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Effect of *in ovo* Feeding and Dietary Feeding of *Silybum marianum* Extract on Performance, Immunity and Blood Cation-Anion Balance of Broiler Chickens Exposed to High Temperatures

Research Article

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ABSTRACT

This experiment was conducted to investigate the effect of *in ovo* feeding (IOF) and dietary feeding of *Silybum marianum* (SM) extract on the immunity of chicken under heat stress conditions. A total of 360 fertile eggs from a commercial broiler breeder (Ross-308) farm was used for *in ovo* feeding of 0, 100 and 200 mg/L SM water extract at day 17.5 of incubation. After hatching, 240 chicks were transferred to the experimental cages. Two types of diet were used; one of them without extract and the other one containing 100 mg/kg of SM extract. Then chicks were exposed to elevated temperatures 4 ˚C above optimum from 7 to 28 days of age for 4 h daily and then they were kept at the optimum temperature. The chicks were divided into six treatments with four replicates as a completely randomized design with 2 × 3 factorial arrangement. The results showed that chicks fed diet containing the extract had the highest feed intake, daily weight gain and final body weight and lowest feed conversion ratio ($P<0.01$). At 28 and 42 days, dietary feeding of the extract increased the relative weights of bursa ($P<0.05$), thymus and spleen ($P<0.01$) significantly. Results showed a significant increase ($P<0.05$) in antibody titer against infectious bursal disease (IBD) by feeding extract. White blood cell counts was significantly higher ($P<0.01$), and heterophile/lymphocyte (Het/Lym) ratio was lower in dietary-feeding SM extract treatments ($P<0.01$). The dietary feeding of extract increased blood cationic charge (Na^+ and K^+) at 28 d and led to increase cation-anion equation ($P<0.01$). However at 42 d, effect of dietary feeding of extract on these parameters was not significant. The ash percentage was significantly higher in chicks fed 100 mg/kg of extract at 28 d ($P<0.01$). It seems that dietary feeding of the extract to broiler chickens considerably increased immunity response under elevated temperatures, but *in ovo* feeding of the extract had no much impact on immunity parameters.

KEY WORDS antibody titer, cation-anion equation, elevated temperatures, incubation.

INTRODUCTION

The pursuant to improvement of avian embryos and hatched chicks are affected by the yolk nutrient situation (Al-Murrani, 1982). Many nutrients have considerable structural, physiological, and immunological functions in avian embryogenesis and growth performance. *In ovo* feeding of nutrients may help beard any limitations of insufficient egg nutrition. During early development, there is rapid oxidative metabolism that results in production of a large

amount of free radicals in many tissues that will make them more susceptible to oxidative damage. Antioxidants are a crucial defense against these free radicals. The developing embryo may use antioxidants found in the yolk in order to lessen the influence of free radicals. Some researchers represented that *in ovo* feeding of vitamin E, at day 14 of incubation at the time of increasing fatty acid oxidation, could be instrumental in decreasing the production of free radicals (Cherian *et al*. 1997; Cherian and Sim, 1992; Cherian and Sim, 1997). Tullett (1990) reported that incubated eggs and their embryos may be subjected to stress caused by extreme production of metabolic heat during the latter part of egg incubation, producing some internal environmental stress for embryo, which may be not proper for the embryo to hatch successfully. In addition, one of the most important stressors in poultry production, especially in tropical regions is hot weather, and the poultry are susceptible to environmental challenges correlated to temperature, particularly heat stress. In addition, the modern poultry genotypes are producing more body heat because of their further metabolic activity (Deeb and Cahaner, 2002). Therefore *in ovo* feeding of some herbs as an anti-stress factor may be useful.

One of these herbs is known as *Silybum marianum* (Milk thistle) and its polyphenolic compound extract from fruits and seeds that is known as Silymarin Loguercio and Festi (2011). Its active components are flavonolignans (Ding *et al*. 2001). It has been reported that flavonoid components are caused to sustain immunity system by increasing vitamin C activity and antibacterial impact in animals (Cook and Samman, 1996); also Silymarin stimulates the immunity to infectious diseases (Wilasrusmee *et al*. 2002). Studies showed that using Silymarin in poultry diets decreases aflatoxin toxicity (Tedesco *et al*. 2004). In recent studies, *Silybum marianum* has been distinguished as an effective component for treating many diseases such as cancer, bone hollow, fatty liver and diabetes (Thelen *et al*. 2004; Lucena *et al*. 2002). The seeds of this plant contained betaine, 3-methyl Glycine and a large amount of oil (about 20%) that have an anti-inflammatory effect and will decrease hepatic disorders (Hadolin *et al*. 2001).

Natural antioxidants corroborate body defense system against oxidative stresses and consequently decreasing damages due to free radicals; also antioxidant substances are preventing fat peroxidation of membrane immunity cells and by keeping cell membrane mobility are inhibiting body immunity system weakness (Yang *et al*. 2001). Liu (1999) expressed that broiler nutrient needs for immunity may not be parallel to those for growth, so application of immunostimulants is one solution to improve the immunity of animals and to reduce their susceptibility to infectious diseases.

MATERIALS AND METHODS

Silybum marianum water extract composition was analyzed in advance via HPLC by Qingdao BNP Co., Ltd (Qingdao, China). This commercial silymarin extract contains Silymarin (≥80%) and Silybin isomers (≥30%) and its solvents are acetone and water.

The preparation of *in ovo* solutions was done by dissolving the extract in distilled water. So that the stock solution (200 mg/L density) (w/v) was obtained by dissolving 200 mg of the extract in 1 liter of distilled water. Then for providing the 100 mg/L density solution, a proportion of the stock solution was poured in a separate vessel and was diluted by twice volume of distilled water.

A total number of 360 eggs were gained from broiler breeder strain (Ross-308) at 43 weeks of age and used for *in ovo* feeding (IOF) of *Silybum marianum* water extract. Eggs were incubated at 37.8 ˚C and 63% RH, then at day 17.5 of incubation the eggs were candled, the infertile ones were omitted, the fertilized eggs were weighed on a balancer to 0.1 g precision.

The eggs were distributed into three groups (each group 120 eggs) with an equal weight frequency of 55 ± 1 g and then 1 mL of the mentioned solutions injected into amniotic fluid using a 21-gauge needle. The IOF procedure was performed as described by Tako *et al*. (2004).

On the completion of the injections, the injection holes were sealed with molten paraffin and the eggs were located in hatching trays. Eventually, all the eggs were returned to the hatcher until the day of hatching.

After hatching, chicks (n=240) were transferred to the experimental cages with floor pens bedded with soft wood shavings and given *ad libitum* access to water and a commercial diet for the first (from d 1 to d 21) and second (from d 22 to d 42) periods (Table 1).

The diet included of two types; one of them without extract and the other one containing 100 mg/kg of *Silybum marianum* extract.

The diets were formulated on the basis of Ross strain recommendations (NRC, 1994). The chicks were distributed into six treatments with four replicates (10 chicks/replicate) each. The experimental dietary treatments were as follows:

1. *In ovo* feeding of no extract solution (distilled water) with a control diet (without extract)
2. *In ovo* feeding of no extract solution (distilled water) with a diet containing 100 mg extract/kg
3. *In ovo* feeding of 100 mg/L extract solution with a control diet (without extract)
4. *In ovo* feeding of 100 mg/L extract solution with a diet containing 100 mg extract/kg

5. *In ovo* feeding of 200 mg/L extract solution with a control diet (without extract)
6. *In ovo* feeding of 200 mg/L extract solution with a diet containing 100 mg extract/kg

Table 1 Ingredient composition and calculated values of the basal diets (as fed basis)

Ingredient (g/kg)	0–21 d	21-42 d
Corn	547.0	622.5
Fish meal	30. 0	20. 0
Soybean meal	355.0	297.3
Soybean oil	35.0	30
Oyster shell-flour	12.0	12.5
Dicalcium phosphate	11.2	9
Sodium chloride	3.9	3
Methionine	1.4	0.7
Trace mineral-vitamin permix[1]	5.0	5
Calculated values		
Metabolisable energy (kJ/kg)	12.6	12.8
Crude protein (g/kg)	216.8	192.6
Calcium (g/kg)	9.43	8.67
Available phosphorus (g/kg)	4.24	3.37

[1] Supplied per kg of diet: Antioxidant (ethoxyquin):100 mg; Biotin: 0.2 mg; Calcium pantothenate: 12.8 mg; Cholecalciferol: 60 g; Cyanocobalamin: 0.017 mg; Folic acid: 5.2 mg; Menadione: 4 mg; Niacin: 35 mg; Pyridoxine: 10 mg; Trans-retinol: 1240 mg; Riboflavin: 12 mg; Thiamine: 3.0 mg; DL-tocopheryl acetate: 60 mg; Choline chloride: 638 mg; Co: 0.3 mg; Cu: 3 mg; Fe: 25 mg; I: 1 mg; Mn: 125 mg; Mo: 0.5 mg; Se: 200 g and Zn: 60 mg.

The elevated temperature condition (optimum temperature+4 ˚C) was set from 7 days of age for all chicks for 4 hours a day (12-16 o'clock). The high temperature was discontinued after 28 days, but the diets were not changed. The birds were kept according to the Iranian Council of Animal Care guidelines (1995).

During the experiment, body weight (BW), feed intake, and feed conversion were recorded every week. All chicks were vaccinated against IBD (Infectious Bursal Disease) (Cevac Sant Animal) on d 16 and Newcastle Disease (ND) (Lohmann Animal Health Gmbh and Co. KG) for 2 periods on days 8 and 22 via drinking water. At 28 and 42 days of age birds were starved for 6 h before slaughtering and two chicks from each pen were slaughtered.

Chickens were slaughtered by decapitation, plucked and eviscerated, then, the relative weights (% of BW) of immunity segments (bursa, thymus and spleen) of chickens were noted. At 42 day of age, four blood samples were randomly taken from each treatment after slaughtering from the neck artery; the samples were centrifuged at 1500 g for 10 min and the serum was transferred into vials and stored at -20 ˚C. The serum samples were analyzed for antibody titer, Na, K and Cl values. Also 4 blood samples contain anticoagulant per each treatment were taken for evaluating blood cell counts of broilers. Antibody titer against NDV was measured using Hemagglutination Inhibition (HI) test according to Brugh *et al.* (1978) and by ELISA method for IBD vaccine titer.

At 28 and 42 days of age, after slaughtering, the left tibia of each carcass was separated completely from meat and then their length was measured by a caliper (in mm). Also for measuring the ash percentage, bones were placed in the oven with 80 ˚C temperature for 24 h, afterward their fat was extracted by Soxhlet apparatus and again the free fat bones were placed in the oven for 24 h. The weight of these dry free fat bones is the initial weight of samples. Thereupon bones were crushed and placed in an electrical oven with 600 ˚C temperature for 8 h when the bones became white, again samples were weighed and the ash weight (g) was obtained. Finally the ash percentage was calculated by the following formula:

Bone ash (%)= Ash weight (g) / dry sample weight (g)

Data were analyzed using the generalized linear models (GLM) procedures of SAS (2005) to determine statistical differences between the treatments. This test was designed for all parameters as a completely randomized 2 × 3 factorial design. Differences between the treatments were compared by Tukey test following ANOVA and the values were considered statistically significant at ($P<0.05$).

RESULTS AND DISCUSSION

Average feed consumption, daily BW gain, feed conversion, and final body weight of the chickens in the two different periods of feeding are provided in Table 2. No differences were observed over the 7-42 d period for the daily feed consumption in IOF treatments. Also, daily feed consumption was not influenced by dietary feeding of the extract between the 7-42 d periods. Investigating interactions between IOF and dietary feeding of the extract between 7-42 d, there were no differences for feed consumption. It seems that the presence of the extract in the diet decreases the effects of high temperature on feed consumption. No differences were observed for the effect of IOF of the extract on daily weight gain (DWG) between 7-42 d. However dietary feeding of the extract increased DWG between 7-42 d ($P<0.01$).

Also the interaction effects showed that DWG at different periods was influenced by dietary feeding of extract and was higher in dietary feeding of extract groups than those without feeding extract ($P<0.01$). Chickens fed the extract containing diet had the highest final BW ($P<0.01$). The feed conversion of chickens was not affected by IOF extract and no significant differences were observed between the treatments. However feed conversion was declined by dietary feeding of the extract between 7-42 d which was due to higher final BW ($P<0.01$) of the birds at similar diet intakes.

Table 2 The effect of dietary feeding and *in ovo* feeding of *Silybum marianum* extract on performance of broiler chickens maintained under high temperature conditions for 7-42 d[1]

Item	Daily feed intake (g/day) (7-42 d)	Daily weight gain (g) (7-42 d)	Feed conversion ratio (7-42 d)	Final BW (42 d)
***In ovo* injection**				
0 mg/L injection	96.99	59.23	1.63	2179.96^{a}
100 mg/L injection	96.18	58.05	1.65	2125.20^{b}
200 mg/L injection	97.86	58.57	1.67	2142.54^{ab}
SEM	0.93	0.42	0.012	14.78
The significance level	NS	NS	NS	*
Feeding				
Diet without extract	96.21	56.63^{b}	1.69^{a}	2079.97^{b}
Diet with mg/kg extract	97.82	60.61^{a}	1.61^{b}	2218.50^{a}
SEM	0.75	0.35	0.009	12.06
The significance level	NS	**	**	**
Interaction				
0 mg/L injection × no feeding	96.38	57.6^{b}	1.67^{ab}	2124.46^{b}
0 mg/L injection × 100 mg/kg feeding	97.60	60.83^{a}	1.60^{c}	2235.46^{a}
100 mg/L injection × no feeding	94.67	55.49^{c}	1.70^{a}	2036.01^{c}
100 mg/L injection × 100 mg/kg feeding	97.69	60.62^{a}	1.61^{c}	2214.38^{a}
200 mg/L injection × no feeding	97.57	56.76^{bc}	1.72^{a}	2079.43^{bc}
200 mg/L injection × 100 mg/kg feeding	98.16	60.39^{a}	1.62^{bc}	2205.65^{a}
SEM	1.31	0.60	0.01	20.90
The significance level	NS	**	**	**

[1] Data are means of 4 replicate pens of 10 birds each.
The means within the same column with at least one common letter, do not have significant difference (P>0.05 or P>0.01).
* (P<0.05) and ** (P<0.01).
BW: body weight.
NS: non significant.
SEM: standard error of the means.

The lowest feed conversion was observed in chickens fed with extract between 7-42 d (P<0.01). Recently some researchers reported that broilers under chronic heat stress conditions experienced a significant reduction in feed intake (-16.4%), lower body weight (-32.6%) and higher feed conversion ratio (+25.6%) at 42 days of age (Sohail *et al.* 2012; Deng *et al.* 2012). However, the results of this study showed that dietary feeding of *Silybum marianum* extract could increase final body weight and daily weight gain at elevated temperatures and improve feed conversion while the IOF of the extract did not have any effect. These results are in agreement with the findings of Tedesco *et al.* (2004) who reported that addition of silymarin phytosome® at 600 mg/kg feed for reducing the toxicity of B1 aflatoxin resulted in increasing body weight. Similar results were also reported by other researchers who fed milk thistle to broilers and observed a more efficient feed conversion ratio (Chakarverty and Parsad, 1991; Zahid and Durrani, 2007). Zahid and Durrani (2007) fed 15 g of milk thistle/kg feed to broilers and found improved FCR (2.20) as compared to the control group (2.40).

Gowda and Sastry (2000) showed significant effects of milk thistle on body weight gain and attributed the effect to the antioxidant activity that provoked protein synthesis by the bird's enzymatic system. Higher weight gain was also reported by Chakarverty and Parsad (1991) in a milk thistle supplemented group.

The accurate mechanism of improved body weight gain is not well established; although, this effect might be because of the improved immune function of the birds (e.g. higher liver, spleen and bursa weights) receiving *Silybum marianum* extract.

The relative weights (% BW) of immune segments at 28 and 42 days are presented in Table 3. The results showed that at 28 and 42 days the weights of bursa, thymus and spleen were not affected by extract IOF, however the interaction effects showed that dietary feeding of the extract increased the relative weights of bursa (P<0.05), thymus and spleen (P<0.01) significantly. Also lower relative weights of thymus, spleen (Ghazi *et al.* 2012) and other lymphoid organs (Quinteiro-Filho *et al.* 2010; Niu *et al.* 2009) have been reported in laying hens and broilers under heat stress conditions. Some researchers reported that feeding flavonoid compounds in broiler chickens stimulated the growth of immune organs and caused an increase in their weights (Takahashi *et al.* 2000). Also Tedesco *et al.* (2004) and Kalorey *et al.* (2005) have been demonstrated the protective role of *Silybum marianum* on immune organ weights (bursa, thymus and spleen) against aflatoxin in broiler chickens.

The results showed that extract IOF did not have any effect on antibody titer against ND and IBD, but the dietary feeding of extract increased antibody titer against IBD significantly (P<0.01).

Table 3 The effect of dietary feeding and *in ovo* feeding of *Silybum marianum* extract on relative weight (% of BW) of immunity segments of broiler chickens under elevated temperature after 28 and 42 days[1]

Item	28 d			42 d		
	Bursa	Thymus	Spleen	Bursa	Thymus	Spleen
***In ovo* injection**						
0 ppm injection	0.22	0.88	0.087	0.080	0.61	0.127
100 mg/L injection	0.21	0.87	0.073	0.077	0.61	0.131
200 mg/L injection	0.22	0.85	0.078	0.077	0.58	0.125
SEM	0.004	0.016	0.004	0.003	0.009	0.004
The significance level	NS	NS	NS	NS	NS	NS
Feeding						
Diet without extract	0.20^{b}	0.81^{b}	0.069^{b}	0.070^{b}	0.56^{b}	0.110^{b}
Diet with 100 mg/kg extract	0.22^{a}	0.92^{a}	0.090^{a}	0.085^{a}	0.63^{a}	0.145^{a}
SEM	0.004	0.013	0.003	0.002	0.007	0.004
The significance level	**	**	**	**	**	**
Interaction						
0 mg/L injection × no feeding	0.20^{c}	0.83^{b}	0.072^{bc}	0.070^{c}	0.57^{b}	0.112^{b}
0 mg/L injection × 100 mg/kg feeding	0.23^{a}	0.94^{a}	0.102^{a}	0.090^{a}	0.64^{a}	0.142^{a}
100 mg/L injection × no feeding	0.20^{c}	0.82^{b}	0.065^{c}	0.067^{c}	0.58^{b}	0.110^{b}
100 mg/L injection × 100 mg/kg feeding	0.22^{ab}	0.92^{a}	0.082^{bc}	0.087^{ab}	0.64^{a}	0.152^{a}
200 mg/L injection × no feeding	0.21^{bc}	0.79^{b}	0.070^{bc}	0.075^{bc}	0.54^{b}	0.107^{b}
200 mg/L injection × 100 mg/kg feeding	0.22^{ab}	0.91^{a}	0.087^{ab}	0.080^{abc}	0.62^{a}	0.142^{a}
SEM	0.006	0.023	0.006	0.004	0.012	0.007
The significance level	*	**	**	*	**	**

[1] Data are means of 4 replicate pens of 10 birds each.
The means within the same column with at least one common letter, do not have significant difference (P>0.05 or P>0.01).
* (P<0.05) and ** (P<0.01).
BW: body weight.
NS: non significant.
SEM: standard error of the means.

Also the interaction effects showed the significant increment (P<0.05) of antibody titer against IBD by feeding extract (Table 4). Chand *et al*. (2011a) reported that *Silybum marianum* extract had effect on immune cells and support immune system through the strong antioxidants and collecting free radicals. In addition Wilasrusmee *et al*. (2002) and Thyagarajan *et al*. (2002) emphasized the importance of immunity stimulant role of *Silybum marianum*. Some researchers demonstrated that using water soluble extract of medicinal plants in broiler chickens improve blood antibody titer against ND and IBD (Maroof *et al*. 2012). Also, Chand *et al*. (2011b) expressed that adding 10 g *Silybum marianum* per kg diet of broiler chickens improved immune level against the ND and IBD. On the other hand, the results of cell blood counts confirm the antibody titer results, so that WBC counts were not influenced by extract IOF, whereas the Het/Lym ratio was significantly (P<0.05) lower in 100 and 200 mg/L IOF treatments. The dietary feeding of extract increased WBC counts and decreased Het/Lym ratio (P<0.01).

Het/Lym ratio increases under stress conditions but it has been observed that this ratio decreased in dietary feeding treatments. Regarding to using Het/Lym ratio as an index for stress tolerance, our findings are in agreement with the results of Borges (1997) and Zulkifli and Siegel (1995). The interaction effects showed that WBC counts were significantly higher (P<0.01) and Het/Lym ratio was lower in dietary feeding extract treatments (P<0.01).

The results of Table 5 showed that the extract IOF did not have any significant effect on the amount of Na^{+}, K^{+}, Cl^{-} and dietary cation anion difference (DCAD) of broiler chickens blood serum at 28 and 42 days, but at 28 d the dietary feeding of extract increased blood cationic charge (Na^{+} and K^{+}) and led to increase cation-anion equation (P<0.01).

However, the effect of the dietary extract on these parameters was not significant at day 42. The interaction effects also showed a similar trend and these parameters were influenced by dietary feeding of the extract.

At 42 d no significant differences observed between treatments for interaction effects of IOF and the dietary extract. Since some researchers reported that certainly followed by heat stress, a reduction in blood Na and K level occurs (Ait-Boulahsen *et al*. 1995; Borges, 1997), but our findings express that dietary feeding of extract was effective as a factor in moderating negative effects of heat stress such as reducing blood electrolyte level and blood cationic charge until 28 days of age that heat stress conditions was existed.

The length and ash percentage of tibia at 28 and 42 days is given in Table 6. Results showed that the IOF of extract did not have any significant effect on tibia measurements.

Table 4 The effect of dietary feeding and *in ovo* feeding of *Silybum marianum* extract on humoral immunity of broiler chickens under elevated temperature after 42 days[1]

Item	Anti-body titer		Blood cell count	
	ND	IBD	WBC	Het/Lym
***In ovo* injection**				
0 mg/L injection	2.66	4824.0	5.11	0.65[a]
100 mg/L injection	2.50	4879.8	4.41	0.61[b]
200 mg/L injection	2.83	4610.5	4.51	0.60[b]
SEM	0.419	239.69	0.323	0.009
The significance level	NS	NS	NS	*
Feeding				
Diet without extract	2.55	2478.5[b]	3.71[b]	0.74[a]
Diet with 100 mg/kg extract	2.77	5264.3[a]	5.65[a]	0.51[b]
SEM	0.342	195.70	0.263	0.008
The significance level	NS	**	**	**
Interaction				
0 mg/L inject × no feed.	2.33	4353.0[ab]	4.16[bc]	0.78[a]
0 mg/L inject × 100 mg/kg feed.	3.00	5295.0[a]	6.06[a]	0.52[c]
100 mg/L inject × No feed.	2.33	4435.3[ab]	3.36[c]	0.73[b]
100 mg/L inject × 100 mg/kg feed.	2.66	5324.3[a]	5.46[ab]	0.50[c]
200 mg/L inject × No feed.	3.00	4047.3[b]	3.60[c]	0.71[b]
200 mg/L inject × 100 mg/kg feed.	2.66	5173.6[a]	5.43[ab]	0.50[c]
SEM	0.593	338.97	0.456	0.013
The significance level	NS	*	**	**

[1] Data are means of 4 replicate pens of 10 birds each.
The means within the same column with at least one common letter, do not have significant difference (P>0.05 or P>0.01).
* (P<0.05) and ** (P<0.01).
NS: non significant.
SEM: standard error of the means.
ND: Newcastle disease; IBD: infectious bursal disease; WBC: white blood cell and Het/Lym: heterophile/lymphocyte.

Table 5 The effect of dietary feeding and *in ovo* feeding of *Silybum marianum* extract on blood cation-anion balance of broiler chickens under elevated temperature after 28 and 42 days[1]

Item	28 d				42 d			
	Na	K	Cl	DCAB	Na	K	Cl	DCAB
***In ovo* injection**								
0 mg/L injection	146.33	5.31	105.16	46.48	163.50	5.95	115.16	54.28
100 mg/L injection	143.50	5.03	104.50	44.03	162.66	5.88	115.00	53.55
200 mg/L injection	145.33	5.23	105.16	45.40	162.66	5.90	114.66	53.90
SEM	1.536	0.182	1.269	1.813	1.468	0.089	0.844	1.407
The significance level	NS	NS	NS	NS	NS	NS	NS	NS
Feeding								
Diet without extract	137.44[b]	4.67[b]	106.00	36.12[b]	162.77	5.87	115.44	53.21
Diet with 100 mg/kg extract	152.66[a]	5.71[a]	103.88	54.48[a]	163.11	5.94	114.44	54.61
SEM	1.254	0.149	1.036	1.481	1.199	0.072	0.689	1.149
The significance level	**	**	NS	**	NS	NS	NS	NS
Interaction								
0 mg/L injection × no feeding	140.00[b]	4.90[bc]	105.67	39.23[b]	163.00	5.93	115.67	53.26
0 mg/L injection × 100 mg/kg feeding	152.66[a]	5.73[a]	104.67	53.73[a]	164.00	5.96	114.67	55.30
100 mg/L injection × no feeding	134.00[b]	4.53[c]	106.00	32.53[b]	162.33	5.86	115.33	52.86
100 mg/L injection × 100 mg/kg feeding	153.00[a]	5.53[ab]	103.00	55.53[a]	163.00	5.90	114.67	54.23
200 mg/L injection × no feeding	138.33[b]	4.60[c]	106.33	36.60[b]	163.00	5.83	115.33	53.50
200 mg/L injection × 100 mg/kg feeding	152.33[a]	5.86[a]	104.00	54.20[a]	162.33	5.96	114.00	54.30
SEM	2.173	0.258	1.795	2.56	2.077	0.126	1.194	1.990
The significance level	**	**	NS	**	NS	NS	NS	NS

[1] Data are means of 4 replicate pens of 10 birds each.
The means within the same column with at least one common letter, do not have significant difference (P>0.01).
** (P<0.01).
NS: non significant.
SEM: standard error of the means.
DCAB: dietary cation-anion balance.

Table 6 The effect of dietary feeding and *in ovo* feeding of *Silybum marianum* extract on the length (cm) and ash percentage of broiler chickens tibia under elevated temperature at 28 and 42[1]

Item	28 d		42 d	
	Length	Ash	Length	Ash
***In ovo* injection**				
0 mg/L injection	7.44	46.07	8.48	45.20
100 mg/L injection	7.50	45.44	8.55	45.53
200 mg/L injection	7.49	46.57	8.47	45.54
SEM	0.112	0.596	0.072	1.22
The significance level	NS	NS	NS	NS
Feeding				
Diet without extract	7.43	43.86[b]	8.52	45.09
Diet with 100 mg/kg extract	7.52	48.20[a]	8.47	45.75
SEM	0.092	0.486	0.059	0.998
The significance level	NS	**	NS	NS
Interaction				
0 mg/L injection × no feeding	7.39	44.07[b]	8.52	45.32
0 mg/L injection × 100 mg/kg feeding	7.49	48.08[a]	8.44	45.08
100 mg/L injection × no feeding	7.47	43.16[b]	8.57	44.73
100 mg/L injection × 100 mg/kg feeding	7.52	47.72[a]	8.52	46.32
200 mg/L injection × no feeding	7.44	44.35[b]	8.48	45.21
200 mg/L injection × 100 mg/kg feeding	7.55	48.79[a]	8.46	45.87
SEM	0.159	0.843	0.103	1.73
The significance level	NS	**	NS	NS

[1] Data are means of 4 replicate pens of 10 birds each.
The means within the same column with at least one common letter, do not have significant difference (P>0.01).
** (P<0.01).
NS: non significant.
SEM: standard error of the means.

However, at 28 d the ash percentage of tibia was significantly higher in chicks fed 100 mg extract/kg diet (P<0.01). Our results regarding the retarding effect of heat stress on tibia measurements are in agreement with the findings of Bruno *et al*. (2000), Bruno *et al*. (2007), who reported that the growth of tibia, femur and humerus was reduced by environmental temperature.

In contrast, Leeson and Caston (1993) have reported a 10% increase in tibia length of 14 weeks leghorn pullets exposed to heat stress. A reduction in weights of tibia and humerus was also reported by Yalcin *et al*. (1996) in heat stress condition.

CONCLUSION

The results of present study showed that dietary feeding of *Silybum marianum* extract to broiler chickens under elevated temperatures increased considerably their performance, relative weights of immune segments, immunity response, ash percentage of tibia and ionic balance. It also reduced antibody titers against IBD and Het/Lym ratio. Whereas, *in ovo* injection of the extract had little impact on these parameters.

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