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Effect of Aqueous-Alcoholic Extract of *Hibiscus sabdariffa* Calyx and Leaf Calyx and Leaf on Performance, Egg Quality, Immune System and Antioxidant Balance of Laying Hens

Research Article

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ABSTRACT

This experiment was conducted to obtain information on how aqueous-alcoholic extract of *H. sabdariffa* plant can affect laying hen performance, egg quality, immune system and antioxidant balance during thermo-neutrals conditions. Two hundred hens (23-wk-old) were divided into 5 experimental treatments with 4 replicates each, 10 white-egg Hy-Line (W36) hens per cage. Treatment 1 (control) birds were fed with a corn and soybean meal-based diet without extract, whereas treatments 2 and 3 received, 300 and 700 mg/kg of *H. sabdariffa* leaf extract (HSLE) and treatments 4 and 5, received 300 and 700 mg/kg of *H. sabdariffa* calyx extract (HSCE), respectively. The weekly recorded performance data included egg production, feed intake, egg mass and feed conversion ratio. The egg quality parameters were evaluated at each 28 days of the experimental periods. Two hens from each replicate were selected and the blood was gathered to determine the immune system and plasma malondialdehyde (MDA). Also, two egg yolks from each replicate were used to investigated yolk MDA, cholesterol and triglyceride. Comparing the results with the control showed, HSCE at 700 mg/kg promoted laying rate during peak production (P<0.05) up to 8%. However, antioxidant balance and immune function did not differ between treatment groups. Eggshell strength, eggshell thickness, eggshell weight and egg-shape index were affected by treatments (P<0.05). Hens receiving 700 mg/kg of HSLE significantly decreased yolk cholesterol (P<0.05) compared to the control group (44.74 *vs.* 47.67). The results suggest that the *H. sabdariffa* can improve laying performance, egg qualitative characteristics and reduce egg yolk cholesterol during peak production period. The beneficial effects of the *H. sabdariffa* should be further studied in commercial production conditions.

KEY WORDS egg shell, *H. sabdariffa*, malondialdehyde, yolk cholesterol.

INTRODUCTION

In conditions to imbalances between oxidants and antioxidants that leads to oxidative stress (Sahin *et al.* 2001), there are decreases in antioxidant status (Sahin *et al.* 2004) and impair of immune system (McReynolds *et al.* 2009). During the peak laying period, due to the high metabolism of laying hens for egg formation, reducing oxidative stress, retaining health status of the birds and consequently egg quality are very important issues (Wang *et al.* 2017). However, there is little information on how we can overcome this natural condition with minimal cost and no side effects. In recent years, dietary extracts or medicinal plants powder were considered by nutritionists and researchers (Kahraman, 2009). *Hibiscus sabdariffa* is an annual, erect and bushy plant, with brown seeds, red sepals (calyces) and green leaves, which are the three major components of this plant. The leaves are alternate with reddish veins. The

fleshy and red calyces, fully encloses the velvety capsule which contains kidney-shaped, light-brown seeds (Morton, 1987). Apart from estrogenic (Saeed *et al.* 2013), antibacterial (Olaleye, 2007) effects and cholesterol reduction property (Carvajal-Zarrabal *et al.* 2005), that decreases cholesterol deposition in poultry products (Kwari *et al.* 2011), calyx and leaf of *H. sabdariffa* are known as potent antioxidant (Ochani and Mello, 2009; Ologundudu *et al.* 2010). Five major antioxidant compounds including neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, rutin and isoquercitrin are known in *H. sabdariffa* leaf (Wang *et al.* 2014) and four major antioxidant compounds including anthocyanins, quercetin, protocatechuic acid and flavonol glycosides in *H. sabdariffa* calyx have been identified (Hirunpanich *et al.* 2005). According to literature (Rice-Evans and Miller, 1996; Wang *et al.* 1997), these antioxidants have much more activity than vitamin A, vitamin E, carotenoids (Rice-Evans and Miller, 1996) and ascorbate (Wang *et al.* 1997).

In laying hens, *H. sabdariffa* reduced the negative effect of heat stress on mortality rate, heterophile to lymphocyte ratio (Minka *et al.* 2007) and level of thiobarbituric acid reactive substances (TBARS) in egg yolk and blood plasma (Sukkhavanit *et al.* 2011). Also, *H. sabdariffa*, especially its leaves, which are discarded in most countries, showed the significant beneficial effects, such as anti-lipid effects, improvement of total plasma antioxidant and oxidative balance of old laying hens (Sabet Sarvestani *et al.* 2019). However, there is not enough information available to help in maximizing production with slightest depression of production quality and immunity, regarding supplementation of laying hens diet with *H. sabdariffa* calyx extract (HSCE) or *H. sabdariffa* leaf extract (HSLE) during stressful condition of peak production. Since stressful situations increases the need for antioxidants (Cheng *et al.* 1990), it decreases the performance and quality of the eggs (Al-Batshan *et al.* 1994; Sahin *et al.* 2010) and weakens immune system status of laying hens (McFarlane and Curtis, 1989), therefore, this research was designed to investigate the effect of aqueous-alcoholic extract of *H. sabdariffa* calyx and leaf on production performance, egg quality traits, egg yolk cholesterol, triglyceride, antioxidant balance, total plasma antioxidant capability and immune status of laying hens.

MATERIALS AND METHODS

Experimental birds and dietary treatments

All procedures used in this experiment were approved by the Animal Care and Ethics Committee of Birjand University (Birjand, Iran) and complied with the Guidelines for the Care and Use of Animals in Research.

Two hundred laying hens were purchased from a local company (Morghak Company, Tehran, Iran), allocated into 5 experimental treatments with 4 replicates each including 10 white-egg Hy-Line (W36) hens. Treatment 1 (control) birds were fed with based diet without extract, treatments 2 and 3 received, 300 and 700 mg/kg of leaf extract respectively and treatments 4 and 5 birds, received 300 and 700 mg/kg calyx extract of *H. sabdariffa*, respectively. The laying hens had similar initial body weight (1500±10 g) and the same age (23-wk-old). On the bases of homogenous production, birds were selected and their initial weights were recorded, randomly assigned to each cage as experimental unit that was equivalent to each treatment replicate. Birds were housed in stainless cages (40 cm-width×50 cm-length×45 cm-height). Room environmental temperature was controlled at 23 ˚C and a daily lighting schedule of 16 h light and 8 h dark was used. The experiment started at peak production period and continued for a period of 12 weeks (23 to 35 weeks of age). Water was available *ad libitum* throughout all the experimental period. One hundred and ten g of mash feed for each bird was given on daily bases. A corn-soybean meal-based commercial laying diet was chosen as the control and basal diet (Table 1). In this study, aqueous-alcoholic extract of *H. sabdariffa* calyx and leaf, due to better extraction of components in comparison with aqueous or alcoholic extracts (Fakeye *et al.* 2008), were prepared and sprayed on feed at either level of 300 or 700 mg/kg. For extraction preparation, 1 liter of ethanol 96%/distilled water mixture (30:70) was used to infuse every 100 g of plant powder material (either calyx or leaf) for 24 h (Fakeye *et al.* 2008).

The obtained extracts were collected using filter paper and stored in a dark and cool place until use. The yield percentage of *H. sabdariffa* aqueous-alcoholic extract was either 40 or 30 of calyx and leaf, respectively. These extracts were mixed with either dietary levels of 300 or 700 mg/kg. Before starting the experiment, the total phenolic compounds (Chuah *et al.* 2008), anthocyanin (Swain, 1965), total antioxidants (Turkmen *et al.* 2005), vitamin C (Smith *et al.* 2003), calcium and chemical analyses for *H. sabdariffa* were carried out according to methods of AOAC (AOAC, 2007) and are reported in Table 2.

Egg quality, laying performance and egg yolk cholesterol and triglyceride

The production performance (feed intake, egg mass, egg weight and feed conversion ratio) were recorded weekly. To determine the qualitative characteristics of eggs, two eggs from each replicate were randomly used every 28 days of the experimental period. Haugh unit score was applied using egg weight and albumin height.

Table 1 Ingredients of basal (control) diet

Diet components (%)	
Corn	47.00
Soybean meal	28.00
Barley	8.50
Organic herbal powder[1]	1.65
Soybean oil	2.00
Dicalcium phosphate	1.80
Calcium carbonate	8.50
Salt	0.200
Sodium bicarbonate	0.150
Bentonite	1.00
Vitamin and mineral premix[2]	0.600
Vitamins (A, E, D_3, K, B-complex)	0.250
Methionine	0.150
Lysine	0.075
Threonine	0.075
Choline	0.050
Chemical composition (%)	
Energy (kcal/kg)	2663.05
Protein	17.77
Fiber	3.78
Linoleic acid	2.14
Lysine	0.948
Methionine	0.411
Methionine + cysteine	0.828
Calcium	3.82
Phosphorus	0.449

[1] Each kg of herbal organic powder contains: Energy: 2440 kcal/kg; Protein: 10.9%; Fat: 3.3%; Fiber: 22.4%; Ash: 14.9%; Moisture: 5.8%; Dry matter: 94.2%; vitamin B_6: 182.79 mg/kg; vitamin B_5: 49.63 mg/kg; vitamin A: 3702.27 IU/kg; vitamin E: 601.025 IU/kg; vitamin D_3: 90088.74 IU/kg; vitamin B_2: 158.17 IU/kg; vitamin B_3: 3221.42 IU/kg; Manganese 62.956 mg/kg, Zinc: 15.725 mg/kg; Copper: 9.942 mg/kg; Calcium: 12104.2 mg/kg; Phosphorus: 1.838 mg/kg; Iron: 606.539 mg/kg and Magnesium: 3574.9 mg/kg.

[2] Each kg of minerals and vitamins supplement contains: Manganese: 36000 mg/kg; Zinc: 32000 mg/kg; Copper: 3200 mg/kg; Iodine: 480 mg/kg; Selenium: 88 mg/kg; Iron: 16000 mg/kg; vitamin A: 3200000 IU/kg; vitamin D_3: 1320000 IU/kg; vitamin K_3: 1000 mg/kg; vitamin E: 8000 IU/kg; vitamin B_6: 1600 mg/kg; vitamin B_1: 100 mg/kg; vitamin B_2: 2200 mg/kg; vitamin B_9: 360 mg/kg; vitamin B_{12}: 9 mg/kg; Niacin: 12000 mg/kg; Calpan: 3200 mg/kg; Biotin: 30 mg/kg; Antioxidant: 3000 mg/kg and Choline: 44000 mg/kg.

Table 2 Chemical composition and antioxidant activity of *H. sabdariffa*

H. sabdariffa	Total antioxidants (%)	Phenol (mg/100 g)	Anthocyanin (mg/L)	Vitamin C (mg/100 g)	Ca (%)	Crude energy (cal/g)	Crude protein (%)	Crude fat (%)	Dry matter (%)
Calyx	61.71	0.801	183.63	1.40	1.40	2797.66	10.42	1.06	93.01
Leaf	61.21	0.642	18.33	2.55	2.57	3355.84	11.00	3.35	93.58

The yolk index was obtained by dividing the height of yolk at the central point of the yolk diameter, the yolk color score was obtained by matching the yolk with one of the 15 bands of Roche, specific gravity was determined by using buckets containing water and salt with different densities (specific gravity range of 1.06 to 1.099), resistance and thickness of egg shell, respectively, were measured by egg shell strength tester (Ogawa Seiki Co., LTD. OSK 13473 R, with 0.01 kg/cm^2 accuracy) and using an egg shell thickness meter (Ogawa Seiki Co., LTD. OSK 13469, with 0.001 mm accuracy) and the relative weight of white, yolk and shell were obtained by dividing the weight of each of these by weight of the whole egg multiplied by 100.

Also, egg shape index was obtained by dividing the egg width by egg length multiplying by 100 (North, 1984). In order to measure the level of yolk cholesterol and triglyceride, at the end of the experimental period, 2 random samples from each replicate were analyzed, after separating and mixing the yolks, by auto-analyzer spectrophotometer (Chem Gesan 2000, Italy) and laboratory kits (Pars Azmoon Inc., Tehran, Iran) using enzymatic method of Luhman *et al.* (1990). In order to measure the level of yolk cholesterol and triglyceride, exactly 1.00 g of yolk with 50 mL of NaOH (0.05 molarity) was thoroughly mixed. In the next step, this mixture was neutralized by adding 50 ml of hydrochloric acid (0.25

normality) to actually looked like blood plasma. Then, the obtained solution was centrifuged for 15 min with 3000 rpm and about 1 mL of this solution was injected into spectrophotometer (Luhman *et al*. 1990).

Antioxidant balance, total plasma antioxidant capacity and immune system

The malondialdehyde (MDA) measurement method is based on reaction with reactive substances (ThioBarbituric Acid and TriChloroacetic Acid, Merck, Darmstadt, Germany), spectrophotometric absorption measurements and is used to compare absorption with standard curve. At the end of the experimental period, blood samples from the wing vein of the birds (2 birds per replicate) were obtained and centrifuged at 3000 × g for 10 min. Plasma total antioxidant capacity using commercially available kit (Randox Total Antioxidant Control Cat. No. NX 2331, UK.), according to method of Miller *et al*. (1993) and plasma MDA level using a colorimetric method (Yoshioka *et al.* 1979) were analyzed, respectively. Blood plasma samples (200 µL) were mixed with 2 cc of TBARS reagent and shaken for 20 min in a hot water bath of 80 ˚C.

After cooling and transferring of 2 cc butanol were added to the test tubes and were centrifuged at 3000 rpm for 10 min to separate the two phases. MDA was measured with a spectrophotometer at a wavelength of 532 nm (Yoshioka *et al.* 1979).

In addition to plasma MDA, egg yolk antioxidant index (Cherian *et al.* 1996), were determined for each of 2 replicate samples. Two g of egg yolk were completely homogenized with 18 mL of 3.86% trichloroacetic acid solution and were filtered using filter paper (no. 41, Whatman International Ltd., Maidstone, UK). Then, 2 cc of this solution were mixed with 2 cc of the thiobarbituric acid (0.8%) and were incubated for 30 min in a hot water bath 80 ˚C. After cooling, the mix was analyzed by spectrophotometer at 531 nm (Cherian *et al.* 1996). Antibody titer to the Newcastle virus and heterophile to lymphocyte ratio were calculated and used to compare the immune system status between treatments. After coagulation of blood samples, the serum was collected and used to assess antibodies titer for Newcastle disease virus (NDV) by hemagglutination inhibition (HI) test (Allan and Gough, 1974). For the HI method, the volume of antigen virus was reacted with the antibody. The HI titers were defined on log 2 based of the highest dilution reciprocal which was hemagglutinated (Allan and Gough, 1974). To determine heterophile to lymphocyte ratio (H:L), blood samples were collected using ethylenediaminetetraacetic acid (EDTA) anticoagulant. A thin smear from each blood sample was prepared on clean microscope slides.

Blood smears were prepared by allowing air-drying and then, were stained with Greenwald-Giemsa stain after fixation with methyl alcohol. Finally, the ratios of heterophiles to lymphocytes from 100 cells were determined by dividing the numbers of heterophiles by lymphocytes (Gross and Siegel, 1983).

Statistical analysis

Statistical analysis of the data was carried out using the SAS statistical program (SAS, 2015) in a completely randomized design and differences between means were tested with Tukey-Kramer tests at $P \leq 0.05$. The statistical models used for the analysis of repeated (performance and egg quality) and unrepeated data (yolk cholesterol and triglyceride, antioxidant balance, total plasma antioxidant capacity and immune system) were, respectively:

$$Y_{ijk} = \mu + T_i + W_j + (T\times W)_{ij} + e_{ijk}$$
$$Y_{ij} = \mu + T_i + e_{ij}$$

Where:
Y_{ijk} and Y_{ij}: studied trait.
μ: trait mean.
T_i: treatment effect.
W_j: effect of period or week.
$(T\times W)_{ij}$: interaction between treatment and week.
e_{ijk} and e_{ij}: effect of experimental error.

RESULTS AND DISCUSSION

As Table 2, numerically, shows; fat, energy and vitamin C were higher in the leaf compared to calyx whereas the antioxidant activity including total antioxidants, phenols and anthocyanins in calyx were higher than the leaf. Data in Table 3 indicate that HSCE at 700 mg/kg significantly promoted laying production rate during peak production, probably due to quercetin and daidzein phytoestrogens found in *H. sabdariffa* calyx (Omotuyi *et al*. 2011; Saeed *et al*. 2013). These compounds have the ability to bind to estrogen receptors and thus can exert their effects on the physiological processes of the body, like an increase in egg yolk precursors (vitellogenin) synthesis, which resulted in increases in egg production (El-Ghalid, 2009). In other similar studies, supplementation of daidzein improved duck laying performance (Zhao *et al.* 2005) and laying hen (Ni *et al*. 2007). The highest percentage of production, after the HSCE, was observed at 700 mg/kg of HSLE and in other treatments was showed numerical increase compared with control treatment. Also, the lowest feed conversion ratio (1.87) and the highest egg mass (52.44 g/d) were observed by 700 mg/kg of HSCE (Table 3).

Table 3 Effect of *H. sabdariffa* plant extract on laying performance (23-35 weeks of age)[1]

H. sabdariffa	Level (mg/kg)	Feed conversion ratio	Feed intake (g/d)	Egg mass (g/d)	Egg weight (g)	Egg production (%)
Control	0	2.04	98.37	48.63	57.72	84.20[b]
Leaf	300	1.99	97.12	48.98	56.82	86.18[ab]
Leaf	700	1.89	96.03	51.32	56.24	91.08[ab]
Calyx	300	1.97	96.44	49.22	56.43	86.85[ab]
Calyx	700	1.87	97.20	52.44	56.85	92.58[a]
SEM	-	0.045	1.414	1.084	0.353	1.919
P-value	-	0.0791	0.8079	0.0995	0.0802	0.0377

[1] Data are means from 4 replicates of 10 hens.
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.

The extracts in comparison with the control group, caused a numerical decrease in feed intake and egg weight which were dose-dependent by leaf extract, but were dose-independent by the calyx extract. This appeared to be due to high rate of metabolism and excretion of anthocyanins as the most important calyx polyphenols (Vanzo *et al.* 2008), compared to the polyphenols present in the leaf. The numerical reduction of feed intake may be due to low absorption of *H. sabdariffa* polyphenols in the gastrointestinal tract and thus their inhibitory impact on digestive enzymes (Renard *et al.* 2017), that could decrease digestibility of protein, carbohydrates, lipids and thus could reduce feed intake. Also, because of the positive relationship between dietary protein level and egg weight (Calderon and Jensen, 1990), reduction of protein bioavailability, following the disturbances in the digestion process, may reduce egg weight.

The effect of HSCE and HSLE on egg quality traits of laying hens is reported in Table 4. Among egg quality traits, thickness and shell resistance were significantly increased by 700 mg/kg of HSLE and 300 mg/kg of HSCE (P<0.05) in comparison with the control, that is probably due to the weight loss of eggs in these two experimental groups (Calderon and Jensen, 1990). In addition, hens receiving 700 mg/kg of HSLE (treatment 3), significantly, obtained the highest egg shape index and eggshell weight, regarding to the control group. This improvement in egg shell quality, also, can be attributed to the phytoestrogens (Picotto *et al.* 1996; Omotuyi *et al.* 2011), the antioxidants (Sahin *et al.* 2001; Ologundudu *et al.* 2010; Wang *et al.* 2014), the organic acids (Zhou *et al.* 2009; Lin *et al.* 2011) and the higher content of calcium found in the leaf of *H. sabdariffa* in comparison with the calyx (2.5% *vs*. 1.4%).

Phytoestrogens by up-regulation of ATP-dependent calcium pumps in the duodenum (Picotto *et al.* 1996), the antioxidant compounds by increasing the mineral absorption capacity (Sahin *et al.* 2001) and organic acids by increasing the solubility of minerals (Zhou *et al.* 2009) improve the absorption of dietary calcium and subsequently the quality of the egg shell.

The best egg shape index obtained by HSLE at 700 mg/kg (73.85) can be attributed to vitamin C and natural antioxidants found in *H. sabdariffa.* Similarly, an increase in egg shape index of laying hens has been attributed to natural antioxidants (Radwan *et al.* 2008) and vitamin C (Keshavarz, 1996; Saki *et al.* 2010). Other qualitative traits of eggs were not affected by extracts.

As reported in Table 5, HSLE at 700 mg/kg significantly reduced egg yolk cholesterol compared to control group, which is in agreement with literature (Olatunji *et al.* 2005; Hirunpanich *et al.* 2006; Ochani and Mello, 2009).

The reason for this decrease could be due to the inhibition of lipid synthesis by *Hibiscus* acid which generates a substance in the gut in order to inhibit the citrate lyase (Sullivan *et al.* 1972; Carvajal-Zarrabal *et al.* 2005). This decrease could also be attributed to the antioxidant activity of several compounds such as isoquercitrin and protocatechuic acid contained in the *H. sabdariffa* extract or β-sitosterol and pectin found in *H. sabdariffa* (Tseng *et al.* 1997; Hirunpanich *et al.* 2006).

Additionally, the synergetic effect of L-ascorbic acid, pectin (Ginter *et al.* 1979) and adrenocortical hormones activating compounds (Lin *et al.* 2011) that have been reported for *H. sabdariffa*, could explain the anti-lipid effect. On the other hand, according to Kim *et al.* (2007) the inhibition of fat synthesis by *H. sabdariffa* is not attributed to hormones but to the inhibition of adipogenic transcription factors.

The results showed that there was no significant effect of extracts on H:L or antibody titer, however, in all experimental treatments, there were a numerical increase in the antibody titre against the Newcastle virus and heterophile to lymphocyte ratio.

Relative improvement of immunity is in consistent with the fact of *H. sabdariffa*, except antioxidants such as polyphenol, contain vitamin C (Wong *et al.* 2002), whereas, the synthesis of vitamin C is inadequate in adult stressed birds. Stability of leucocytes membrane and improving phagocytosis of neutropils are also vitamin C activities (Khan *et al.* 2012).

Table 4 Effect of *H. sabdariffa* plant extract on egg quality traits of laying hens

H. sabdariffa	Level (mg/kg)	Egg shape index	Yolk color score	Yolk index	Yolk weight (%)	White weight (%)	Haugh unit	Shell weight (%)	Shell thickness (mm)	Shell resistance (MPa)	Specific gravity (g/cm³)
Control	0	71.34[b]	6.00	43.10	27.51	59.46	87.71	13.02[b]	0.376[b]	0.359[b]	1.075
Leaf	300	72.10[ab]	6.58	43.48	27.16	59.22	89.86	13.60[ab]	0.385[ab]	0.378[ab]	1.076
Leaf	700	73.85[a]	6.83	44.09	26.53	59.20	90.06	14.26[a]	0.399[a]	0.406[a]	1.078
Calyx	300	72.71[ab]	6.79	44.44	26.75	59.24	90.96	13.99[ab]	0.397[a]	0.400[a]	1.077
Calyx	700	72.00[ab]	6.62	43.14	27.37	59.41	87.85	13.20[ab]	0.384[ab]	0.376[ab]	1.076
SEM	-	0.524	0.211	0.505	0.501	0.692	0.911	0.262	0.0034	0.0067	0.0008
P-value	-	0.0418	0.0868	0.2866	0.6182	0.9981	0.0878	0.0230	0.0013	0.0013	0.2823

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 5 Effect of *H. sabdariffa* plant extract on egg yolk lipid, antioxidant balance, total plasma antioxidant capability and immune system of laying hens (week 35)[1]

H. sabdariffa	Level (mg/kg)	Yolk triglyceride (mg/g)	Yolk cholesterol (mg/g)	MDA Yolk (µg/g)	Blood MDA (µg/L)	Total antioxidant capability (mmol/L)	H:L	Antibody titer
Control	0	276.42	47.67[a]	1.41	0.822	1.57	0.218	9.37
Leaf	300	251.33	45.73[ab]	1.35	0.758	1.67	0.232	9.87
Leaf	700	230.37	44.74[b]	1.40	0.765	1.59	0.246	9.50
Calyx	300	269.57	46.82[ab]	1.30	0.722	1.84	0.271	9.62
Calyx	700	254.14	46.30[ab]	1.32	0.751	1.72	0.281	9.25
SEM	-	18.298	0.627	0.041	0.0584	0.244	0.0280	0.360
P-value	-	0.4586	0.0477	0.3335	0.8157	0.9380	0.4973	0.7751

[1] Data are means of 4 replicates of 2 samples at the end of the experimental period.
H:L: heterophile to lymphocyte ratio.
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.

Also, improvement in immunity can be due to stimulation of lymph organs by compounds such as alkaloids, saponins, tannins, glycosides, flavonoids, phenols, steroids and other vitamins that are found in the plant (Mungole and Chaturvedi, 2011). With regard to the low bioavailability of the components of *H. sabdariffa*, it seems that its effect on the immune system is mainly related to stimulation of the intestinal immune system (Pandey and Rizvi, 2009). Similarly, Okoko and Ere (2012) introduced *H. sabdariffa* as a plant with immunoprotective effect, which could be exploited for pharmacological advantages. Fakeye (2008) reported that extract of *H. sabdariffa* flowers at 50 to 100 mg/kg level could have the potential for stimulating immune system of rat. Besides, our attempts to increase antioxidant capacity of plasma and egg yolk by HSLE and HSCE did not generate a statistically significant difference (Table 5). However, all extracts, especially calyx at 300 mg/kg level, showed a numerical reduction in the MDA that is probably due to higher level of antioxidants in the calyx compared to the leaf (Table 2). Contrary to our findings, in laying hens, *H. sabdariffa* calyx reduced the level of TBARS in egg yolk and blood plasma (Sukkhavanit *et al*. 2011).

Overall, the effect of *H. sabdariffa* was dose-independent in some traits that probably increasing the activity of extracts at a low dose (300 mg/kg) compared to a high dose (700 mg/kg) could be attributed to increase in the polarity of water molecular moiety, which increases the solubility of the compounds in the extract and provides effective absorption of extract from the gastrointestinal tract in dilute form (Lubega *et al*. 2013).

CONCLUSION

It can be concluded that *H. sabdariffa* had significant beneficial effects on laying rate, egg shell quality and yolk cholesterol level. Although only calyx of *H. sabdariffa* have been used optimally until now, the full knowledge of the bioactive components of different parts of this plant, especially leaf that is usually ignored and discarded around the world, is of great importance given that under *in vivo* conditions the leaf can extend the range of its effects. Since the cellular, biological and epigenetic mechanisms of the reported effects for this plant are still unknown, it is necessary to carry out more studies to fully understand them. Additionally, finding of effective dose of HSLE and HSCE

is important to achieve maximum efficacy according to the test conditions.

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