

Effect of the Three Herbal Extracts on Growth Performance, Immune System, Blood Factors and Intestinal Selected Bacterial Population in Broiler Chickens

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ABSTRACT

A research study was conducted to evaluate the effects of three herbal extracts and an antibiotic –virginiamycin- on growth performance, immune system, blood factors and selected intestinal bacterial populations in broiler chickens. A total of four hundred and eighty 1-day old male broiler chicks were assigned to the basal diet (control) , basal diet supplemented with 15 ppm of virginiamycin, basal diets with a 0.1% dose of either thyme (*Thymus vulgaris*), coneflower (*Echinacea purpurea*), garlic (*Allium sativum*) or a blend of the three extracts in the drinking water. The highest and the lowest body weight and weight gain were related to virginiamycin and coneflower, ($P < 0.05$) respectively. The lowest and the highest feed conversion rates were respectively related to virginiamycin and coneflower ($P < 0.05$). Relative weight of bursa Fabricius in the garlic group showed a significantly more increase as compared with other groups, while the relative weight of spleen was unaffected by treatments. Cutaneous basophils hypersensitivity response (to phytohemagglutinin injection) and antibody response to Sheep Red Blood Cells (SRBC) was higher in coneflower group ($P < 0.05$). Antibody responses to Newcastle Disease vaccine (LaSota) was unaffected by treatments but coneflower improved antibody levels ($P > 0.05$). Garlic (*Allium sativum*) significantly reduced the serum levels of cholesterol, LDL, and triglyceride as well as significantly increasing the level of HDL. Thyme (*Thymus vulgaris*) improved hematocrit percentage and hemoglobin concentration, but not significantly. The colony forming units of *Escherichia coli* in digesta of ileo-cecum in the blend group showed a significantly lower number compared with control. However, there was no difference observed in *E. coli* counts between blend group and others, except for control. The lactic acid bacteria counts in the thyme group increased as compared to other groups, except for coneflower ($P < 0.05$).

Keywords: Blood factors, Coneflower, Garlic, Immune system, Performance, Thyme.

INTRODUCTION

A number of feed additives including antibiotics have been widely employed in the poultry industry for several decades. A manipulation of gut function and microbial habitat of domestic animal with feed additives has been recognized as an important tool for improving growth performance and feed efficiency (Collington *et al.*, 1990).

Approximately 80% of domestic animals have been fed synthetic compounds for the purpose of either medication or growth promotion (Lee *et al.*, 2001). Recently, the concerns about possible antibiotic residues and antibiotic resistance have aroused great caution in the usage of antibiotics in the animal industry.

The banning of the use of antibiotics as feed additives has accelerated and led to investigations of alternative feed additives in

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animal production. As one of the alternatives, herbal extracts are already being used as feed supplements to improve growth performance under intensive management systems (William and Losa, 2001). Plant extracts and spices as single compounds or as mixed preparations can play a role in supporting both performance and health status of the animal (Janssen, 1989; Horton *et al.*, 1991; Bakhiet and Adam, 1995; Skrabka Blotnicka *et al.*, 1997; Gill, 2000; Manzanilla *et al.*, 2001). Beneficial effects of herbal extracts or active substances in animal nutrition may include the stimulation of appetite and feed intake, the improvement of endogenous digestive enzyme secretion, activation of immune response and antibacterial, antiviral, antioxidant and antihelminthic actions. Isoprene derivatives, flavonoids, glucosinolates and other plant metabolites may affect the physiological and chemical function of the digestive tract. The stabilizing effect on intestinal microflora may be associated with intermediate nutrient metabolism (Horton *et al.*, 1991; Baratta *et al.*, 1998; Jamroz *et al.*, 2003). The pharmacological action of active plant substances or herbal extracts in humans is well known, but in animal nutrition the number of precise experiments is relatively low.

Volatile oil from thyme (*Thymus vulgaris*) was assessed for antibacterial and antiviral activity as inhibitors of microbial growth (Dorman and Deans, 2000). Garlic (*Allium sativum*) is widely distributed and used in all parts of the world as a spice and herbal medicine for the prevention and treatment of a variety of diseases, ranging from infections to heart diseases. Garlic is thought to have various pharmacologic properties. For example, it has been found to lower serum and liver cholesterol (Qureshi *et al.*, 1983), inhibit bacterial growth (Cavallito and Bailey, 1994), inhibit platelet growth and reduce oxidative stress (Horie *et al.*, 1992). Tollba and Hassan (2003) found that garlic as a natural feed additive, improved broiler growth and Feed Conversion Ratio (FCR), and decreased mortality rate. *Echinacea* plant extract is widely used as a self prescribed agent against such upper respiratory tract infections as

common cold (O'Hara *et al.*, 1998). According to many *in vitro* studies, it is thought to stimulate macrophage activity and hence the immune system (Stimpel *et al.*, 1984; Fry *et al.*, 1998). In recent years, much effort has been made to identify the potential components in *Echinacea* plant extract that could account for its *in vitro* immunostimulatory effects (Bauer and Wagner, 1991). Some of these bioactives include polysaccharides, cichoric acid and alkyamides. Despite the *in vitro* immunomodulatory potential of these compounds in *Echinacea* plant extract, the principle active(s), its optimal dose level and its action *in vitro*, have not been well known. In older animals the effectiveness of plant extract supplementation was relatively low, but higher digestibility of nutrients and reduction of *E. coli* and *Clostridium* sp. in intestinal content were stated (Jamroz *et al.*, 2003).

The objective of this study was to compare the effects of three commercial herbal extracts as alternative to antibiotic on growth performance, immune system, blood factors and intestinal microflora in broiler chickens.

MATERIALS AND METHODS

Experimental Animals and Design

A total of 480 one-day old chicks (Ross male-308) were purchased from a local hatchery. On arrival, chicks were weighed and randomly housed in wood shavings covered floor pens (20 chicks per pen). Continuous lighting was provided throughout the experiment. The ambient temperature was gradually decreased from 30°C on day 7 to 25°C on day 21 and was then kept constant. There were 6 treatments, each consisting of 4 replicates. The replicate was a pen with 20 male chicks so that each treatment was comprised of 80 animals. Chicks were assigned to the basal diet (control) and basal diet supplemented with 15 ppm virginiamycin, 0.1% aqueous extract of: thyme (*Thymus vulgaris*), coneflower (*Echinacea purpurea*),

garlic (*Allium sativum*) and blend of extracts with the same dose in drinking water. The ingredients and composition of the basal diet (starter from 7 to 21, grower from 21 to 35 and finisher from 36 to 42 days of age) are presented in Table 1. All birds employed in the experiment were fed according to applicable recommendations of the National Research Council (NRC, 1994).

Thyme (*Thymus vulgaris*) extract, coneflower (*Echinacea purpurea*) extract, garlic (*Allium sativum*) were commercially purchased supplements (Zardband Pharmaceutical Co., Tehran).

Performance Data

Body weight, feed intake and feed conversion were assessed on days 21 and 41 or during the age ranges of 1-21 and 22-41 days. Mortality rate was reported. For

performance data, pen means served as the experimental unit for statistical analysis. For data on relative weight, individual birds were considered as the experimental unit.

Immune System

Toe-Web Hypersensitivity

At 42 days of age, cutaneous basophils hypersensitivity response (to phytohemagglutinin injection) was determined through the methodology of Corrier and Deloach (1990). Briefly, the right foot was cleansed with 70% ethanol and the thickness of the toe web between the third and fourth digits measured, using a micrometer. One hundred microliters of a 100 $\mu\text{g mL}^{-1}$ solution of phytohemagglutinin (PHA) (Pars-Azmoon Co., Tehran) in sterile 0.85% saline was injected intradermally.

Table 1. Composition of the basal (control) diet.

Ingredients (% diet)	Starter	Grower	Finisher
Maize, yellow	58.4	85.3	61.5
Soybean meal (48%)	27.9	23.8	16.7
Wheat	4.6	9.4	13.9
Fish meal	4.6	2.8	2.8
Bagasse	1.8	1.88	2.8
Limestone	0.9	0.94	0.93
Mineral premix (0.25%) ^a	0.25	0.25	0.25
Vitamin premix (0.25%) ^a	0.25	0.25	0.25
DL-Metionine	0.094	0.095	0.093
L-Lysine	0.094	0.096	0.093
Oil	0.9	1.88	-
Salt	0.25	0.26	0.26
Calculated chemical composition (%) ^b			
ME (Kcal kg ⁻¹)	2850	2950	3050
Crude protein	21.5	19.5	17.5
Calcium	1	0.95	0.9
Available phosphorus	0.5	0.46	0.43
Sodium chloride	0.18	0.16	0.14
Lysine	1.2	1.15	1
Methionine	0.53	0.48	0.43
Methionine+cystine	0.95	0.85	0.75

^a The 10 g of premix consisted of 24.0 mg of vitamin A (500,000 IU g⁻¹); 6.0 mg of vitamin D3 (100,000 IU g⁻¹); 60.0 mg of vitamin E (500 IU g⁻¹); 6.6 mg of vitamin K3 (purity, 22.7%); 100.0 mg of vitamin B12 (purity, 0.1%); 2,000.0 mg of biotin (purity, 0.01%); 1,100.0 mg of choline chloride (purity, 50%); 1.1 mg of folic acid (purity, 90%); 65.2 mg of nicotinic acid (purity, 100%); 16.3 mg of d-pantothenate (purity, 92%); 4.5 mg of vitamin B6 (purity, 100%); 12.5 mg of riboflavin (purity, 80%); 2.5 mg of vitamin B1 (purity, 100%); 32.00 mg of CuSO₄H₂O; 333.20 mg of FeSO₄H₂O; 166.80 mg of MnO; 1.0 mg of Na₂SeO₃H₂O; 220.00 mg of ZnSO₄H₂O; 4.80 mg of CoSO₄ 7H₂O; 0.56 mg of KI; 100.00 mg of ethoxyquin, and 5,742.94 mg of corn meal as carrier.

^b The values were calculated from NRC (1994).



After 24 hours, the toe webs were cleansed and measured again. Cutaneous basophil hypersensitivity response was determined as the difference in skin thickness pre and post (24 hours) injection.

Sheep Red Blood Cells

At 27 and 36 days of age SRBC suspension (5% in sterile PBS) was injected in breast muscle of 12 birds per treatment. Total antibody (Ab) titers to SRBC were determined by agglutination according to Van der Zijpp and Leenstra (1980) in serum from all 72 birds. Therefore, 7 days after each sensitization (35 and 42 days) antibody titers against SRBC were measured and expressed as the \log_2 of the reciprocal of the highest serum dilution giving complete agglutination.

Lymphoid Organ Weights.

At 42 days of age relative weights of immune organs (spleen and bursa of Fabricius) as two immune indexes were determined.

Newcastle Disease Vaccine Response

Newcastle disease vaccine (LaSota) was administered with drinking water at 23 days of age. Blood samples were collected on 7 and 14 days after vaccination and antibody response was determined by using HI method.

Blood Factors

Total Cholesterol (TC), LDL cholesterol, Triglycerides (TG), HDL cholesterol, hematocrit percentage and hemoglobin

concentration were measured at 42 days of age. Concentrations of these parameters except for LDL cholesterol were determined by enzymatic kits (Pars-Azmoon Co., Tehran). To measure LDL cholesterol, Friedwald (Soltani *et al.*, 2007) formula was used.

Selected Bacterial Population in the Intestinal Contents

For a determination of some selected micro-organisms in intestinal digesta from 48 birds (8 birds per treatment and pen) the contents of the distal part of the small intestine (10 cm anterior to the junction with caecum and rectum) and whole caeca of two birds per replicate pen were separately collected, cooled and used for microbial assays. The populations of *Escherichia coli* and lactic acid bacteria (LAB) were then estimated as CFU g^{-1} . Sterilized PBS (99 mL) was added (1:100) to 1 g of fresh material, and then subsequent dilutions prepared. *E. coli* was cultured on MacConkey agar (Merck, Germany) at 37°C for 24 hours, and the presence of *E. coli* then determined. LAB was enumerated on MRS (Merck, Germany) agar after incubation under anaerobic condition for 72 hours at 37°C.

Statistical Analysis

A complete randomized model was employed to analyze data for weight gain, feed intake, and efficiency, characteristics of carcass, immune system, blood factors, as well as intestinal microflora. The data were analyzed using the GLM procedure of the statistical software (SAS Institute Inc, 1989). Mean differences among treatments were evaluated through Duncan multiple range test at $P < 0.05$. The original data for microbial counts were transformed to \log_{10} CFU g^{-1} of intestinal content for statistical analysis.

RESULTS

Performance

In the present study, feed intake and feed conversion ratio were significantly influenced by the addition of herbal extracts to diets and at the 42 days of age (Table 2). The coneflower group presented a significantly lower feed intake (4,125.6 g vs. 4,376.3 g) and body weight (2,269.00 g vs. 2,595.40 g) and while a higher FCR (1.86 vs. 1.71) as compared with the virginiamycin group. At 21 days of age all the herbal extracts except coneflower improved the FCR as compared with the control group, although this difference was not significant. Also at the end (1-42 days) of the experimental period, among herbal extracts, thyme presented a lower FCR as compared with the other treatments except for virginiamycin ($P < 0.05$). Relative weights of the carcass, abdominal fat pad, and digestive organs except small intestine weight were not affected by treatments. Virginiamycin treatment presented a lower small intestinal weight than that in the other treatments (Table 2). The effects of three herbal extracts on relative weight (% body weight) of some internal organs of broilers are shown in Table 3.

Immune System

Relative weight of bursa Fabricius in the garlic group showed a significant increase as compared with that in other groups, but relative weight of spleen was unaffected by the different treatments. Cutaneous basophils hypersensitivity response (to phytohemagglutinin injection) and antibody responses to SRBC were recorded as higher in coneflower group ($P < 0.05$). Antibody response to Newcastle disease vaccine (LaSota) was unaffected by the treatments but the antibody levels were improved in the coneflower group ($P > 0.05$) (Table 4).

Table 2. The effects of the three herbal extracts and antibiotic on body weight (g), feed intake (g), FCR (feed conversion ratio) and mortality (%) in broilers.

Treatments	Period	Control	Coneflower	Garlic	Blend of extracts	Thyme	Antibiotic	SEM
Initial body weight (g)		45.90	46.57	46.10	45.70	46.27	46.32	0.37
Body weight (g)	1 to 21	669.90 ^{bc}	644.00 ^c	693.40 ^b	680.10 ^{bc}	656.40 ^c	736.80 ^a	8.74
	1 to 42	2404.20 ^b	2269.00 ^c	2421.60 ^b	2423.58 ^b	2423.30 ^b	2595.40 ^a	38.20
	1 to 21	625.18 ^{bc}	600.10 ^c	648.60 ^b	635.72 ^b	612.70 ^c	692.00 ^a	8.74
Weight gain (g)	21 to 42	1734.30 ^{bc}	1625.00 ^c	1728.20 ^{bc}	1724.60 ^{bc}	1766.90 ^{ab}	1858.60 ^a	37.26
	1 to 42	2359.50 ^b	2225.10 ^c	2376.80 ^b	2378.32 ^{bc}	2379.60 ^b	2550.60 ^a	38.26
	1 to 21	953.60 ^b	948.60 ^b	957.70 ^{ab}	951.95 ^b	925.00 ^b	993.46 ^a	10.92
Feed intake (g)	21 to 42	3316.11	3177.00	3334.00	3301.94	3314.60	3368.00	39.88
	1 to 42	4290.00 ^a	4125.60 ^b	4291.80 ^a	4264.56 ^{ab}	4240.30 ^{ab}	4376.30 ^a	42.36
	1 to 21	1.52 ^{ab}	1.58 ^a	1.48 ^{bc}	1.50 ^{abc}	1.50 ^{abc}	1.43 ^c	0.023
FCR	21 to 42	1.89 ^{ab}	1.96 ^a	1.93 ^a	1.91 ^a	1.87 ^{ab}	1.82 ^{ab}	0.025
	1 to 42	1.82 ^{ab}	1.86 ^a	1.81 ^{ab}	1.79 ^b	1.78 ^{bc}	1.71 ^c	0.021
	1 to 42	1.25	0.50	0.50	0.51	0.00	0.50	0.18
Mortality %								

* Values in the same row with no common superscript differ significantly.

**Table 3.** The effect of three herbal extracts and antibiotic on relative weight (% body weight) of some internal organs of broilers at 42 days of age.

Treatments	Control	Coneflower	Garlic	Blend of Extracts	Thyme	Antibiotic	SEM
Relative weight (%)							
Carcass yield	70.45	72.15	69.78	69.53	69.93	71.90	0.313
Fat pad	2.09	2.05	1.92	2.04	2.19	2.05	0.066
Liver	2.26	2.27	2.35	2.15	2.43	2.39	0.034
Pancreas	0.255	0.260	0.260	0.230	0.230	0.242	0.005
Proventriculus	0.376	0.415	0.395	0.390	0.327	0.355	0.01
Gizzard	1.48	1.43	1.41	1.88	1.35	1.39	0.063
Small intestine	2.84 ^a	2.70 ^{ab}	2.68 ^{ab}	2.81 ^a	2.48 ^{bc}	2.29 ^c	0.059
Heart	0.504	0.531	0.536	0.490	0.541	0.491	0.01

* Values in the same row with no common superscript differ significantly.

Blood Factors

Garlic (*Allium sativum*) significantly reduced the serum levels of TC, LDL, and TG and significantly increased the level of HDL. There was no significant difference observed for triglyceride levels among extracts in all of which a significant reduction from the control and antibiotic group was observed. Cholesterol level was recorded as the same in groups thyme (*Thymus vulgaris*) and coneflower (*Echinacea purpurea*). Thyme (*Thymus vulgaris*) improved

hematocrit percentage and hemoglobin concentration, but not significantly (Table 5).

Selected Bacterial Population

The colony forming units of *Escherichia coli* in digesta of ileo-cecum in the blend group showed a significantly lower number compared with control group. However, there was no difference observed in *E. coli* counts between blend group and others, except for control. The LAB counts in the thyme group increased as compared with the other groups, except for coneflower ($P < 0.05$) (Table 6).

Table 4. The effect of three herbal extracts and antibiotic on immune system of broilers.

Treatments	Control	Coneflower	Garlic	Blend of extracts	Thyme	Antibiotic	SEM
Antibody response to SRBC							
27day	3.20 ^c	5.00 ^a	4.75 ^{ab}	3.75 ^{bc}	4.00 ^{abc}	3.75 ^{bc}	0.189
42 days	6.50 ^b	8.25 ^a	7.50 ^{ab}	7.00 ^b	6.75 ^b	6.75 ^b	0.198
Antibody response to Newcastle vaccine							
36 d	2.82	3.20	2.92	2.97	3.02	3.17	0.088
42 d	2.07	2.57	2.60	2.40	2.40	2.12	0.088
Bursa Fabricious relative weight (%)	0.22 ^b	0.20 ^b	0.27 ^a	0.21 ^b	0.20 ^b	0.19 ^b	0.007
Spleen relative weight (%)	0.113	0.099	0.133	0.129	0.125	0.113	0.006
Hypersensitivity cutaneous basophiles response (mm)	0.53 ^{bc}	0.80 ^a	0.58 ^{bc}	0.74 ^{ab}	0.53 ^{bc}	0.43 ^c	0.043

* Values in the same row with no common superscript differ significantly.

Table 5. The effect of three herbal extracts and antibiotic on blood factors of broilers.

Treatments	Control	Coneflower	Garlic	Blend of Extracts	Thyme	Antibiotic	SEM
(mg/dl)							
Triglycerid	44.92 ^a	35.50 ^b	30.98 ^b	32.27 ^b	32.15 ^b	50.73 ^a	1.84
Cholesterol	93.92 ^b	86.75 ^{bc}	76.13 ^d	81.17 ^{cd}	85.77 ^{bc}	102.78 ^a	2.19
LDL	47.03 ^a	32.01 ^{bc}	16.85 ^d	24.95 ^{cd}	28.11 ^c	39.37 ^{ab}	2.31
HDL	45.56 ^c	47.64 ^{bc}	53.08 ^a	49.76 ^{ab}	51.22 ^{ab}	45.60 ^c	1.20
Hematocrit	27.28	28.49	27.50	29.19	30.40	28.74	0.447
(%)							
Hemoglobin	7.69	8.88	7.88	8.46	9.45	7.81	0.387

* Values in the same row with no common superscript differ significantly.

DISCUSSION

Over the entire period (1-42 days) coneflower group had significantly lower body weight and weight gain when compared with the other groups especially virginiamycin. However, during the period of 21-42 days, daily weight gain presented no significant difference between thyme (*Thymus vulgaris*) and virginiamycin. Thus, when comparing coneflower with virginiamycin in relation to body weight and weight gain, it appears that coneflower decreases feed intake, leading to lower body weight and weight gain. Among herbal extracts employed, coneflower (*E. purpurea*) did not improve FCR. Findings in this study are in agreement with those of Dora *et al.* (2008) who reported that use of 2.4% *Echinacea* cob or 10 mg kg⁻¹ feed flavomycin showed a significantly lower feed intake (2,713 g vs. 2,995 g) and body weight (1,719 g vs. 1,895 g) as compared to flavomycin and control group. Roth-Maier *et al.* (2005), who fed cobs prepared from *E. purpurea* aerial parts to healthy broiler and

layer chickens, found no beneficial effect of the treatment on feed intake and growth performance of the animals. They concluded that *E. purpurea* cobs cannot be considered as a suitable alternative for antibiotics as a growth promoter in the animals' feed.

Results of the experiment indicated that thyme extract has improved FCR in comparison with other groups except for virginiamycin (P < 0.05). This finding is in agreement with results of Lee *et al.* (2003) and Ocak *et al.* (2008) who reported that use of 2% thyme supplement could significantly improve the growth of broilers. Results of studies by some other investigators are also in agreement with the present findings (Cross *et al.*, 2002; 2007; Bampidis *et al.*, 2005; Demir *et al.*, 2003; Lee *et al.*, 2003). There was no significant difference in relative weight of carcass, fat pad, or digestive organs among treatments except for the small intestine. These findings are in agreement with the results of Ocak *et al.* (2008) who found no differences in carcass and organs' weight of broilers fed a diet containing 2% thyme powder. Similar results were observed by Hernandez (2004)

Table 6. The effect of three herbal extracts and antibiotic on *E. coli* and LAB numbers in ileal contents of broilers.

Treatments	Control	Coneflower	Garlic	Blend of extracts	Thyme	Antibiotic	SEM
<i>E. coli</i> (log CFU g ⁻¹)	7.10 ^a	4.82 ^b	5.20 ^b	4.32 ^b	4.40 ^b	4.42 ^b	0.248
Lactic acid bacteria (log CFU g ⁻¹)	4.52 ^c	6.20 ^a	5.47 ^{ab}	5.82 ^a	5.95 ^a	4.92 ^{bc}	0.149

* Values in the same row with no common superscript differ significantly.



who found no difference in weight of organs of broiler chickens fed diets containing an extract from thyme and oregano.

In contrast, it has been proposed that immunostimulation may have adverse effects on growth performance, because more nutrients will be repartitioned to synthesize antibodies and develop the immune organs, thereby decreasing the nutrients available for growth (Hevener *et al.*, 1999; Takahashi *et al.*, 2000). Coneflower increased production of such acute phase proteins as interleukin (IL)-1 and IL-6, or tumor necrosis factor (TNF- α) - like substances. Cachectins are produced by such extra vascular effector cells as macrophages in response to invasive stimuli. Among various multiple effects, cachectins induce a state of anorexia, as occurs during injecting antigens. Consequently cachectins induce a reduction of growth and feed utilization in chickens, and an accelerated muscle protein degradation (Klasing and Johnstons, 1991); whereas a negative relationship between BW and Ab titers is found in commercial broilers (Martin *et al.*, 1990). Intraperitoneal stimulation of chickens with IL-1, SRBC resulted in reduced growth and feed intake, probably due to the cachectin activities of IL-1, IL-6, TNF- α (Klasing *et al.*, 1987). These cytokines are the earliest mediators secreted by the host in response to antigens and other injurious stimuli (Van Miert, 1995). Acute phase proteins act in concert with a decrease in feed intake (partially as a result of loss of appetite) (Grimble, 1994), increase resting expenditure, gluconeogenesis, glucose oxidation, and synthesis of fatty acids as well as acute phase proteins by the liver. Also, metabolism of circulating insulin, glucagons, and corticosterone are affected (Klasing, 1988). Amino acids and glutamine released from muscle, and glucose derived from increased hepatic gluconeogenesis may provide endogenous sources of nutrients for immune system. Diversion of nutrient pool of the host as a consequence of cachectin release may also lead to retarded growth (Grimble, 1994).

Negative correlations between BW and antibody response might be based on pleiotropic effects for genes associated with immunoresponsiveness (Martin *et al.*, 1990). In the present experiment, growth of birds was significantly retarded by coneflower.

In poultry production, it's very important to improve immunity so as to prevent infectious diseases. A variety of such factors as vaccination failure, infection by immune suppressive diseases, and abuse of antibiotics can induce immunodeficiency. Utilization of immunostimulants is one solution to improve the immunity of animals and to decrease their susceptibility to infectious disease (Liu, 1999). The study of the immune system showed that coneflower (*Echinacea purpurea*) was most effective in immune system improvement. Increasing response to phytohemagglutinin injection (hypersensitivity on toe-web) and SRBC in coneflower group was expected, because this herb increased stimulation of non-specific immune system. Rehman *et al.* (1999) showed production of IgG in rats increasing after being challenged with antigens. Schraner *et al.* (1989) studied the effect on the humoral immune response of a complex drug containing *E. angustifolia* homeopathic mother tincture and *E. angustifolia* mother tincture alone on normal and on immunodeficient chickens. The administration of the complex drug enhanced humoral immune parameters in both group of chickens. Herbs that are rich in such flavonoids as thyme (*Thymus vulgaris*) extend the activity of vitamin C, act as antioxidants and may therefore enhance the immune function (Manach *et al.*, 1996; Cook and Samman, 1996). It is thought that immune enhancement of *Echinacea* is provided by certain polysaccharides, flavonoids, and isobutylamides (Bruneton, 1995).

Garlic lowers total and LDL-cholesterol as well as triacylglycerol concentrations without affecting HDL-cholesterol concentrations (Warshafsky *et al.*, 1993; Kleijnen *et al.*, 1989). Terpenoids (from essential oil components of medicinal

plants) also elicit a significant reduction in total and LDL-cholesterol concentration (Elson and Yu, 1994; Zheng *et al.*, 1992; Pearce *et al.*, 1992).

This study showed that the lowest *E. coli* counts were related to thyme group, but there was no significant difference observed among coneflower, garlic and virginiamycin supplemental groups. In earlier studies (Jamroz *et al.*, 2005) significant reduction of *E. coli* number has been obtained following an application of natural plant extract.

It has been documented that garlic extracts exert a differential inhibition between beneficial intestinal microflora and potentially harmful enterobacteria (Rees *et al.*, 1993). Inhibition observed in *E. coli* was more than 10 times greater than that seen in *Lactobacillus casei* for the same garlic dose (Skyrme, 1997). Exactly why this differential inhibition should occur is not clear, but it may be due to differing composition of bacterial membranes and their permeability to allicin (Miron *et al.*, 2000). Garlic extract and allicin have been shown to exert bacteriostatic effects on some vancomycin-resistant enterococci. Decreasing number of such viable Gram-positive bacteria, as *Lactobacilli* and *Bifidobacteria*, may increase the presence of Gram-negative species. Antibiotics decrease the number of viable Gram-positive bacteria in gastrointestinal tract of chickens (Ferket *et al.*, 2002). Polystylenes from coneflower showed bacteriostatics against *E. coli* (Schulte *et al.*, 1967).

Jang (2006) reported that birds fed diets containing antibiotics showed a significant ($P < 0.05$) reduction in CFU of *E. coli* when compared to those fed the basal diet (control). There was a similar CFU number of *E. coli* among birds fed the basal diets fortified with antibiotics and two levels of essential oil. Canan Bolukbasi and Kuddusi Erhan (2006) showed that control group and the 1% thyme group had the highest average concentration of *E. coli* in feces. The group fed 0.1% and 0.5% thyme had a significantly lower *E. coli* count than the control and the 1% thyme group. Average *E. coli* counts

significantly differed ($P < 0.05$) from each other, with the 0.1% thyme group bearing the lowest concentration. Thymol (from thyme essential oil) has been shown to reduce the number of coliforms within the digesta of chickens (Cross *et al.*, 2004). Furthermore, it has been suggested that supplementation with oligosaccharides may have a prebiotic effect through an increase in production of lactic acid, thus increasing the proliferation of beneficial bacteria and reducing the presence of Gram-negative bacteria (Savage *et al.*, 1996). Polysaccharides in coneflowers include: Echinacein, echinacosid, echinalon. Carvacrol is the thyme essential oil component that has a stimulating effect on *Lactobacillus proliferation* (Tschirch, 2000). Jamroz *et al.* (2005) reported that plant extract supplement also significantly increases the *Lactobacillus* numbers.

In conclusion, use of herbal extracts specially garlic improved FCR comparable to virginiamycin in broilers. This effect could be attributed to improvement of digestive enzymes secretion. According to the obtained results in this study, it can be concluded that coneflower is not a suitable feed additive alternative for antibiotic as a growth promoter in broilers. Among the herbal extracts employed, coneflower and garlic were the most effective in immune function enhancement. Also these extracts were able to reduce the serum lipids. Improvement of selected intestinal bacterial populations through these extracts was remarkable. However, more trials are needed to clarify the effect of different medicinal plants on performance of broilers with regard to varied management conditions, including different stress factors, herbal extracts and their optimal dietary inclusion levels, active substances of extracts, dietary ingredients and nutrient content.

REFERENCES

1. Bakhiet, A. O. and Adam, S. E. I. 1995. Therapeutic Utility, Constituents and



- Toxicity of Some Medicinal Plants: A Review. *Veter Human Toxicol.*, **37**: 255-258.
2. Bampidis, V. H., Christodoulou, V., Florou-Paneri, P., Christaki, E., Chatzopoulou, P. S., Tsilingianni, T. and Spais, A. B. 2005. Effect of Dietary Dried Oregano Leaves on Growth Performance, Carcass Characteristics and Serum Cholesterol of Female Early Maturing Turkeys. *Brit. Poult. Sci.*, **46**: 595-601.
 3. Baratta, M. T., Dorman, H. J. D., Deans, S. G., Biondi, D. M. and Ruberto, G. 1998. Chemical Composition, Antimicrobial and Antioxidative Activity of Laurel, Sage, Rosemary, Oregano and Coriander Essentials Oils. *The J. Essent. Oil Res.*, **10**: 618-627.
 4. Bauer, R. and Wagner, H. 1991. Echinacea Species as Potential Immunostimulating Drugs. In: Wagner H, Farnsworth N, editors. *Eco-386 V. Goel et al. / International Immunopharmacology 2 (2002)* 381-387 agronomic and medicinal plant research. London, England: Academic Press; p. 253-321.2002.
 5. Bruneton, J. 1995. *Pharmacognosy, Phytochemistry, Medicinal Plants*. (Translator): Hatton, C. K., Lavoisier Publishers, Paris.
 6. Canan Bolukbasi, S. and Kuddusi Erhan, M. 2006. Effect of Dietary Thyme (*Thymus vulgaris*) on Laying Hen Performance and *Escherichia coli* (*E. coli*) Concentration in Feces. *J. Natur. Engin. Sci.*, **1(2)**: 55-58.
 7. Cavallito, C. J. and Bailey, J. H. 1994. Allicin, the Antibacterial Principle of *Allium sativum*. I. Isolation, Physical Properties and Antibacterial Action. *J. A. C. S.*, **66**: 1950-1951.
 8. Collington, G. K., Park, D. S. and Armstrong, D. G. 1990. The Influence of Inclusion of Both an Antibiotic and a Probiotic in the Diet on the Development of Digestive Enzyme Activity in the Pig. *Br. J. Nutr.*, **64**: 59-70.
 9. Cook, N. C. and Samman, S. 1996. Flavonoids-chemistry, Metabolism, Cardioprotective Effects, and Dietary Sources. *J. Nutr. Biochem.*, **7**: 66-76.
 10. Corrier, D. E. and Deloach, J. R. 1990. Evaluation of Cell Mediated Cutaneous Basophile Hypersensitivity in Young Chickens by an Interdigital Skin Test. *Poult. Sci.*, **69**: 403-408.
 11. Cross, D. E., Acamovic, T., Deans, S. G. and Cdevitt, R. M. 2002. The Effects of Dietary Inclusion of Herbs and Their Volatile Oils on the Performance of Growing Chickens. *Brit. Poult. Sci.*, **43**: S33-S35.
 12. Cross, D. E., Hillman, K., Fenlon, D., Deans, S. G., McDevitt, R. M. and Acamovic, T. 2004. Antibacterial Properties of Phytochemicals in Aromatic Plants in Poultry Diets. In: "*Poisonous Plants and Related Toxins*", (Eds.): Acamovic, T., Stewart and Phennycott, T. W.. CAB International, Wallingford, Oxon, PP. 175-180.
 13. Cross, D. E., McDevitt, R. M., Hillman, K. and Acamovic, T. 2007. The Effects of Herbs and Their Associated Essential Oils on Performance, Dietary Digestibility and Gut Microflora in Chickens from 7 to 28 days of Age. *Brit. Poult. Sci.*, **48**: 496-504.
 14. Demir, E., Sarica, S., Ozcan, M. A. and Suicmez, M. 2003. The Use of Natural Feed Additives as Alternatives for Antibiotic Growth Promoter in Broiler Diets. *Brit. Poult. Sci.*, **44**: S44-S45.
 15. Dora, A., Roth-Maier, B., Bohmer, M. and Brigitte, R. 2008. *Efficiency of Echinacea purpurea on Performance of Broilers and Layers*. Fachbereich, Tierernahrung, TU Munchen, Freising Weihenstephan Germany. Instit. fur. Tierhaltung und Tierschutz, Kitzingen, Germany.
 16. Dorman, H. J. D. and Deans, S. G. 2000. Antimicrobial Agents from Plants: Antimicrobial Activity of Plant Volatile Oils. *J. Appl. Microbiol.*, **88**: 308-316.
 17. Duncan, D. B. 1995. Multiple Ranges and Multiple *F* Test. *Biometrics*, **1**: 1-42.
 18. Elson, C. E. and Yu, S. G. 1994. The Chemoprevention of Cancer by Vevalonate-derived Constituents of Fruits and Vegetables. *J. Nutr.*, **124**: 607-14.
 19. Ferket, P. R., Parks, C. W. and Grimes, J. L. 2002. Benefits of Dietary Antibiotic and Mannanooligosaccharide Supplementation for Poultry. *Multi-State Poultry Meeting*, May14-16.
 20. Fry, J., Carr, R. I. and Lee, T. D. G. 1998. *Echinacea* stimulation of mouse macrophages. *FASEB J. (Abstract)*, **12(5)**: A299.
 21. Gill, C. 2000. Botanical Feed Additives. *Feed. Int.*, April 14-17.

22. Grimble, R. F. 1994. Malnutrition and the Immune Response. 2. Impacts of Nutrients on Cytokine Biology in Infection. *Trans. Royal. Soc. Trop. Med. Hygiene*, **88**: 615-619.
23. Hernandez, F., Madrid, J., Garcia, V., Orengo, J. and Megias, M. D. 2004. Influence of Tow Plant Extracts on Broiler Performance, Digestibility, and Digestive Organ Size. *Poult. Sci.*, **83**: 169-174.
24. Hevener, W. P., Routh, A. and Almond, G. W. 1999. Effects of Immune Challenge on Concentrations of Serum Insulin-like Growth Factor-I and Growth Performance in Pigs. *Can. Vet. J.*, **40**: 782-786.
25. Horie, T., Awazu, S., Itakura, Y. and Fuwa, T. 1992. Identified Diallyl Polysulfides from an Aged Garlic Extract which Protects the Membranes from Lipid Peroxidation. *Planta Med.*, **58**: 468-469.
26. Horton, G. M. J., Fennell, M. J. and Prasad, B. M. 1991. Effect of Dietary Garlic (*Allium sativum*) on Performance, Carcass Composition and Blood Chemistry Changes in Broiler Chickens. *Can. J. Anim. Sci.*, **71**: 939-942.
27. Jamroz, D., Orda, J., Kamel, C., Williczkiewicz, A., Wartelecki, T. and Skorupin'Ska, J. 2003. The Influence of Phytogenic Extract on Performance, Nutrients Digestibility, Carcass Characteristic and Gut microbial Status in Broiler Chickens. *J. Anim. Feed Sci.*, **12(3)**: 583-596.
28. Jamroz, D., Williczkiewicz, A., Warteleck, T., Orda, J. and Skorupinska, J. 2005. Use of Active Substances of Plant Origin in Chicken Diets Based on Maize and Locally Grown Cereals. *Brit. Poult. Sci.*, **46(4)**: 458-493.
29. Jang, I. S., Ko, Y. H., Kang, S. Y. and Lee, C. Y. 2006. Effect of Commercial Essential Oil on Growth Performance, Digestive Enzyme Activity and Intestinal Microflora Population in Broiler Chickens. *Anim. Feed. Sci. Technol.*, **134**: 304-315.
30. Janssen, A. M. 1989. Antimicrobial Activities of Essential Oils: A Pharmacognostical Study. Dissertation, Rijksuniversiteit e Leiden.
31. Klasing, K. C. 1988. Nutritional Aspects of Leukocytic Cytokines. *J. Nutr.*, **118**: 1436-1446.
32. Klasing, K. C. and Johnstone, B. J. 1991. Monokines in Growth and Development. *Poult. Sci.*, **70**: 1781-1789.
33. Klasing, K. C., Laurin, D. L., Peng, R. K. and Fry, D. M. 1987. Immunologically Mediated Growth Depression in Chicks: Influence of Feed Intake, Corticosterone and Interleukin-1. *J. Nutr.*, **117**: 1629-1637.
34. Kleijnen, J., Knipschild, P. and Ter Riet, G. T. 1989. Garlic, Onions and Cardiovascular Risk Factors. A Review of the Evidence from Human Experiments with Emphasis on Commercially Available Preparations. *Br. J. Clin. Pharmacol.*, **28**: 535-44.
35. Lee, K. W., Everts, H., Kappert, H. J., Yeom, K. H. and Beyen, A. C. 2003. Dietary Carvacrol Lowers Body Weight Gain but Improve Feed Conversion in Female Broiler Chickens. *J. Appl. Poult. Res.*, **12**: 394-399.
36. Lee, M. H., Lee, H. J. and Ryu, P. D. 2001. Public Health Risks: Chemical and Antibiotic Residues Review. *Asian-Aust. J. Anim. Sci.*, **14**: 402-413.
37. Liu, X. Y. 1999. Stress and Immunity. In: "Poultry Immunology", (Ed.): Yin, T. B.. China Agriculture Press, Beijing, China. PP. 230-252
38. Manach, F., Regerat, F. and Texier, O. 1996. Bioavailability, Metabolism and Physiological Impact of 4-oxo-flavonoids. *Nutr Res.*, **16**: 517-44.
39. Manzanilla, E. G., Baucells, F., Kamel, C., Morales, J., Perez, J. F. and Gasa, J. 2001. Effects of Plant Extracts on the Performance and Lower Gut Microflora of Early Weaned Piglets. *J. Anim. Sci., Suppl.*, **1**: 473. (Abstract)
40. Martin, A. E. A., Dunnington, W. B., Gross, W. E., Briles, R., Briles, W. and Siegel, P. B. 1990. Production Traits and Alloantigen Systems in Lines of Chickens Selected for High or Low Antibody Responses to Sheep Erythrocytes. *Poult. Sci.*, **69**: 871-878.
41. Miron, T., Rabinkov, A., Mirelman, D., Wilckek, H. and Weiner, L. 2000. The mode of Action of Allicin: It's Ready Permeability through Phospholipids Membranes May Contribute to It's Biological Activity. *Biochem. Biophys. Acta*, **1463**: 20-30.
42. National Research Council. 1994. *Nutrient Requirements of Poultry*. 9th Rev. Ed., Natl. Acad. Press, Washington, DC.
43. O'Hara, M. A., Kiefer, D., Farrelle, K. and Kemper, K. 1998. A Review of Commonly



- Used Medicinal Herbs. *Arch. Fam. Med.*, **7**: 523-36.
44. Ocak, N., Erener, F., Burak, A.K., Sungu, M., Altop, A. and Ozmen, A. 2008. Performance of Broilers Fed Diets Supplemented with Dry Peppermint (*Mentha piperita L.*) or Thyme (*Thymus vulgaris L.*) Leaves as Growth Promoter Source. *Czech. J. Anim. Sci.*, **53(4)**: 169-175.
 45. Pearce, B. C., Parker, R.A., Deason, M. E., Qureshi, A. A. and Wright, J. J. 1992. Hypocholesterolemic Activity of Synthetic and Natural Tocotrienols. *J. Med. Chem.*, **35**: 3595-606.
 46. Qureshi, A. A., Abuirmeileh, N., Din, Z. Z., Elson, C. E. and Burger, W. C. 1983. Inhibition of Cholesterol and Fatty Acid Biosynthesis in Liver Enzymes and Chicken Hepatocytes by Polar Fractions of Garlic. *Lipids*, **18**: 343-348.
 47. Rees, L. P., Minney, S. F., Plummer, N. T., Slater, J. H. and Skyrme, D. A. 1993. A Quantitative Assessment of the Antimicrobial Activity of Garlic (*Allium sativum*). *World J. Microbiol. Biotechnol.*, **9**: 303-307.
 48. Rehman, J., Dillow, J. M., Carter, S. M., Chou, J., Le, J. B. and Maisel, A. S. 1999. Increased Production of Antigen-specific Immunoglobulins G and M Following *In vivo* Treatment with Medicinal Plants *Echinacea angustifolia* and *Hydratis canadensis*. *Immuno. Lett.*, **68(2-3)**: 391-395.
 49. Roth-Maier, D. A., Böhrer, B. M., Maaß, N., Damme, K. and Paulicks, B. R. 2005. Efficiency of *Echinacea purpurea* on Performance of Broilers and Layers. *Arch. Geflügelk.*, **69(3)**: 123-127.
 50. SAS Inst. 1989. *SAS/STAT User's Guide*, Version 6, 4th Ed., Vol. 2. SAS Inst., Cary, NC.
 51. Savage, T. F., Cotter, P. F. and Zakrzewska, E. I. 1996. The Effect of Feeding Mannan Oligosaccharide on Immunoglobulins, Plasma Ig G and Bile Ig A, of Wrolstad MW mal Turkeys. *Poult. Sci.*, **75**: 143.
 52. Schraner, I., Würdinger, M., Klumpp, N., Lösch, U. and Okpanyi, S. N. 1989. Beeinflussung der aviären humoralen Immunreaktionen durch Infuex und *Echinacea Angustifolia* Extrakt. *J. Vet. Med.*, **36**: 353-364.
 53. Schulte, K. E., Rucker, G. and Perlick, J. 1967. The Presence of Polyacetylene Compounds in *Echinacea purpurea* and *Echinacea angustifolia* DC. *Arzneimittelforschung*, **17**: 825-829.
 54. Skrabka-Blotnicka, T., Rosin'ski, A., Przysie-Zzna, E., Woloszyn, J. and Elminowska-Wenda, G. 1997. Effect of Dietary Formulation Supplemented with Herbal Mixture on Goose Breast Muscle Quality. Report I. The Effect on the Chemical Composition. *Archiv fu'r Geflu'gelkunde*, **61(3)**: 135-138.
 55. Skyrme, D. A. 1997. The Antimicrobial Activity of *Allium sativum*. Ph.D. Thesis, Cardiff University.
 56. Soltani, N., Keshavarz, M. and Dehpour, A. R. 2007. Effect of Oral Magnesium Sulfate Administration on Blood Pressure and Lipid Profile in Streptozocin Diabetic Rat. *European J. Pharmacol.*, **560**: 201-205.
 57. Stimpel, H., Proksch, A., Wagner, H. and Lohmann-Matthes, M. L. 1984. Macrophage Activation and Induction of Macrophage Cytotoxicity by Purified Polysaccharide Fractions from Plant *Echinacea purpurea*. *Infect. Immun.*, **46**: 845-849.
 58. Takahashi, K. T., Mashiko, Y. and Akiba, Y. 2000. Effect of Dietary Concentration of Xylitol on Growth in Male Broiler Chicks during Immunological Stress. *Poult. Sci.*, **79**: 743-747.
 59. Tollba, A. A. H. and Hassan, M. S. H. 2003. Using Some Natural Additives to Improve Physiological and Productive Performance of Broiler Chicks under High Temperature Conditions, Black Cumin (*Nigella sativa*) or Garlic (*Allium sativum*). *Poult. Sci.*, **23**: 327-340.
 60. Tschirch, H. 2000. The Use of Natural Plant Extracts as Production Enhancers in Modern Animal Rearing Practices. *Zeszyly Naukowe Akademicy Rolniczej Wroclaw, Zootechnik*, **XXV (376)**: 25-39. (in Polish)
 61. Van der Zijpp, A. J. and Leenstra, F. R. 1980. Genetic Analysis of the Humoral Immune Response of White Leghorn Chicks. *Poult. Sci.*, **59**: 1363-1369.
 62. Van Miert, A. S. J. P. A. M. 1995. Pro-inflammatory Cytokines in a Ruminant Model: Pathophysiological, Pharmacological, and Therapeutic Aspects. *J. Vet. Med.*, **17**: 41-50.
 63. Warshafsky, S., Kramer, R. S. and Sivak, S. L. 1993. Effect of Garlic on Total Serum Cholesterol: A Meta-analysis. *Ann. Intern. Med.*, **119**: 599-605.

64. William, P. and Losa, R. 2001. The Use of Essential Oils and Their Compounds in Poultry Nutrition. *World's Poult.*, **17(4)**: 14-15.
65. Zheng, G. Q., Kenney, P. M. and Lam, L. K. T. 1992. Myristicin: A Potential Cancerchemopreventive Agent from Parsley Leaf Oil. *J. Agric. Food. Chem.*, **40**: 107-10.

اثر سه عصاره گیاهی بر عملکرد رشد، سیستم ایمنی، فاکتورهای خونی و جمعیت باکتریایی انتخاب شده روده کوچک جوجه های گوشتی

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چکیده

این تحقیق به منظور ارزیابی اثرات سه عصاره گیاهی (آویشن باغی، سرخارگل و سیر) و آنتی بیوتیک ویرجینامایسین بر عملکرد رشد، سیستم ایمنی، فاکتورهای خونی و جمعیت باکتریایی انتخاب شده در جوجه های گوشتی انجام گردید. ۴۸۰ قطعه جوجه گوشتی یکروزه نر به گروههای جیره پایه (کنترل) و جیره پایه با ۱۵ ppm ویرجینامایسین، ۰/۱ درصد عصاره آویشن، سرخارگل، سیر، و مخلوطی از عصاره ها با دوز مشابه تقسیم شدند. بیشترین و کمترین وزن بدن بترتیب مربوط به تیمارهای ویرجینامایسین و سرخارگل بودند ($P < 0/05$). همچنین کمترین و بیشترین ضریب تبدیل غذایی بترتیب مربوط به تیمارهای ویرجینامایسین و سرخارگل بودند ($P < 0/05$). وزن نسبی بورس فابریسیوس در گروه سیر افزایش معنی داری را در مقایسه با دیگر گروهها نشان داد، اما وزن نسبی طحال به وسیله تیمارها تحت تأثیر قرار نگرفت. پاسخ به افزایش حساسیت بازوفیل پوستی (تزریق فیتوهماکلوتینین) و عیار آنتی بادی بر علیه گلبول قرمز گوسفند در گروه سرخارگل بالاتر بود ($P < 0/05$). عیار آنتی بادی بر علیه واکسن نیوکاسل (لاسوتا) به وسیله تیمارها تحت تأثیر قرار نگرفت اما سرخارگل سطوح آنتی بادی را بهبود داد ($P > 0/05$). عصاره سیر به طور معنی داری سطوح کلسترول، LDL و تری گلیسرید سرم را کاهش و HDL را افزایش داد ($P < 0/05$). عصاره آویشن درصد هماتوکریت و میزان هموگلوبین را افزایش داد اما این بهبود معنی دار نبود ($P > 0/05$). شمارش باکتریهای ای.کولای در محتویات ایلنو-سکوم در گروه مخلوط عصاره ها کمترین تعداد را در مقایسه با گروه کنترل داشت، اگر چه اختلاف معنی داری در شمارش ای.کولای بین گروه مخلوط عصاره ها و دیگر گروهها بجز کنترل وجود نداشت. شمارش باکتریهای اسید لاکتیک در گروه آویشن در مقایسه با سایر گروهها بجز سرخارگل افزایش داشت ($P < 0/05$).