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Effect of Shirazi thyme on oxidant status and absorptive surface area of the intestine in cold-induced pulmonary hypertensive broiler chickens

Mohammad Ali Faraji[1], Hossein Hassanpour[2]*, Shahab Bahadoran[3], Waranyoo Kaewduangta[4], Hamed Zarei[5], Morteza Hosseininejad[3], Tahere Karimi Shayan[2]

[1] Department of Basic Sciences, Faculty of Veterinary Medicine, Islamic Azad University, Garmsar Branch, Garmsar, Iran; [2] Department of Basic Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran; [3] Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran; [4] Department of Agricultural Technology, Faculty of Technology, Mahasarakham University, Mahasarakham, Thailand; [5] Department of Physiology, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.

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Abstract

The effects of Shirazi thyme as a medicinal plant on oxidant status (lipid peroxidation, protein oxidation, total antioxidant capacity, and catalase activity) and absorptive surface area were measured in three segments of the small intestine in cold-induced pulmonary hypertensive chickens. Birds were reared at four groups (thyme 0, 0.25, 0.50, and 1.00 % of diet) for 42 days. To induce pulmonary hypertension, the temperature was gradually decreased. The body weight was increased in thyme-0.25% birds compared to control ones, while it was decreased in thyme-1% of birds. The feed consumption was only increased in thyme-1.00% birds. The feed conversion ratio was lower in thyme-0.25% birds and higher in thyme-1.00% birds than control ones. The duodenal and jejunal villus surface area was lower in thyme-1.00% birds than control ones, while it was greater in the thyme-0.50% birds. The ileal villus surface area and duodenal lamina propria thickness were also greater in thyme-0.50% birds. Lipid peroxidation was only decreased in the duodenum and ileum of thyme-0.50% birds compared to control ones, whereas it was increased in the duodenum and jejunum of thyme-1.00% birds. Catalase activity was only elevated in the duodenum and jejunum of thyme-1.00% fed chickens. Total antioxidant capacity was increased in the duodenum, jejunum, and ileum of thyme-0.50% birds. It is concluded that the Shirazi thyme has beneficial effects on growth performance, intestinal absorptive surface area / secretory system, and pulmonary hypertension response at low doses (0.25 and 0.50% of diet), whereas high dose (1.00% of diet) of this plant may be toxic.

Introduction

Shirazi thyme *(Zataria multiflora* Boiss) is a valuable medicinal and condimental plant from the Lamiaceae family.[1] The chemical and pharmacological characteristics of this plant are similar to *Thymus vulgaris* (a well-known medicinal plant). This thyme-like plant is also known as Shirazi thyme. Previous studies have reported that Shirazi thyme has different biological effects including anti-nociceptive, antimicrobial, spasmolytic, anti-inflammatory, anti-leishmaniasis, antiaphthous, anti-ulcerogenic, anti-diabetic, immunostimulatory, hepatoprotective and antioxidant properties.[2-7] It has also been confirmed that Shirazi thyme protects against gastric ulceration via increasing of mucosal secretion and against ϒ irradiation-induced genotoxicity in lymphocytes via scavenging of free radicals.[8-9] This plant stimulates the respiratory burst activity of neutrophils and elevates red blood cell count and hematocrit levels.[10]

The components of Shirazi thyme have been determined in different studies.[4,11-13] The main components were monoterpenes, carvacrol, thymol, zatariol, zataroside A, zataroside B, multiflotriol, multiflorol, linalool, caryophyllene, g-terpinene, and borneol. This plant also contains various alkanes, fatty acids, phytosterols, triterpenes, hydroxycinnamic acids, flavonoids, tannins, resins, and saponins. In mammals and chickens, a wide range of herbs including thyme plant are

***Correspondence:**

Hossein Hassanpour. DVM, PhD
Department of Basic Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran
E-mail: hassanpour-h@vet.sku.ac.ir

known to provide useful effects within the digestive tract and improve growth performance.[14] Stimulation of digestive secretion (enzymes, acid, bile, and mucus) for better digestion and absorption is important in the nutritional action.[15] In broilers, it has been reported that phytogenic feed additives increase intestinal secretion of mucous, decrease the adhesion of pathogens, and even are alternatives for replacement of antibiotic growth promoters.[16] The antimicrobial/antifungal and antioxidant activities of these additives have also been confirmed.[17-18]

In modern broiler chickens with a high growth rate, the respiratory system is not able to supply efficient ventilation and gas exchange leading to hypoxia. Compensatory effects of the cardiovascular system result in increased blood flow, increased stroke volume, increased cardiac output, elevated vasculature pressure in the lung and pulmonary arteries, right ventricular hypertrophy, and increased pressure in the pulmonary arteries, arterioles, and capillaries. This situation finally results in the occurrence of pulmonary hypertension syndrome (PHS) and ascites.[19-20] It has been confirmed that a high level of oxidants is produced in this syndrome leading to aggravating the disease, especially impairs the normal function of digestive tract.[21-22] Several studies have reported that antioxidants could modulate the pathologic process of PHS.[23-25] This study aimed to evaluate the antioxidant effects of *Z. multiflora* Boiss (Shirazi thyme) on the intestine of broiler chickens with cold-induced pulmonary hypertension.

Materials and Methods

Birds and treatments. The experiment was conducted at Shahrekord University, Shahrekord, Iran in accordance with the recommendations of the Guide for the Care and Use Committee of Shahrekord University. Ethical approval was obtained from the Animal Ethics Committee of Shahrekord University (PSG-234/4434). A total of 96, one-day-old fast-growing Ross 308 chickens (average body weight: 44.50 ± 1.25 g) were purchased from Behjoojeh Co. (Shahrekord, Iran) and randomly assigned to four groups with three replicates per group (24 birds per group; eight birds per pen; one control and three thyme groups of birds as treatments). All pens had similar initial weights.

All pens were equipped with a bell drinker and trough feeder. The access of water and feed was *ad libitum* in the floor pens covered with wood shaving litter and chickens were provided with 23 L: 1D lighting program throughout the trial. Chicks were reared under standard conditions except for the temperature and a standard ration recommended by National Research Council (Table 1) for six weeks.[26] Cold stress was gradually induced in all experimental birds according to the temperature program of Hassanpour *et al.*[27] According to that program, the temperature was gradually decreased about 17.00 ˚C from days 1 to 21 and then maintained at 15.00 ˚C until the end of the experiment (42 days). In the treated birds, Shirazi thyme was added to basal diets from day 1 at three dose rates of 0.25, 0.50, and 1.00%. The recording of mortality was done daily after the first week. The dead broilers were autopsied to observe signs of heart dilation, hydro-pericarditis, and fluid accumulation in the ventral hepatic and peritoneal spaces.

Table 1. Composition of basal starter (1-10 days), grower (11-25 days) and finisher (26-42 days) diets.

Item	Starter	Grower	Finisher
Ingredient (g kg^{-1})			
Corn	526.30	542.60	578.90
Soybean meal	526.30	542.60	578.90
Soybean oil	45.00	59.00	54.00
Limestone	12.40	10.10	10.10
Dicalcium phosphate	20.80	18.20	17.10
Vitamin mixture[1]	5.50	5.50	5.50
Mineral mixture[2]	5.00	5.00	5.00
Salt	2.20	2.20	2.20
DL-Methionine	3.10	2.30	2.00
Bicarbonate Na	1.70	1.70	1.70
Threonine	0.70	0.10	0.10
L-Lysine-HCl	2.40	0.60	0.50
Calculated chemical composition			
Metabolizable energy (MJ kg^{-1})	12.72	12.97	13.15
Crude protein (g kg^{-1})	221.00	212.00	191.00

[1] Supplied per kg of diet: Vitamin A (trans retinyl acetate) 9,000 IU; Vitamin D3 (cholecalciferol) 1,500 IU; Vitamin E (dl-α-tocopheryl acetate) 10.00 IU; Vitamin K 0.50 mg; Cobalamin 0.007 mg; Thiamin 0.40 mg; Riboflavin 6.00 mg; Folic acid 1.00 mg; Biotin 0.15 mg; Pantothenic acid 12.00 mg; Niacin 35.00 mg; Pyridoxine 4.00 mg; Cholin chloride 1,000 mg.

[2] Supplied per kg of diet: Mn (from $MnSO_4.H_2O$) 60.00 mg; Cu (from $CuSO_4.5H_2O$) 5.00 mg; Zn (from ZnO) 50.00 mg; I (from $Ca(IO_3)_2.H_2O$) 0.35 mg; Se (from sodium selenite) 0.10 mg; Fe (from $FeSO_4.7H_2O$) 40.00 mg.

Measurement of growth performance. At the end of the rearing period (42 days), body weight and feed consumption were measured in the broilers of all groups on a pen basis and feed conversion ratio was estimated and improved for mortality.

Dissection of the heart and estimation of the ratio of right to total ventricular weight (RV/TV). Four birds from each pen (12 chickens per group) were randomly selected and decapitated at day 42. The heart of each bird was removed and RV/TV was calculated according to Hassanpour *et al.*[21] This ratio is an index to show a progressive rate of pulmonary hypertension syndrome (PHS) and elevation of this ratio more than 0.28 is indicative of induced PHS.[28] Accordingly, the morbidity of this syndrome could be determined via this index.

Estimation of intestinal villus surface area and lamina propria thickness. To evaluate the morphology of small intestine, the midpoint of the duodenum, jejunum (between the bile duct entry and Meckel's diverticulum) and ileum (distal end) were separated at the size of 2.00 cm

from decapitated chickens (12 chickens per group), then fixed in the Clark solution (25.00% acetic acid + 75.00% ethyl alcohol) for 45 min and long maintenance, the samples were transferred to 55.00% ethyl alcohol. Villus surface area was calculated by measurement of height/width of villi and using of the following formula:[24,29]

$$Villus\ surface\ area = \pi \times VW \times VL$$

where, *VW* is the villus width and *VL* is the villus height.

According to Hassanpour *et al*.,[29] the intestinal segments were stained by periodic acid-Schiff reagent for 30 sec, muscle layers were separated from mucosa, and rows of villi were cut, placed between the glass slide and cover-slipped. The height and width of villi were assessed by a light microscope equipped with eyepiece graticules under 10× magnification. The villus height was estimated from the top of the villus to top of the lamina propria. The villus width was measured from the widest part of villus. The lamina propria thickness was also determined at the base of the villus.

Malondialdehyde (MDA), oxidized protein (carbonyl), ferric ion reducing antioxidant power (FRAP), and catalase activity assays. The mucosa of mid-duodenum, mid-jejunum, and mid-ileum was washed with phosphate-buffered saline (pH: 7.40; PBS) and then scratched by a scalpel. The collected mucosa was transferred to PBS solution, homogenized/sonicated for cell lysate, then frozen in liquid nitrogen and stored at –70.00 ˚C for thiobarbituric acid reactive substances (TBARS), oxidized protein (carbonyl) and catalase activity assays. All reagents and kits for the mentioned assays were purchased from Sigma-Aldrich (St. Louis, USA). According to Hassanpour *et al*.,[22] lipid peroxidation of mucosal samples was assessed by TBARS via measurement of produced MDA. In this assay, thiobarbituric acid was added to the mucosal lysate and then incubated in the boiling water bath for 10 min. The samples were centrifuged and supernatants were collected. The absorbance of collected supernatants was measured spectrophotometrically at 532 nm.

The FRAP assay was used to estimate the antioxidant capacity. As this assay has previously been described by Hassanpour *et al*.,[22] it is based on the reduction of ferric tripyridyl triazine (TPTZ) to ferrous colored form in the presence of antioxidants. After adding the FRAP reagent (TPTZ, $FeCl_3$, and acetate buffer) to each sample and incubation at 37.00 ˚C for 4 min, its absorbance was measured spectrophotometrically at 593 nm. The catalase activity and concentration of oxidized proteins containing carbonyl groups were measured in the homogenized/sonicated mucosal samples using spectrophotometer according to Hassanpour *et al*.[22]

The TBARS, FRAP, and oxidized protein data were expressed as micromoles in g total protein of tissue sample (μmol per g protein). Catalase activity data were expressed as units in g total protein of tissue sample (U per g protein). Unit definition: One unit of catalase will decompose 1.00 μL of hydrogen peroxide to oxygen and water per min at pH 7.00 at 25.00 ˚C at a substrate concentration of 50.00 mM hydrogen peroxide. The mass protein of cells was assessed by Bradford method.[22]

Results

Assessment of growth performance. The initial body weight did not differ between experimental groups (data not shown). The growth performance of chickens in the experimental groups is offered in Table 2. The body weight was increased in thyme-0.25% birds compared to control ones ($p < 0.05$), while it was decreased in thyme-1.00% birds and did not change in thyme-0.50% birds. The feed consumption was only increased in thyme-1.00% birds compared to control ones ($p < 0.05$), but it did not change in other birds. The feed conversion ratio was lower in thyme-0.25% birds and higher in thyme-1.00% birds than control ones ($p < 0.05$), while it did not change in thyme-0.50% birds.

Measurement of RV/TV. As shown in Table 2, RV/TV (evidence of pulmonary hypertension) was only less than 0.28 in thyme-0.25% and thyme-0.50% birds, while it was greater in control and thyme-1.00% birds. Statistically, this index was increased in thyme-1.00% birds compared to control ones ($p < 0.05$), while it did not significantly change in thyme-0.25% and thyme-0.50% birds.

Assessment of villus surface area and lamina propria thickness. As shown in Table 3, the duodenal and jejunal villus surface area was lower in thyme-1.00% birds than control ones, while it was greater in the thyme-0.50% birds. This parameter did not change in the duodenum and jejunum of thyme-0.25% birds. The ileal villus surface area

Table 2. Effect of Shirazi thyme on broiler growth performance and the ratio of right to total ventricular weight (RV/TV).

Groups	No.	BW (g)	FC (g)	FCR	RV/TV
Control	24	2057.00[a]	3987.00[a]	1.79[a]	0.31[a]
Thyme-0.25%	24	2133.00[b]	4088.00[a]	1.73[b]	0.27[a]
Thyme-0.50%	24	2087.00[b]	4100.00[a]	1.81[a]	0.27[ab]
Thyme-1.00%	24	1981.00[c]	4212.00[b]	1.88[c]	0.38[b]
Pooled SEM	-	19.70	37.10	0.05	0.08
***p*-value**	-	0.01	0.04	0.04	0.03

BW: Body weight; FC: Feed consumption; FCR: Feed conversion ratio; No.: Total number of chickens (8 chickens per pen).
[abc] Means with different superscripts within each column different are significantly different ($p < 0.05$).

Table 3. Oxidant and morphometrical parameters of intestine in broiler chickens.

parameters	Duodenum					Jejunum					Ileum				
	Control	Thyme			SEM	Control	Thyme			SEM	Control	Thyme			SEM
		0.25%	0.50%	1.00%			0.25%	0.50%	1.00%			0.25%	0.50%	1.00%	
Surface area (mm^2)	3.12^{a}	3.67^{ab}	3.89^{a}	2.61^{c}	0.20	2.52^{a}	2.69^{ab}	2.91^{b}	1.81^{b}	0.22	0.66^{a}	0.63^{a}	0.84^{b}	0.60^{a}	0.15
Lamina propria (mm)	0.41^{a}	0.45^{a}	0.67^{b}	0.48^{a}	0.07	0.39	0.34	0.42	0.30	0.09	0.27	0.31	0.36	0.25	0.09
MDA (µmol g^{-1} protein)	0.49^{a}	0.40^{a}	0.28^{b}	0.62^{c}	0.02	0.39^{a}	0.33^{a}	0.34^{a}	0.53^{b}	0.04	0.39^{a}	0.34^{a}	0.19^{b}	0.40^{a}	0.02
PC (µmol g^{-1} protein)	3.78	3.69	3.45	3.95	0.65	3.98	3.77	3.54	4.23	0.86	2.66	2.54	2.01	2.98	0.65
CAT activity (U g^{-1} protein)	420.10^{a}	431.30^{a}	439.10^{a}	523.60^{b}	21.30	428.50^{a}	441.20^{a}	438.20^{a}	560.10^{b}	23.40	312.30	321.60	344.10	318.90	19.30
FRAP (µmol g^{-1} protein)	0.97^{a}	0.99^{a}	1.66^{b}	1.12^{ab}	0.08	0.94^{a}	0.90^{a}	1.55^{b}	1.11^{ab}	0.08	0.78^{a}	0.69^{a}	1.43^{b}	1.03^{ab}	0.07

MDA: Malondialdehyde, PC: Protein carbonyl, CAT: Catalase, FRAP: Ferric ion reducing antioxidant power.
^{abc} Means with different superscripts within each column different are significantly different ($p < 0.05$).

was also greater in thyme-0.50% birds than control ones ($p < 0.05$), while it did not change in the thyme-0.25% and thyme-1% birds. The duodenal lamina propria thickness was greater in thyme-0.50% birds than control ones ($p < 0.05$), while it did not change in the thyme-0.25% and thyme-1.00% birds. The jejunal and ileal lamina propria thickness did not significantly change in the thyme-treated birds compared to control ones.

Assessment of TBARS, catalase activity, and oxidized protein (carbonyl). Results of TBARS (lipid peroxidation), carbonyl (protein oxidation), and FRAP (total antioxidant capacity) assays as well as catalase activity in the duodenum, jejunum, and ileum are presented in Table 3. Lipid peroxidation was only decreased in the duodenum and ileum of thyme-0.50% birds compared to control ones ($p < 0.05$), whereas it was increased in the duodenum and jejunum of thyme-1.00% birds ($p < 0.05$). Lipid peroxidation did not change in the intestine of thyme-0.25% birds. Catalase activity was only elevated in the duodenum and jejunum of thyme-1.00% group compared to control one ($p < 0.05$), while this parameter did not change in the intestine of other treated birds.

Total antioxidant capacity was increased in the duodenum, jejunum and ileum of thyme-0.50% birds compared to control ones ($p < 0.05$), whereas it did not change in the intestine of other birds. Protein oxidation was not different between thyme-treated birds and control ones.

Discussion

This research was designed to investigate growth performance, pulmonary hypertensive response, oxidant status, and absorptive surface area of the intestine in the chickens with pulmonary hypertension syndrome induced by cold stress and treated by Shirazi thyme. Lipid peroxidation, protein oxidation, antioxidant capability, and catalase activity were evaluated as an index of oxidant status. The present study showed that different doses of Shirazi thyme could imply different effects on growth performance of chickens which is in agreement with the previous studies.[30-32] Those studies also confirmed positive, negative, or insignificant effects of thyme on chicken performance.

The results showed that the Shirazi thyme could modulate pulmonary hypertensive response and decrease hypertrophy and dilation of the heart in the low doses. This effect of the Shirazi thyme was predictable because of its confirmed antioxidant aspect. Previous studies have shown that the Shirazi thyme acts as nitric oxide and MDA scavenger and thus decreases nitrosative stress and lipid peroxidation.[2,5] In our study, the results of the oxidant and antioxidant status of three parts of the intestine were also evidenced that Shirazi thyme (0.50% of diet) could improve the antioxidant capability of enterocytes and reduce lipid peroxidation in the intestine of pulmonary hypertensive chickens. As our data showed, this anti-oxidant effect of the Shirazi thyme was able to improve the absorptive surface area of the intestine and increase the capability of intestinal secretion due to the increase of lamina propria thickness in the duodenum. Lamina propria is the main place of intestinal glands, so the change of its thickness could be an index for growth and development of the intestinal glands and subsequent intestinal secretion.[33,34] Zamani Moghaddam *et al.*[24] have determined the failure in the intestine of chickens with pulmonary hypertension and they have showed that dietary antioxidant could improve intestinal function via gut morphology improvement. Miller *et al.*[35] have reported that dietary antioxidants preserve intestinal epithelia from pro-apoptotic oxidant stress, and increase epithelial cell growth. Ronco *et al.*[36] have suggested that antioxidants reduce lipid peroxidation and apoptosis and increase cell proliferation. Parish *et al.*[37] have reported that vitamin C increases epidermal cell mitosis and Pirmohamammadi *et al.*[38] and Abdulkarimi *et al.*[39] have determined that dietary supplementation of thyme improves the meat quality of broiler chickens through decreasing the MDA concentration. However, these studies could partially justify the improved absorptive area of intestine via Shirazi thyme in the pulmonary hypertensive chickens. According to our data, growth performance was improved in the chickens treated by low dose of Shirazi thyme, while at its high dose, the performance was diminished. The high dose of Shirazi thyme produced the high amount of MDA as an index of lipid peroxidation in the intestine which may confirm the adverse effect of Shirazi thyme during its high consumption. The defect of total antioxidant capacity to

elevate against this lipid peroxidation could also be another evidence that high dose of Shirazi thyme might be toxic. The toxic effect of Shirazi thyme has been reported previously.[4] The prior study was disclosed that both the oil and extract form Shirazi thyme have cytopathologic effects on the cultured cells at specific concentrations.[40] It has also been reported that high intake of this plant shows side effects such as mucosal membranes stimulation, dizziness, nausea, vomiting and dermatitis in human. However, our data confirmed that Shirazi thyme in the high dose not only decreases absorptive surface area but also exacerbates pulmonary hypertension in chickens.

It was concluded that Shirazi thyme has beneficial effects on the broiler chicken performance (i.e., body weight and feed consumption rate), intestinal absorptive surface area/secretory system and pulmonary hypertension response at low doses (0.25 and 0.50% of diet), whereas a high dose of this plant (1.00% of diet) not only doesn't show any useful effect but also diminishes growth performance/gut morphology and exacerbates pulmonary hypertension in chickens.

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Conflict of interest

The authors declare no potential conflict of interest.

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