In vitro and in vivo evaluation of thyme (*Thymus vulgaris*) essential oil as an alternative for antibiotic in quail diet[1]

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ABSTRACT: The thyme (*Thymus vulgaris*) essential oil was assessed as antibiotic growth promoter replacement in quail chick diet and in vitro test. In total, 250-d-old Japanese quail chicks (mixed sex) were allocated into 5 dietary treatments of 5 replications (6 females and 4 males in each cage with the size of 40× 90× 25 cm) under a completely randomized design. The dietary treatments were included the control diet, control diet without any additive, control diet plus 100 ppm flavophospholipol as an antibiotic growth promoter, control diet plus 200 ppm TVE, control diet plus 300 ppm TVE, and control diet plus 400 ppm *T. vulgaris* essential (**TVE**) oil. Feed intake, BW gain, feed conversion ratio (**FCR**), organs weight, morphology of intestine, serum lipids, and microbial population were measured on day 35. Lipid oxidation of stored muscle tissue was measured by TBARS test. GC–MS assay, DPPH method, and well diffusion method were evaluated for determination of components, antioxidant, and antimicrobial properties, respectively. FCR improved significantly in 400 ppm TVE compared with 200 and 300 ppm TVE ($P < 0.05$). The serum triglyceride decreased significantly in both sexes receiving 400 ppm TVE compared with control. Villi height increased significantly in duodenum accompanied by decreasing crypt dept at all TVE levels compared with control and antibiotic. The breast muscle tissue of quail fed on 300 and 400 ppm TVE reduced the rate of oxidation during refrigerated storage compared with control. Thymol was the main component (35.40%) of the thymus oil. The considerable antioxidant activity of TVE was identified by IC50 of 58.48 µg/mL. Moreover, zones of growth inhibition of Gram-positive bacteria and *Escherichia coli* were numerically greater in different doses of TVE than antibiotics. Therefore, The TVE is suitable alternative component for antibiotic growth promoters by dosing consideration. However, it is possible that antibiotic resistance would increase for these natural compounds along the time.

Key words: antioxidant, morphology, performance, quail, serum lipids, thyme oil

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INTRODUCTION

Application of synthetic antibiotics in poultry husbandry can result in increased antibiotic resistance bacteria. Hence, researchers are seeking new natural feed additive alternatives to improve growth performance, modulate microbial populations, and improve meat quality and animal health. Phytogenic essential oils are one of the natural feed additives that their main effects and modes of action on poultry production have been reviewed previously (Brenes and Roura, 2010). It has been reported that the type and application dosage of essential oils have the major influence

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on determining the performance parameter and nutrient availability on the birds (Chowdhury et al., 2018; Liu et al., 2018). In addition, it is claimed that phytogenic essential oils improved intestinal health indices in laying hens (Mousavi et al., 2018).

Among the plants, one of the large plant families is the Lamiaceae family, and the genus *Thymus* is part of this family. The most famous species of *Thymus* is *vulgaris* that commonly was called thyme (Stahl-Biskup and Saez, 2004). Chemical analyses have shown the presence of different compounds in the essential oil of this plant. These compounds are flavonoids, tannins, saponins, and phenols, and it is rich in the monoterpene thymol and its isomer carvacrol (Fachini-Queiroz et al., 2012; Walentowska and Flaczyk, 2013). This medical plant can display antimicrobial, antifungal, antioxidant, antiviral, anti-cancer, and anti-inflammatory activities due to the existence of thymol and carvacrol (Pina-Vaz et al., 2004; Behnia et al., 2008; Cetinus et al., 2013; Ferreira et al., 2016; Nabavi et al., 2015).

The production of Japanese quail (*Coturnix japonica*) as a meat-type bird for human food has been popular in many countries (Jeke et al., 2018). The cholesterol value and skin fat is low in quail meat while it is rich in vitamins like folate, E, K, and B complex as well as other micronutrients (Manafi et al., 2016). This bird has been used as an animal model in numerous studies due to its short lifespan and high growth rate (Huss et al., 2008). The feeding requirement of Japanese quail is about 20 to 25 g/d and reaches to the weight of 140 to 180 g between 5 to 8 wk of age (Altine et al., 2016).

The purposes of this study was first to evaluate the effects of different dosage of thyme essential oil on quail physiology and nutrition, and second to compare this oil with antibiotics used in poultry farming as a natural alternative, and third to identify whether the thyme essential oil has antioxidant or antibacterial properties in vitro.

MATERIALS AND METHODS

The experimental protocols were approved by the Research Ethics Committee of the Shahid Bahonar University of Kerman, Iran (License number: 1396/137).

In Vivo Tests

Providing essential oil. Hydrodistillated *T. vulgaris* essential (**TVE**) oil was purchased from Barij essence Kashan factory in Iran.

Housing. This study was carried out in standard quail breeding buildings in Yazd agricultural and natural resources research center (Yazd, Iran). Two halls (A and B) were prepared for the trial. In first 15 d, chicks were kept in the hall A at 36 °C temperature and in the cages containing 40 W lamps, baby drinker, and chicken feeding tray. After day 15, chicks moved to hall B equipped with automatic linear feeder and nipple drinker with 32 °C temperature and 50% relative humidity. The hall temperature was dropped 2° weekly and maintained at 26 °C in last week. Quail were maintained on 24 h lighting regimen and had free access to water.

Birds, diets, and experimental design. A total of 250-d-old Japanese quail chicks (mixed sex) were allocated to 5 dietary treatments of 5 replications (6 females and 4 males in each cage with the size of 40× 90× 25 cm) under a completely randomized design. The dietary treatments were included the control diet, control diet without any additive, control diet plus 100 ppm flavophospholipol (Iranian Veterinary Pharmaceuticals Company, Iran, Tehran) as an antibiotic growth promoter, control diet plus 200 ppm TVE, control diet plus 300 ppm TVE, and control diet plus 400 ppm TVE. Corn–soy meal mash diets contained 2,900 kcal ME/kg and 22% CP were formulated using nutritional requirements of quail during growing and developing phases (Table 1 Rostagno et al., 2011). The essential oil was mixed with the required oil of the diets and added to control diet. Quail chicks were fed on experimental rations from the beginning of trial and were allowed ad libitum access to both feed and water throughout the study period of 35 d.

Performance data collection. Birds and the rest of the feed per cage were weighed weekly and feed intake (**FI**), BW gain (**BWG**), and feed conversion ratio (**FCR**) per bird calculated weekly as well as the end of the trial (35 d). Mortalities were recorded daily to adjust performance data. Total FI, total BWG, and total FCR were reported as total performance.

Weighing and blood sampling. At final day of trial (35 d), feed was removed 3 h before slaughtering. One bird per cage (random sex) nearest to the average weight of same cage was selected to measure organs weight and also two birds (1 male and 1 female) from each cage were separately selected to measure serum lipids due to variance in serum lipid ranges between sexes. Each bird was exsanguinated by cutting the jugular vein, and 5 mL of

Table 1. Feed ingredients and nutrient composition of basal diets during different production phases

Ingredient, % DM	Grower and developer phases
Corn, yellow	55.34
Soybean meal	39.21
Soybean oil	1.92
Limestone	1.21
Dicalcium phosphate	1.34
Common salt	0.41
Vitamin premix[1]	0.25
Mineral premix[2]	0.25
DL-Methionine	0.08
Nutrient composition	
ME_n, kcal/kg	2,900
CP, %	22.00
Calcium, %	0.90
Non-phytate P, %	0.37
Na, %	0.17
Methionine, %	0.42
Lysine, %	1.19
Threonine, %	0.79
Tryptophan, %	0.21
Arginine, %	1.19

[1]Vitamin premix provided the followings per kilogram of diet: vitamin A, 12,000 IU; cholecalciferol, 5,000 IU; vitamin E, 45 IU; vitamin K_3, 2.4 mg; thiamine, 2.6 mg; riboflavin, 6.6 mg; pantothenic acid, 25 mg; niacin, 55 mg; choline chloride, 500 mg; biotin, 0.1 mg; folic acid, 1.5 mg; pyridoxine 5.5 mg; vitamin B_{12}, 0.015 mg; BHT, 1 mg.

[2]Mineral premix provide the followings per kilogram of diet: iron, 50 mg; zinc, 85 mg; manganese, 90 mg; iodine, 1 mg; copper, 10 mg; selenium, 0.25 mg.

blood samples were collected by small plastic tubes. Viscera were removed immediately; thereafter the weights of liver, heart, tights, carcass, breast, and gastrointestinal tract were taken. Carcass yield and relative weights of organs were calculated as a percentage of live BW. After transferring blood samples to the lab, they were centrifuged at 3,000 rpm for 10 min (HETTICH Rotafix32A, Germany) and sera stored at –20 °C until further analysis. Serum triglyceride and cholesterol concentrations were measured using a biochemical analyzer (Autolab, PM 4000, Auto analyzer, Medical System, Rome, Italy) according to the procedures recommended by producing company.

Tissue sampling. During visceral separation, 1 cm segments of the duodenum (proximal intestine), jejunum (before the Meckel's diverticulum), and ileum (distal intestine) were excised, washed in physiological saline solution, and fixed in 10% buffered formalin. The tissue samples were later embedded in paraffin, and a 2-µm section of each sample was placed on a glass slide and stained with hematoxylin and eosin according to Baurhoo et al. (2007) procedure. Pictures of villus and crypts were obtained with a video camera (SPoT idea, Diagnostic Instrument Inc., Sterling Heights, MI), and then measurements were made by a computer using the Spot Basic (Diagnostic Instruments Inc., Sterling Heights, MI) imaging software.

Microbial population. The contents of the ileum were collected in the tubes and transferred to the laboratory for counting the microbial population (total coliforms and the lactic acid bacteria). The contents of ileum were diluted in normal saline. Plates containing MRS agar (deMan, Rogosa and Sharpe agar) were prepared for counting lactic acid producing bacteria and plates containing MacConkey agar were prepared for counting total coliforms. After bacteria culture, plates incubated for 24 ho in 37 °C temperature. All plates were counted during 24 h after incubation (Li, 1991).

TBARS assay. Thiobarbituric acid reactive substances (TBARS) test is one of the most accurate tests to determine lipid oxidation in animal tissues. In general, this test expresses the concentration of malondialdehyde, which is a good indicator of oxidation. For measurement of lipid stability in quail storage muscle tissue, the quail breast muscle tissue (5 replicates) was defrosted at room temperature after 60 d storage in –22 °C and homogenized with phosphate buffer (0.1 molar, containing 5 mM EDTA, pH = 7) on liquid nitrogen. Then, 40 µL of homogenized breast tissue was added to 40 µL of 0.9% sodium chloride plus 40 µL distilled water and placed at 37 °C for 20 min. Then, the reaction was stopped by using 600 mL hydrochloric acid (0.8 molar, containing 12.5% trichloroacetic acid). In the next step, 780 mL 1% thiobarbituric acid were added to the solution, boiled for 20 min and cooled to 4 °C. The cooled solution was centrifuged at 1,500 × *g* for 20 min. Finally, the absorbance of the above solution was measured at 532 nm and the data were expressed in milligram malondialdehyde per kilogram (Subbarao et al., 1990; Botsoglou et al., 2003).

In Vitro Tests

GC–MS system. Three samples of thyme oil were injected to GC–MS system for determining the components. The GC–MS system was used for analysis of the essential oil was the CP 3800 model (Varian BV company) developed by Australia and equipped by a capillary column of DB-5 (30 m × 0.25 mm × 0.25 µm). The injector temperature

equal to 240 °C and detector was 230 °C. Electron impact was 70 eV and helium was the carrier gas at a flow rate of 1.0 mL/min with split ratio of 1/20. Temperature was held at 60 °C and programmed at 3 °C/min to 240 °C and volume injected was 1 µL. Finally, for identifying the components of the oil, mass spectra of the components were compared with data of the computer library (Nist 62 lib database) and Kovats retention index (Adams, 2007).

Well diffusion assay. The bacteria selected for microbial test, including Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028) and Gram-positive bacteria *Staphylococcus aureus* (PTCC 1337) and *Bacillus cereus* (PTCC 1015) were provided from the faculty of veterinary medicine, Shahid Bahonar University of Kerman, Iran. Pathogenic bacteria transferred from –20 °C to MHB (Mueller Hinton Broth) medium and were renewed after 24 h of incubation at 37 °C. The inoculums density was set to 0.5 McFarland standards (10^8 CFU/mL). 0.5 McFarland suspension of each bacterium inoculated on plates (60 mm in size) contained MHA (Muller Hinton Agar) by swab. The wells of 6 mm diameters were punched in agar surface and the amounts of 1,100, 2,200, 4,400, and 8,800 µg/mL of TVE added to the wells. Control plates were attached to ceftriaxone (CRO-30) and tetracycline (Te-30) antibiotic disks for each bacterium. Finally, after incubating plates at 37 °C for 18 h (Memmert incubator, Germany), inhibition zones diameters were measured in millimeter by a ruler. This experiment was repeated 3 times for each strain and mean values with standard deviations of inhibition zones were recorded as final results (Jorgensen and Turnidge, 2015).

Antioxidant activity determination. In this experiment, we used DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich, Germany) as free radical to test antioxidant activity of TVE on the basis of Brand-Williams et al., 1995. Further, beta-hydroxy toluene (**BHT**, Sigma-Aldrich, Germany) antioxidant potential was determined as a standard antioxidant. At first, 0.004% DPPH methanolic solution was prepared. Then, 50 µL of the oil different dilutions in methanol were added to test tubes contained 2.5 mL DPPH solution (final concentrations were 294, 147, 73.5, 36.7, 18.3, and 9 µg/mL). In parallel, there was a control to contain all reagents except the essential oil. After 60 min, data were read at a wavelength of 517 nm by a spectrophotometer (Biotek Instruments). The following equation was used for the calculation of the antioxidant activity:

$$\%I = A\ \text{blank} - A\ \text{sample}/A\ \text{blank} \times 100$$

where %I is the inhibition percentage of DPPH, "A blank" is the absorbance of DPPH with methanol at 517 nm and "A sample" is the absorbance of different TVE dilutions in 517 nm. Finally, the IC50 parameter was used to compare the activity of TVE with BHT standards (IC50 is the concentration of essential oil that inhibits 50% of free radicals).

Statistical Analysis

All the data obtained in this experiment were analyzed by one-way analysis of variance using ANOVA procedures of SAS statistical software (SAS, 1999). A completely randomized design was applied in the analysis of all traits. The experimental unit was a cage. $Y_{ij} = \mu + A_j + e_{i(j)}$, where Y_{ij} is the observed value for a particular character, µ is the overall mean, A_j is the effect of A treatment level j, and $e_{i(j)}$ is the random error associated with the ijth recording. Data averages were compared by using Duncan's multiple range tests at the $P < 0.05$ significant level.

RESULTS

In Vivo Tests

Performance traits (BWG, FI, and FCR). The performance data of quail fed with different levels of TVE are illustrated in Table 2. FI increased significantly in T200 treatment compared with antibiotic ($P < 0.05$). However, different levels of TVE could not decrease FI than control or antibiotic. Birds fed with different levels of TVE revealed no remarkable BWG difference than control or antibiotic ($P > 0.05$). FCR improved significantly in 400 ppm TVE compared with 200 and 300 ppm TVE ($P < 0.05$) although it was not significant than control or antibiotic. The contrast between antibiotic and 200 ppm TVE was significant ($P < 0.05$).

Organs weight. In this study, different levels of TVE and antibiotic had no marked effect on the relative weight of the liver, heart, carcass, breast, thighs and gastrointestinal tract (Table 3). No differences were observed when organs weight data were compared between treatments ($P > 0.05$).

Serum lipids. Changes in the pattern of serum lipids were evaluated separately in male and female quail at the age of 35 due to difference in lipids ranges

between sexes (Table 4). The level of triglyceride in male birds decreased significantly in T400 treatment compared with control and antibiotic ($P < 0.05$). In females, the amount of triglyceride in treatments containing 300 and 400 ppm TVE decreased significantly compared with control ($P < 0.05$) but not to antibiotic. Significant difference ($P < 0.05$) was observed when female triglyceride was compared between control and T400 treatments. However, blood cholesterol level was not affected by treatments in both sexes.

Table 2. Effect of different levels of thyme essential oil and antibiotic on total performance of quail during 35 d rearing[1]

Treatments	FI[2], g/d per bird	BWG[2], g/d per bird	FCR[2], g feed/g gain
Control	18.40[ab]	6.29	2.92[ab]
Antibiotic[3]	18.32[b]	6.45	2.84[b]
T200	18.61[a]	6.14	3.03[a]
T300	18.47[ab]	6.20	2.98 [a]
T400	18.24[b]	6.39	2.82[b]
P-value[4]	0.035	0.168	0.009
SEM	0. 089	0.107	0.045
P-value for contrasts[4]			
Control vs. antibiotic	0.951	0.785	0.623
Control vs. T200	0.373	0.780	0.372
Control vs. T300	0.962	0.945	0.829
Control vs. T400	0.619	0.958	0.479
Antibiotic vs. T200	0.116	0.191	0.028
Antibiotic vs. T300	0.650	0.363	0.140
Antibiotic vs. T400	0.950	0.990	0.997

[1]All data are average of 5 replicates.

[2]FI = feed intake; BWG = BW gain; FCR = feed conversion ratio.

[3]Antibiotic is 100 ppm flavophospholipol. T200, T300, and T400 indicate the levels of 200, 300, and 400 ppm of *Thymus vulgaris* essential oil.

[4]Significance level ($P < 0.05$) by 95% confidence interval.

[ab]Different superscripts indicate significant differences ($P < 0.05$) within the same column.

Morphology of intestine tissue. Table 5 presents the effects of different levels of TVE and antibiotic on the morphology of duodenum, jejunum, and ileum cells. The villi height, villi width, and the crypt depth of the intestine cells were influenced by the experimental treatments. The villi height and width increased in treatments contained different levels of TVE and antibiotics when compared with the control. There was a significant increase in duodenum and jejunum villi height ($P < 0.0001$) in treatments containing different levels of TVE compared with control and antibiotic. However, villi height in ileum showed more fluctuation between treatments so that it increased significantly only in T200 and T300 treatments ($P < 0.05$). The duodenum, jejunum, and ileum villi width were also affected by experimental treatments so that in duodenum a significant decrease in villi width were observed in the treatments containing different levels of TVE. The greatest villi width in ileum related to 300 ppm TVE which differed significantly with control an T200 groups ($P < 0.05$). The crypt

Table 3. Effect of different levels of thyme essential oil and antibiotic on carcass characteristics in 35-d aged quail (expressed as % of live BW)[1]

Treatments	Liver	Heart	Carcass	Breast	Tights	Gastrointestinal tract[3]
Control	2.25	0.80	59.39	29.97	20.75	8.28
Antibiotic[2]	2.00	0.85	61.44	29.71	20.89	8.73
T200	2.07	0.84	62.02	30.74	21.10	7.39
T300	2.41	0.84	59.03	29.24	20.75	8.88
T400	2.56	0.82	58.66	27.81	20.30	8.00
P-value[4]	0.701	0.858	0.671	0.546	0.701	0.786
SEM	0.352	0.041	2.203	0.834	0.659	0.663
P-value for contrasts[4]						
Control vs. antibiotic	0.977	0.864	0.945	0.999	0.999	0.996
Control vs. T200	0.993	0.915	0.876	0.980	0.997	0.950
Control vs. T300	0.996	0.891	0.999	0.985	1.000	0.987
Control vs. T400	0.957	0.986	0.998	0.554	0.992	0.999
Antibiotic vs. T200	0.999	0.999	0.999	0.945	0.999	0.815
Antibiotic vs. T300	0.880	1.000	0.905	0.997	0.999	0.999
Antibiotic vs. T400	0.718	0.989	0.852	0.665	0.978	0.976

[1]All data are average of 5 replicates.

[2]Antibiotic is 100 ppm flavophospholipol. T200, T300, and T400 indicate the levels of 200, 300, and 400 ppm of *Thymus vulgaris* essential oil.

[3]Gastrointestinal tract includes the total weight of the crop, gizzard, caecum, and intestines.

[4]Significance level ($P < 0.05$) by 95% confidence interval.

dept showed only significant differences in the duodenum ($P < 0.0001$). The lowest crypt dept was related to the treatment contained 200 ppm TVE which was significantly lower than antibiotic and control treatments. The crypt dept in jejunum and ileum did not show any significant difference.

Table 4. Effect of different levels of thyme essential oil and antibiotic on triglyceride and cholesterol (mg/dL) based on the sex of quail in 35 d[1]

	Triglyceride		Cholesterol	
Treatments	Male	Female	Male	Female
Control	255.70[a]	1,564.0[a]	215.93	268.07
Antibiotic[2]	243.33[ab]	1,161.1[b]	188.60	257.73
T200	170.07[abc]	1,245.7[ab]	189.10	316.50
T300	156.27[bc]	1,091.7[b]	179.33	298.67
T400	143.93[c]	999.0[b]	185.90	309.00
P-value[3]	0.039	0.031	0.509	0.186
SEM	23.060	92.18	12.980	16.180
	P-value for contrasts[3]			
Control vs. antibiotic	0.997	0.128	0.703	0.994
Control vs. T200	0.229	0.285	0.716	0.408
Control vs. T300	0.135	0.063	0.461	0.774
Control vs. T400	0.082	0.024	0.631	0.557
Antibiotic vs. T200	0.355	0.977	1.000	0.246
Antibiotic vs. T300	0.218	0.989	0.991	0.557
Antibiotic vs. T400	0.135	0.814	0.999	0.358

[1]All data are average of 5 replicates.

[2]Antibiotic is 100 ppm flavophospholipol. T200, T300, and T400 indicate the levels of 200, 300, and 400 ppm of *Thymus vulgaris* essential oil.

[3]Significance level ($P < 0.05$) by 95% confidence interval.

[abc] Different superscripts indicate significant differences ($P < 0.05$) within the same column.

Microbial population. In this study, the number of lactobacilli and total coliforms were not significantly affected by experimental treatments (Table 6). However, 400 ppm TVE was better in increasing lactobacilli related to other treatments numerically. Some edible additives derived from herbal products have direct or indirect effect on digestive tract microflora. Although birds take little nutritional advantage from intestinal microflora compared with other species, quail fed on the diets contained different levels of TVE showed lower coliforms compared to birds fed with antibiotic ($P > 0.05$). No differences were observed when lactobacilli and total coliforms data were compared between the treatments.

Lipid oxidation in tissue. The amount of lipid oxidation in breast muscle of quail is shown in Table 7 by TBARS factor. The greatest amount of TBARS produced in quail breast muscle tissue was related to control treatment, which was significantly different from antibiotic, T300, and T400 treatments ($P < 0.05$).

In Vitro Results

TVE components. The composition of TVE used in this experiment is shown in Table 8. In TVE, 29

Table 5. Effect of different levels of thyme essential oil and antibiotic on intestine morphology of quail in 35 d[1]

	Villi height, µm			Villi width, µm			Crypt dept, µm		
Treatments	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
Control	993.3[c]	806.0[c]	750.0[b]	93.3[a]	86.6[ab]	85.3[bc]	84.0[a]	74.6	68.6
Antibiotic[2]	1,007.3[c]	864.0[b]	808.6[ab]	94.6[a]	89.3[b]	89.3[a]	84.0[a]	77.3	68.0
T200	1,051.3[b]	904.6[a]	848.6[a]	84.0[b]	87.3[ab]	84.6[b]	56.0[c]	72.6	62.6
T300	1,059.3[ab]	918.0[a]	870.6[a]	86.0[b]	90.6[ab]	90.6[a]	62.0[b]	78.0	68.0
T400	1,074.6[a]	924.6[a]	758.0[b]	88.0[b]	91.3[a]	88.0[ab]	60.0[b]	74.6	66.0
P-value[3]	<.0001	<.0001	0.023	0.002	0.089	0.011	<.0001	0.185	0.291
SEM	4.61	6.74	21.65	1.31	1.06	0.93	1.00	1.36	1.77
				P-value for contrasts[3]					
Control vs. antibiotic	0.396	0.002	0.496	0.968	0.565	0.137	1.000	0.754	0.999
Control vs. T200	0.0001	<.0001	0.107	0.010	0.994	0.991	<.0001	0.891	0.300
Control vs. T300	<.0001	<.0001	0.041	0.041	0.221	0.035	<.0001	0.587	0.999
Control vs. T400	<.0001	<.0001	0.999	0.171	0.126	0.447	<.0001	1.000	0.881
Antibiotic vs. T200	0.001	0.026	0.787	0.171	0.777	0.070	<.0001	0.294	0.401
Antibiotic vs. T300	0.0003	0.004	0.447	0.015	0.934	0.899	<.0001	0.998	1.000
Antibiotic vs. T400	<.0001	0.001	0.622	0.067	0.777	0.899	<.0001	0.754	0.953

[1]All data are average of 5 replicates.

[2]Antibiotic is 100 ppm flavophospholipol. T200, T300, and T400 indicate the levels of 200, 300, and 400 ppm of *Thymus vulgaris* essential oil.

[3]Significance level ($P < 0.05$) by 95% confidence interval.

[abc] Different superscripts indicate significant differences ($P < 0.05$) within the same column.

Table 6. Effect of different levels of thyme essential oil and antibiotic on intestine microbial population of quail in 35 d (mean log 10 cfu Coliform/g sample)[1]

Treatments[2]	Lactobacilli	Coliforms
Control	6.70	5.20
Antibiotic	7.07	5.49
T200	7.00	5.38
T300	6.46	5.21
T400	7.67	5.12
P-value[3]	0.74	0.53
SEM	0.294	0.368
P-value[3] for contrasts		
Control vs. antibiotic	0.963	0.770
Control vs. T200	0.981	0.999
Control vs. T300	0.993	1.000
Control vs. T400	0.917	0.999
Antibiotic vs. T200	1.000	0.569
Antibiotic vs. T300	0.836	0.652
Antibiotic vs. T400	0.999	0.560

[1]All data are average of 5 replicates.

[2]Antibiotic is 100 ppm flavophospholipol. T200, T300, and T400 indicate the levels of 200, 300, and 400 ppm of *Thymus vulgaris* essential oil.

[3]Significance level ($P < 0.05$) by 95% confidence interval.

components were identified by GC/–MS technique, which represented about 97.5% of the total detected constituents. The main constituents of TVE were thymol (35.40%), durenol (31.09%), *p*-cymene (6.70%), and carvacrol (3.30%) which embraced 76.50% of the essential oil and the rest of the oil consisted of some trace products.

TVE antioxidant properties. As shown in Table 9, IC50 of TVE was 58.48 µg/mL while IC50 of BHT was 20.28 µg/mL. These results were clearly revealed in Figure 1 and showed that by increasing the concentration of essential oil, inhibition percentage of free radicals increased logarithmically while in BHT increased linearly. However BHT indicated greater response relative to its concentration than thyme oil selected levels. TVE had weaker free radical scavenging capacity compared with BHT (IC50 of 58.48 vs. 20.28 µg/mL).

TVE antimicrobial properties. Table 10 shows antibacterial variables of TVE by the well diffusion method that is compared with standard antibiotics. As seen in the data of well diffusion, TVE could inhibit the growth of all bacteria, especially Gram-positive bacteria in all dosage. All the bacteria were removed from the plates contained 4,400 and 8,800 µg/mL of TVE so that the diameter of inhibition zone was more than 60 mm. However, the least inhibition of TVE was related to the plates contained *S. typhimuriumin* with 1,100 µg/mL TVE.

DISCUSSION

The effects of thyme and its active compound thymol on poultry performance parameters have been reported in current research publications and variable results observed (Hoffman-Pennesi and Wu, 2010; Khaksar et al., 2012; Mehdipour et al., 2014; Oceľová et al., 2018). This variability may be due to the basal diet formulation, environment condition, the application dosage of the TVE, and the type of the selected bird (broiler, hen layer, quail, etc.). For example, performance parameters in broilers received 0.01%, 0.05%, 0.1% (w/w) thyme essential oils were not influenced significantly (Oceľová et al., 2018), while the quail fed on 1 g/kg of thyme oil showed greater BWG related to control (Khaksar et al., 2012). Similar to our results, the effects of 60, 100, and 200 mg/kg thymol and carvacrol combination were examined on broiler chicks and revealed that FI reduced linearly, while the birds received 200 mg/kg of this combination received the greatest BWG and feed efficiency (Hashemipour et al., 2013b). By the way, the TVE used in this experiment was low in carvacrol content.

According to Brenes and Roura (2010), essential oils stimulate oronasal sensing and digestive conditioning. The phenolic terpene content in thyme oil may give the diets a taste that was disagreeable to chicks during the early weeks (Cross et al., 2003). Nevertheless, compared with mammals, birds have lower taste bud numbers and fewer taste receptor genes, thus, they have most likely lower taste acuity (Roura et al., 2013). Therefore, reducing feed intake in 400 ppm TVE could not be most likely related to the taste of the essential oil. It seems that the positive effects of thyme on performance may be, in part, attributed to the content of thymol and its effects on improvement of feed consumption efficiency. It is pointed that thymol absorb intensively in the initial sections of the digestive tract (Oceľová et al., 2018). Hereon, it is reported evidences of antioxidant and antimicrobial properties (Botsoglou et al., 2002, Jang et al., 2007), development of nutrients digestibility (Hernandez et al., 2004), and stimulation of digestive enzymes secretion (Jang et al., 2007) in response to substances contained thymol and carvacrol. Therefore, in our experiment 400 ppm TVE may be involved in improving birds' performance by reducing feed

intake in response to efficacy of thymol on quail's health.

Similarly to our findings, thyme essential oil did not influence significantly quail carcass characteristics in the reports of Denli et al. (2004). Nevertheless, it was reported that the percentages of breast and carcass in Japanese quail fed on thyme essential oil were greater than control (Khaksar et al., 2012).

Hypolipidemic effects of some medical plants, their essential oils, or their main components have been reported in several papers (Akbari et al., 2014; El-Ghousein and Al-Beitawi, 2009; Khaksar et al., 2012, Mehri et al., 2015). A study on male broilers fed on a high-viscosity diet supplemented with thymol + carvacrol revealed that the concentration of cholesterol decreased significantly ($P < 0.05$) in broilers received 200 mg/kg thymol + carvacrol though triglyceride did not change (Hashemipour et al., 2013a). However, essential oils or their effective compounds have the ability to decrease the plasma cholesterol and triglyceride levels (Edris, 2007). This event may be due to inhibition of enzymes involved in synthesis of these lipids by monoterpenes (Goldstein and Brown, 1990; Elson, 1995). It has been suggested that the hypocholesterolemic effect of essential oils is due to compounds in essential oil that have the ability to inhibit hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, a key regulatory enzyme in cholesterol synthesis (Yu et al., 1994). However, according to our results, Khattak et al. (2013) observed no differences between the treatments when cholesterol values were compared.

Different parts of small intestine are the areas for absorption of ingested feed and the structure of them is related to their function. So, changes in feed and feed additives may affect the morphology of intestine different parts. In this case, Mohiti-Asli and Ghanaatparast-Rashti (2018) compared

Table 7. Lipid oxidation (Thiobarbituric acid reactive substances, TBARS, mg malondialdeyde/kg) of quail breast muscle tissue after 60 d storage at –22 °C[1]

Treatments	TBARS
Control	0.37[a]
Antibiotic[2]	0.12[b]
T200	0.23[ab]
T300	0.10[b]
T400	0. 10[b]
P-value[3]	0.028
SEM	0.179
P-value for contrasts[3]	
Control vs. antibiotic	0.034
Control vs. T200	0.216
Control vs. T300	0.021
Control vs. T400	0.022
Antibiotic vs. T200	0.332
Antibiotic vs. T300	0.827
Antibiotic vs. T400	0.834

[1]All data are average of 5 replicates.

[2]Antibiotic is 100 ppm flavophospholipol. T200, T300, and T400 indicate the levels of 200, 300, and 400 ppm of *Thymus vulgaris* essential oil.

[3]Significance level ($P < 0.05$) by 95% confidence interval.

[ab]Different superscripts indicate significant differences ($P < 0.05$) within the same column.

Table 8. *Thymus vulgaris* essential oil compounds

Compounds	% Total[1]	compounds	% Total
Thymol	35.40	Bicyclo[3.1.0] hexane, 4-methyl-1-(1-methylethyl)-, didehydro deriv.	0.29
Durenol	31.09	α-Terpineol	0.28
P-Cymene	6.70	4-Terpinenol	0.28
3-Benzylsulfonyl-2,6,6-trimethylbicyclo(3.1.1)heptane	4.58	(−)-Spathulenol	0.26
Carvacrol	3.34	Caryophyllene oxide	0.25
3-Carene	2.99	(+)-Gamma-gurjunene	0.24
Caryophyllene	2.09	Cineole	0.19
Propioin	1.62	Isoborneol	0.09
Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl-, acetate, (1S-exo)-	1.62	Apiol	0.08
2-Carene	1.40	Espatulenol	0.05
Benzene, 1-methoxy-4-methyl-2-(1-methylethyl)-	1.32	Isopropyl myristate	0.05
Thymol, acetate	1.22	(S)-(−)-Limonene	0.05
Caryophyllene	0.95	Isoaromadendrene epoxide	0.04
Methoxy-*p*-cymene	0.50	Unknown	0.01
Eucalyptol	0.45		

[1]Relative percentage obtained from peak area.

the effects of a combined phytogenic feed additive with oregano essential oil on intestinal morphology in broilers. They reported that broilers fed 300 ppm individual oregano essential oil in their diet had larger villi height, villi surface area, villi height to crypt dept ratio, and lower crypt dept in jejunum than those fed either control diet or combined phytogenic feed additive. Also, similar to our findings, Hashemipour et al. (2013b) reported that the inclusion of 100 and 200 ppm thymol and carvacrol increased villi height, surface area, and villi height to crypt dept ratio of jejunum and ileum in broilers. Villi development provided greater absorption surface for availability of nutrients (Awad et al., 2008). It is well known that development of nutrient absorption occurs by increasing the villi height and decreasing crypt depth. Therefore, thyme, essential oil may increase the availability of nutrients by affecting the height, width and crypt dept of the intestine villi. In the current study, 200, 300, and 400 ppm TVE reduced villi width in duodenum of quail. In contrast, the results of a study on rats showed that the villi width improved significantly in duodenum and jejunum of the rats fed thyme volatile oil (Sepehri Moghadam et al., 2014). However, crypt dept did not significantly influence by the treatments in jejunum and ileum but only in duodenum. Based on these results, it seems that supplementation of different levels of TVE in quail feed due to its thymol content has improved intestinal morphology. Accordingly, Du et al. (2016) reported that in the pathogen challenged birds, thymol and carvacrol increased significantly the villi height to crypt depth ratio compared with the basal diet.

Antimicrobial activity is recognized as one of the most important beneficial effects of essential oils, although their precise antimicrobial mechanism is still not fully understood. A large number of in vitro studies have shown that essential oils containing thymol and carvacrol exhibited antimicrobial activity against intestinal microbes such as *Clostridium perfringens*, *S typhimurium*, and *E. coli* (Helander et al., 1998; Hammer et al., 1999). The antimicrobial effect of essential oils is due to their lipophilic properties, which make them easily enter the bacterial and release cell membrane compounds to the external environment (Helander et al., 1998). On the other hand, it seems that the effects of essential oils on gut microflora are not stable, although the essential oils are generally recognized as antimicrobial agents. Therefore, the antimicrobial activity of essential oils in poultry is

Table 9. Antioxidant activity of *Thymus vulgaris* essential oil[1]

Essential oil	*T. vulgaris*, µg/mL	BHT[2], µg/mL
IC50[3]	58.48	20.28
STD	0.039	0.97

[1]Data are average of triplicates.

[2]BHT= beta-hydroxy toluene.

[3]IC50 = the concentration of essential oil that inhibits 50% of free radicals.

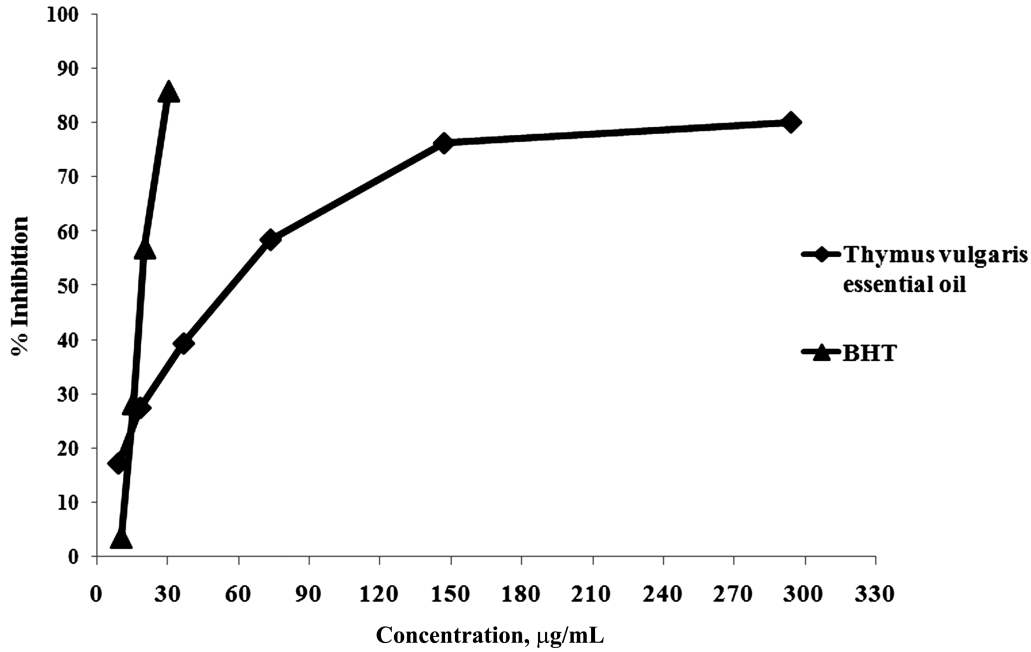

Figure 1. Comparison of % inhibition of free radicals by *Thymus vulgaris* essential oil and BHT. All data are average of triplicates. BHT = β-hydroxy toluene.

Table 10. Zones of growth inhibition (mm) showing antibacterial activity of *Thymus vulgaris* essential oil against 4 bacterial strains assessed by well diffusion method[1]

		Gram (−) bacteria		Gram (+) bacteria	
Compound	Amount, µg/mL	*S. typhimurium* (ATCC 14028)	*E. coli* (ATCC 25922)	*B. cereus* (PTCC1015)	*S. aureus* (PTCC 1337)
T. vulgaris	1,100	10 ± 0.0	28 ± 2.8	12 ± 0.0	50 ± 0.0
	2,200	12 ± 0.0	30 ± 0.0	24 ± 2.8	56 ± 2.8
	4,400	60 <	60 <	60 <	60 <
	8,800	60 <	60 <	60 <	60 <
Positive controls	disk code				
Tetracycline	Te-30[2]	15 ± 1.4	20 ± 0.0	24 ± 1.4	25 ± 4.2
Cephtriaxone	CRO-30[3]	24 ± 2.8	8 ± 0.0	10 ± 1.4	37 ± 0.0

[1]Plate diameter = 60 mm; All data are average of triplicate ± SD.

[2]Te-30 = disks contained 30 µg tetracycline.

[3]CRO-30 = disks contained 30 µg cephtriaxone.

supposed to be influenced by the basal diet and environmental conditions (Jang et al., 2007).

Poultry meat due to containing high concentrations of unsaturated fatty acids with multiple bands is especially susceptible to oxidative degradation (Luna et al., 2010). It has been reported that the essential oils contain phenolic compounds have increasing regenerative capacity, since phenolic compounds prevent oxidation by disabling free fatty radicals and proxy radicals (Huange et al., 2011). Similar to our results, Luna et al. (2010) reported that TBARS of chicken thigh meat samples in control group were larger than the groups fed with 342 mg/kg thymol and carvacrol. In addition, it was reported that the thyme oil, similar to β-hydroxy anisole and β-hydroxy toluene, decreased TBARS in the samples (Saricoban and Tahsin Yilmaz, 2014). In this study, the antioxidant activity of thyme oil may be related to thymol content. In this case, Yanishlieva et al. (1999) proposed that thymol is a more effective and more active antioxidant than carvacrol, because thymol has greater steric hindrance of the phenolic group than carvacrol.

Thymol is a natural monoterpene phenol derivative of *p*-cymene, $C_{10}H_{14}O$, found in oil of thyme and some other species (Austgulen et al., 1987). The thyme essential oil components found here were approximately homogenous with the results of the most studies. For example, Boskovic et al. (2015) showed that thymol, *p*-cymene, linalool, γ-terpinene, and 1,8-cineole were the most dominant compounds of thyme essential oil (50.48%, 24.79%, 4.69%, 4.14%, and 4.35% of the oil, respectively). Additionally, the results of other previous studies revealed that thymol was the most common components of *T. vulgaris* oil (Lee et al., 2005; Ferreira et al., 2016; Foe et al., 2016; Moghaddaszadeh-Ardebili, 2016; Mohammed et al., 2016; Szczepanik et al., 2012). In contrast, Quesada et al. (2016) used a chemotype of TVE with low odor intensity that did not have any thymol and had a very low content of linalool with the major component of 1,8 cineole. This incompatibility also approved by another study that used *T. vulgaris* oil of Eastern Morocco (Imelouane et al., 2009). Therefore, the analysis obtained here showed that our purchased TVE got from a chemotype was rich in thymol and durenol.

In a study, Lee et al. (2005) evaluated antioxidant activity of *T. vulgaris* compounds and found that all chemicals in thyme exhibited dose-dependent inhibitory activity. They found that among the chemicals were identified in the extracts of thyme, the exhibition of potent antioxidant activities was related to thymol, carvacrol (isothymol) and eugenol. It seems that the power of the antioxidant activity of TVE in this study is related to high thymol content of TVE. It is proved that phenolic components like thymol could act as an antioxidant due to high reaction with peroxyl radicals. They deposed of free radicals by hydrogen atom transfer (Amorati et al., 2013). TVE had weaker free radical scavenging capacity compared with BHT. However, BHT is a pure powder without any additional ingredients, while natural essential oils are mixtures of several components, the different types of antioxidants or oxidizable terpenoid components. In fact, the overall performance of thyme oil as antioxidant depending on the complex interplay among components and the oxidizable material, the exact essential oil composition and experimental conditions, and synergistic or antagonistic behavior (Amorati et al., 2013).

It is proved that the effect of essential oils on Gram-positive bacteria is relatively substantial (Hyldgaard et al., 2012). In addition, it is exhibited

that phenolic compounds like thymol are able to disintegrate the outer membrane of Gram-negative bacteria and increase the permeability of the cytoplasmic membrane to ATP (Lambert et al., 2001). In accordance with our results, thyme essential oil could inhibit *E. coli* and *S. typhimurium* growth by the microdilution method (Boskovic et al., 2015). Also, thyme essential oil efficiently killed Salmonella in suspension and prevented biofilms formation (Miladi et al., 2016). Compared with antibiotics (tetracycline and cephtriaxone), TVE acted weaker in removing or inhibiting of bacteria at low concentrations. In fact, the antibacterial effects of TVE may mostly be related to the thymol content, since in many studies it is proved that this substance could inhibit bacterial growth (Trombetta et al., 2005; Mathela et al., 2010; Wattanasatcha et al., 2012).

This study approved antimicrobial and antioxidant properties of TVE oil in vitro and evaluated this oil as feed additive in quail's nutrition for improving performance, organs weight, serum lipids, intestinal morphology, microbial population, and lipid oxidation in storage meat. Comparisons were made between TVE and synthetic substances. Based on the results obtained here, the main component of this essential oil was thymol that may be the main reason for advantages of this essential oil. In addition, although some gut health variables were affected by the treatments, the response for animal growth and efficiency was not found. The effect of TVE on performance was dose dependant. So, TVE in high doses may have the potential to improve quail performance by lowering FI. Also, TVE decreased serum triglycerides dose dependently. The decreased TBARS in storage muscle tissue of quail fed with TVE showed that TVE could reduce the rate of oxidation of quail's muscle tissue during refrigerated storage. TVE could compete with synthetic substances in all aspects in high dose. Therefore, by dosing consideration, TVE is a suitable alternative for synthetic substances especially antibiotic growth promoters without having any adverse effect on quail's health. However, it should be noted that these compounds are antibiotics with both short- and long-term effects. So, it is possible that antibiotic resistance would increase for these natural compounds along the time.

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