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Effect of Cocoa and Thyme Powder Alone or in Combination on Humoral Immunity and Serum Biochemical Metabolites of Broiler Chicks

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Abstract. This experiment was conducted to evaluate the effect of cocoa and thyme powder separately or in combination on serum biochemical parameters and humoral immune responses in broiler chicks. Two hundred and forty day-old broiler chicks (Ross 308) were randomly assigned to four treatments with four replicates of 15 chicks based on a completely randomized design. The dietary treatments included the basal diet as control, supplemented groups receiving 3 g/kg cocoa powder, 5 g/kg thyme powder and 2+3 g/kg a combination of cocoa and thyme added to the basal diet. Blood samples were taken at 28 d to quantify antibody titer production against Newcastle and Influenza viruses and SRBC. Serum biochemical metabolites and leucocyte types were determined at 42 d of age. Serum total protein and globulin concentration increased significantly ($p<0.05$) in birds fed diets supplemented with a combination of additives. Diet supplementation resulted in a marked reduction of serum total and HDL cholesterol compared to control group ($p<0.05$). Dietary treatments did not induce any significant impacts on antibody titer production, heterophil, lymphocyte, monocyte and eosinophil counts, albumin to globulin and heterophil to lymphocyte ratios. In conclusion application of thyme and cocoa powder, particularly their combination in broiler diets has the potential to improve serum biochemical values but failed to have favourable impact on humoral immune responses of the birds.

Keywords: Broiler, Thyme, Cocoa, Serum biochemistry, Antibody titer

1. Introduction

Due to the European Union ban on subtherapeutic use of antibiotics as growth promoters in animal diets and growing pressure on livestock producers in other parts of the world, alternative substances for animal growth promotion are being investigated, among which phytogenic and herbal products have received increased attention. Phytogenic additives, plant extracts, essential oils, prebiotics, and probiotics have largely been investigated as possible in-feed antibiotic substitutions, and some have proved satisfactory results.

Thyme is a popular medicinal plant mostly grown in Mediterranean regions and is among the herbal plants which have received increased attention due to its antioxidant and antibacterial properties. The herb has also been reported to have antibacterial activities against a wide range of pathogenic microbial organisms [1]. The major components of thyme essential oil are thymol and carvacrol, which both have been shown to possess potent antioxidant properties [2] in addition these phenolic compounds exhibit considerable antimicrobial and antifungicidal activities [3]. Thymol has used to inhibit oral bacteria (Twetman and Peterson, 1997). Furthermore, Allen et al. [5], Denil et al. [6] and Cross et al. [7] reported the beneficial effects of thyme in poultry production.

Cocoa has recently become a matter of interest as a therapeutic natural product due to its flavonoid content. Cocoa polyphenols have been reported in many studies as bioactive compounds, with antioxidant,

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antiradical and anticarcinogenic properties [8],[9]. Oligomeric procyanidins isolated from cocoa have been shown to possess biological activities potentially relevant to oxidant defences and immune function [10]. Also, it is well-known that methylxanthines have physiological effects on various body systems, including the central nervous, cardiovascular, gastrointestinal, respiratory and renal systems [11].

In view of all mentioned positive biological effects of thyme and cocoa, the current study was conducted to investigate the effect of foregoing herbal additives on serum biochemistry and some humoral immune reponses of broiler chicks.

2. Material and Methods

2.1. Animals and diets

Three hundred, day-old male broiler chicks (Ross 308) purchased from a local hatchery, were weighed on arrival and randomly assigned to one of four treatments with five replicates of 15 chicks based on a completely randomized design. The dietary treatments included the basal diet as control, supplemented groups receiving 3 g/kg cocoa powder, 5 g/kg thyme powder and 2+3 g/kg a combination of cocoa and thyme added to the basal diet. The basal corn-soybean diets were formulated to meet or exceed the nutrient requirements of broilers provided by Ross Manual Catalogue (2007); also, the same batch number of ingredients, which contained no antibacterial or anticoccidial supplements, was used to formulate the diets for different periods. Chicks were raised on floor pens covered by sawdust as litter (10 birds/m2) for 6 weeks. Food and water were provided for ad libitum consumption throughout the experiment. The lighting program consisted of a period of 23 hours light and 1 hour of darkness. The house ambient temperature was initially set at 33°C by the first week and gradually decreased until 25°C was reached by the third week and was then kept constant.

2.2. Immune parameters

Birds were intramuscularly vaccinated against Influenza and Newcastle viruses at day 18 of experiment. At 22 d of age, 15 wing banded birds from each treatment groups were injected i.v. with 1 ml of a 1% suspension of sheep red blood cell (SRBC) prepared in phosphate-buffered saline. To assess the systemic antibody response to Influenza, Newcastle, and SRBC blood samples were collected from brachial vein of vaccinated (three birds per replicate), and challenged birds on 28 d respectively. Blood samples were kept at room temperature for 2 hours and then at 4°C overnight. Antibody titers against Newcastle and Influenza viruses were measured using Hemagglutination Inhibition Test. Anti-SRBC titers were measured by the microtiter procedure of Wegmann and Smithies [12]. All titers were expressed as the $\log_2$ of the reciprocal of the highest dilution giving visible hemagglutination. At day 42, three birds per replicate were selected and blood samples were collected by syringes containing heparin to avoid blood clot formation. Blood films were air dried (unfixed) and stained in concentrated May-Greenwald stain for 6 min, 1:1 May-Greenwald stain distilled water for 1.5 min and 1:9 Geisma stain for 15 min [13]. A minimum of one hundred leukocytes per samples were counted by heterophil to lymphocyte separation under an optical microscope (Nikon, Japan) with 100x oil immersion lens, then heterophil to lymphocyte (H/L) ratio was calculated and recorded.

2.3. Serum biochemical metabolites

After 12 h fasting, on day 42 of the experimental period, 3 ml of blood was collected by puncturing the brachial vein from three birds per pen. Serum samples were isolated by centrifugation at 2000×g for 10 minutes. Individual serum samples were analyzed for total protein, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, triglyceride, by an automatic biochemical analyzer following the instructions of the corresponding reagent kit (Pars Azmoon Co., Tehran, Iran). Globulin concentration in serum was computed by subtracting albumin concentration from proteins, and consequently albumin to globulin ratio was calculated.

2.4. Statistical analysis

Data analysis for the sampled traits were performed using Mixed Model procedure of of SAS Institute (2008) followed by considering birds within experimental unit as repeated measures. Tukey–Kramer method was used to assess any significant differences at the probability level of $P \leqslant 0.05$ among the experimental treatments.

3. Results and Discussion

Table 1 summarizes the effect of treatments on serum biochemical parameters of chicks determined at 42 d of age. Supplementing diets with thyme and cocoa powder significantly ($p<0.05$) reduced serum albumin concentration; however, application of a combination of additives resulted in a marked elevation on serum total protein and globulin level compared to the other treatments ($p<0.05$). Albumin to globulin ratio was calculated to be numerically lower in cocoa and cocoa + thyme group but the differences observed did not reach statistical significance. Diet supplementation also, caused a statistical decrease in total cholesterol and accordingly HDL cholesterol levels ($p<0.05$).

Table 1. Effect of dietary treatments on serum biochemical metabolites at 42 d of age.

Serum Parameters	Treatments				P-Value
	Control	Thyme	Cocoa	Cocoa + Thyme	
Total Protein (g/dL)	3.92±0.084 ^{ab}	3.72±0.109 ^{b}	3.85±0.086 ^{ab}	4.07±0.100 ^{a}	0.084
Globulin (g/dL)	1.97±0.05 ^{b}	1.92±0.08 ^{b}	2.04±0.05 ^{ab}	2.17±0.07 ^{a}	0.068
Albumin (g/dL)	1.95±0.048 ^{a}	1.80±0.043^{b}	1.81±0.042 ^{b}	1.90±0.041 ^{ab}	0.054
A/G ratio	1.011±0.03	1.011±0.06	0.897±0.02	0.893±0.02	0.057
Cholesterol (mg/dL)	165.7±3.66 ^{a}	152.7±2.81 ^{b}	151.1±3.47 ^{b}	155.9±3.37 ^{b}	0.012
HDL cholesterol (mg/dL)	95.20±1.88 ^{a}	87.50±2.40 ^{b}	86.33±2.69 ^{b}	89.86±2.86 ^{ab}	0.063
LDL cholesterol (mg/dL)	11.87±2.16	13.51±2.46	10.98±2.00	10.94±1.99	0.254
Triglyceride (mg/dL)	62.53±3.43	63.60±3.45	60.03±3.27	60.86±3.05	0.871

Values in a row not sharing a common superscript differ significantly ($p<0.05$)

Similar to current findings Ruzaidi et al. [14] showed that supplementation of 1 and 3% cocoa extract had significantly reduced the level of total cholesterol in diabetic rats. However, the authors reported lowered triglycerides concentration of serum by addition of 1, 2, and 3% cocoa extract to the diets which was not evident in our study. Abrokwah et al. [15] investigated the effect of consumption of natural cocoa powder on some biochemical and hematological indices in the rat. The results obtained showed significant reduction of total cholesterol, LDL cholesterol and triglyceride levels, WBC count was also elevated in cocoa fed animals but no marked differences were observed on serum protein fractions. The significant decrease in the total cholesterol levels by cocoa supplementation may have resulted from the antioxidant properties of the polyphenols in cocoa. Since, these antioxidants have the ability to increase the synthesis of nitric oxide which has the ability to cause vasodilation, resulting in the clearance and prevention of the deposition of excess cholesterol in the blood vessels. The beneficial effects of nitric oxide modulation include the regulation of blood pressure, lowering of NO-affected hypercholesterolemia and monocyte adhesion, all of which are involved in the progression of atherosclerosis [16].

The results reported by Ali et al. [17] indicated that adding thyme to hen's rations significantly decreased plasma HDL and total cholesterol. The reduction of triglycerides and cholesterol noticed by thyme in animal studies has been attributed to the lowering effect of thymol or carvacrol on HMG-Co A reductase the rate-limiting enzyme of cholesterol synthesis [18]. In addition, combination of cocoa and thyme revealed the synergic effects of additives on serum biochemical parameters tested. So that, the differences obseved for protein and albumin concentrations in cocoa+thyme goup rechead statistical significance and for other parameters such as triglyceride and LDL better values were obtained in this treatment.

As Table 2 and 3 exhibit dietary treatments failed to induce any significant impact on antiboy production against Newcastle, Influenza and sheep red blood cells at different ages. In addition, leucocyte types counted did not differ markedly among treatments. Although the dietary treatments did not induce any significant effect on the immune related parameters measured in this study, no deleterious impact was also detected as a result of treatments.

A/G ratio has been used as an indicator of immune responses so that high globulin level and low A/G ratio signify better disease resistance and immune responses [19]. In addition, the reliability of H/L Ratio as a biological index of stress in avian species is also, well documented [20]. Although the lower H/L and A/G ratios observed in cocoa and cocoa + thyme supplemented groups did not reach statistical significance compared to control birds but it could imply the positive influence of additives and their synergic effects on stress and immunity.

Cocoa flavonoids are potent anti-oxidants [21], and its intake has been reported to be valuable in certain pathological and/or physiological states associated with free radical production, in vitro studies have shown the ability of cocoa flavonoids to modulate immune responses such as lymphocyte activation [22] and down-regulating inflammatory mediators produced by stimulated macrophages [23]. Thyme has aslo been reported to have antibacterial and antifungicidal activities [1,3] and the major components of thyme essential

oil thymol and carvacrol, have been indicated to possess potent antioxidant properties; and consequently, elevated immune responses of chicks were expected.

Table 2. Effect of dietary treatments on antibody titer against Newcastle, Influenza and SRBC.

Antibody titer	Treatments				P-Value
	Control	Thyme	Cocoa	Cocoa + Thyme	
New castle (log_2)	5.13±0.23	5.13±0.30	4.85±0.32	4.53±0.27	0.424
Influenza (log_2)	5.13±0.40	5.40±0.33	5.71±0.36	5.00±0.33	0.529
SRBC (log_2)	6.73±0.42	5.86±0.49	6.07±0.51	6.06±0.39	0.561

The lack of statistically significant effects of the thyme and cocoa on immune responses might be related to the inclusion levels of the additives in the diets. Additives supplementation levels in the present experiment may not be optimal for enhancing immune responses in chickens. Nevertheless, the health status of birds, hygienic status of experimental site, external challenges and basal diets composition and digestibility may to a great extent account for the contradictory and inconsistent reports in the literature regarding the immunomodulatory impact of thyme and cocoa. Moreover, different types of cocoa, different countries of origin, degree of fermentation and roasting might have different composition of polyphenol content [24].

Table 3. Effect of dietary treatments on Leucocyte type counts at 42 d of age.

Leucocyte type	Treatments				P-Value
	Control	Thyme	Cocoa	Cocoa + Thyme	
Heterophil	29.33±1.09	27.86±1.51	28.66±1.22	27.40±1.03	0.694
Lymphocyte	67.86±1.17	70.00±1.40	68.86±1.30	69.73±1.14	0.628
H/L	0.437±0.02	0.405±0.02	0.422±0.02	0.397±0.02	0.663
Monocyte	1.93±0.11	1.93±0.11	1.93±0.15	2.00±0.16	0.979
Eosinophil	1.30±0.15	1.20±0.13	1.00±0.03	1.18±0.12	0.455

4. Conclusions

Considering the results of the current study it could be concluded that application of thyme and cocoa powder, particularly their combination in broiler diets has the potential to improve serum biochemical values but may not exert any favourable impact on humoral immune responses of the birds.

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