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Extract and leaf powder effect of *Artemisia annua* on performance, cellular and humoral immunity in broilers

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Summary

The effects of methanolic extract and leaf powder of *Artemisia annua* were studied on performance, cellular and humoral immunity in 240 Cobb broiler chicks in a completely randomized design. Control group did not receive any treatment. The chicks in the second and third groups were fed with feed which contained 2000 and 4000 ppm plant extract in diet and in the groups 4, 5 and 6 chicks received 0.5, 1 and 1.5% of dietary *Artemisia annua* leaf powder, respectively. Daily feed intake, daily weight gain and feed conversion ratio (performance) were measured. Skin response to phytohemagglutinin-P (PHA-P) injected intradermally on day 16 was measured 24 and 48 h after injection. The birds were immunized with sheep red blood cells (SRBC) on days 8 and 22 of age and serum antibody levels produced in response to SRBC were measured on days 21, 28, 35 and 42. The weights of thymus and bursa of fabricius were also measured after slaughter. The results indicated that plant extract and leaf powder increased daily weight gain and reduced daily feed intake and feed conversion ratio ($P<0.05$). Plant extract and leaf powder increased cellular immunity on PHA-P injection after 24 and 48 h ($P<0.05$). *Artemisia annua* extract and leaf powder increased total anti-SRBC and IgG titer in experimental groups compared to control group ($P<0.05$). Thymus and bursa of fabricius weights were increased in treatment groups ($P<0.05$). It is concluded that *Artemisia annua* extract and leaf powder increases performance, cellular and humoral immunity of broilers.

Key words: *Artemisia annua*, Cellular immunity, Humoral immunity, Broiler

Introduction

Over the past several years the use of prebiotics, probiotics and natural products have been substituted by antibiotics in order to improve immune system and to defense against pathogens in human and animal life. In contrast to antibiotics, these products seem to have lower side effects and can be regarded safer in food chain. One of the possible candidates in natural products is antioxidants and flavonoids, which occur naturally (Taheri *et al*., 2005) and are stored in different parts of some plants (Bhakuni *et al*., 2001; Brisibe *et al*., 2009).

Artemisia annua is an aromatic annual herb which belongs to *Asteraceae* family, endemic in the north of Iran. *Artemisia annua* is an important medicinal plant (Bhakuni *et al*., 2001). The plant produces a beautiful portfolio of bioactive compounds including flavonoids, coumarins, steroids, phenolics, purines, lipids, aliphatic compounds, monoterpenoids, triterpenoids and sesquiterpenoids (Bhakuni *et al*., 2001; Brisibe *et al*., 2009). Biological activities reported for the compounds isolated from *A. annua* are antimalarial, anticoccdiosis, antibacterial, anti-inflammatory, angiotensin converting enzyme inhibitory, cytokinin-like and antitumour (Bhakuni *et al*., 2001).

There are numerous reports which confirm the positive effects of natural flavonoids on immune system of different species. These studies mostly focus on antibody synthesis. T lymphocyte stimulation, increasing blood lymphocytes, phagocytosis activity, thymus and bursa of

fabricious weight are several factors which have been considered in this regard (Kong *et al*., 2004; Taheri *et al*., 2005). Beginning of the humoral and cellular immune response is mainly related to the cytokines released from activated T cells (Taheri *et al*., 2005). Recent clinical trials have been found to show that antioxidant supplementation can significantly improve certain immune responses. Supplementation with the antioxidants also protects immune responses of individuals exposed to certain environmental sources of free radicals (Bendich, 1993).

Moreover, broilers that were fed with *A. annua* leaves showed a higher feed intake, which resulted in higher weight gain (Brisibe *et al*., 2008; Dragan *et al*., 2010).

However, there is not much information available on the effects of *A. annua* on immune system of broilers. The objective of the present study was to evaluate the effects of methanolic extract and leaf powder of *A. annua* on performance, cellular and humoral immunity in broilers.

Materials and Methods

Collection of *Artemisia annua*, extract and powder preparation

Fresh green leaves of *A. annua* were collected from Ramsar (36°54´ N latitude, 50°40´ E longitude) in July. Leaves were washed with distilled water and dried at room temperature in the shade, they were also powdered and stored in a refrigerator (-80°C). Crude methanolic extraction was carried out according to the procedure described by Warthen *et al*. (1984).

Broilers and diets

One-day-old broiler chicks (240 Cobb 500) were divided into 6 treatments with 4 replicates and 10 chickens per cage. Chickens had free access to water and food *ad libitum*. The diets were based on soybean corn (Table 1) according to the 1994 Council procedure (NRC, 1994). The chicks in group 1 did not receive any treatment (control). The chicks in groups 2 and 3 were fed with the food containing 2000 and 4000 ppm of *A. annua* extract (E_{2000} and E_{4000}) and groups 4, 5 and 6 were fed with food containing 0.5, 1 and 1.5% leaf powder ($P_{0.5}$, P_1 and $P_{1.5}$) of *A. annua* in diet during the entire feeding period.

Table 1: Ingredients and composition of broilers diet

Nutrient (%)	Starter (1-11)	Growth (11-25)	Finisher (25-42)
Corn	55.3	62.73	67.07
Soya meal	38.2	31.28	27.25
DCP	2.04	2.02	1.89
Sunflower oil	2.2	1.8	1.6
Ca carbonate	0.88	0.85	0.8
Salt	0.3	0.3	0.3
Na bicarbonate	0.25	0.13	0.1
Mineral	0.3	0.3	0.3
Vitamins	0.3	0.3	0.3
Methionin	0.18	0.19	0.22
Lysine	0.05	0.1	0.17
Total	100	100	100
Calculated analysis			
Metabolizable energy (Kcal/kg)	2988	3083	3176
Protein (%)	21	19	18
Lysine (%)	1.20	1.10	1.05
Methionin (%)	0.46	0.44	0.43
Methionin + Cystein (%)	0.89	0.84	0.82
Calcium (%)	1.00	0.96	0.90
Available phosphorus (%)	0.5	0.48	0.45
Arginine (%)	1.26	1.17	1.13
Tryptophan (%)	0.20	0.19	0.19
Na (%)	0.20	0.17	0.16
Cl (%)	0.20	0.20	0.20

Daily feed intake, daily weight gain and feed conversion ratio were evaluated according to Oyegoke *et al*. (2006).

Evaluation of cellular immunity

In order to evaluate the cellular immunity responses, chicks were injected in the right wing web with the mitogen, 0.1 ml of 1 mg/ml PHA-P (Sigma, L8754, St. Louis Mo, USA) in a phosphate buffered saline (PBS) solution, after plucking and marking the injection site on day 16. The left wing web (control) was injected with 0.1 ml of PBS. The thickness of the wing web at the injection site was measured with a digital Caliper just prior to injection of PHA-P (time 0), 24 and 48 h after injection of PHA-P. The PHA-P stimulation index was calculated as the increase in wing web thickness caused by PHA-P (right wing) minus the increase caused by PBS (left wing) after 24 and 48 h (Grasman, 2010).

Evaluation of humoral immunity

In order to assess the systemic antibody response, chicks were immunized intra-muscularly with 0.1 ml of 5% sheep red blood cell (SRBC) in PBS on days 8 and 22

post hatching. Blood samples were collected from birds via the wing vein on days 21, 28, 35 and 42 of age. Blood samples were kept at room temperature for 2 h and then at 4°C overnight. They were centrifuged for 10 min at 2500 × g, and the sera were decanted and frozen (-80°C) until serological examinations were performed. Antibody levels to SRBC were determined by microhemagglutination assay technique. Serum samples were incubated at 56°C for 30 min to inactivate the complement. The 2-mercaptoethanol (2-ME, Sigma, St. Louis Mo, USA) resistant antibody levels, immunoglobulin (Ig) G, were determined by incubating the serum with an equal volume (50 µl) of 1.4% (vol/vol) 2-ME in PBS at 37°C for 30 min prior to hemagglutination test. The 2-mercaptoethanol-sensitive anti-body levels (IgM) were determined by subtracting the 2-ME resistant antibody titers from the total antibody titer. The antibody titers are expressed as the $\log_2$ of highest dilution of serum that agglutinated an equal volume of 0.5% red blood cells (Kamran Azad *et al*., 2009; Grasman, 2010).

The birds were scheduled for slaughter on day 42 of age and the weights of thymus and bursa of fabricius were measured.

Statistical analysis

Data were analysed using the GLM procedure of SAS (1997) in a completely randomized design and means were compared using Duncan's multiple range test at P<0.05.

Results

The effects of extract and leaf powder on broiler's performance are depicted in Table 2. As shown in the Table 2, differences in daily feed intake, daily weight gain and feed conversion ratio of the five treatment groups were significant (P<0.05) compared to the control group. The highest daily feed intake was observed in the control group and the lowest in $P_{1.5}$ (P<0.05). On the other hand, the lowest daily weight gain was seen in control group and the highest in E_{4000} and P_1 (P<0.05). The best and worst values of feed conversion ratio were registered in P_1 and in the control group, respectively.

Table 2: Effects of methanolic extract and leaf powder of *A. annua* on broilers performance

	Daily feed intake (g)*	Daily weight gain (g)	Feed conversion ratio
C	125.10^{a}	63.5^{e}	1.969^{a}
E_{2000}	121.19^{b}	66.9^{c}	1.812^{b}
E_{4000}	120.09^{c}	68.4^{a}	1.756^{d}
$P_{0.5}$	120.08^{c}	67.1^{b}	1.762^{d}
P_1	118.98^{d}	68.4^{a}	1.743^{e}
$P_{1.5}$	117.87^{e}	66.6^{d}	1.771^{c}
SEM	0.072	0.082	0.0025

*Different superscript in each column indicate significant difference (P<0.05). C: Control group, E_{2000}: Diet containing 2000 ppm extract, E_{4000}: Diet containing 4000 ppm extract, $P_{0.5}$: Diet containing 0.5% leaf powder, P_1: Diet containing 1% leaf powder, $P_{1.5}$: Diet containing 1.5% leaf powder

Results of PHA-P stimulation index after 24 and 48 h are shown in Table 3. Differences in stimulation index in the five treatment groups were significant compared to the control (P<0.05). Treatment groups increased stimulation indices 24 and 48 h after the injection of PHA-P (P<0.05).

Table 3: Effects of methanolic extract and leaf powder of *A. annua* on PHA-P stimulation index after 24 and 48 h

	Stimulation index (mm)* 24 h	Stimulation index (mm) 48 h
C	0.6150^{b}	0.5900^{b}
E_{2000}	0.8375^{a}	0.7975^{a}
E_{4000}	0.9075^{a}	0.8575^{a}
$P_{0.5}$	0.9325^{a}	0.8825^{a}
P_1	0.9400^{a}	0.9150^{a}
$P_{1.5}$	0.9175^{a}	0.8675^{a}
SEM	0.040	0.043

*Different superscript in each column indicate significant difference (P<0.05). C: Control group, E_{2000}: Diet containing 2000 ppm extract, E_{4000}: Diet containing 4000 ppm extract, $P_{0.5}$: Diet containing 0.5% leaf powder, P_1: Diet containing 1% leaf powder, $P_{1.5}$: Diet containing 1.5% leaf powder

Results of the antibody tests on days 21, 28, 35 and 42 of age are shown in Table 4. The lowest total anti-SRBC, IgG and IgM were observed in the control group on days 21, 28, 35 and 42 of age (P<0.05). Total anti-SRBC was highest in E_{4000} and P_1 on day 21 (P<0.05), on this day IgG and IgM levels of the five treatments were not significantly different from control chicks (P>0.05). Total anti-SRBC and IgM were increased by treatment groups and IgG was

Table 4: Effects of methanolic extract and leaf powder of *A. annua* on antibody responses to sheep red blood cell (SRBC) on days 21, 28, 35 and 42 of age

	Antibody titer (log_2)											
	Day 21			Day 28			Day 35			Day 42		
	Total*	IgG	IgM	Total	IgG	IgM	Total	IgG	IgM	Total	IgG	IgM
C	3.25^{b}	0^{a}	3.25^{a}	3.75^{c}	0.5^{b}	3.25^{c}	5.25^{c}	3.5^{d}	1.75^{a}	5.25^{b}	4.50^{b}	0.75^{a}
E_{2000}	3.75^{ab}	0.5^{a}	3.25^{a}	5.75^{b}	1.25^{ab}	4.50^{b}	7.00^{b}	5.00^{bc}	2.00^{a}	7.00^{a}	6.25^{a}	0.75^{a}
E_{4000}	4.00^{a}	0.5^{a}	3.5^{a}	7.5^{a}	1.5^{a}	6.00^{a}	8.25^{a}	6.00^{a}	2.25^{a}	7.75^{a}	6.50^{a}	1.25^{a}
$P_{0.5}$	3.5^{ab}	0.5^{a}	3.0^{a}	6.5^{ab}	1.25^{ab}	5.25^{ab}	7.25^{ab}	4.75^{c}	2.50^{a}	7.25^{a}	6.25^{a}	1.00^{a}
P_1	4.00^{a}	0.5^{a}	3.5^{a}	7.5^{a}	1.5^{a}	6.00^{a}	8.25^{a}	6.00^{a}	2.25^{a}	7.75^{a}	6.75^{a}	1.00^{a}
$P_{1.5}$	3.75^{ab}	0.5^{a}	3.25^{a}	6.75^{ab}	1.25^{ab}	5.50^{ab}	7.50^{ab}	5.00^{bc}	2.50^{a}	7.00^{a}	6.25^{a}	0.75^{a}
SEM	0.204	0.166	0.263	0.354	0.270	0.338	0.333	0.300	0.493	0.391	0.312	0.391

*Different superscript in each column indicate significant difference (P<0.05). C: Control group, E_{2000}: Diet contain 2000 ppm extract, E_{4000}: Diet contain 4000 ppm extract, $P_{0.5}$: Diet contain 0.5% leaf powder, P_1: Diet contain 1% leaf powder, $P_{1.5}$: Diet contain 1.5% leaf powder

increased by E_{4000} and P_1 on day 28 of age (P<0.05). Treatments increased total anti-SRBC and IgG on day 35 of age (P<0.05). Extract and leaf powder increased the total anti-SRBC and IgG on day 42 of age (P<0.05) but there was no significant differences between treatment groups (P>0.05). IgM titer of treatment groups was not significantly different from control birds on days 35 and 42 of age (P>0.05).

Table 5 shows the effects of extract and leaf powder on the ratio of thymus and bursa of fabricius weight to carcass weight. The highest ratio was observed in E_{4000} and P_1 (P<0.05).

Table 5: Effects of methanolic extract and leaf powder of *A. annua* on the ration of thymus and bursa of fabricius weights to carcass weight

	Thymus (%)*	Bursa of fabricius (%)
C	0.1780^{c}	0.1077^{e}
E_{2000}	0.2442^{b}	0.1167^{d}
E_{4000}	0.2617^{a}	0.1465^{a}
$P_{0.5}$	0.2415^{b}	0.1365^{c}
P_1	0.2585^{a}	0.1451^{a}
$P_{1.5}$	0.2465^{b}	0.1370^{c}
SEM	0.0017	0.0016

*Different superscript in each column indicate significant difference (P<0.05). C: Control group, E_{2000}: Diet containing 2000 ppm extract, E_{4000}: Diet containing 4000 ppm extract, $P_{0.5}$: Diet containing 0.5% leaf powder, P_1: Diet containing 1% leaf powder, $P_{1.5}$: Diet containing 1.5% leaf powder

Discussion

The observed increased daily weight gain and decreased daily feed intake and feed conversion ratio in this experiment are consistent with previous reports by administration of *A. annua* (Baciu *et al*., 2006; Brisibe *et al*., 2008; Dragan *et al*., 2010). *Artemisia annua* leaves have high levels of crude protein, essential amino acids, minerals, vitamins, antioxidants and flavonoids. These are very crucial for growth and development of poultry as well as the enhancement of weight gain in animals (Brisibe *et al*., 2008). Therefore, it is possible that the performance of the broilers seen in the current study may have been enhanced following the addition of *A. annua* leaves powder and extract in the diets, probably because of its high protein content and presence of essential minerals such as sodium, potassium, zinc and manganese, amino acids and vitamins (Brisibe *et al*., 2008).

Additionally, *A. annua* has a high concentration of antioxidants (Bhakuni *et al*., 2001; Brisibe *et al*., 2009) as has been reported in some other plants (Bahorun *et al*., 2004) which are known for containing high levels of vitamins A, C, and E and flavonoids such as quercetin. Antioxidants are very important as they help to block the action of free radicals which have been implicated in several stresses related to gastrointestinal mucosal injuries (Brisibe *et al*., 2008) and in the pathogenesis of many diseases (Coruh *et al*., 2007). Aside from antioxidants, and compared to traditional forages, *Artemisia* species also have high concentration of essential oils which are useful in the maintenance of a favourable microfloral balance, suppression of protozoa, increasing nitrogen uptake and reducing methane production (Brisibe *et al*.,

2008). It is speculated that *Artemisia* leaves powder and extract in poultry rations have the potential to enhance daily weight gain and better feed conversion ratio.

PHA-P stimulation index after 24 and 48 h and total anti-SRBC, IgG and IgM were increased in treatment groups (Tables 3, 4). The phytohemagglutinin and SRBC tests are nonlethal, minimally invasive methods and are amenable to studies of many different avian species. The PHA skin response test is an *in vivo* method of measuring T lymphocyte-mediated immunity. It measures the swelling caused by inflammatory leukocyte and fluid infiltration after an intradermal injection of PHA. Mitogens such as PHA are derived from lectins, which are plant or bacterial proteins that bind to specific sugar components of glycoproteins attached to the surface of cells (Grasman, 2010). Increased PHA-P stimulation index in the present study is the result of increase in cellular immunity by *A. annua* treatment.

The SRBC hemagglutination assay measures the antibody response to immunization with SRBC antigens, integrating the functions of lymphocytes, helper T lymphocytes and macrophages (Grasman, 2010). Stimulation of immune system by natural products has already been reported (Kong *et al*., 2004; Taheri *et al*., 2005). The effects of natural products on immune system of different species are interesting and complicated. The direct effect might be related to stimulating the lymphatic tissue in the digestive system, and indirect effect via changing the microbial population of the lumen of gastrointestinal tract. Some natural products in numerous experiments have revealed different actions on immune system. For example increasing the macrophage activity (Dimov *et al*., 1991), increasing the IL1 (Orsolic and Basic, 2003), IL2 and IL4 (Park *et al*., 2004).

Increased total anti-SRBC, IgG and IgM in the present study indicate increase in humoral immunity. In this regard, increasing the humoral responses in broilers could be related to a combination of these responses. It is very obvious that in the immune system B lymphocytes are stimulated by these cytokines, and are then changed to plasma cells which are able to produce antibodies (Taheri *et al*., 2005).

On the other hand, *A. annua* possess antioxidant and anti-inflammatory effects, and these are related to inhibition of prostaglandin synthesis (Bhakuni *et al*., 2001; Brisibe *et al*., 2009) as an anti-immune substance resulting in better humoral response.

Treatment by *A. annua* increased weight of thymus and bursa of fabricius (Table 5). The masses, cellularity and histological characteristics of primary organs such as the bursa of fabricius (the site of B lymphocyte maturation in birds) and thymus (the site of T lymphocyte maturation) give important data regarding immunological development (Grasman, 2010). Increased weight of thymus and bursa of fabricius in the present work indicate that the effect of *A. annua* on primary immune organs is consistent with the results of SRBC and PHA tests.

Finally, the present study showed that *A. annua* leaf powder and methanolic extract affected performance, cellular and humoral immunity of broilers, especially by E_{4000} and P_1. Based on the data presented here, leaf powder and plant extract increased daily weight gain and reduced feed conversion ratio. Thymus and bursa of fabricius weights, total anti-SRBC, IgG titer and cellular immunity on PHA-P injection were increased by experimental groups compared to the control group. Further experiments are needed to explore other indigenous plants having the same effects on experimental broilers.

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