

Effect of Peppermint (*Mentha piperita*) Powder on Immune Response of Broiler Chickens in Heat Stress

Research Article

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ABSTRACT

To study the effect of different levels of peppermint (*Mentha piperita*) plant powder, on immune system of broilers under heat stress condition, 192 one-day old chickens (Ross, 308) were randomly allocated to 4 dietary treatments with 4 replicates of 12 chicks each, using a completely randomized design. The four groups were characterized by a basal diet (control), basal diet supplemented with 1 and 2 percent peppermint powder and basal diet supplemented with 300 mg/kg vitamin E. Heat stress was created by setting room temperature at 34 °C for 8 hour/day from the 35th to the 42nd day of experiment. Results showed differences ($P<0.05$) for feed conversion ratio (FCR) at 21 days and body weight (BW) at 42 days of the experiment. Birds treated by 2 percent peppermint powder and 1 percent peppermint powder showed higher and lower body weight gain, respectively, at 21 days of age, when compared with birds fed basal diet and vitamin E. A significantly higher level of total Ig, IgM and IgG was found for peppermint powder than other treatment groups at 35 days and 42 days of age. Significant interactions were observed between diet and sex on IgG at 35 days of the experiment ($P<0.05$). There were significant ($P<0.05$) differences among the treatments for total white blood cells, lymphocytes, heterophils, heterophils to lymphocytes ratio at 42 days of experiment and 2 percent peppermint powder increased total white blood cells values compared to basal diet and vitamin E. The peppermint powder significantly made a difference for serum concentrations of total protein, albumin, globulin, triglyceride, total cholesterol, high-density lipoprotein-cholesterol (HDL_C), low-density lipoprotein-cholesterol (LDL_C) and very low-density lipoprotein-cholesterol (VLDL_C) at 21 days and 42 days of age ($P<0.05$). Blood serum concentration of HDL_C increased by peppermint powder treatment, whereas they were lower for basal diet and vitamin E at 42 days of age. Liver weight was higher and lower in 1 percent peppermint powder and basal diet treated groups, respectively ($P<0.05$). In general, results indicated that supplementation of peppermint powder in the diet did not improve bursa of fabricius and spleen weight of broiler chicken, but has an antioxidative potential to improve oxidative stability and immune response.

KEY WORDS broiler, heat Stress, immune system, peppermint.

INTRODUCTION

In commercial production of poultry, any disruption in health and welfare of the birds will have an important impact on economic efficiency. Health is also a significant factor in determining the performance and flock uniformity

and durability due to resistance to various diseases. Strong immune system improves resistance to diseases. Large herds are constantly exposed to stress and stressors. High ambient temperature results in an enormous annual economic loss in the poultry industry. Most notably among them are the reductions in feed intake, weight loss, de-

creased carcass quality and weakening of the immune system's defenses. When the ambient temperature is higher than normal temperatures, the bird cools down the body using energy, which reduces performance and productive traits and causes hormonal and biochemical imbalance (Lin *et al.* 2006). Cardiovascular system is involved in the regulation of body temperature. When the birds are housed under heat stress, cardiovascular system changes occur including the balance of acid-base, blood pH, respiratory alkalosis and decreased levels of blood viscosity, hematocrit and plasma protein concentration (Teeter *et al.* 1985). Blood proteins, triglycerides and cholesterol can serve as indicators for the evaluation of the antioxidant power of body under heat stress conditions. Due to the increasing amount of plasma protein and cell wall protein denaturation under heat stress these parameters are used in the assessment of heat stress (Hosseini-Mansoub *et al.* 2010). Due to the abundance of the content of unsaturated fatty acids in membrane lipids, there is high sensitivity to peroxidation and degradation. Therefore, destruction of tissue lipids and release of triglycerides and cholesterol in the blood will be greater by reducing the antioxidant capacity under heat stress conditions (Leeson and Summers, 2001). Stresses such as heat stress leads to excessive production of free radicals, which reduce antioxidant capacity (Robert *et al.* 2003). In fact, heat stress affects the sympathetic nerves and causes catecholamine release thereby leading to an increase in free radicals in the blood and tissues of the body. Free radicals attack the structure of unsaturated fat, hence damaging cell membranes (Curi *et al.* 2003). Free radicals give rise to peroxidation in cells and thereby increase lipoperoxide concentrations in the tissues. Lipoperoxide surplus leads to reduced enzyme activity of glutathione peroxidase, superoxide dismutase and catalase (Du *et al.* 2000). In these conditions, the plasma levels of certain vitamins and minerals involved in the antioxidant system decreases and the amount of active oxygen radicals (ROS) increases. Heat stress also reduces the number of white blood cells, lymphocytes, antibody secretion and changes various components of the immune system such as killer T-cell activity, cytokines secretion, multiplication of lymphocytes and the level of immunoglobulins in broiler chickens (Bartlett and Smith, 2003; Mashaly *et al.* 2004; Shephard, 1998). In addition, heat stress leads to cell wall degradation, protein denaturation and reduction of hematocrit, increased glucose, cholesterol and heterophil to lymphocyte ratio (Puvadolpirod and Thaxon, 2000). In addition, increased concentrations of pharmacologic corticosteroids, via attenuation of poultry lymphoid tissues (bursa fabricius, spleen and thymus), weakens the immune system (Borges *et al.* 2004). Thus, heat stress increases the need for antioxidants. Since birds do not have the ability to synthesize

antioxidant in the event of heat stress and given the fact that antioxidants are reduced under oxidative stress, the addition of antioxidants to poultry diets is essential (Tatli *et al.* 2006). Therefore, to cope with the adverse effects of heat stress; vitamins, minerals and plant antioxidant are currently being used (Bartlett and Smith, 2003).

Today, heart-cardiovascular and liver-biliary diseases are the most common causes of death in the world (Gebhardt, 1995). Researchers have concluded that diets containing high levels of fat and cholesterol can increase blood cholesterol and this causes atherosclerosis and artery diseases. Liver malfunctions include hepatitis, liver failure, liver dysfunction and hepatic colic (Pang *et al.* 1992). Since liver damage is usually caused by free radicals and lipid peroxidation, researchers have attempted to use certain compounds such as membrane stabilizers, natural and synthetic antioxidants as well as metabolic pathways inhibitors as protective substances in case of liver toxicity (Hall *et al.* 1994). Studies show that medicinal plants due to their antioxidant and flavonoid compounds could play an important role in improving cardiovascular health and liver diseases (Pouramir *et al.* 2006).

Vitamins are organic compounds whose existence is essential for metabolic reactions (Shaker Hoseini and Azadbakht 2004). Lipid oxidation causes the production of hydroperoxides. Hydroperoxides cause tissue damage and the cell's structural integrity destruction in the body and thus interrupts the cell metabolism. Vitamin E as an antioxidant is of great importance in the prevention of oxidation of the cellular and intracellular membranes (Afshar Mazandarani and Rajab, 2002). The addition of 300 mg vitamin E per kg feed improves phagocytosis in the immune system (Effati *et al.* 2012). Vitamin E, like many other nutritional factors, directly through their effects on immune cells and indirectly through changes in metabolic and endocrine parameters, effectively improves immune system function, thus leading to the complementarity of the immune system (Gershwin *et al.* 2004).

Given the adverse effects of synthetic antioxidants and antibiotics such as toxicity, mutagenicity and carcinogenicity (Hertampf, 2001), the need for alternative factors to create a margin of safety during culture and in the face of stressful situations is inevitable, hence emphasis is given on the use of natural antioxidants (Hertampf, 2001). It has been proven that medicinal herbs, extracts or their active components can be a good alternative to synthetic antioxidants to be used in livestock and poultry rations (Greathead, 2003; Chaves *et al.* 2008). Among secondary metabolites, flavonoids due to the powerful antioxidant property are of particular importance in such a way that their antioxidant activity *in vitro* was found to be higher than vitamins E and C (Manashi *et al.* 1999). Hofshagen and Kaldhosal (1992)

stated that the peppermint ingredients not only appeared to have antibacterial and antioxidant properties but also, by stimulating the activity of digestive enzymes of pancreas and intestine and increasing liver activity, to improve nutrient digestibility and feed efficiency in broiler chickens.

Edens *et al.* (1983) and Zulkifi *et al.* (2000) reported that heat stress reduces antibody production. This reduction happens indirectly due to increased inflammatory cytokines under stress condition. These substances stimulate the production of corticotrophin, and through the secretion of adrenal cortical stimulating hormone and corticosterone, antibody production is reduced. Yang *et al.* (2010) investigated the effects of using 2% of medicinal plants on the immune system in broilers. They reported that the herb significantly increased antibody titers than the control group, resulting in augmented activity of the immune system. Antibody titer is a proper marker for the humoral immune system (Yang *et al.* 2010). Zadeh Amiri *et al.* (2014) reported that compared to the control group, essential oil of *Satureja* causes an increase in the number of serum immunoglobulin against SRBC in broilers. Mahmoudi *et al.* (2012) reported that lavender and black pepper increases blood serum immunoglobulin against sheep red blood cells (SRBC). Medicinal plants probably with increased plasma glutamine levels can increase serum immunoglobulin. Plant extracts which increase immunoglobulin production, also increase humoral immune response by stimulating macrophages and an increase in cytokines production (Mativan and Kalaiarasi, 2007). Also, Nie and Zhang (1999) argued that plant polysaccharides improve antibodies secretion. Menthol, menthone and phenyl acetate as well as less important compounds like flavonoids, polymerized polyphenols, carotenoids, tocopherols, betaine and choline are the constituent polysaccharides of peppermint.

Today applied research on living organisms especially livestock and poultry, comprise much of the research activities of life sciences form. Since blood and biochemical parameters give a full index of the physiological status of living organisms, furthermore, relative weight of lymphoid organs give an index of immune system status. So researchers can indirectly study the influence of a food, anti-nutritional or other environmental factors that can affect the body's physiological and immune systems, according to blood factors and relative weight of lymphoid organs. In consideration of the arguments above described, the aim of this study was to investigate the antioxidant properties of peppermint on immune system of broilers under heat stress.

MATERIALS AND METHODS

Plant preparation

Peppermint plant used in this experiment was collected in the summer season when the plant was in a vegetative

stage, from the research farm (36 °00'-16" north latitude and 59 °00'-36" east longitude; altitude: 985 m) of Mashhad University of Ferdowsi, Mashhad, Iran. Collected leaves were shadow dried and grounded with a laboratory hammer mill (Iran khodsaz gristmill, ELS 300C, Iran). The total values of phenolic compounds were measured by colorimetry, using the Folin-Ciocalteu method (Guo *et al.* 2000).

Chickens, diets and experimental design

A total of 192 one-day-old broiler chickens (Ross, 308) were purchased from a local commercial hatchery and raised over a 42-day experimental period. The chickens were housed in an environmentally controlled poultry house with wood shavings as litter at the research farm of Animal Faculty, Gorgan University of Agricultural Science and Natural Resources, Gorgan, Golestan province, Iran. The temperature was set at 32 °C at 1 day of age and then decreased by 1 °C every 2 days until a permanent temperature of 24 °C was reached at 35 day of experiment. The heat stress was applied once daily (from 0800 to 1600 h=8 h/d) during the last experimental week by increasing room temperature to reach 34 °C. From 1800 to 0800 h, the environmental temperature was reduced to 21 °C. The lighting schedule provided 24 hrs of light per day. A 2-phase feeding program was used, with a starter diet until 21 day and a finisher diet until 42 day of age. The composition of the two basal diets is shown in Table 1. Diets for each period were prepared with the same batch of ingredients and all diets within a period had the same compositions. Diets were formulated to meet or exceed requirements by the NRC (1994) for broilers of this age. The experiment was performed as a completely randomized design in a factorial arrangement (4×2) with 4 replicats of 12 broilers each. Experimental groups were as follows: 1) basal diet; 2) basal diet + 1% peppermint powder; 3) basal diet + 2% peppermint powder and 4) basal diet + 300 mg of vitamin E per kilogram. During this time, broilers were provided with unlimited water and food *ad libitum*.

Antibody titers against SRBC

Duration of the experimental period was 42 days in order to evaluate immune system function in broilers under treatment. At days 28 and 35 of each experimental unit, 2 chickens (1 male and 1 female) were selected and 0.1 mL of 25% SRBC (sheep red blood cell) was injected into the breast muscle of the birds. Then to determine the antibody titer against SRBC, 2 cc of blood was taken from the wing vein of the chickens at 35 and 42 days.

Hemagglutination test

For measurement of antibody against SRBC, the hemagglutination method was used (Arshami *et al.* 2010).

Table 1 Composition and calculated analyses of the basal diets¹

Item (% unless noted)	0 to 21 days	22 to 42 days
Ingredient:		
Corn	56.5	60.56
Soybean meal	37.27	32.33
Soybean oil	2.38	3.69
Dicalcium phosphate	1.44	1.09
Calcium carbonate	1.28	1.38
Vitamin premix ²	0.25	0.25
Mineral premix ³	0.25	0.25
DL-methionine	0.15	0.07
Salt	0.43	0.33
Calculated analysis:		
Metabolizable energy (kcal/kg)	2950	3100
Crude protein	21.2	19.38
Calcium	0.92	0.87
P (available)	0.41	0.34
Sodium	0.18	0.15
Lysine	1.15	0.03
Methionine	0.48	0.37
Cysteine	0.83	0.69
Teroonin	0.81	0.73

¹ Calculated composition according to [NRC \(1994\)](#).

² Vitamin premix (each kg contained): vitamin A: 3600000 IU; vitamin D₃: 800000 IU; vitamin K₃: 1600 mg; vitamin B₁: 720 mg; vitamin B₂: 3300 mg; vitamin B₃: 4000 mg; vitamin B₅: 15000 mg; vitamin B₆: 150 mg; vitamin B₉: 500 mg; vitamin B₁₂: 600 mg and Biotin: 2000 mg.

³ Mineral premix (each kg contained): Mn: 50000 mg; Fe: 25000 mg; Zn: 50000 mg; Cu: 5000 mg; Iodine: 500 mg and Choline chloride: 134000 mg.

Serum was exposed for a 30 minutes to a temperature of 56 °C. Then 50 µL of serum and 50 mL PBS buffer were added to the first well of 96-well ELISA (U-shaped) and incubated at 37 °C for 30 minutes. After half an hour the pellets were removed from the incubator and 50 mL PBS was added to the other wells and thus 1/2 to 1/4096 dilutions were prepared for each sample. After preparing the solution, 50 mL SRBC 2% was added to each well and then incubated for 30 minutes at 37 °C temperature and lysed wells recorded. Titers were reported by log₂, as the highest dilutions showing complete agglutination ([Munnz and Lamont, 1991](#)). To determine IgG, 50 µL of serum was added to the first well of the 96 pellets ELISA (U-shaped) and the pellets were transferred to an incubator at 37 °C for 30 min. After half an hour, 50 µL of 0.01% mercaptoethanol solution was added to the first well of the pellets and again incubated for 30 minutes at 37 °C. After half an hour 50 µL of PBS dilutions was then added to the other wells and then other dilutions of (1/2-1/496) were prepared. Thereafter, 25 µL SRBC 2% solution was added to each well. The pellets were incubated for 30 minutes at a 37 °C temperature and then the number the first lysed well was recorded. Titers were reported by log₂ as the highest dilutions showing complete agglutination. IgM evaluation was obtained, once the difference between total antibody titer and resistance to mercaptoethanol was established ([Cheema et al. 2003](#)).

White blood cell count (WBC), lymphocytes, heterophils, heterophils to lymphocytes ratio

On day 28 and 42, one male chicken from each experimental unit, (other than those that received SRBC) was randomly selected for white blood cells count. Blood was drawn from a vein and fixed via methanol 99.5% on the slide.

The blood smears were then covered with water-diluted Giemsa staining. After 50 minutes, the slides were washed and by 100 × magnification, the type of white blood cell was recorded and counting was performed up to 100 white blood cells ([Gross and Siegel, 1983](#)).

Serum biochemistry

On day 21 and 42, two chickens of each group, close to the average weight were selected. Blood samples were taken from the wing veins and transferred to test tubes free of heparin for further laboratory analysis. Samples were centrifuged at 3000 rotations per min for 7 min (HERMLE-Z323K, Germany) to separate the serum. After separation blood constituents were measured using available commercial kit.

For this purpose, serum total protein and albumin concentrations, which are indicators of liver damage, were measured, together with globulin, triglycerides, total cholesterol and serum HDL-cholesterol. The method of measuring LDL-cholesterol and VLDL-cholesterol is as follows ([Friedewald et al. 1972](#)):

$$\text{VLDL} = \text{triglycerides} / 5$$

$$\text{LDL} = \text{TC} - \text{HDL} - (\text{TG}/5)$$

The relative weight of lymphoid organs

On day 42, for each experimental unit, two chickens (1 male and 1 female) close to the average weight were selected and lymphoid tissues (such as the spleen and bursa fabricius) and livers were separated from the body and weighed by a digital scale with an accuracy of one thousandth of a gram.

Statistical analysis

This study was conducted as a completely randomized design. A GLM procedure was performed ([SAS, 2003](#)) and the difference among the mean values was tested, using the Duncan multiple range test at (P<0.05). Mean values and SEM are reported.

RESULTS AND DISCUSSION

Total phenol, flavonoids and antioxidants

The amounts of total phenol, flavonoids and antioxidants of alcoholic extract peppermint leaf are shown in Table 2.

Table 2 Total phenol, flavonoids and antioxidant of alcoholic extract peppermint

Compounds	Dry weight
Total phenol	2.719 (mg/g)
Flavonoids	1.835 (mg/g)
Antioxidants	72.531 (%)

Growth performance

The effects of peppermint powder on growth performance of broilers are shown in Table 3. There were significant ($P<0.05$) differences among the treatments for feed conversion ratio (FCR) on day 21 and body weight (BW) on day 42 of the experiment. Vitamin E supplemented group showed significantly ($P<0.05$) higher BW than the other treatments at 42 day of age. A significantly lower ($P<0.05$) FCR was observed in control group at 21 day of age.

Antibody titers against SRBC

The effect of dietary treatments on antibody titer against SRBC is shown in Table 4. Peppermint powder increased TIg, IgM and IgG titer against SRBC ($P<0.05$). The birds fed a diet supplemented with 1% peppermint powder had higher total antibody titer TIg, IgM and IgG compared to other treatments on day 42 of the experiment. Significant interactions were observed between diet and sex on IgG at day 35 ($P<0.05$). Significant interactions between diet and sex on TIg and IgM were not observed at 35 and 42 days ($P>0.05$).

Total white blood cells, lymphocytes, heterophils, heterophils to lymphocytes ratio

The effects of dietary treatments on total white blood, lymphocytes, heterophil and H:L ratio are shown in Table 5. Results showed significant differences in total white blood, lymphocyte, heterophil and H:L ratio among treatments at day 42 of experiment. The chickens fed a diet supplemented with 2% peppermint powder had higher total white blood. Vitamin E supplemented chicken showed a higher and lower H:L ratio and lymphocyte, respectively.

Blood biochemical parameters

Data on the levels of blood serum (total protein, albumin and globulin) are presented in Table 6 and data on the levels of blood serum (triglyceride, total cholesterol, HDL_C, LDL_C and VLDL_C) are presented in Table 7. Significant differences were observed on total protein, albumin, globulin, triglyceride, total cholesterol, HCL_C, LDL_C and VLDL_C ($P<0.05$). Results showed that peppermint powder increased blood serum concentrations of HDL_C at 21 day and 42 day of age. The levels of total protein in the serum of 1% peppermint powder and basal diet-treated broilers were significantly higher and lower, respectively on day 21 of experiment.

The results in this study show that the use of 2% peppermint powder and basal diet supplemented with 300 mg/kg vitamin E significantly increased and decreased blood serum concentrations of globulin, respectively, at 42 day of age.

The relative weight of lymphoid organs

Data on the relative weight of lymphoid organs are presented in Table 8. Liver weight was significantly different among treatments ($P<0.05$). Thus, the relative weight of liver in chicks receiving 1% peppermint powder was higher than other treatments. Significant interactions between diet and sex on bursa of fabricius, spleen and liver weight were not observed ($P>0.05$). Our results show that peppermint powder ameliorate the stress effects on BWG and FCR. Consistent with our findings, Helander *et al.* (1998), other than highlighting the antimicrobial activity, argued that compounds present in peppermint, improve growth, appetite and BW by restricting the growth of harmful microorganisms. Data reported by (Galib and Al-Kassi, 2010) showed an improvement in BWG and FCR under dietary treatment with peppermint powder. Results of this study are in agreement with some previous research indicating that herbs, plant extracts, essential oil and / or the main components of essential oil, did not affect FI or feed efficiency in broilers (Cross *et al.* 2002; Cross *et al.* 2007; Hernandez *et al.* 2004; Bampidis *et al.* 2005).

Findings in this study on antibody titers against SRBC, are in agreement with other studies. Peppermint has a very high antioxidant property and peppermint (*Mentha piperita*) is one of those medicinal plants belonging to the *Lamiaceae* family. Its essential oils are made up mainly of menthone, menthol, methyl acetate and flavonoid (Murray, 1995).

This plant is usually used as an antiseptic, antispasmodic, carminative, mild tonic, antimicrobial and for the treatment for irritable bowel syndrome, inflammatory bowel disease, disorders of the biliary system and liver problems (Foster, 1996; Taylor, 1984; Boukra *et al.* 2005). Taylor (1984) reported most of the effects of peppermint are related to its action on bile flow and liver function. Peppermint has also been demonstrated for compounds like eugenol, caffeic acid, rosmarinic acid, flavonoids and α -tocopherol shaping its antioxidant and anti-peroxidant trait (Rastogi and Mehrotra, 1991).

The anti-fungal (Aqil *et al.* 2001), antiviral (Hirobe *et al.* 1994), anti-bacterial (Yang *et al.* 2010), anticancer (Lirio *et al.* 1998), anti-mutagenic (Vokovic-Gacic and Simic, 1993) and other pharmaceuticals (Samman *et al.* 1998) properties of this plant, have been proven. Peppermint increases phagocytic activity of macrophages and the level of nitric oxide in the blood serum.

Table 3 The effects of peppermint on growth performance of broiler chickens

Treatment	0 to 21 day			0 to 42 day		
	BW	FI	FCR	BW	FI	FCR
Control	643.36 ^a	956.65 ^a	1.48 ^b	2005.02 ^{ab}	4209.73 ^a	2.09 ^a
1% peppermint	619.48 ^a	951.57 ^a	1.53 ^{ab}	1992.17 ^b	4210.64 ^a	2.10 ^a
2% peppermint	650.32 ^a	975.12 ^a	1.49 ^{ab}	2100.56 ^{ab}	4291.60 ^a	2.03 ^a
300 mg vitamin E	626.15 ^a	968.76 ^a	1.54 ^a	2121.36 ^a	4361.59 ^a	2.05 ^a
Pooled SEM	15.24	17.72	0.02	38.60	51.78	0.03
P-value	0.469	0.774	0.097	0.079	0.169	0.427

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

BW: body weight gain (g); FI: feed intake (g) and FCR: feed conversion ratio (g of feed/g of BW gain).

SEM: standard error of the means.

Table 4 The effect of dietary treatments on antibody titer against SRBC of broiler chickens at 35 and 42 days

Treatment	35 day			42 day		
	Tlg	IgM	IgG	Tlg	IgM	IgG
Control	3.00 ^b	1.13 ^{bc}	1.87 ^b	2 ^b	0.43 ^b	1.57 ^b
1% peppermint	5.87 ^a	2.63 ^a	3.24 ^a	4.25 ^a	1.75 ^a	2.50 ^a
2% peppermint	4.50 ^{ab}	0.38 ^c	4.12 ^a	2.20 ^b	0.33 ^b	1.87 ^{ab}
300 mg vitamin E	5.25 ^a	1.75 ^{ab}	3.50 ^a	2.37 ^b	0.62 ^b	1.75 ^{ab}
SEM	0.53	0.40	0.53	0.52	0.28	0.52
Gender						
Male	4.56	1.93	2.63	2.86	0.73	2.13
Female	4.57	1.00	3.57	2.50	0.75	1.75
SEM	0.60	0.28	0.60	0.37	0.17	0.37
Interactions						
Control × male	3.25	1.27	2.00 ^b	2.30	0.36	2.00
Control × female	2.75	1.00	1.75 ^c	1.70	0.50	1.14
1% peppermint × male	6.50	2.75	3.74 ^{ab}	4.00	1.25	2.75
1% peppermint % female	5.25	2.52	2.75 ^b	4.50	2.25	2.25
2% peppermint × male	3.50	0.52	3.00 ^{ab}	2.20	0.25	1.75
2% peppermint % female	5.50	0.25	5.25 ^a	2.20	0.41	2.00
Vitamin E × male	5.00	3.25	1.75 ^c	3.00	1.00	1.70
Vitamin E × female	5.50	0.25	5.25 ^a	1.75	0.25	1.80
SEM	0.80	0.57	0.74	0.75	0.38	0.74
Significance						
Diet	0.009	0.005	0.04	0.02	0.002	0.67
Gender	0.74	0.03	0.04	0.55	0.88	0.49
Diet × gender	0.20	0.05	0.02	0.69	0.19	0.92

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

SEM: standard error of the means.

Studies have shown that flavonoids have anti-allergic activity, anti-viral and anti-inflammatory properties and may also cause dilation of blood vessels. These pharmacologic effects are related to the flavonoid antioxidant properties. Flavonoids can also restrain enzymes that are responsible for producing superoxide (such as xanthine oxidase). Many flavonoids also play an important role in oxygen metabolism by chelating rare metals and preventing the onset of lipoxygenase reaction (McAnlis, 1997).

Results of this study indicate that white blood cells under heat stress condition are increased by supplementation of 2 percent peppermint in dose related fashion. Data of this study are in agreement with those of Amin Dousti *et al.* (2012), on peppermint supplementation, with similar compounds like thyme and cinnamon Al-Kassie *et al.* (2009). The increase in white blood cells may be attributed to epinephrine, because intensified catecholamines and cortisol increase the number of leukocytes.

Table 5 The effects of peppermint on total white blood cells (WBC), lymphocyte (L), heterophil (H) and H/L of broiler chickens at 28 and 42 days

Treatment	28 day				42 day			
	WBC ($\mu\text{L}/10^3$)	L (%)	H (%)	H/L (%)	WBC ($\mu\text{L}/10^3$)	L (%)	H (%)	H/L (%)
Control	22300 ^a	80.50 ^a	19.50 ^b	0.24 ^b	20230.0 ^b	73.33 ^a	26.67 ^b	0.36 ^b
1% peppermint	22570 ^a	80.60 ^a	19.40 ^b	0.24 ^b	20213.8 ^b	72.50 ^a	27.50 ^b	0.37 ^b
2% peppermint	23040 ^a	75.90 ^b	24.10 ^{ab}	0.31 ^{ab}	20700.0 ^a	66.50 ^c	33.50 ^{ab}	0.50 ^{ab}
300 mg vitamin E	22417 ^a	70.53 ^c	29.47 ^a	0.41 ^a	20187.5 ^b	62.80 ^b	37.20 ^a	0.59 ^a
Pooled SEM	36.80	0.78	2.10	0.03	80.04	0.60	3.07	0.03
P-value	0.52	0.0001	0.04	0.01	0.0001	0.0001	0.07	0.01

The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).
SEM: standard error of the means.

Table 6 The effects of peppermint on blood serum of total protein, albumin and globulin of broiler chickens at 21 and 42 days

Treatment	21 day			42 day		
	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Total protein (g/dL)	Albumin (mg/dL)	Globulin (mg/dL)
Control	3.60 ^c	1.53 ^b	2.06 ^b	3.38 ^c	1.82 ^b	2.06 ^b
1% peppermint	3.87 ^a	1.94 ^a	1.92 ^c	3.91 ^a	1.96 ^a	1.97 ^{bc}
2% peppermint	3.82 ^a	1.34 ^c	2.47 ^a	3.88 ^a	1.52 ^c	2.47 ^a
300 mg vitamin E	3.70 ^b	1.90 ^a	1.79 ^d	3.75 ^b	1.95 ^a	1.85 ^c
Pooled SEM	0.09	0.01	0.02	0.04	0.01	0.04
P-value	0.0001	0.0001	0.0001	0.001	0.0001	0.0001

The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).
SEM: standard error of the means.

Table 7 The effects of peppermint on blood serum triglyceride, cholesterol, HDL_C, LDL_C and VLDL_C of broiler chickens at 21 and 42 days

Treatment	21 day					42 day				
	TG (mg/dL)	TC (mg/dL)	HDL _C (mg/dL)	LDL _C (mg/dL)	VLDL _C (mg/dL)	TG (mg/dL)	TC (mg/dL)	HDL _C (mg/dL)	LDL _C (mg/dL)	VLDL _C (mg/dL)
Control	58 ^a	109.95 ^a	40.90 ^d	57.45 ^a	11.60 ^a	98.30 ^a	184.75 ^a	62.62 ^d	102.46 ^a	19.66 ^a
1% peppermint	55 ^b	94.37 ^d	51.45 ^a	31.90 ^d	11.00 ^{ab}	75.25 ^c	161.90 ^c	86.00 ^a	60.85 ^b	15.05 ^c
2% peppermint	54 ^b	98.90 ^b	47.62 ^c	40.55 ^b	10.72 ^{bc}	95.35 ^b	165.47 ^b	80.25 ^b	65.12 ^b	19.07 ^b
300 mg vitamin E	51 ^c	96.67 ^c	49.62 ^b	36.85 ^c	10.20 ^c	97.42 ^a	160.75 ^c	66.42 ^c	69.76 ^b	19.48 ^a
Pooled SEM	2.88	0.42	4.60	11.08	0.57	10.95	11.20	11.10	19.88	2.19
P-value	0.002	0.0001	0.0001	0.0001	0.002	0.0001	0.01	0.0001	0.002	0.0001

The means within the same column with at least one common letter, do not have significant difference ($P>0.05$).
SEM: standard error of the means.

TG: triglyceride; TC: total cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein and VLDL: very low density lipoprotein.

Also, herb protects the lymphocytes against the damages due to free radicals produced during heat stress. Improved activity of lymphocytes, macrophages and natural killer cells as an effect of herb use, stimulate the production of interferon and enhances phagocytosis (Lavinia *et al.* 2009). Peppermint strengthens the immune system, which can be attributed to the presence of various active compounds (Fallah *et al.* 2003). Many researchers have reported that the illness and stress increases the number of heterophiles (Zulkifli *et al.* 2000; Borges *et al.* 2004). Thaxton *et al.* (1968) reported that high environment temperature affects the development of the immune system in broilers. Consequential effects include a decrease in the number of lym-

phocytes, white blood cells and an increase in the heterophils to lymphocytes ratio, that is a proper sign of high temperature.

During the rise in temperature, the number of heterophils released from the bone marrow increases, while the level of lymphocyte goes down, hence the heterophil to lymphocyte ratio rises as a result.

Close relationship exists between the number of white blood cells and stress in birds. Stressors stimulate ACTH and the adrenal glands secretion, that increase the relative frequency of the number of lymphocytes in birds. Stresses such as heat stress, increase heterophil to lymphocyte ratio (Aydin *et al.* 2008).

The results of this study suggest the important role of peppermint in controlling the liver function. Such findings are consistent with the results of, [Fallah *et al.* \(2013\)](#) who reported that peppermint had increased albumin, total protein, HDL-cholesterol and significantly reduced total cholesterol, triglycerides, LDL-cholesterol in broilers. It seems that some components of peppermint, including menthol and menthone, have a potential to decrease blood lipids in broilers ([European Scientific Cooperative on Phytotherapy, 2003](#)), also in this case, increased albumin:globulin ratio is believed to be linked to the improved liver function because of the administration of peppermint extract.

[Barbalho *et al.* \(2009\)](#) investigated the effect of peppermint extract on plasma lipids in mice, showing that the use of peppermint extract improves plasma lipids profile in mice. They stated that peppermint extract significantly decreases triglycerides, total cholesterol, LDL-cholesterol, VLDL-cholesterol and increases HDL-cholesterol in blood serum. The menthol and thymol, which are components of essential oils of peppermint leaves, at the level of 200 mg per kg in broilers fed with diets containing cholesterol or no cholesterol, reduced serum total cholesterol and triglyceride concentrations. The presence of compounds such as thymol and menthol in peppermint extract has a lowering role for serum triglycerides and total cholesterol ([Clegg *et al.* 1980](#)). [Al-Harathi *et al.* \(2004\)](#) reported that the addition of some herbs such as peppermint extract to broiler diets reduces the concentration of serum total cholesterol and triglyceride. This would increase the total protein in the blood of broiler chickens. [Abdolkarimi and Mirzaaghazade \(2010\)](#) found that peppermint extract reduces levels of cholesterol, triglycerides and serum LDL-cholesterol in broilers. Stress increases synthesis of adrenocortical hormones that will be followed by blood glucose levels and body fat.

Activity of some of the compounds in the volatile oil of peppermint (menthol and thymol) decreases the enzymatic activity of hydroxymethyl glutaryl coenzyme A (HMG-CoA) and hepatic reductase that regulates synthesis of cholesterol. It seems that one of the reasons for the decrease in total cholesterol in the presence of phenolic compounds such as peppermint extract is the presence of volatile phenolic compounds such as essential oils: menthol, menthone, menthyl acetate, menthofuran, limonene, polygen, cineole and azolen.

On the other hand, the active ingredients in peppermint by increasing the activity of liver cells, give rise to the concentration of bile acids. The high concentration of bile acids in the small intestine, facilitates digestion of fats and fat-soluble vitamins, because bile acids are essential for fat emulsion ([Crossland, 1980](#)). [Mimica Dukic *et al.* \(2003\)](#) during a trial showed that peppermint, due to its antioxidant and antibacterial properties, may increase the flow of bile in

the gallbladder. As a result serum total cholesterol can be reduced. The use of peppermint is useful in the treatment of patients with cholesterol stones in the gallbladder and the bile duct. The active ingredients in peppermint extract can prevent or reduce the absorption of cholesterol by the intestines, resulting in reduced serum cholesterol and serum fat ([Crossland, 1980](#)). The results of this research show the important role of peppermint in liver weight. This result is consistent with the results of [Khorramshahi and Samadi \(2015\)](#) who reported that the incorporation of artichoke in Japanese quail's diets had no significant effect on spleen and bursa of fabricius. [Khaligh *et al.* \(2011\)](#), reported that polysaccharides extracted from a number of plants stimulate the growth of organs such as the spleen, thymus and bursa of fabricius and also increase the number of immune cells such as T- cells and lymphocytes and macrophages resulting in humoral and cellular immune responses. [Khaligh *et al.* \(2011\)](#) observed no significant differences in liver weight of chicks fed with herb mixtures. In contrast, [Guo *et al.* \(2000\)](#) reported that the use of medicinal plants has led to the increased weight of the lymphoid organs such as thymus, spleen and bursa of fabricius in broiler chickens. [El-Iraqi *et al.* \(2013\)](#) reported that peppermint can reduce the relative percentage of liver weight and increase that of spleen and bursa of fabricius's in broilers under thermal stress. [Abdolkarimi and Abdollahzade \(2011\)](#) also reported that the peppermint extract improves immune system and increase the relative weight of the bursa of fabricius in broilers. [Galib and Casey \(2010\)](#) reported that the relative percentage of liver weight in the group fed with peppermint extract was smaller than control's. Assessing the effect of peppermint extract on broilers, [Malekian *et al.* \(2011\)](#) reported that it has no effect on weight gain and lymph organs. [Khaligh *et al.* \(2011\)](#) observed no significant differences in the liver weight of chicks fed with a mixture of several herbs, which contradicts the results of this study. [Kusandi and Djulardi \(2011\)](#) demonstrated that high temperature reduces the weight of the bursa of fabricius, liver and spleen. This growth inhibition leads to increased secretion of corticosterone. As other reasons suggest, heat stress reduces feed intake, body weight and the weight of the lymphoid organs. The lymphatic organs weight loss can be due to reduced feed intake in broilers under heat stress ([Niu *et al.* 2009](#)).

CONCLUSION

Based on the results of this experiment, it can be said that peppermint, due to antioxidant properties, besides adjusting oxidative stress induced by thermal stress, can improve immune system in broilers under heat stress and has a protective effect against oxidative stress, such as heat stress.

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