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Research Paper

Antioxidant Effects of Marjoram (*Origanum Majorana* L.) Extract on Intestinal Morphology in Chickens With Pulmonary Hypertension

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ABSTRACT

Background: Marjoram is an herbal plant with different medicinal effects. This study evaluates its impact on enzymatic antioxidant status, growth performance, intestinal mucosa morphology, and pulmonary hypertensive response in cold-induced pulmonary hypertensive chickens.

Materials and Methods: Broiler chicks were reared for 35 days under cold stress and treated with 0.05% vitamin C (positive control) and 0 (control), 0.1%, or 0.2% marjoram extracts. Serum malondialdehyde (MDA) and activity of catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD) were assayed on day 35. Meanwhile, gene expression of these enzymes was evaluated in the duodenum.

Results: The right ventricle to total ventricles (RV:TV) ratio was lower in all treatments of chickens than control ($P<0.05$). The feed conversion ratio was only decreased in the chickens fed marjoram-0.2%. Lipid peroxidation was reduced in all groups, while the CAT activity was increased in the marjoram-0.2% group compared to the control ($P<0.05$). In the lung, *SOD*, *CAT*, *and* GPX transcripts were decreased in the marjoram-0.2% group compared to the control ($P<0.05$). In the right ventricle of the heart, *SOD* and *CAT* transcripts were increased in the marjoram-0.2% group compared to other groups of chickens, whereas *GPX* transcript was decreased ($P<0.05$). In comparison to the control, the chickens fed vitamin C and marjoram had longer duodenal villus and more surface area, and their villus lamina propria was thicker ($P<0.05$).

Conclusion: Supplementation of marjoram could modulate pulmonary hypertensive response and ameliorate intestinal morphology through its antioxidant effects.

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Introduction

Herbal plants are among the principal sources of human and animal drugs and are essential to the world's healthcare systems. Increasing interest has been recently developed in animal nutrition and the potential impact of medicinal plants and herbs on the growth performance and treatments of diseases [1]. Herbal plant extracts had been applied in times BC in the Middle East to treat different disorders. Among these herbs, marjoram (*Origanum majorana* L.) of the Lamiaceae family is the endemic origanum species in Iran (Common Persian name: Marzanjoosh or Marzangoosh) [2, 3], and its main components are terpinene-4-oil, cis-sabinene hydrate, p-cymene, and δ-terpinene [4]. Some species of this genus have several medicinal effects, such as antibacterial, antifungal, antiviral, antiparasitic, antiseptic, antispasmodic, expectorant, digestive, carminative, and antioxidant activity [5].

In many experiments, marjoram has been supplemented to diet to evaluate its impact on the growth performance and feed conversion of chickens [6-8]. While some of these studies indicated that marjoram did not influence feed conversion or growth, many of them demonstrated the plant's beneficial effects on the mentioned parameters.

Because of the beneficial effects of marjoram in different diseases and their antioxidant effect, it is permissible that this medicinal plant improves pulmonary hypertension syndrome (PHS; ascites), which is a severe metabolic disorder in broiler chickens. In this syndrome, the fast growth of broiler chickens elevates the oxygen demand and then increases cardiac output. The vascular capacity of the lungs in the chickens is anatomically unable to respond to this raised cardiac output. Then, it causes a progressive elevation of pulmonary vasculature resistance, which is associated with an imbalance between pulmonary vasoconstrictors and vasodilators [9-11]. This phenomenon finally results in hypoxemia, right-sided congestive heart failure, major venous congestion, cirrhosis of the liver, and accumulation of ascitic fluid into the abdominal cavity [12]. A critical pathophysiologic factor contributing to PHS is the production of free radicals. High production of free radicals could damage the heart and lungs and exaggerate PHS [12, 13]. According to previous studies, free radicals negatively impact the gastrointestinal and cardiovascular systems of pulmonary hypertensive chickens [14, 15]. Marjoram with its antioxidant and other valuable effects may modulate the cold-induced pulmonary hypertensive response. This study investigates whether two concentrations (0.1% and 0.2%) of marjoram extracts could reduce ascites incidence and improve antioxidant status, growth performance, and intestinal morphology.

Materials and Methods

Plant preparation and extraction

The marjoram plant used in this experiment was collected in the summer when the plant was in the vegetative stage in Chahar Mahal Bakhtiari Province, Iran. Collected leaves were shadow-dried and ground with a laboratory hammer mill. The dried leaf powder was mixed with methanol 80% with a ratio of 2:10. Then, it was shaken for 15 min to be thoroughly mixed and kept for 24 h at room temperature, after that passed through a Whatman filter paper no. 1, and then dried at 40 ˚C for 48 h. Then, 11 g of extract was obtained from every 100 g of marjoram powder. Gas chromatography-mass spectrometry was utilized to identify the chemical constituents of the marjoram extract.

Rearing and treatment of broiler chickens

A total of 144 one-day-old broiler chickens (Ross 308 strain) were randomized across 12-floor pens measuring 1.5 m × 1 m × 1 m each. Chicks were assigned to four groups (two controls and two treatments) with three replicate pens per group and twelve chickens per pen. Chickens were housed on the floor in a deep litter system with wood shaving, reared in standard conditions (temperature, ventilation, and light) for 35 days, and had free access to water and feed. A mash form of normal basal diet was formulated for the starting (1–10 days), growing (11–24 days), and finishing (25–35 days) growth stages which were mainly composed of corn and soybean meal according to Bahadoran et al. [16]. For the treatments, the marjoram was added to the starter, grower, and finisher basal diets at concentrations of 0.1% and 0.2%. A cold temperature program was implemented in the rearing room to induce PHS in all groups of chickens [17, 18]. This program gradually reduced the temperature from 30 ˚C to 15 ˚C on days 1 to 35. In each pen, feed consumption (FC) and body weight were recorded every day to calculate weight gain (WG) and feed conversion rate (FCR). We recorded the mortality rate of broilers for each group.

Assessment of pulmonary hypertension index

All chickens of each group were euthanized at the end of rearing (35 days of age). The heart was dissected and

weighed, and then the index of the heart (right ventricle to total ventricle ratio; RV:TV) was calculated for each chicken. Right ventricular hypertrophy and pulmonary hypertension were estimated by this index as described by previous studies [19, 20]. According to this index, broilers with an RV:TV ratio of more than 0.25 have developmental pulmonary hypertension, while chickens with an RV:TV ratio ≥0.29 indicate clinical pulmonary hypertension syndrome, i.e. ascites with considerable right ventricular dilation [21].

Morphometric analysis of duodenum

According to Hassanpour et al. [22], morphometric variables of the duodenum (villus length, width, surface area, and lamina propria thickness) were assessed in the nine chickens per group. Briefly, in this method, the midpoint piece of the duodenum was fixed in Clark fixative solution and then ethyl alcohol. After staining with periodic acid-Schiff, sagittal cutting of the villus rows was provided and then placed between a glass slide and cover slip. To measure villus parameters, a microscope with the magnification of an eyepiece graticule (10×) and objective lens (10×) was used. The villus length was identified as the top of the villus to the top of the lamina propria. The villus surface area was calculated as "π × villus width × villus length". The lamina propria thickness (the site of Lieberkuhn glands) was measured from the base of the villus to the top of the muscular layer of mucosa.

Measurement of malondialdehyde (MDA) and serum catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD) activities

Serum CAT, GPX, SOD activities (indicators of enzymatic antioxidant defense system), and MDA (an indicator of lipid peroxidation), levels were assayed on day 35 (nine chickens per diet) according to Hassanpour et al. [23]. CAT activity was measured based on catalysis of the decomposition of hydrogen peroxide per minute per total serum protein. GPX activity was estimated as the amount of nicotinamide adenine dinucleotide phosphate consumed per minute per milligram of total serum protein in the sample. SOD activity was measured as the percent of inhibition of reduction in nitro blue tetrazolium chloride by SOD per total serum protein of the sample.

Ribonucleic acid extraction, complementary DNA synthesis, and quantitative real-time polymerase chain reaction analysis

Lung and heart (right ventricle) tissues were processed for total ribonucleic acid (RNA) isolation by RNX-Plus solution (Sinaclon Bioscience, Karaj, Iran), according to Pirany et al. [24]. To ensure that probable contamination (genomic DNA) of extracted RNA is removed, the RNase-free DNase (Sinaclon Bioscience, Iran) was used. Only RNA samples exhibiting an A260/A280 ratio of more than 1.9 were used for the complementary DNA (cDNA) synthesis. PrimeScript TM RT reagent Kit (Takara Bio Inc., Japan) was used to synthesize cDNA according to the previous study [25].

The specific primers of CAT, GPX, SOD, and *YWHAZ* (as an endogenous standard) [26] were prepared according to Hassanpour et al. [23] to determine their relative amounts by quantitative real-time polymerase chain reaction (PCR) (Table 1).

Previously, it has been checked that *YWHAZ* is the best housekeeping gene in chicken pulmonary hypertension [26].

SYBR®PremixExTaq™II (Tli RNaseH Plus) kit (Takara BioInc., Japan) was used to do PCR. The thermal program for 40 cycles was 95 ˚C for 40 s, 66 ˚C for 30 s, and 72 ˚C for 30 s. [23, 27]. The mRNA level of each target gene relative to *YWHAZ* was estimated for each

Table 1. Primers used for quantitative real-time polymerase chain reaction analysis of chicken messenger ribonucleic acids

Target	Primers	PCR Product	Accession No.
YWHAZ	5′-AGGAGCCGAGCTGTCCAATG-3′ 5′- CTCCAAGATGACCTACGGGCTC-3′	84 bp	NM_001031343.1
SOD	5′-CACTGCATCATTGGCCGTACCA-3′ 5′-GCTTGCACACGGAAGAGCAAGT-3	233 bp	NM_205064.1
GPX	5′-GCTGTTCGCCTTCCTGAGAG-3′ 5′-GTTCCAGGAGACGTCGTTGC-3′	118 bp	NM_001277853.1
CAT	5′-TGGCGGTAGGAGTCTGGTCT-3′ 5′-GTCCCGTCCGTCAGCCATTT-3′	112 bp	NM_001031215.1

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Abbreviations: SOD1: Superoxide dismutase 1; CAT: Catalase; GPX: Glutathione peroxidase; YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta; bp: Base pair.

sample with the Pfaffl method, as described previously by Hassanpour et al. [17].

Statistical analysis

The data are represented as Mean±SE. Data normality was assessed using the Shapiro-Wilk test, and then statistical comparisons were made using Duncan's multiple range test following a one-way analysis of variance. All statistical analyses were computed by SPSS software version 26 (SPSS Inc, Chicago, IL, USA). P<0.05 was considered significant.

Results

Marjoram compositions

Table 2 represents the major compositions of marjoram. Specific constituents of terpinen-4-ol, spathulenol, c-terpinene, α-terpineol, neophytadiene, β-caryophyllene, and p-cymol were most present in the leaf extract, respectively.

RV:TV ratio and mortality rate

The RV:TV ratio as an index of PHS was decreased to less than 0.25 in vit C (0.23±0.017), marjoram-0.1% (0.24±0.020), and marjoram-0.2% (0.23±0.025) groups of chickens compared to the control group (0.35±0.035) at 35 days of age (P<0.05). The RV:TV ratio was not significant between different treated groups (P>0.05). Ascites mortality was 11.1%, 5.6%, 8.3%, and 8.3% in the control, vitamin C, marjoram-0.1% and 0.2% groups of chickens, respectively.

Growth performance

The growth performance of all groups of chickens is given in Table 3. The initial body weight was similar between groups (data not shown). Parameters of growth performance (WG, FC, and FCR) did not significantly change in the experimental groups at 3-21 and 3-35 days of the rearing period. The WG of chickens in all treatments was increased in days 22-35 compared to control (P<0.05), while the FCR was only decreased in the chickens fed marjoram-0.2%. FC did not differ among groups, and also FCR and WG were not significant between vitamin C and marjoram groups of chickens (P>0.05) (Table 3).

Table 2. The major compositions of leaf extract of marjoram (*O.majorana* L.)

Compound	T
Terpinen-4-ol	33.1
Spathulenol	10.2
c-terpinene	6.5
α-terpineol	5.1
β-caryophyllene	2.9
p-cymol	2.8
cis-sabinene hydrate	1.3
Terpenyl-ester	1.9
Linalool	1.7
α-terpinene	1.5
Neophytadiene	3.9
β-pinene	0.3
β -myrcene	0.2
Camphene	0.2
Not identified	24.4

Table 3. Values of growth performance (WG, FC, and FCR) for chicken in four groups in different days

Days	Group	WG (g)	FC (g)	FCR
3 to 21	Control	671.8[a]	1048.4[a]	1.56[a]
	Vitamin C	666.4[a]	986.2[a]	1.48[a]
	Marjoram (0.1%)	676.3[a]	1021.2[a]	1.51[a]
	Marjoram (0.2%)	682.9[a]	990.3[a]	1.45[a]
	Pooled SEM	8.22	10.90	0.45
22 to 35	Control	855.8[b]	1890.8[a]	2.21a
	Vitamin C	1018.8[a]	1976.6[a]	1.94[ab]
	Marjoram (0.1%)	1017.3[a]	2014.3[a]	1.98[ab]
	Marjoram (0.2%)	1074.2[a]	1869.2[a]	1.74[b]
	Pooled SEM	10.46	11.47	0.09
3 to 35	Control	1544.2[a]	2980.2[a]	1.93[a]
	Vitamin C	1591.4[a]	2896.3[a]	1.82[a]
	Marjoram (0.1%)	1627.8[a]	3042.1[a]	1.87[a]
	Marjoram (0.2%)	1741.4[a]	2976.1[a]	1.71[a]
	Pooled SEM	13.30	14.23	0.51

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Abbreviations: WG: Weight gain; FC: Feed consumption; FCR: Feed conversion ratio; Pooled SEM: Pooled standard error of the mean.

Notes: Each mean represents values from 30 replicates. Superscripts in the same column with different letters are significantly different (P<0.05).

Morphometric assessment of duodenum

Morphologic parameters of duodenal villi in the different groups of chickens are given in Table 4. The duodenal villus length and surface area in the chickens fed vitamin C were higher than control (P<0.05). The villus lamina propria thickness in the chickens fed vitamin C and marjoram was also increased compared to the control (P<0.05). The duodenal villus width did not change between the experimental groups (P>0.05).

Assessment of lipid peroxidation and SOD, serum catalase, and GPX activities

Data on lipid peroxidation (MDA measurement) and antioxidant enzyme activities in the serum samples of chickens are presented in Table 5. MDA was decreased in all treatments compared to control (P<0.05). SOD and GPX activities were only increased in the vitamin C group, whereas CAT was only increased in the marjoram-0.2% group compared to the control (P<0.05). Three enzyme activities did not change in the group of marjoram-0.1%.

Gene expression of antioxidant enzymes

Relative expression of *SOD*, *CAT*, and *GPX* genes in the lung and heart tissues is presented in Table 6. In the lung, the relative expression of *CAT*, *SOD*, and *GPX* genes was decreased in the marjoram-0.2% group compared to other groups of chickens (P<0.05). The expression of these three genes did not change in the vitamin C and marjoram-0.1% groups compared to the control (P>0.05).

In the right ventricle of the heart, the relative expression of *SOD* and *CAT* genes were increased in the marjoram-0.2% group compared to other groups of chickens (P<0.05), whereas the relative expression of the *GPX* gene was decreased in the marjoram-0.2% group compared to other groups (P<0.05). The relative expression of these three genes did not change in the marjoram-0.1% group of chickens compared to the control and Vit C groups (P>0.05).

Table 4. Morphologic parameters of duodenal villi in the different groups of chickens

Diet	Length (mm)	Width (mm)	Lamina Propria (mm)	Surface Area (mm²)
Control	0.74^{bc}	0.34^{a}	0.78^{b}	0.80^{b}
Vitamin C	0.92^{a}	0.45^{a}	0.87^{a}	1.28^{a}
Marjoram (0.1%)	0.80^{c}	0.35^{a}	0.88^{a}	0.87^{b}
Marjoram (0.2%)	0.77b^{c}	0.37^{a}	0.87^{a}	0.89^{b}
Pooled SEM	**0.016**	**0.043**	**0.012**	**0.034**

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Pooled SEM: Pooled standard error of the mean.

Notes: Superscripts in the same column with different letters are significantly different (P<0.05). Length indicates from the top of the villus to the top of the lamina propria, width shows the width of the villus at the base. The lamina propria is the space between the base of the villus and the top of the muscularis mucosa. Villus surface area is calculated as "π × length × width". Each mean represents values from 9 replicates.

Table 5. Effects of vitamin C and marjoram extracts on mean plasma oxidant and antioxidant status of Ross 308 broiler chickens*

Diet	MDA (µmol/mL)	CAT (U/mg Protein)	SOD (% Inhibition)	GPX (U/mg Protein)
Control	36.1^{b}	107.4^{a}	17.1^{a}	12.1^{a}
Vitamin C	22.3^{a}	111.1^{a}	28.1^{b}	18.3^{b}
Marjoram (0.1%)	20.7^{a}	89.7^{a}	12.8^{a}	14.7^{ab}
Marjoram (0.2%)	20.2^{a}	157.1^{b}	14.3^{a}	15.3^{ab}
Pooled SEM5	**5.13**	**10.26**	**4.36**	**3.88**

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Abbreviations: MDA: Malondialdehyde; CAT: Catalase; GPX: Glutathione peroxidase; SOD: Superoxide dismutase; Pooled SEM: Pooled standard error of mean.

Notse: Superscripts in the same column with different letters are significantly different (P<0.05). Each mean represents values from 9 replicates.

Discussion

In the present study, vitamin C was used as a positive control. Our previous study has already validated its positive impacts on chicken growth performance, pulmonary hypertensive response, and intestinal morphology [28]. Then, a comparison between the effects of vitamin C and marjoram could prove beneficial. The findings, as indicated by the RV:TV ratio, demonstrated that marjoram could decrease pulmonary hypertensive response in broilers. Chen and Chen [29] reported that phenolic acids and tanshinones improve atherosclerosis, thrombosis, and myocardial injuries. Bina and Rahimi [5] also determined that marjoram inhibits lipid peroxidation and nitric oxide synthesis in the heart tissue to improve cardiovascular dysfunction.

Although many reports have shown that marjoram improves body WG, food consumption, or feed conversion ratio [7, 8, 30], there are also conflicting reports [6, 31, 32]. Various forms of marjoram products (including oil, essential oil, powder, and extract) at different concentrations were utilized in these reports. Indeed, the compounds and characteristics of marjoram would be different in these products and then considerably change the effects of marjoram in the chickens. In this study, marjoram extract was employed in the context of developmental pulmonary hypertension, with only the 0.2% marjoram extract demonstrating improved FCR in 22-35 days.

In the studies of Solis de Los Santos et al. [33] and Zamani Moghaddam et al. (2009), it was reported that digestive system damages occur in the PHS. Many studies suggested different additives and supplements (e.g. vitamin C, L-arginine, garlic) in the diets of pulmonary hy-

Table 6. Effects of vitamin C and marjoram extracts on mean relative gene expression (target/*YWHAZ*) of Ross 308 broiler chickens

Group		CAT	SOD	GPX
Lung	Control	0.09[a]	3.75[a]	4.65[a]
	Vitamin C	0.10[a]	4.16[a]	5.63[a]
	Marjoram (0.1%)	0.08[ab]	3.30[a]	4.24[a]
	Marjoram (0.2%)	0.05[b]	1.18[b]	1.06[b]
	Pooled SEM	0.007	0.097	0.184
Right ventricle of heart	Control	0.19[a]	1.31[a]	4.39[a]
	Vitamin C	0.27[a]	3.67[a]	3.35[a]
	Marjoram (0.1%)	0.27[a]	2.33[a]	4.96[a]
	Marjoram (0.2%)	1.83[b]	6.24[b]	1.76[b]
	Pooled SEM	0.089	0.261	0.153

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MDA: Malondialdehyde; CAT: Catalase; GPX: Glutathione peroxidase; SOD: Superoxide dismutase; Pooled SEM: Pooled standard error of mean.

Notes: Superscripts in the same column with different letters are significantly different ($P<0.05$). Each mean represents values from 9 replicates.

pertensive chickens for improving the PHS and intestinal damage [21, 22, 28, 34]. In our study, we determined that oral supplementation of marjoram extracts changes the intestinal morphology (duodenum) and increases the thickness of lamina propria in pulmonary hypertensive broilers. The effect of marjoram on lamina propria thickness could be evidence of its improvement in intestinal secretion. The beneficial effect of marjoram on the intestine may be associated with its antioxidant and anti-apoptotic effects [2, 30, 35]. Dietary antioxidants, such as vitamin C protect enterocytes against oxidative stress, promote epithelial proliferation, and improve villus morphology [28, 36]. Therefore, the antioxidant effect of marjoram could be an essential factor in protecting the intestine against oxidative damage in PHS. This useful effect of marjoram may also be observed in the heart, lung, and blood vessels which are crucial organs in PHS. Different studies have determined that compounds such as terpinen-4-ol, spathulenol, c-terpinene, and α-terpineol have strong anti-oxidative, anti-inflammatory, antifungal, and antibacterial effects [37-40]. These compounds were considered the major constituents of marjoram leaf extract in our study. Therefore, the potent influence of marjoram constituents on the oxidant and antioxidant status may account for its enhancement in developmental PHS and its additional benefits in the intestine. In our study, the decrease in lipid peroxidation and increase in CAT activity could partially confirm the antioxidant properties of marjoram in PHS [16]. Abdel-Wahab [30] reported that high doses of marjoram (1%-2.5% of diet) increased GPX activity in healthy chickens. Kamel [41] found that marjoram increased CAT, SOD, and GPX activities in the diabetic rats. However, the antioxidant effect of marjoram may be related to its dose and cell condition. There is a complex antioxidant protection system in the cells and organs. This system consists of enzymatic and non-enzymatic antioxidants with several members. The members work together and regulate each other to neutralize and scavenge free radicals. Enzymatic antioxidants (such as SOD, CAT, and GPX) are the essential members of this protective system that play a crucial role in cellular defense [42]. In this study, marjoram did not significantly affect the enzymatic antioxidants (except for CAT). Marjoram even down-regulated the gene expression of those enzymes in the lung and heart. Therefore, marjoram may also influence the other members of the total antioxidant system to reduce lipid peroxidation.

Conclusion

The findings of this study suggest that dietary supplementation of marjoram improves pulmonary hypertension and ameliorates intestinal secretion in broiler chickens through its antioxidant effects.

Ethical Considerations

Compliance with ethical guidelines

All the procedures in this study were approved by the Institutional Animal Care and Use Committee of Shahrekord University, Shahrekord, Iran (Code: IR.SKU.REC.1396.088), based on the welfare standard of the 1964 Declaration of Helsinki.

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Authors' contributions

Study and Experimental design: Shahab Bahadoran; Major experimental work, Data analysis, and writing: Hossein Hassanpour, Younes Teymouri, and Abdonnaser Mohebbi.

Conflicts of interest

The authors declared no conflict of interest.

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