Single or combined effects of dietary supplemental vitamin C and ethanol extracts of propolis on productive traits, egg quality and some blood biochemical parameters of laying hens

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\begin{abstract}
This 8-week study was conducted to determine the effects of adding ethanol extract of propolis (EEP) and vitamin C (VitC) to diet on performance, egg quality characteristics and blood biochemical of laying hens. A total number of 144 Lohmann LSL-Lite laying hens (59 weeks of age) were assigned to six experimental diets with four replicate cages and six hens per cage. Based on a 3 × 2 factorial arrangement of treatments, 6 iso-caloric and iso-nitrogenous diets consisting 3 levels of EEP (0, 150 and 300 mg/kg) and 2 levels of VitC (0 and 250 mg/kg) were fed. EEP and VitC had no significant effect on overall egg production, egg weight, abnormal egg and feed intake. Improved egg mass and feed conversion ratio were observed in the EEP group. Increased shell thickness was seen in birds fed the diets included 150 and 300 mg/kg EEP compared to the control. VitC decreased the blood level of glucose, but had no significant effect on performance and egg quality characteristics. Decreased serum triglycerides were observed in birds fed 300 mg EEP/kg. In conclusion, EEP could improve productive performance, shell thickness and decreased blood plasma triglycerides; besides, diet inclusion of VitC decreased serum level of glucose.
\end{abstract}

\section*{Introduction}
Antibiotics, used in poultry diets to improve production performance, have been prohibited because of their negative subsequences in birds and human health (Zulkifli et al. 2000; Caswell et al. 2003; Apata 2009). Stressors (regardless of their sources) have a highly detrimental influence on laying hens (Puthpongsiriporn et al. 2001; Belloni et al. 2015), which depresses feed intake (FI), body weight, egg production (EP) and egg quality (Puthpongsiriporn et al. 2001). Bee glue or propolis, which contains wax (30%), resin (50%), essential oils (10%), pollen (5%) and some other substances (Kumova et al. 2002; Arpasova et al. 2016) and looks an adhesive, dark yellow to brown coloured balsam, is one of the products from beekeeping which is found in the hives (Galal et al. 2008); since bees collect propolis from various origins, namely buds, leaves and similar parts of trees and other plants such as pine, oak, eucalyptus, poplar and chestnut (Valle 2000; Seven et al. 2010) its composition differs depending on a wide variety of factors including collecting location, time and plant source. Propolis, beside honey and royal jelly, has introduced as a functional food (Khan et al. 2017), may be due to its beneficial composition (e.g. polyphenols, steroids terpenoids, amino acids and inorganic compounds). The beneficial effect of dietary supplemental propolis on weight gain, feed consumption, Feed conversion ratio (FCR) and mortality rate of broilers has shown (Havsteen 2002). A wide variety of beneficial properties, namely immunomodulatory, hepatoprotective, antioxidative, antiviral, antibacterial, anti-fungal, anti-inflammatory, anti-ulcer, anti-tumour and cardio-protective and local anaesthetic properties is detected for propolis, and has made it widespread in medical science, apitherapy and in the biocosmetology (Acikgoz et al. 2005; Tatli Seven et al. 2008; Saeed et al. 2017). Propolis has valuable pharmaceutical, antioxidative (Seven et al. 2010) and disinfection effects (Belloni et al. 2015). High flavonoid, phenolic acid and terpenoid contents of propolis have introduced as responsible for some beneficial properties of propolis (e.g. cytostatic, anti-mutagenic and immunomodulatory properties) (Prytzyk et al. 2003; Wang et al. 2004). Since free radicals may result in the pathogenesis of lipid peroxidation of the plasma membrane (Khan et al. 2011), they are mainly focused on animal and human studies. Propolis (which contains flavonoids) is shown as a potent antioxidant which protects the cell membrane from damage due to oxidation by ascorbate (Havsteen 2002).

Adrenal glands are stimulated by ACTH (secreted by the pituitary) to secrete cortisol in response to stress (Padayatty et al. 2007). During prolonged periods of stress adrenal glands secret hormones which may exert harmful effects; vitamin C (VitC) may play a role to reduce their harmful effects. Besides, VitC plays a role in collagen and bone minerals’ production (McDowell 1989; Abdel-Tawwab et al. 2004; Tatli Seven et al. 2008). In addition to the role of VitC in a great number of biochemical processes (Abdel-Tawwab et al. 2004), the beneficial
effects of this vitamin on various growth and production parameters in laying hens or broilers, namely growth, EP, eggshell strength and eggshell thickness have demonstrated (Bains 1996; Tatlı Seven 2006; Tatlı Seven et al. 2006); moreover, it has been indicated that VitC synthesis may be inadequate under stress conditions such as abnormal environmental temperatures and high productive rate (Khan et al. 2012).

Keeping in view the mentioned pharmaceutical advantages of propolis and VitC, the authors predict adding propolis and/or VitC to diet may exert positive effects on performance, egg quality characteristics and blood parameters of laying hens. Assessing the probable synergistic interaction between dietary propolis and VitC can be mentioned as novelty of the current study.

Materials and methods

Birds, diets and propolis preparation

All experimental protocols were in accordance with the Animal Ethics Committee of Razi University (Kermanshah, Iran) and the guidelines on animal welfare (The number of approval letter: AD-197-2014). In this study, 144 Lohman-LSL Lite laying hens (59 weeks of age) were weighed individually and randomly assigned to 6 treatments with 4 replicates and 6 birds in each replicate in a completely randomized design. Based on a 3 × 2 factorial arrangement of treatments, the birds were supplied with six iso-caloric and iso-nitrogenous diets including three levels of ethanol extract of propolis (EEP) (0, 150 and 300 mg/kg) and VitC (0, 250 mg/kg) (Table 1). Feed and water were ad libitum during the 8-week trial period. Propolis was collected and extracted as previously explained by Seven et al. (2010).

Performance and egg traits

Daily EP, egg weight (EW) and FI were daily recorded throughout the experimental period and summed up to calculate monthly EP. FCR was calculated by dividing FI on egg mass (EM), (FI/EM). EM was calculated by EP multiplied by EW values (EP × EW). Fresh eggs were collected and weighed using an Aculab electronic (sensitivity 0.01 g) top-loading scale. The broken, cracked and shell-less eggs were separated, counted and considered as abnormal eggs. Egg shape index (egg diameter/egg length) was calculated. The eggs were broken open onto a flat surface, then yolk diameter, yolk and albumen heights were measured and yolk index was calculated (yolk height /yolk diameter). The eggshells were carefully washed, dried (at room temperature) and weighed (to within 0.01 g). An Ames micrometer was used to measure eggshell thickness, without inner membranes and at three different points (air cell, equator, and sharp end), besides, USDA Interior Egg Quality Measure (USDA Chart for scoring broken-out eggs, Catalog 4-4200 American Instrument Co. Inc. Silver Spring, MD) was used to determine Haugh unit (HU) (Abd El-Hack et al. 2018, 2019). The yolk colour was scored with the aid of Roche Yolk Color Fan. The egg specific gravity was determined using 11 gradient saline solutions varying in specific gravity of 1.060–1.100 at 0.005-unit increments (Holder and Bradford 1979).

Blood parameters

Blood samples were collected from the wing vein from four randomly selected birds of each treatment (n = 24) at the last week of the experiment. Blood samples were immediately centrifuged (4000 rpm and 10 min), and the plasma was frozen and stored (−20°C) until assayed for blood parameters glucose, cholesterol, triglycerides (TG), albumin, total protein and cortisol via the enzymatic colorimetric method using a commercial kit supplied by Pars Azmoon Company, Karaj, Iran.

Statistical analysis

All data were subjected to analysis of variance using general linear models procedure of SAS software (SAS 2003). Differences among the treatment means were compared using Duncan’s multiple range tests. Results were considered as significant when P < 0.05. Analysis of variance was conducted according to the following model: $Y_{ijk} = \mu + A_i + B_j + (AB)_{ij} + \mu_{ijk}$ where $Y_{ijk}$ is the measured parameter, $\mu$ is the overall mean, $A_i$ is the main effect of VitC, $B_j$ is the main effect of dietary EEP, $(AB)_{ij}$ is the interaction between VitC and EEP and $\mu_{ijk}$ is the effect of experimental error.

Results

Production performance

Table 2 shows the results of the dietary EEP and VitC supplementation effects on EP, EM, FI and FCR. EM was positively influenced ($P < 0.05$) by adding 150 and 300 mg of EEP/kg diet either week 1–4 and overall periods when compared to the control group. Concerning the FCR values, addition of 150 mg of EEP/kg diet improved FCR on 5–8-week experimental period when compared to the control group ($P < 0.05$). EP and FI values were not affected by dietary EEP or VitC ($P > 0.05$). There was no significant interaction between dietary EEP and VitC on productive performance throughout the experimental periods ($P > 0.05$).

### Table 1. Composition and calculated chemical analysis of the experimental diet

| Ingredients                  | Percentage |
|------------------------------|------------|
| Corn                        | 66.07      |
| Soybean meal                | 20.91      |
| Alfa alfa                   | 2.84       |
| Limestone                   | 3.98       |
| DCP                         | 1.23       |
| Iodine common salt          | 0.29       |
| Oyster                      | 4.00       |
| Vitamin premix*             | 0.25       |
| Mineral premix              | 0.25       |
| Di-Met                      | 0.18       |

Calculated composition:

- ME (kcal/kg): 2720
- Crude protein (%): 14.58
- Ether extract (%): 4.52
- Crude fibre (%): 2.84
- Calcium (%): 3.75
- Available phosphorus (%): 0.30

*Vitamin and mineral mixture provides per 2.5 kg of diet: vitamin A, 7700; vitamin D3, 3300; vitamin E, 6600 mg; vitamin K3, 550 mg; thiamine, 2200 mg; riboflavin, 4400 mg; vitamin B6, 4400 mg; Ca pantothenate, 550 mg; nicotinic acid, 200 mg; folic acid, 110 mg; choline chloride, 275,000 mg; biotin, 55 mg; vitamin B12, 8.8 mg; Trace mineral (milligrams per 2.5 kg of diet): Mn, 6600; Zn, 6600; Fe, 33000; Cu, 8800; Se, 300; I, 900.
## Table 2. Effects of dietary EEP and supplemental VitC on egg production, egg mass, feed conversion ratio and feed intake of the laying hens.

| EEP (mg/kg diet) | Vitamin C (mg/kg diet) | Interaction | Egg production (%) | Egg mass (g egg/hen/day) | Feed intake (g Feed/hen/day) | FCR (FI/EM) | Egg weight (g) |
|-----------------|------------------------|-------------|-------------------|--------------------------|-----------------------------|-------------|---------------|
| 0               | 0                      |             | 2.01              | 66.3                    | 66.49                       | 66.71       | 66.42         |
| 0               | 250                    |             | 2.04              | 65.3                    | 66.65                       | 67.01       | 66.49         |
| 0               | 300                    |             | 2.05              | 65.3                    | 66.65                       | 67.00       | 66.49         |
| 150             | 0                      |             | 2.06              | 64.9                    | 66.20                       | 67.28       | 66.50         |
| 150             | 250                    |             | 2.07              | 64.9                    | 66.20                       | 67.28       | 66.50         |
| 150             | 300                    |             | 2.08              | 64.9                    | 66.20                       | 67.28       | 66.50         |
| 300             | 0                      |             | 2.09              | 64.9                    | 66.20                       | 67.28       | 66.50         |
| 300             | 250                    |             | 2.10              | 64.9                    | 66.20                       | 67.28       | 66.50         |
| 300             | 300                    |             | 2.11              | 64.9                    | 66.20                       | 67.28       | 66.50         |

### Egg characteristics

Effects of dietary EEP and supplemental VitC on EW, egg gravity, shell weight, shell thickness, HU, egg shape index, yolk index, yolk colour and abnormal egg are shown in Table 3. EW, egg gravity, shell weight, HU, egg shape index, yolk index, yolk colour and abnormal egg were not affected by dietary EEP and VitC ($P > 0.05$). Propolis inclusion at 150 and 300 mg EEP/kg significantly increased shell thickness ($P < 0.05$), so that the lowest shell thickness was seen in the control group.

### Hematological parameters

The effect of dietary EEP and VitC supplementation on the plasma biochemicals are presented in Table 4. Plasma concentration of glucose decreased in hens fed VitC-supplemented diets. The highest and the lowest plasma triglyceride concentrations were identified in the control group and in the group given 300 mg EEP/kg diet with or without VitC, respectively; which difference between them was significant ($P < 0.05$). The other parameters were not influenced by dietary treatments ($P > 0.05$).

### Discussion

#### Production performance

EM was significantly influenced by dietary EEP ($P < 0.05$), except for 5–8 weeks of the trial period. The present results indicated that the EM of laying hens fed the diets with 150 and 300 mg propolis/kg was significantly higher than of hens fed control diet. In a previous study, the addition of 30 ppm of propolis to the rations of laying hens increased EP by 6.07% compared with the control group (Bonomi et al. 1976). The higher EM of hens fed propolis-included diet in the current study is in agreement with previous reports (Bonomi et al. 1976; Ghisalberti et al. 1983; Khojasteh Shalmany and Shivazad 1979). Improvement in EM is due to the positive effects of propolis on the laying hen. Several reports are available showing the roles of flavonoids. Flavonoids can prevent blood coagulation; besides they protect veins and decrease levels of harmful oestrogen (Duarte et al. 1993; Hanasaki et al. 1994; Middleton and Kandaswami 1994). In addition, propolis is assumed to have antimicrobial effects. Improved EM in propolis groups may be attributed to these properties of propolis (Taylor and Sulaiman 2014).

Regarding the effect of propolis addition to laying hen diets on FI values there are contrary reports. In the current study, in accordance with Ozkok et al. (2013) and Shreif Effat and El-Saadany Amina (2016), propolis supplementation to laying hen diets had no significant effect on the FI values. Significant increase in propolis supplemented diet consumption by laying hens fed the diet containing propolis could be attributed to the positive effects of propolis on the laying hen.
Table 3. Effects of dietary EEP and supplemental vitamin C on egg weight, egg gravity, shell weight, shell thickness, Haugh unit, egg shape index, yolk index, yolk colour and abnormal egg of the laying hens.a

| EEP      | Overall | 1–4 (week) | 5–8 (week) |
|----------|---------|------------|------------|
| 0 mg/kg diet | 65.03 | 66.45 | 66.22 |
| 150 mg/kg diet | 66.45 | 66.71 | 66.57 |
| 300 mg/kg diet | 66.38 | 66.04 | 65.78 |
| Vitamin C 0 mg/kg diet | 66.04 | 66.26 | 65.98 |
| 250 mg/kg diet | 66.04 | 66.26 | 65.98 |
| Interaction | 0.089 | 0.15 | 0.26 |

| Vitamin C | Overall | 1–4 (week) | 5–8 (week) |
|-----------|---------|------------|------------|
| 0 mg/kg diet | 0.816 | 0.98 | 0.84 |
| 150 mg/kg diet | 0.846 | 0.96 | 0.85 |
| 300 mg/kg diet | 0.846 | 0.96 | 0.85 |
| Vitamin C × EEP | 0.089 | 0.15 | 0.26 |

*Means within columns with different superscripts are significantly different, P < 0.05.

Values are means of six replicates ± SEM.

The insignicant effects of VitC and propolis on EW, egg gravity, shell weight,HU, egg shape index, yolk index, yolk colour and

**Egg characteristics**

The insignificant effects of VitC and propolis on EW, egg gravity, shell weight, HU, egg shape index, yolk index, yolk colour and
abnormal egg have previously reported. Addition of propolis did not influence egg shell, shape index, egg yolk index, albumen% and yolk colour score (Shreif Effat and El-Saadany Amina 2016). In agreement with our result in the current study, Arpasova et al. (2016) and Tatli Seven et al. (2008) reported that VitC or propolis had no effect on yolk index. Belloni et al. (2015) concluded that propolis supplementation to laying hen diets had no significant effect on egg specific gravity. Abdel-Kareem and El-Sheikh (2017) reported that propolis supplementation to the layer diets (250, 500 and 1000 mg) did not influence EW. Besides, Arpasova et al. (2016), Yang et al. (2003), Belloni et al. (2015) and Ting et al. (2011) did not find significant effect of propolis supplementation to laying hen diets on EW. In agreement with our result in the current study, Belloni et al. (2015) reported that VitC or propolis had no effect on EW. Arpasova et al. (2016) and Tatli Seven et al. (2006) indicated insignificant influence of propolis supplemented diets on albumen index. HU which has assumed as an index to estimate egg quality (Monira et al., 2000) assumed several reasons for improvement in egg shell thickness due to increased calcium digestibility and absorption (Shreif Effat and El-Saadany Amina 2016) quoted from Foucher (1982)). Presence of minerals, namely iron, calcium, copper, magnesium, zinc, manganese and chromium in the propolis has shown (Burdock 1998). In the current study, these minerals, at least partly, may lead to thicker egg shell in eggs laid by hens fed with diets contain propolis.

**Hematological parameters**

Beneficial effects of supplementation diet with VitC on various blood parameters, namely cholesterol, glucose, urea, TG, protein and albumin concentrations, and alkaline phosphates are reported (Kutlu and Forbes 1993). It is detected that serum and liver concentrations of VitC are reduced under environmental stress condition; this may show that stress has some prooxidative effects in birds (Klasing 1998). Khan (2011) showed that exogenous supplementation of antioxidants may decelerate negative consequences of free radicals in the body. VitC may play a dual role: antioxidative or prooxidative; VitC acts as a free radical chain terminator in high level of VitC and relatively low concentrations of free or activated metal ions. The oxidation–reduction potential of VitC is one of the important factors in elucidating its intracellular physiological action such as multiple oxidation function involving oxygen incorporation in the substrate (McDowell 1989). In the current study, decreased corticosteron (catabolic) and increased insulin (anabolic) concentrations (Kucuk et al. 2003) in the birds received supplemental VitC, may be at least in part, responsible for suppressed glucose concentration in those birds. In contrary with our results, Abdel-Kareem and El-Sheikh (2017) showed that level of total protein increased, while cholesterol concentration decreased significantly as a result of propolis addition to laying hen diet. These different results may be, at least in part, due to different composition of propolis, which depends on collecting location, time and plant source (Tatli Seven et al. 2008; Arpasova et al. 2016). Suppressed plasma triglycerides

| Interaction | EEP Vitamin C | Vitamin C | EEP × Vitamin C | SEM | P values |
|-------------|--------------|-----------|-----------------|-----|----------|
| 0 0         | 293.8        | 226.25    | 130.00          | 3626.3 | 5.625    | 3.000 | 0.803 |
| 0 250       | 277.5        | 226.25    | 130.00          | 3626.3 | 5.625    | 3.000 | 0.803 |
| 0 250       | 247.5        | 210.00    | 97.500          | 2626.3 | 5.125    | 3.000 | 1.293 |
| 300 0       | 283.8        | 190.00    | 95.000          | 2250.0 | 5.250    | 2.870 | 0.810 |
| 300 0       | 242.5        | 213.75    | 106.25          | 2293.8 | 5.375    | 3.000 | 1.293 |
| 150 0       | 285.0        | 214.17    | 109.58          | 2840.8 | 5.375    | 3.000 | 0.952 |
| 250 0       | 253.3        | 210.83    | 101.67          | 2667.5 | 5.375    | 3.000 | 0.838 |
| 0 250       | 281.8        | 217.50    | 115.63          | 3354.4 | 5.625    | 3.125 | 0.791 |
| 150 250     | 262.5        | 218.13    | 100.63          | 2636.3 | 5.187    | 2.812 | 1.267 |
| 300 250     | 263.1        | 201.88    | 100.63          | 2271.9 | 3.735    | 3.000 | 0.626 |

*Means within column with different superscripts are significantly different, *P* < 0.05.

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due to propolis is reported (Fuliang et al. 2005); in line with previous report, significantly lower TG was detected in the broilers fed propolis compared with the broilers fed propolis plus lead (as lead acetate) (Seven et al. 2010); this result may be related to the flavonoid content of propolis which may exert regulatory effect on blood circulation and stimulatory effect on triglyceride degradation to generate energy (Tekeli et al. 2011).

**Conclusion**

In conclusion, EEP could improve productive performance, shell thickness and decreased blood plasma triglycerides; besides, diet inclusion of VitC decreased serum level of glucose.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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