INTRODUCTION

Heat stress (HS) is one of major problems in the poultry industry, especially in hot regions, because of its adverse effects on economic components (Niu et al. 2009b). Studies have shown that HS reduces growth performance and antibody production in young chickens (Niu et al. 2009b; Niu et al. 2009a). High environmental temperatures enhance the plasma concentration of corticosterone and heterophil to lymphocyte ratio (Yalçın et al. 2003). Heat stress also decreases relative weight of lymphoid organs (Niu et al. 2009b) and has negative effects on some blood metabolites in broiler chicks (Habibian et al. 2014). Heat stress could disturb the balance between the production of reactive oxygen species (ROS) and the antioxidant systems, resulting in increased production of ROS (Feng et al. 2008). The produced ROS can cause oxidative changes such as lipid peroxidation and oxidative damages to proteins and DNA.
Thymus vulgaris is a medicinal herb belonging to Lamiaceae family which mainly cultivated worldwide as culinary uses, cosmetic perennial and medical herb. Thyme contains high amount of polyphenols which are responsible for antioxidant activity in its essential oils (Dahal and Farran, 2011).

Numerous studies have shown that flavonoids and polyphenolic substances have several pharmacological effects, such as antioxidant activity, preventing histamine release and arachidonic acid metabolism (Amresh et al. 2007). Studies have also shown that the use of antioxidant plants in diet can prevent the oxidative changes created by free radicals and other reactive species (Soler-Rivas et al. 2000). Antioxidants may also prevent oxidation of low-density lipoproteins (Aoudi et al. 2014). Dietary inclusion of thyme essential oil (TEO) and peppermint essential oil, combined form, reduced the serum concentration of cholesterol in laying hens submitted to cold stress condition (Akbari et al. 2015).

Thyme products are known to have hypocholesterolemic and antihyperlipidemic activities in broiler chickens (Abdulkarimi et al. 2011; Dahal and Farran, 2011). On the other hand, plant derivatives are utilized in animal feeding as growth promoter, because of their antioxidant, antimicrobial and digestion properties (Abdulkarimi et al. 2011; Assiri et al. 2016). Attia et al. (2017) have reported that dietary inclusion of TEO improved growth performance in broiler chicks.

Raggar et al. (2016) have also shown that dietary inclusion of TEO increased weight gain and immune system in broiler chicks. It was hypothesized that TEO may alleviate adverse effects of HS on growth performance, immune responses and blood biochemical variables. Thus, this study was conducted to evaluate the effects of dietary inclusion of TEO on growth performance, blood biochemical variables and immune responses of heat-stressed broilers.

One-d-old broiler chicks were randomly allocated in five treatments with six cages (6 replicates) each with 10 birds. They were allocated in two chambers; chamber A (4 treatments) and chamber B (1 treatment). The recommended brooding temperatures were applied until 21 days of experiment, i.e., the temperature was gradually decreased from 33 to 23.9 °C during 1 to 21 d of age in the both chambers. From 22 to 42 d of age, the birds in chamber A were exposed to HS (23.9-38 °C daily) and those in chamber B were reared under thermoneutral condition (TN; 23.9 °C).

The heat-stressed birds (chamber A) were fed with basal diet (control-HS), 100 mg TEO/kg of diet (100 TEO-HS), 150 mg TEO/kg of diet (150 TEO-HS) and 200 mg TEO/kg of diet (200 TEO-HS), from 1 to 42 days. The birds in chamber B were fed with basal diet (control-TN).

Feed, in mash form, and water were provided ad libitum during the experiment. Two iso-caloric and iso-nitrogenous experimental diets were formulated to meet the nutrient requirements for starter and grower periods as recommended by NRC (1994) (Table 1).

The calculated amounts of TEO were firstly mixed with small amounts of the basal diet, as a small batch. The small batch was subsequently mixed with larger amount of the basal diet to obtain a homogenized mixture. The lighting schedule was 23 h of light/1 h dark cycle with an average light intensity of 15 lx which maintained until the end of the experiment.

Temperature and treatments
After 3 weeks of age, the birds in stress group were daily exposed to temperatures (regulatory) as follows: 12 h of 23.9 °C, 3 h of 23.9 to 38 °C, 5 h of 38 °C, and 4 h of 38 to 23.9 °C [2 and 29] and those in chambers (control group) were reared in constant temperature (23.9 °C). The relative humidity was maintained in 50-55%.

Component analysis of thyme essential oil
TEO was prepared from Gareban Company (Kermanshah-Iran). Components of essential oil were analysed by using gas chromatography as described by Juliano et al. (2000) and the data are presented in Table 2.

Growth performance
The broiler chicks were weighed at 1, 21 and 42 days of age, and the body weight gain (BWG, g/bird) per replicate was calculated.

Feed intake (FI) was recorded for each replicate (g/bird) as well as feed conversion ratio (FCR) was calculated. Mortality rate was considered to calculate the growth performance parameters during experiment.
Determination of blood biochemical variables

On d 42 and after 12-hours fasting, blood samples were collected in non heparinised tubes from two birds per each cage (3 mL per bird) via brachial vein and centrifuged at 2500 × g for 15 min (SIGMA 4-15 Lab Centrifuge, Germany) and serum samples were obtained. The serum samples were individually analysed for cholesterol, triglyceride using Pars Azmoon commercial kit package (Pars Azmun, Tehran, Iran).

Corticosterone concentration was evaluated as explained by manufacture protocol kit. Malondialdehyde (MDA) was evaluated, as lipid peroxidation index, as described by Richards et al. (1992). The MDA is one of the thiobarbituric acid reactive substances (TBARS). Mixture of these substances is in biological specimens and it is an index for oxidative stress.

Immune system variables

On day 26, three ml blood was taken of two birds per replicate to pre-challenge antibody titre analysis which followed to determine the presence of antibodies prior to challenge with sheep red blood cells (SRBC). At d 28, two birds per replicate were intravenously treated with 1ml of 7% SRBC suspension administrated to right wing (Habibian et al. 2015). On d 35, the same birds were bled through brachial venipuncture, and 3 mL blood was taken for primary antibody response. Blood samples were centrifuged at 2500 × g at 4 °C for 15 minutes and the obtained sera were stored in -20 °C for further analysis. On day 35, 1 mL from 7% SRBC suspension were intravenously administrated to same birds and blood samples (3 mL per bird) were collected for secondary responses on day 42. Blood samples were centrifuged and stored as explained for primary responses. Samples were investigated for IgM and IgG by the 2-mercaptoethanol (ME) method as previously explained by Lepage et al. (1996).

Summary, sera samples were inactivated at 56 °C by water bath and 50 μL phosphate buffer saline (PBS) was then added in the first row from wells in a 96-well V-bottom microtitration plate. Subsequently, 50 μL of serum was added to same wells and those were sealed and incubated in 37 °C to 30 min. The plates were subsequently ejected from incubator and other wells, 11 remaining same row, were treated with 50 μL PBS.
Other wells were serially diluted with a 2-fold dilution and 50 μL 2.5% SRBC suspension was administrated to each well, and plates were again sealed and incubated for 0.5 h. Titres were expressed by holding plates on a lighted mirror to see wells for agglutination. Antibody titres were reported as log 2 of the reciprocal of the last dilution in which agglutination was seen.

On d 35, blood samples (2 drops per bird) were taken from 2 birds per replicate and smeared on glass slides (one drop on per slide). The prepared smears were then stained by May–Grünwald-Giesma stains (Lucas and Jamroz, 1961), about 3 h after methyl alcohol fixation. Heterophil (H) count, lymphocyte (L) count and heterophil/lymphocyte ratio (H/L) was estimated. The data were presented as mean of two slides.

On d 42, 2 birds per replicate were weighed, killed and lymphoid organs including bursa, spleen and thymus were weighted. Organ weights were expressed as a percentage of body weight.

Statistical analysis
The data were analysed using the ANOVA procedure from SAS (2001) to assign the significant differences among groups. Means were subsequently compared using Duncan’s least significance multiple-range test. All data were expressed as means ± standard deviation (±SD). The log2 transformations were done on antibody titres before statistical analysis.

RESULTS AND DISCUSSION

Growth performance
Effects of dietary inclusion of TEO on growth performance are shown in Table 3. Results showed that HS significantly increased FCR and mortality and decreased BWG and FI (control-HS vs. control-TN) (P<0.05). However, dietary inclusion of TEO (150 and 200 mg/kg) alleviated negative effects of HS on growth performance (FI, BWG and FCR) (P<0.05). Lower levels of TEO (100 mg/kg) could not alleviate adverse effects of HS on growth performance (P>0.05).

Blood biochemical variables
Effects of HS and dietary inclusion of TEO on the blood biochemical variables of broiler chickens are presented in Table 4. HS increased the serum contents of triglycerides, cholesterol, corticosterone and malondialdehyde (P<0.05) when compared with TN condition (control-HS vs. control-TN). The broiler chicks treated with TEO (150 and 200 mg/kg) showed lower the serum concentrations of cholesterol, triglycerides, corticosterone and MDA when compared with other birds (P<0.05; Table 4).

It was not observed significant difference between 200 TEO-HS and control-TN for the serum concentrations of cholesterol and triglycerides (P>0.05).

Immune system variables
The data for effects of HS and dietary inclusion of TEO on immune system are displayed in Tables 5 to 7. Sera samples for pre-challenge antibody titre were negative. Comparing control-HS and control-TN shows that heat stress suppressed immune responses (P<0.001); but the both primary and secondary immune response against SRBC were affected by TEO treatments (P<0.01). The highest antibody titre against SRBC was observed in birds treated with 200 mg/kg of TEO (P<0.01). However, broiler chicks at 100 TEO-HS group was similar to antibody titre compared with control-HS; broiler chicks at 150 TEO-HS group had higher antibody titre compared with control-HS (P<0.01; Table 5). Heterophil count and H/L ratio were increased, while lymphocyte count and relative weight of lymphoid organs were decreased in control-HS and 100 TEO-HS group in comparison to control-TN group (P<0.0001; Tables 6-7). Dietary inclusion of TEO (150 and 200 mg/kg) reduced heterophil count and H/L ratio and increased lymphocyte count and relative weight of lymphoid organs in comparison to control-HS (P<0.001).

Our results showed that HS supressed growth performance but dietary inclusion of high levels of TEO improved growth performance.olfati et al. (2018) have reported that HS supressed growth performance in broiler chicks. Thermal stress influences the productive performance of poultry by affecting nutrient metabolism and digestibility (Zhang et al. 2012) while increasing corticosterone blood levels (Sahin et al. 2002). Corticosterone reduces nutrient utilization and digestibility and finally decreases performance. In addition, HS-exposed birds showed a reduced villus-height to crypt-depth ratio (Deng et al. 2012). Thus, HS by increasing corticosterone decreases growth performance which was confirmed by our findings. Results showed that corticosterone concentration was significantly higher in control-HS in comparison to control-TN. With regards to dietary inclusion of TEO, Pournazari et al. (2017) have reported that dietary inclusion of TEO and probiotic, separately, increased FI. Similar to our findings, Attia et al. (2017) have reported that dietary inclusion of TEO improved growth performance in broiler chicks during summer season.

Ragga et al. (2016) also showed that dietary inclusion of TEO increased BWG in broiler chicks. Improved growth performance in high levels of TEO can be due to antioxidant properties and phenolic components of EOs that reduces effects of pathogens on intestinal system and help to absorb the amino acids (Lee et al. 2003).
TEO not only helps to absorb amino acids but also increases secretion of the digestive enzymes which improves growth (Lee et al. 2003). Growth performance can also be suppressed because of increased corticosterone. Our findings also indicated high levels of TEO decreased the levels of corticosterone. Thus, TEO can improve growth performance by decreasing corticosterone levels under HS condition.

Results also showed that HS increased cholesterol, triglycerides, corticosterone and malondialdehyde concentrations. Previous studies have reported that HS increases the serum concentrations of MDA (Tawfeek et al. 2014), cholesterol and total lipids (Nawalany et al. 2010) in broiler chicks. Stress enhances free radicals production and formation of ROS, thus it increases lipid peroxidation and subsequently MDA levels in blood and tissues (Ates et al. 2006).

HS reduces food consumption in birds and they compensate their need to energy through lipolysis (Rashidi et al. 2010).

It can be concluded that HS increases lipolysis through increased corticosterone secretion, production of free radicals and formation of ROS and decreased feed intake. Dietary inclusion of TEO at high levels, 150 and 200 mg/kg, reduced the serum concentration of corticosterone, triglycerides, and cholesterol. Results are in agreement with those reported by Abdulkarimi et al. (2011). The TEO could reduce cholesterol content through its effect on hepatic 3-hydroxy-3-methyl glutaryl CoA reductase, the limiting enzyme in cholesterol biosynthesis (Lee et al. 2003). Rahim et al. (2011) reported that the TEO, as an antioxidant, increases the synthesis of nitric oxide, a vasodilator, which may prevent excess of cholesterol in the blood vessels.
On the other hand, the decreased cholesterol synthesis by TEO can be responsible for decreased corticosterone synthesis because cholesterol is precursor for corticosteroid hormones. MDA, end product of lipid peroxidation, is index for level of ROS-induced biological damage (Popova and Popov, 2002) which is isolated from urine, blood, and tissues. In the present study, the decreased triglycerides concentration is paralleled with decreased MDA content. This phenomenon can be explained by antioxidant theory. In this study, the decreased MDA concentration confirms antioxidant activity of TEO. Antioxidant theory states that the decreased antioxidant vitamins increase lipid peroxidation. On the other hand, HS excretes antioxidant minerals and vitamins. The TEO, as an antioxidant, may compensate antioxidant deficiencies and prevent the lipid oxidation under HS condition. This idea was confirmed by other researchers who indicated that essential oils prevented oxidative changes produced by ROS production (Soler-Rivas et al. 2000) and inhibited oxidation of low-density lipoproteins (Aoudi et al. 2014). It is possible that the TEO, at high levels, reduces lipid peroxidation by decreasing corticosterone concentration because corticosterone increases lipid peroxidation.

Comparing control-HS and control-TN showed that HS reduces antibody titres against SRBC and increases mortality. HS suppressed anti-SRBC antibody responses in broiler chicks (Bartlett and Smith, 2003; Niu et al. 2009b). Studies have shown that HS suppresses immune system through increasing inflammatory cytokines (Ogle et al. 1997), and increases corticosterone concentrations (Trout and Mashaly, 1994). Some researchers have shown that HS activates the hypothalamic-pituitary-adrenal axis and increases the plasma concentration of corticosterone which enhances ROS production, thus it suppresses the immune system (Manach et al. 1996). The TEO alleviated negative effects of HS on antibody titre and mortality. Similarly, Ragga et al. (2016) have reported that dietary inclusion of TEO improved immune system in broiler chicks. Takazadeh and Mayahi (2017) have reported that water inclusion of thyme extract improved immune response in broiler chickens. In contrast to these findings, other researchers reported that TEO, alone and in combination with other feed additives, had no significant effect on antibody titre in poultry (Ozek et al. 2011; Hosseini et al. 2013). Hashemipour et al. (2013) reported that diet supplementing with thymol + carvacrol increased the cellular and humoral immune responses in broilers. Flavonoids and other phenolic components, present in essential oils, increased activity of vitamin C as immunostimulator (Manach et al. 1996). Amresh et al. (2007) showed flavonoids and polyphenolic compounds help immune system through their antioxidant activity. As mentioned, HS increases ROS which subsequently causes injuries in cells (Flanagan et al. 1998), or induces cytotoxicity (Mujahid et al. 2005). ROS also damages immunity organs in broilers (Pamok et al. 2009) which finally suppress immunity system. It seems that the TEO, as an antioxidant, alleviates negative effects of HS on immune system through blocking or preventing ROS production.

### Table 6
Effects of dietary inclusion of thyme essential oil (TEO) on the relative weight of lymphoid organs of 42-day-old broilers

<table>
<thead>
<tr>
<th>Groups</th>
<th>Heterophil (%)</th>
<th>Lymphocyte (%)</th>
<th>Heterophil/lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.19±0.005</td>
<td>0.13±0.001</td>
<td>0.29±0.013</td>
</tr>
<tr>
<td>Control-HS</td>
<td>0.11±0.009</td>
<td>0.08±0.014</td>
<td>0.22±0.010</td>
</tr>
<tr>
<td>100 TEO-HS</td>
<td>0.12±0.014</td>
<td>0.08±0.005</td>
<td>0.22±0.010</td>
</tr>
<tr>
<td>150 TEO-HS</td>
<td>0.16±0.007</td>
<td>0.11±0.002</td>
<td>0.27±0.009</td>
</tr>
<tr>
<td>200 TEO-HS</td>
<td>0.17±0.010</td>
<td>0.12±0.001</td>
<td>0.27±0.009</td>
</tr>
</tbody>
</table>

The data were presented as means ± standard deviation (Mean±SD). SEM: standard error of the means.

### Table 7
Effects of dietary inclusion of thyme essential oil (TEO) on count of heterophil, lymphocyte or their ratio (in 100 cells) of 35-day-old heat-stressed broilers

<table>
<thead>
<tr>
<th>Groups</th>
<th>Heterophil (%)</th>
<th>Lymphocyte (%)</th>
<th>Heterophil/lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.33±1.21</td>
<td>75.50±1.18</td>
<td>0.24±0.02</td>
</tr>
<tr>
<td>Control-HS</td>
<td>37.16±1.50</td>
<td>55.16±1.89</td>
<td>0.67±0.04</td>
</tr>
<tr>
<td>100 TEO-HS</td>
<td>36.00±1.35</td>
<td>55.50±2.19</td>
<td>0.64±0.03</td>
</tr>
<tr>
<td>150 TEO-HS</td>
<td>31.66±2.00</td>
<td>61.81±1.54</td>
<td>0.51±0.03</td>
</tr>
<tr>
<td>200 TEO-HS</td>
<td>26.16±1.66</td>
<td>67.83±1.94</td>
<td>0.38±0.02</td>
</tr>
</tbody>
</table>

The data were presented as means ± standard deviation (Mean±SD). SEM: standard error of the means.
The idea is confirmed by other researchers who showed that essential oils, or antioxidants, prevent ROS production through interaction with peroxide radicals (Yamshliyeva \textit{et al.} 1999) and prevents oxidative injuries to immune cells (Nicksels, 1996). In addition, HS provides opportunities for infectious factors which dominate on immune system (Deying \textit{et al.} 2005), but essential oils balance gut microbiobial ecosystem (Williams and Losa, 2001). Thus, TEO, at high levels (150 and 200 mg/kg), may help immune responses by intestine microbial balance.

HS increased heterophil count and H/L ratio and it reduced L count and relative weight of lymphoid organs (control HS vs. control TN). These findings were confirmed by other researchers who showed HS disturbs leucocytes count and H/L ratio (Yalçın \textit{et al.} 2003) and reduces relative weights of lymphoid organs of birds (Niu \textit{et al.} 2009b).

Glucocorticoid hormones reduce lymphocytes count, since circulating lymphocytes in response to glucocorticoids join to the endothelial cells and subsequently emigrates from circulation into other tissues (Dhabhar, 2002). Similarly, corticosterone decreases food consumption and it may reduce relative weight of lymphoid organs (Niu \textit{et al.} 2009b) because of organs need to more feed for the proper development.

TEO at high levels, 150 and 200 mg/kg, improved leucocytes count and increased relative weight of lymphoid organs. Diet supplementing with TEO and other essential oils improved leucocytes count, but they had not significant effect on relative weight of lymphoid organs (Parvar \textit{et al.} 2013). As mentioned, HS influences lymphocyte count and relative weight of lymphoid organs through glucocorticoid hormones. Results showed that TEO at high levels, 150 and 200 mg/kg, reduced negative effects of HS on corticosterone concentration. Thus, it is reasonable that TEO improves leucocytes count and relative weight of lymphoid organs.

**CONCLUSION**

Results indicated that HS has negative effects on growth performance, the serum concentrations of lipid profile, antioxidant system and immunity parameters. Dietary inclusion of TEO at levels of 150 or 200 mg/kg alleviated negative effects of HS on growth performance, blood biochemical variables and immune responses. On the basis findings, it can be advised dietary inclusion of TEO in broiler chicks, at levels of 150 or 200 mg/kg, help to improve the immune system in tropical regions and summer season.

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**REFERENCES**


