks of 28 weeks of age. The eggs were randomly distributed into five treatment groups which included three replicates for each one (30 eggs each group) in a completely randomized design at day 10 of embryogenesis. Treatment groups included a control group (P1: without any injection), the group received an injection of 0.5 ml deionized water (P2: sham group), and the groups injected with 0.1 ml cinnamon, thyme, and clove extracts (P3, P4, P5, respectively). The hatchlings from each treatment were randomly assigned to five replicates of 10 chickens, and reared until 35 days of age. The results showed no significant differences among groups in terms of feed consumption, serum albumin, and immunoglobulin's A (IgA). Nevertheless, using extracts resulted in a significant increase in body weight and weight gain, and improved feed conversion ratio and immunoglobulin's G and M (IgG and IgM), compared to the control and sham groups at 35 days of age. The injected extracts had significantly positive effects on serum lipids profile, liver functions (AST, ALT, and ALP) values, and antioxidant activity, compared to the control groups. Furthermore, serum concentrations of triiodothyronine and thyroxine were significantly higher in the group injected clove-extracted than in other experimental groups. According to the results, it can be concluded that in-ovo injection of herbal extracts, especially clove extract on day 10 of incubation has a positive effect on the broiler chickens' weight at hatch and post-hatch performance as well as physiological, immunological, and anti-oxidative status of hatched chickens.

Keywords: Antioxidant, Broiler chicken, Herbal extracts, Immune, In-ovo

INTRODUCTION

In-ovo injection (IOI) with exogenous materials could be a technique to boost hatchlings' performance (Kadam et al., 2013). Many years ago, in-ovo technology was firstly became offered for the vaccination of broiler hatcheries (Ricks et al., 1999). Then, it had been wanted to deliver nutrients to embryos, since poultry have a restricted supply of nutrients for the development of the embryo (Uni et al., 2012). Thus, alimentary pack and inhibitor capability are also scarce to produce the embryo needs resulting in poor embryo development, reduced hatchability, and low quality of the chickens. Chicken quality covers all the parameters which directly relate to the ability of the chickens to generate a profit. This deficiency is also resolved by the supply of extra sources of essential nutrients and antioxidants via in-ovo administration (Urso et al., 2015). Nowadays, in-ovo feeding of antioxidants

throughout incubation may enhance the antioxidant status of the chickens' embryo (EL-Saadany et al., 2019) and post-hatch growth phases (Yigit et al., 2014). Also, in-ovo inoculation of extracts of many plant products have improved chicken immune status against the infectious bursal virus, avian influenza virus (H5N1), and fowl poxvirus (Sood et al., 2012; Nyandoro et al., 2014). In recent years, consideration has been given to the utilization of phytochemical substances as antioxidant constituents and growth promoters from spice, herbs, and their products (Oke et al., 2017; Oke, 2018) due to their benefits. Among these photobiotic plants, thyme (*Thymus vulgaris*), cinnamon (*Cinnamomum cassia*), and cloves (*Syzygium aromaticum* L.) attract more interest than else (Toghyani et al., 2011; Saki and Salary, 2015; Al-Mufarrej et al., 2019). Cinnamon is a plant containing several compounds, such as cinnamaldehyde, eugenol, and carvacrol (Chang et al., 2013) which have biological

activities as medical treatment, anti-inflammatory effects, and antioxidant properties (Gurdip *et al.*, 2007). It is also beneficial in poultry production (Sang-Oh *et al.*, 2013); Saeed *et al.*, 2018) and is used as an appetite and digestion stimulant (Toghyani *et al.*, 2011; El-Kholy *et al.*, 2019). Thyme is a plant containing complex mixtures of compounds, such as thymol, carvacrol, tannins, terpenoids, alkaloids, and flavonoids (Levic *et al.*, 2011). Demirel *et al.* (2011) and Levic *et al.* (2011) have reported that thyme is characterized as antimicrobial, antioxidant (Aliyu *et al.*, 2012), and digestive enhancers (Levic *et al.*, 2011). Clove is considered one of the spice herbs containing a large number of biologically active compounds, such as eugenol, eugenol acetate, and β -caryophyllene (Jimoh *et al.*, 2017), which has attracted considerable attention due to the potent antioxidant and antimicrobial activities standing out among the other spices (Shan *et al.*, 2005). Clove extract is commonly used in the food industry because of its special aroma and natural safety. In addition, the essential oil from clove also exhibited strong antibacterial properties. Clove and its ingredients have been shown to have the appetite and digestion stimulant (Kamel, 2001), potent antimicrobial and antifungal (Ehrich *et al.*, 1995), antiparasitic (Kim *et al.*, 2004), and antioxidant (Dragland *et al.*, 2003) properties. Since antioxidants have a major resistance against free radicals, the qualification of the chicken embryo can be improved by IOI with antioxidants (Salary *et al.*, 2014). Cinnamon, thyme, and clove have all been studied for their effects on broiler growth and physiological responses, and reported that their supplementation improves the performance productive organ characteristics, hematology parameters, immune response of broiler chickens, and biochemical blood status of poultry (Mahrous *et al.*, 2017; Pournazari *et al.*, 2017; Menati *et al.*, 2018; Al-Mufarrej *et al.*, 2019). There is currently little information on the effect of IOI of cinnamon, thyme, and clove extracts on broiler chicks under Egyptian condition. Therefore, the current study was conducted to elucidate the effect of IOI of cinnamon, thyme, and clove extracts on the productive performance, immunity, and some physiological responses of broiler chickens.

MATERIALS AND METHODS

Ethical approval

The present research was carried out in accordance with the Animal Care and Use Committee guidelines of the Damietta University, Damietta, Egypt (Approval number: 03/2018/du.edu). The hatching eggs and chickens

in this study were given proper care and management without causing them any unnecessary distress.

Preparation of herbal extracts

The flowers and leaves of the plant thyme, flowers (cloves), and root (cinnamon), purchased from a local market, were cleaned thoroughly. They were then dried at room temperature, then crushed into a coarse powder, each separately. Weighing out (100 g) of each of these herbal powders, soaking them in 400 ml of distilled water in a conical flask, and vigorously stirring with a glass rod produced the aqueous extract. The mixture was then placed in sterile conical flasks with sterile cotton plugs, and shaken for 12 hours at 200 rpm in a Shaking Incubator (Misung Scientific, Korea) to ensure proper extraction. The combinations were allowed to settle for 24 hours at room temperature. The solution was filtered and concentrated using muslin cloth three times/ herb, after which a clear aqueous extract of the plant was extracted. The extracts were then filtered using Whatman no.1 filter paper. Then, for the hot water extract, the residue was taken and soaked separately in 400 ml of boiled distilled water. That mixture was boiled for 30 minutes into a conical flask, then put for 24 hours at room temperature. The filter paper was used to filter the extract, and the process was repeated three times. The hot and cold extracts were mixed in a conical flask, and stirred vigorously with a glass rod, and kept in Shaker Incubator with 200 rpm for 24 hours. The extracts were kept in a refrigerator at 4°C until being used (Harborn, 1973).

Experimental procedures

A total number of 450 fertile broiler breeder eggs (Cobb Avian) were obtained from a local hatchery (Abdel-Baki Company, El-Wastany, Damietta, Egypt) from a maternal flock 53 weeks of age. Eggs were normally incubated at 37.7 °C and 65% Relative Humidity (RH) in an automatic incubator. On day 10 of incubation, eggs were divided into equal mainly five treatment groups which included three replicates (30 eggs each) in a completely randomized design of incubation. The first group was intact non-injected eggs, considered as the negative control (C), and the second group (Sham group) was injected with 0.1 ml of sterile distilled water, while the third, fourth, and fifth groups were injected into the air cell according to the procedure described by Saeed *et al.* (2019) with the same amount (0.1 ml/egg) of cinnamon, thyme, and clove extracts, respectively. The point site of injection was punctured by a hard and thin stylus, and the tested material was injected by using a graded insulin

syringe (1 ml), and the punctured site was sealed with non-toxic glue sticks. On day 18 of incubation, all eggs were transferred to the hatcher and kept till hatching at 36.5°C and 70% RH. The weight of newly hatched chickens was assessed at hatch, and 50 chickens per treatment were selected at random and moved to an experimental house for 35 days (marketing age).

Experimental animals

Chickens of each group were subdivided into five replicates of 10 chickens in each and housed in floor pens (1.2 m × 1.0 m × 3 m), and the ambient temperature during brooding was 34°C ± 1 at two days of age, and gradually reduced to 25°C ± 1 on day 21, and then kept constant. The hatched chickens from the five groups were fed *ad-libitum* on commercial starter (1-25 days old) and grower (26-35 days old) diets. The chemical composition of the basal diet is presented in Table 1. A basal diet was formulated according to NRC (1994).

Table 1. Composition and calculated analysis of starter and grower diets for chickens during the experimental period

Ingredients (%)	Starter	Grower
Yellow corn	58.50	62.50
Soybean meal (44%)	26.00	23.94
Maize gluten meal (62%)	10.00	7.00
Vegetable oil	1.500	2.50
Limestone	1.12	1.23
Di-Calcium Phosphate	1.75	1.70
Premix*	0.30	0.30
NaCl (salt)	0.30	0.30
L-lysine	0.36	0.36
DL-Methionine	0.17	0.17
Total	100	100
Calculated composition**		
ME*** (kcal kg-1)	3058.00	3120
Crude protein	22.45	20.20
Calcium	0.93	0.95
Non phytate phosphorus	0.46	0.45
Methionine	0.62	0.57
Lysine	1.28	1.2

*The premix at 0.30 of the diet supplies, the following per kg of the diet: A, 1000 I.U., Vit D3 2000 I.U., Vit E, 10 mg, Vit K, 1 mg, Vit B1, 5 mg, Vit B2, 5 mg, Vit B6, 1.5 mg, Vit B12, 0.01 mg, folic acid 0.35 mg, Biotin, 0.05 mg, Pantothenic acid 10 mg, Niacin 30 mg, Coline 250 mg, Fe, 30 mg, Zn, 50 mg, Cu, 4 mg and Se, 0.1 mg. **According to NRC (1994). ***ME: Metabolizable Energy.

Performance parameters

They included averages of Body Weight (BW), Body Weight Gain (BWG), Feed Intake (FI), and Feed Conversion Ratio (FCR), evaluated according to the method described as follow: Average daily Body Weight Gain (BWG) was weekly calculated as the difference

between current and previous weight divided by seven days. Daily Feed Intake (FI) and Feed Conversion Ratio (FCR) per bird were calculated weekly. Overall BW gain, FC, and FCR were calculated for the whole duration of the experiment (35 days).

Carcass measurements

At the end of the experiment (35 days of age), five broiler chickens were randomly picked from each replication for carcass evaluation. The birds were slaughtered after being starved (by feed withdrawal overnight) for about 12 hours, then individually weighted to the nearest gram, and slaughtered by severing the jugular veins of the neck with a sharp knife (Siekmann et al., 2018). When complete bleeding was achieved, the hot carcass was weighted. The internal organs (gizzard, Abdominal fat, heart, liver) and lymphoid organs (spleen, thymus, and Bursa) were dissected out, grossly examined, and weighted. The relative weights of these organs were weighted as proportional value to live pre-slaughtering weight.

Biochemical analysis

Blood samples were collected from five chickens per treatment, during their exsanguinations in weatherman tubes from each group, centrifuged at 4000 rpm for 15 minutes. Serum samples were stored at -20°C until analysis according to guidelines of Herling (2016).

Serum total protein and albumin were measured using a commercial kit as described by the manufacturer company (SpinreactCo., Spain) according to guidelines of Buzanovskii (2017) and Doumas and Maume (1977), respectively. Globulin (Glb, g/dl) values were obtained by subtracting albumin values from the corresponding values of total protein. Serum samples were also analyzed for concentrations of aspartate (AST, U/L) and alanine amino transaminases (ALT, U/L), and alkaline phosphatase (ALP, mg/dl) using commercial kits (Linear Chemicals, Barcelona, Spain) according to the manufacturer procedure. Also, the serum was assayed for Total Cholesterol (TC, mg/dl), Total glycerides (TG, mg/dl), High-density Lipoprotein (HDL, mg/dl), and Low-Density Lipoprotein (LDL, mg/dl) using standard protocol methods (Vogel and Vogel, 1997).

Serum Malondialdehyde (MDA, nmol/ml) was measured following the method described by Janero et al. (1990). Superoxide Dismutase (SOD, U/L) activity was measured based on the ability of SOD to inhibit the reduction of nitrobluetetrazolum superoxide (Martin et al., 1987); one unit of SOD is defined as the amount of sample resulting in 50% inhibition of nitrobluetetrazolum

reduction. The serum levels of Immunoglobulin A (IgA), Immunoglobulin G (IgG), and Immunoglobulin M (IgM) were determined by ELISA kits (Kamiya Biomedical Company, USA) following the instructions enclosed in the manufactured kits (Elabsience Company, Wuhan, China). Triiodothyronine (T₃) and thyroxin (T₄) were determined in sera using the ELISA technique according to Walker (1977).

Statistical analysis

Data were subjected to the analysis of variance by using a one-way analysis of variance (SAS, 2004). The following fixed model was used:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where, Y_{ij} is the observation of the jth chickens in the treatment, μ : Overall mean, T_i denotes the effect of the treatments (i: 1, 2, 3, 4, and 5), and e_{ij} stands for random error component. A probability of $p \leq 0.05$ was required for statements of significance. Differences among

treatment means were detected using Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Performance parameters

As shown in Table 2, in-ovo administration of herbal extracts significantly ($p \leq 0.05$) affected the hatching BW, Final BW, daily weight gain, and FCR during different experimental periods. Among these parameters, only feed intake was not significantly affected. Hatching weight was significantly higher when herbal extracts were received as compared to the control and sham groups. Also, chickens from eggs injected with herbal extracts had better BWG and FCR than the chickens hatched from the control and sham groups throughout the experimental rearing period. During the first days of rearing, chickens mobilized progressively the nutrients as an additional substance to the starter diet given that feed intake was not affected.

Table 2. Effect of in-ovo injection of some herbal extracts on the chickens' weight and subsequent performances of newly-hatched chickens

Parameters	Experimental Treatments					p value	SEM*
	Control	Sham	Cinnamon extract (0.1 ml)	Thyme extract (0.1 ml)	Clove extract (0.1 ml)		
Chick weight at hatch (g)	43.90 ^b	42.40 ^b	46.95 ^a	45.38 ^a	47.16 ^a	0.039	4.66
Final body weight (g)	2216.00 ^c	2223.00 ^c	2686.00 ^b	2798.00 ^a	2847.00 ^a	0.015	206.7
Weight gain (g)							
1-21 days of age	873.10 ^{ab}	787.20 ^b	961.89 ^{ab}	1014.13 ^a	1149.40 ^a	0.008	80.30
21-35 days of age	1299.00 ^c	1393.40 ^c	1677.20 ^b	1738.50 ^a	1650.50 ^a	0.043	171.22
1-35 days of age	2172.10 ^c	2180.60 ^c	26390.10 ^b	2752.60 ^a	2799.80 ^a	0.015	262.3
Feed intake (g)							
1-21 days of age	1473.68	1282.20	1400.00	1300.00	1391.67	0.862	140.3
21-35 days of age	2130.39	2183.48	1990.00	2225.50	1973.48	0.739	161.3
1-35 days of age	3604.08	3465.67	3390.00	3525.50	3365.14	0.975	291.5
Feed conversion ratio							
1-21 days of age	1.6879 ^a	1.6288 ^a	1.4555 ^b	1.2907 ^c	1.2138 ^c	0.001	0.04
21-35 days of age	1.65 ^a	1.59 ^a	1.20 ^b	1.29 ^b	1.21 ^b	0.009	0.09
1-35 days of age	1.66 ^a	1.60 ^a	1.29 ^b	1.29 ^b	1.20 ^b	0.001	0.06

*SEM: Standard Error of Mean. ^{a,b,c} Means within the row with different superscripts are significantly different ($p \leq 0.05$).

They have been shown to stimulate bile salt secretion and digestive enzyme activities of the intestinal mucosa and pancreas (Dalkılıç and Güler, 2009). The results of the present study were consistent with those previously reported by Nnanle et al. (2017) who found that IOI of natural antioxidant could be improved the chickens' weight at hatch compared to the non-injected groups. Similar results were confirmed by Elwan et al. (2019). In contrary to the present results, Cross et al. (2007) and Abdel-Ghaney et al. (2017) indicated that herbs, plant extracts, essential oil, and/or the main components of the essential oil did not affect the BWG, or feed efficiency in

broiler chickens. The results of the current study revealed that IOI of herbal extracts on day 10 of incubation resulted in increasing the chickens' weight at hatch, and this increase may be attributed to the improved antioxidant status of embryos. However, the alleviation of the hatch-related oxidative stress may lead to a higher hatch weight and post-hatch performance through the protection of skeletal muscle stem cells from oxidative damages (Choi et al., 2016). Also, aromatic plants and their extracts can favorably stimulate endogenous digestive secretions and establish intestinal epithelial structures to influence gut functions (Jang et al. 2007; Yang et al., 2019). So, the in-

ovo administration of clove extract improved the chick growth performance. The result showed an improvement in the productive performance of broiler chickens due to the present active material in clove (*Eugenia caryophyllus*) which is considered a digestion stimulating factor, and it had an antibiotic effect against organisms in the digestive canal. Mentioned material caused a greater efficiency in utilization of feed, and led to an improvement in the growth performance (Azadegan et al., 2013). In addition, many studies have reported that clove (*Eugenia caryophyllus*) was rich in trace minerals which are essential for protein and carbohydrate metabolism, and could improve broiler chickens' performance (AL-Tabari et al., 2018).

Carcass characteristics

Carcass characteristics of Avian broiler chickens are presented in Table 3, and it was shown that all the examined carcass traits except carcass weight, heart weight, and bursa gland were not affected significantly (p

> 0.05) by in-ovo injection of different herbal extracts. Al-Kassie (2009) reported a significant effect on carcass weight (%) and internal organs' percentage (liver, heart, and gizzard). A large number of biologically active compounds found in cinnamon, thyme, or clove could be responsible to impulse the immune system.

The Spleen, thymus, and bursa of Fabricius are important immune organs for animals, and their status is closely associated with immune functions. Ravis et al. (1988) reported that the relative weight of immune organs could be used to evaluate the immune status, and greater weights of immune organs usually represent stronger immune functions to some extent. In the present study, IOI of different herbal extracts did not affect the weights of immune organs (spleen and thymus), which was in an agreement with the study of Toghiani et al. (2011), and Mohammad et al. (2019) who found that the diet supplemented with different natural antioxidants did not influence weights of spleen of broiler chickens on 42 days of age.

Table 3. Effect of in-ovo injection of some herbal extracts on carcass characteristics of broiler chickens

Parameters	Treatments					p value	SEM*
	Control	Sham	Cinnamon extract (0.1 ml)	Thyme extract (0.1 ml)	Clove extract (0.1 ml)		
Live body weight (g)	2216.00 ^b	2223.00 ^b	2686.00 ^a	2798.00 ^a	2847.00 ^a	0.0001	64.83
Carcass weight (%)	79.66 ^c	79.54 ^c	82.05 ^b	83.18 ^{ab}	84.56 ^a	0.0005	0.77
Liver weight (%)	2.70	6.82	2.52	2.65	2.65	0.3326	0.10
Gizzard weight (%)	1.37	1.48	1.31	1.25	1.16	0.5112	0.13
Heart weight (%)	0.41 ^b	0.51 ^a	0.50 ^{ab}	0.54 ^a	0.47 ^{ab}	0.0540	0.03
Bursa gland weight (%)	0.07 ^b	0.07 ^b	0.11 ^a	0.13 ^a	0.13 ^a	0.0001	0.01
Thymus gland weight (%)	0.29	0.28	0.28	0.26	0.26	0.7251	0.02
Spleen weight (%)	0.17	0.17	0.23	0.22	0.21	0.1044	0.02
Abdominal fat weight (%)	1.04	0.94	0.92	0.92	0.85	0.7130	0.09

*SEM: Standard Error of Mean. ^{a,b,c} Means within the row with different superscripts are significantly different (p ≤ 0.05).

Biochemical parameters

Results of blood biochemical parameters are presented in Table 4. Liver enzymes, globulin fraction, cholesterols, LDL, HDL, and total glycerids levels were significant (p ≤ 0.05) affected by the IOI of herbal extracts in broiler chickens' eggs. The current results were in agreement with Ismail et al. (2019) and Oke et al. (2021) who showed that these blood biochemical traits were significantly affected by IOIs of natural antioxidants (spirulina and black cumin extract). The obtained results also showed that a significant increase in TP and Glb concentration for chickens produced from injected eggs with 0.1 ml clove extract/egg as compared with other experimental groups but these increases were still within

the normal range as indicated by the non-sign of toxicity (Table 4). The Alb/Glb ratio showed an opposite trend to that of Glb results, which was higher in the control and sham groups and lower in herbal extracts groups (Table, 3). This finding agreed with the results of a study conducted by Tag El-Dein et al. (2020). The decrease in Alb/Glb ratio seemed to be due to the increase in Glb rather than the decrease in Alb. This may reflect the positive increase in immunity through the elevation of the gama-globulin (El-Kholy et al., 2019). The IOI of either thyme or clove broiler chickens' eggs had lower lipids profile than those from the control and sham groups. These results also agreed with the experiments by Mehr et al. (2014) and AL-Tabari et al. (2018) who demonstrated that

dietary addition of clove extract decreased cholesterol and LDL in broiler chickens. The results released a significant decrease in cholesterol concentration due to the main component of clove (*Eugenia caryophyllus*), which could be inhibited hepatic 3-hydroxy -3 methylglutaryl coenzyme (HMG-COA) reductase activity, and led to hypocholesterolemia (Mittal et al., 2014; Shima, 2015). In general, hypocholesterolemia might be an indicator that lipid peroxidation was reduced by IOI of either thyme or clove in the broiler chickens' eggs via enhancing anti-oxidative action. Whereas, antioxidant properties of herbal extracts prevented peroxidation of fatty tissue lipid, especially unsaturated fatty acids. Hypertriglyceridemia effects in chickens fed with cinnamon may be due to active ingredients leading to a decrease in the activity of lipogenic enzymes, and thus it was contributed to reducing re-synthesis (de novo) of fatty acids in the liver and subsequently reducing blood LDL level. Also, the hypocholesterolemia and antihyperlipidemic effect of

thyme may be due to the action of thymol and carvacrol on HMG-CoA reductase which reduced fat absorption from the gut or the lipid catabolism for gluconeogenesis (El-Ghousein and Al-Beitawi, 2009; Abdulkarimi et al., 2016). Serum ALT, AST and ALP levels were significantly ($p \leq 0.05$) decreased in the herbal extracts groups in comparison with the control and sham groups. The lowest values were recorded in thyme and clove extracts for ALT and AST, compared to other experimental groups. These results were in partial agreement with koochaksaraie et al. (2011), and Al-Shuwaili et al. (2015) who showed that supplemented groups with garlic 5%, Ginger 5%, and cinnamon 5% reduced ($p \leq 0.05$) AST and ALT significantly. Generally, Hernandez et al. (2004) and Al-Shuwaili et al. (2015) showed that AST and ALT are considered liver enzymes that increase with liver damage (hepatocellular degeneration), so the decrease in AST and ALT may provide evidence for the occurrence of the hepatoprotective effect.

Table 4. Effect of in ovo injection of some herbal extracts on some biochemical parameters of broiler chickens

Parameters	Treatments					p value	SEM*
	Control	Sham	Cinnamon extract (0.1 ml)	Thyme extract (0.1 ml)	Clove extract (0.1 ml)		
Total protein (g/dl)	4.92 ^b	4.90 ^b	5.20 ^{ab}	5.26 ^{ab}	5.48 ^a	0.0544	0.15
Albumin (g/dl)	2.50	2.54	2.56	2.50	2.58	0.8931	0.07
Globulin (g/dl)	2.44 ^b	2.36 ^b	2.64 ^{ab}	2.76 ^a	2.90 ^a	0.0039	0.10
A/G ratio	1.03 ^{ab}	1.09 ^a	0.97 ^{bc}	0.91 ^c	0.89 ^c	0.0004	0.03
Cholesterol (mg/dl)	200.40 ^a	195.20 ^a	184.60 ^{ab}	169.80 ^b	168.40 ^b	0.0032	6.07
Total glycerides (mg/dl)	170.40 ^a	159.60 ^{ab}	139.60 ^{bc}	152.00 ^{abc}	138.20 ^c	0.0119	6.60
High density lipoprotein (mg/dl)	41.80 ^b	38.80 ^b	49.00 ^a	53.40 ^a	55.20 ^a	0.0001	2.08
Low density lipoprotein (mg/dl)	124.52 ^a	124.48 ^a	107.68 ^a	86.00 ^b	85.86 ^b	0.0001	6.20
Aspartate Amino Transaminase (U/L)	27.00 ^{ab}	30.80 ^a	23.20 ^{bc}	21.80 ^c	20.80 ^c	0.0023	1.68
Alanine amino transaminase (U/L)	55.85 ^{ab}	56.50 ^a	52.27 ^{ab}	48.66 ^{bc}	44.31 ^c	0.0096	2.41

*SEM: Standard Error of Mean. ^{a,b,c} Means within the raw with different superscripts are significantly different ($p \leq 0.05$).

Table 5. Effect of in-ovo injection of some herbal extracts on serum Malondialdehyde, Superoxidedismutase, immunoglobulins, and Triiodothyronine and Thyroxine of broiler chickens

Parameters	Treatments					p value	SEM*
	Control	Sham	Cinnamon extract (0.1 ml)	Thyme extract (0.1 ml)	Clove extract (0.1 ml)		
MDA (mg/ml)	17.48 ^a	16.04 ^{ab}	13.98 ^c	15.08 ^{cb}	14.48 ^{cb}	0.0060	0.62
SOD (U/ml)	35.16 ^d	45.04 ^{cd}	50.90 ^{bc}	64.69 ^a	60.38 ^{ab}	0.0001	3.38
IgG (mg/dl)	66.20 ^c	69.00 ^c	86.20 ^b	96.40 ^a	99.60 ^a	0.0001	2.88
IgA (mg/dl)	13.60	13.40	13.60	12.20	12.60	0.9186	1.34
IgM (mg/dl)	23.20 ^c	24.20 ^{bc}	25.20 ^{ab}	24.60 ^{bc}	26.40 ^a	0.0250	2.66
T4 (ng/ml)	17.60 ^b	16.20 ^b	17.60 ^b	17.80 ^b	23.00 ^a	0.0026	0.15
T3 (ng/ml)	0.82 ^b	0.82 ^b	0.86 ^b	0.80 ^b	1.42 ^a	0.0131	0.13

^{a,b,c} Means within the raw with different superscripts are significantly different ($p \leq 0.05$). MDA: Malondialdehyde, SOD: Superoxide Dismutase, IgG: Immunoglobulin's G, IgA: Immunoglobulin's A, IgM: Immunoglobulin's M, T4: Thyroxine, T3: Triiodothyronine.

Serum malondialdehyde, superoxide dismutase, immunoglobulins, Triiodothyronine, and Thyroxine

Table 5 shows the effect of IOI of herbal extracts on serum Malondialdehyde (MDA), Superoxide Dismutase (SOD), Triiodothyronine (T₃), and Thyroxine (T₄) of broiler chickens at market age. The serum MDA and SOD of the birds in the groups that received herbal extracts were lower and higher, respectively than that of sham and control groups. The values of T₃ and T₄ of the chickens injected with herbal extracts, except the group with clove extract, were similar. The levels of T₃ and T₄ of the birds with clove extract were higher than that of other experimental groups. All these results were in agreement with the findings of [Oke et al. \(2021\)](#). Antioxidants have been shown to provide an oxidative defense to the intestines and other organs of developing embryos, protecting them from free radicals that could damage development before hatching ([Surai et al., 1999](#)). The use of antioxidants on developing embryos has been reported to confer oxidative protection on the intestines and other organs from free radicals that could impair development before hatching ([Surai et al., 1999](#)). Indeed, antioxidant protection is an important mechanism on chickens' development at hatching time ([Surai, 2002](#)). Cinnamon Essential Oils (CEO, its main active component is cinnamaldehyde) have been proved to be strong antimicrobials ([Chang et al., 2013](#)). Earlier studies have shown that cinnamon, thyme, or clove could be used as a natural antioxidant for avian ([Abdel-Ghaney et al., 2017](#); [Yang et al., 2019](#)). Malondialdehyde is a biomarker of lipid peroxidation, and it is used to assess oxidative damage ([Jensen et al., 1997](#)). The higher serum SOD in the injected groups than that of the control and sham groups in the present study corroborated the findings of [Mostafa et al. \(2013\)](#) who reported that the use of black cumin as a natural antioxidant resulted in higher SOD in human. The improvement in the oxidative parameters in the chickens received in-ovo herbal extracts in the present study affirmed the observation of [Tollba and Hassan \(2003\)](#) declaring that the use of black cumin relieved the thermal stress effect. The increase in the pattern of the chicken's oxidative parameters at market age as in the present study indicated that the IOI of the herbal extract had a carryover effect on the broiler chickens.

Indeed, previous studies have shown that cinnamon, thyme, and clove possess antioxidant activities which could enhance various enzyme activities including SOD, catalase, and Glutathione-S-transferase which are involved in oxidative stress modulation in broiler chickens. The

effect of IOI of herbal extracts on plasma immunoglobulin (IgA, IgG, and IgM) in avian hatched chickens are presented in Table 5. Plasma IgG was significantly ($p \leq 0.05$) increased in the groups with herbal extracts compared to other groups. On the other hand, plasma concentration of IgG was increased by 30.2, 45.6, and 50.0% for the three herbal extracts treatments (cinnamon, thyme, and clove), respectively compared with the control group. On the other hand, either IgA or IgM was not affected by the injection. These findings were confirmed with [Abdel-Ghaney et al. \(2017\)](#) who found that chickens fed diets supplemented with thyme (0.5%) achieved the highest values of IgG than those fed the control diet. Herbs that are rich in flavonoids as thyme extended the activity of vitamin C, acting as antioxidants and, therefore, enhance the immune function ([Acamovic and Brooker, 2007](#)). [Nadia et al. \(2008\)](#) found that 0.1% thyme-fed to laying hens gave better antibody production response compared to 100 or 200 mg/kg vitamin E which is a potent immunomodulation. It is well accepted that immunoglobulins can be used to evaluate immune status due to their importance in immune functions. The level of triiodothyronine and thyroxine of the birds of clove extract group in the present study suggested that clove exerts its effects through the thyroid axis. In agreement with present findings, a previous study indicated that herbs, as natural antioxidants, enhance the concentration of thyroxine, thereby positively influence the rate of metabolism.

The concentration of serum T₃ and T₄ of the chickens that received thyme and cinnamon extracts, were statistically similar to untreated groups (sham and control), indicating that the levels of thyme or cinnamon extracts did not upregulate this hormone differently.

CONCLUSION

It was concluded that in-ovo injection of herbal extracts, especially clove extract on day 10 of incubation has positive effects on chickens' weight at hatch and post-hatch performance as well as the physiological, immunological, and anti-oxidative status of broiler hatched chickens. The mechanisms of in-ovo injection of herbal extracts on the B-cell and T-cell compartments need to be further investigated, especially in avian species.

Competing Interests

The authors declare that they have no conflict of interest.

Authors contribution

K.H.E., E.A.E., and D.M.A.S. developed the concept of the manuscript. K.H.E. wrote the manuscript. All authors checked and confirmed the final revised manuscript.

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