

Antioxidant effects of marjoram (*Origanum majorana* L.) and its improvement of the intestinal mucosa morphology in pulmonary hypertensive chickens

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Abstract

Background

Marjoram as an herbal plant has different medicinal effects. In this study, the effects of dietary marjoram extract on the enzymatic oxidant (lipid peroxidation)/antioxidant status (enzyme activity and gene expression), growth performance, intestinal mucosa morphology (duodenum), and pulmonary hypertensive response were evaluated in cold-induced pulmonary hypertensive chickens.

Results

Chicks were reared for 35 days and treated with 0.05% vitamin C (positive control) and 0 (control), 0.1, or 0.2% marjoram extracts. Pulmonary hypertension syndrome was induced using cold stress in all groups. RV: TV ratio was lower in all treatments of chickens than in the control ($p < 0.05$). Weight gain of chickens in all treatments was increased in days 22–35 compared to control ($p < 0.05$) while the feed conversion ratio was only decreased in the chickens fed marjoram 0.2%. Lipid peroxidation was decreased in all groups while the activity of catalase (CAT) was increased in the marjoram 0.2% group compared to the control ($p < 0.05$). In the lung, superoxide dismutase (SOD), CAT, and glutathione peroxidase (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 ($p < 0.05$). The duodenal villus length and surface area in the chickens fed vitamin C, and villus lamina propria thickness in the chickens fed vitamin C and marjoram were higher than control ($p < 0.05$).

Conclusion

The dietary supplementation of marjoram could modulate pulmonary hypertensive response in broilers and ameliorate intestinal secretion through its antioxidant effects.

Background

Herbal plants are among the principal sources of human and animal drugs and have an essential role in the world's health care 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 organum 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]. Many of these experiments showed positive effects of this plant on the mentioned parameters, while others concluded no effect of marjoram on growth and feed conversion.

Because of the beneficial effects of marjoram in different diseases and their antioxidant effect, it would be 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 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, 10]. 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 [11]. One of the critical pathophysiologic factors is free radicals in PHS. High production of free radicals could damage the heart and lungs and exaggerate PHS [11, 12]. Previous studies reported that free radicals have harmful effects on the cardiovascular and gastrointestinal systems of pulmonary hypertensive chickens [13, 14]. Marjoram with its antioxidant and other valuable effects may modulate the pulmonary hypertensive response due to cold stress. We investigated whether two concentrations (0.1 and 0.2%) of marjoram extracts could reduce the ascites incidence and improve antioxidant status, growth performance, and intestinal morphology.

Materials And Methods

All procedures, including animal welfare, rearing, and experimental procedures were approved by the Institutional Animal Care and Use Committee of Shahrekord University (approved code: IR.SKU.REC.1396.088).

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 hr at room temperature, after that passed through a Whatman filter paper No. 1, and then dried at 40 °C for 48 hr. From each 100 g marjoram powder, an amount of 11 g extract was obtained. Identification of chemical constituents of the marjoram extract was performed by gas chromatography-mass spectrometry.

Rearing and treatment of broiler chickens

A total of 144 one-day-old broiler chickens (Ross 308 strain) was randomized across 12-floor pens measuring 1.5m × 1m × 1m each. Chicks were assigned to four groups (two controls and two treatments) with three replicates 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. (2022) [15] [15] [15] [15] [15] [15] [15] [15]. For the treatments, the marjoram was added to the starter, grower, and finisher basal diets at concentrations of 0.1 and 0.2%. In the rearing room, a cold temperature program was applied to induce PHS in all groups of chickens [16, 17]. In this program, the temperature was gradually reduced 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, 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 [18, 19]. 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 $\geq$ 0.29 indicate clinical pulmonary hypertension syndrome, i.e. ascites with considerable right ventricular dilation [20].

Morphometric analysis of duodenum

According to Hassanpour *et al.* [21], 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 $\times$) and objective lens (10 $\times$) 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 $(\pi) \times (\text{villus width}) \times (\text{villus length})$. 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 MDA and CAT, GPX, and SOD activities

Serum catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD) activities (indicators of enzymatic antioxidant defense system), and malondialdehyde (MDA, an indicator of lipid peroxidation) concentration were assayed on day 35 (nine chickens per diet) according to Hassanpour et al. [22]. 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 NADPH consumed per minute per milligram of total serum protein in the sample. SOD activity was measured as the percent of inhibition of reduction in NBT by SOD per total serum protein of the sample.

RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis

Lung and heart (right ventricle) tissues were processed for total RNA isolation by RNX-Plus solution (Sinaclon Bioscience, Karaj, Iran), according to Pirany et al. [23]. 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 cDNA synthesis. PrimeScript™ RT reagent Kit (Takara Bio Inc., Japan) was used to synthesize cDNA according to the previous study [24].

The specific primers of CAT, GPX, SOD, and YWHAZ (as an endogenous standard) [25] were prepared according to Hassanpour et al. [22] to determine their relative amounts by quantitative real-time polymerase chain reaction (PCR). SYBR®PremixExTaq™II (Tli RNaseH Plus) kit (Takara Bio Inc., 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 [22, 26]. The mRNA level of each target gene relative to YWHAZ was estimated for each sample with the Pfaffl method, as described previously by Hassanpour et al.[16]

Statistical analysis

Data are represented as means and statistically compared by 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 values less than 0.05 were considered significant.

Results

Marjoram compositions

Table 1 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.

Table 1
The major compositions of leaf extract
of marjoram (*Origanum majorana* L.)

Compound	Percentage (%)
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

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 in the control, Vit C, marjoram 0.1% and 0.2% groups of chickens was 11.1%, 5.6%, 8.3%, and 8.3% respectively.

Growth performance

The growth performance of all groups of chickens was given in Table 2. 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 on days 22–35 compared to the 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 2).

Table 2

Effects of vitamin C and marjoram extracts on mean growth performance of Ross 308 broiler chickens¹

Item	WG (g)	FC (g)	FCR
Days 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
Days 22 to 35			
Control	855.8 ^b	1890.8 ^a	2.21 ^a
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
Days 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
Superscripts in the same column with different letters are significantly different ($p < 0.05$). WG = weight gain; FC = Feed consumption; FCR = feed conversion ratio; Pooled SEM = Pooled standard error of mean. ¹ Each mean represents values from 30 replicates.			

Morphometric assessment of duodenum

Morphologic parameters of duodenal villi in the different groups of chickens were given in Table 3. The duodenal villus length and surface area in the chickens fed vitamin C were higher than the 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 ($p > 0.05$).

Table 3

Effects of vitamin C and marjoram extracts on mean morphologic characteristics of the duodenal villi in Ross 308 broiler chickens¹

Diet	Length (mm)	Width (mm)	Lamina propria (mm)	Surface area (mm ²)
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%)	0.77 ^{bc}	0.37 ^a	0.87 ^a	0.89 ^b
Pooled SEM	0.016	0.043	0.012	0.034
Superscripts in the same column with different letters are significantly different ($p < 0.05$). Length = From top of the villus to top of the lamina propria; Width = The width of villus at the base; The lamina propria = The space between base of the villus and top of the muscularis mucosa; Villus surface area = Calculated as $(\pi) \times (\text{length}) \times (\text{width})$; Pooled SEM = Pooled standard error of mean. ¹ Each mean represents values from 9 replicates				

Assessment of lipid peroxidation and SOD, CAT, and GPX activities

Data on lipid peroxidation (MDA measurement) and antioxidant enzyme activities in the serum samples of chickens are presented in Table 4. MDA was decreased in all treatments compared to the control ($P < 0.05$). SOD and GPX activities were only increased in the Vit 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%.

Table 4
Effects of vitamin C and marjoram extracts on mean plasma oxidant and antioxidant status of Ross 308 broiler chickens¹

Diet	MDA ($\mu\text{mol/mL}$)	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 SEM ⁵	5.13	10.26	4.36	3.88
Superscripts in the same column with different letters are significantly different ($p < 0.05$). <i>MDA</i> = Malondialdehyde; <i>CAT</i> = Catalase; <i>GPX</i> = Glutathione Peroxidase; <i>SOD</i> = Superoxide dismutase; Pooled SEM = Pooled standard error of mean. ¹ Each mean represents values from 9 replicates				

Gene expression of antioxidant enzymes

Relative expression of SOD, CAT, and GPX genes in the lung and heart tissues is presented in Table 5. 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 Vit C and marjoram 0.1% groups compared to the control ($P > 0.05$).

Table 5
Effects of vitamin C and marjoram extracts on mean relative gene expression (target/YWHAZ) of Ross 308 broiler chickens¹

Diet	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
Superscripts in the same column with different letters are significantly different ($p < 0.05$). MDA = malondialdehyde; CAT = Catalase; GPX = Glutathione peroxidase; SOD = Superoxide dismutase; Pooled SEM = Pooled standard error of mean. ¹ Each mean represents values from 9 replicates.			

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 ($P > 0.05$).

Discussion

In the present study, vitamin C was used as a positive control. Its beneficial effects have already been confirmed in chicken growth performance, pulmonary hypertensive response, and intestinal morphology by our previous study [27]. Then, the comparison of vitamin C effects with marjoram could be helpful.

The results based on the RV: TV ratio showed that marjoram could reduce pulmonary hypertensive response in the broilers. Chen and Chen [28] reported that phenolic acids and tanshinones improve atherosclerosis, thrombosis, and myocardial injuries. Bina and Rahimi [5] also determined that marjoram

inhibited lipid peroxidation and nitric oxide synthesis in the heart tissue to improve cardiovascular dysfunction.

Although many reports have shown that marjoram improves body weight gain, food consumption, or feed conversion ratio [7, 8, 29], there are also conflicting reports [6, 30, 31]. In these reports, different products of marjoram (oil, essential oil, powder, and extract) with different concentrations have been used. 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 the present study, marjoram extract was used in developmental pulmonary hypertension, only marjoram 0.2% extract led to improve FCR in 22–35 days.

In the studies of Solis de Los Santos *et al.* [32] 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 hypertensive chickens for improving the PHS and intestinal damage [20, 21, 27, 33]. 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, 29, 34]. It was indicated that dietary antioxidants such as vitamin C protect enterocytes against oxidative stress, promote epithelial proliferation, and improve villus morphology [27, 35]. 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 be also observed in the heart, lung, and blood vessels that 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 [36–39]. These compounds were considered the major constituents of marjoram leaf extract in our study. Thus, this strong effect of marjoram constituents in the oxidant and antioxidant status could justify its improvement in the developmental PHS and its other following advantages in the intestine.

In our study, the decrease of lipid peroxidation and increase of CAT activity could partially confirm the antioxidant property of marjoram in PHS. [15][15][15][15][15][15][15][15] Abdel-Wahab [29] reported that high doses of marjoram (1-2.5% of diet) increased GPX activity in healthy chickens. Kamel [40] found that marjoram increased CAT, SOD, and GPX activities in 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 [41]. 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.

Declarations

Declaration of Competing Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Authors' contributions

SB and HH was the supervisor, designed the study and analyzed data. YT contributed to collecting the samples. YT, HH and AM carried out the experiments and assembled data. HH and SB contributed to writing, reviewing and editing the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The project underwent ethical review and was approved by the local Ethics Committee of Shahrekord University. The care and use of experimental animals complied with local animal welfare laws, guidelines and policies. The study was also carried out in compliance with the ARRIVE guidelines [42].

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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