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Antioxidative, Antihyperlipidemic, and Growth-Promoting Effects of *Kelussia odoratissima* in Meat-type Chickens

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Abstract

Due to the presence of polyphenols and phthalides in *Kelussia odoratissima* Mozzaf (Umbelliferae; *K. odoratissima* hereafter), this medicinal plant might be a robust in-feed additive to modulate lipid peroxidation in meat-type chickens. The present study evaluates antioxidative, antihyperlipidemic, and growth-promoting effects of *K. odoratissima* in meat-type chickens. In a 37-day trial (5-42 days of age), a total of 240 broiler chicks (Ross 308 strain) were randomly assigned to four treatment groups. Treatments included 0, 0.25, 0.50, and 0.75% *K. odoratissima* in feed. Aerial parts of the plant was dried, ground and added to diet. Dietary inclusion of *K. odoratissima* significantly ($P<0.05$) increased villus height and width and its absorptive surface area in different parts of small intestine (duodenum, jejunum, and ileum) compared with the control group. Whole body growth significantly ($P<0.05$) improved when *K. odoratissima* was included in diets at 0.75% (2375 *vs.* 2488 g). Broilers received *K. odoratissima* had significantly ($P<0.05$) lower concentration of malondialdehyde (MDA) compared to the control group. Superoxide dismutase 1 (SOD1) gene has been highly overexpressed (~ 24-fold) in the lung of broilers fed *K. odoratissima* at 0.75%. However, *K. odoratissima* significantly ($P=0.004$) suppressed (~ 8-fold) the expression of inducible nitric oxide synthase (iNOS) gene in the lung of broilers when compared to the control. Feeding *K. odoratissima* at 0.75% caused a significant ($P<0.05$) reductions in serum levels of triglycerides and cholesterol as well as reduction in abdominal fat deposition. In conclusion, *K. odoratissima* showed antioxidative, antihyperlipidemic, and growth-promoting effects in broiler chickens.

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Introduction

Wild celery (*Kelussia odoratissima*, formerly *Apium odoratissima*) is a medicinal plant that belongs to the family of Umbelliferae. It has long been recognized as a medicinal plant to boost immune system and suppress inflammation (Rabbani *et al.*, 2011). The essential oil of *K. odoratissima* consists of bioactive components, including phthalides, mainly *Z*-ligustilide (3-butylidene-4,5-dihydrophthalide) (37.3% of total bioactive components) and sesquiterpenes (14% of total bioactive components) (Shojaei *et al.*, 2011). Phthalides confer potent antioxidant properties to the plant (Ahmadi *et al.*, 2007; Rabbani *et al.*, 2011; Pirbalouti *et al.*, 2013). Most recently, this medicinal herb has shown the potential to prevent pulmonary hypertension (Ahmadipour *et al.*, 2015).

Chickens bred for rapid growth and meat production (broiler chickens) provide an excellent model of experimental study for humans (Wideman *et al.*, 2011). A broiler chick that weighs 40 g at hatch has potential capability to reach to 4,000 g in eight weeks. That means a hundredfold increase in weight in just eight weeks. However, the excessive fat in broiler chickens has been one of the major problems facing the poultry meat industry. More than 85% of total body fat in broiler chickens is not physiologically required for body function. Excessive fat deposition is unfavorable for producers because it is considered to be wasted dietary energy, and for consumers because it affects consumer acceptance and desirability. Majority of fat in the chicken's body is in the form of unsaturated fat that is susceptible to oxidation. Therefore, antioxidant capacity is a pivotal element in maintaining normal chicken metabolism. The present study aims at using *K. odoratissima* as a potential in-feed additive in broiler chicken diets which might suppress lipogenesis and enhance antioxidant capacity and gut function.

Materials and Methods

Birds and experimental facility

The experiment was conducted in the experimental facility of Shahrekord University, Shahrekord, Iran. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use Committee of Shahrekord University.

A total of 240 five-day-old male broilers (Ross 308) were randomized across 20 floor pens measuring 1.5 m^2 (13 birds per pen). Each pen was equipped with a bell drinker and a feed trough. The temperature of the experimental house maintained according to the guideline of the commercial strain (Ross 308). All chicks were exposed to the same environmental conditions (*i.e.* temperature, light, humidity *etc.*).

Treatments

K. odoratissima was collected from Koohrang region located in Chahrmahal-Va-Bakhtiari province, Iran in April 2013 by authors. The plant has unique odour that

make it easy to identify. The aerial parts of *K. odoratissima* consisted of stem and leaves were cut, air-dried, and ground for use in the experimental diets. Analyzed composition of dried powder of *K. odoratissima* showed 9% crude protein, 7.5% crude fiber, 1.8% Ca, 0.6% P, 0.04% Na, 0.04% Cl, 0.28% S, and 2% K. The essential oil of *K. odoratissima* also contained considerable amount of polyphenols (52 mg/g), of which flavonoids consisted 5.2 mg/g. Previous GC/MS analysis of the essential oil also showed *Z*-ligustilide (33.73%), 3-*E*-butyldiene phthalide (20.1%), *E*-ligustilide (6.65%), 2-octen-1-ol, acetate (5.18%), β-selinene (4.58%), kessane (4.09%), 3-*n*-butylphthalide (3.57%), caryophyllene oxide (3.14%); cuparene (2.11%); pentyl benzene (1.74%), and *E*-β-farnesene (1.27%), which comprise 86.16% of all essential oil (Shojaei *et al.,* 2011).

A basal diet was formulated in which substitution of *Kelussia odoratissima* was made for wheat bran. The composition of the basal diet in the starter (5-21 d) and the grower (21-42 d) stages is shown elsewhere (Ahmadipour *et al.,* 2015). Three additional diets were prepared by substituting 0.25, 0.50, and 0.75% *Kelussia odoratissima* in the basal diet. All experimental diets had similar metabolizable energy and protein content.

Measurements

Body weight of birds was obtained at the start (5 d) and end (42 d) of the experiment. At the end of trial (42 d), 10 birds per treatment were randomly selected for blood collection. Blood samples (3 mL) were collected from the brachial vein and centrifuged at 2500 × *g* for 10 min to obtain sera. Serum samples were used for the determination of MDA, triglycerides and total cholesterol. Serum MDA concentration as biomarker of lipid peroxidation was assayed by the method of Nair and Turner (1984). Serum triglycerides and total cholesterol were assayed by commercial kits. The selected birds were then killed and their small intestines were removed for measuring gut morphometry. In order to examine the intestinal morphometry, segments (~2 cm) from the midpoints of the duodenum and jejunum, and the distal ileum were cut, rinsed with phosphate buffered saline (pH=7), fixed in Clarke's fixative for 45 min, and then placed in 50% ethyl alcohol. The segments were then taken in periodic acid Schiff (PAS) for staining. Muscle layers were discarded from mucosa. Villus height, lamina propria, and villus width were measured at 1000 × magnification by means of a computer-assisted microscope (Sigma Scan, Jandel Scientific, San Rafael, CA, USA). Villus height was considered from the top of the villus to the top of the lamina propria. Surface area was determined using the formula = (π) × (VW) × (VL), where VW is villus width and VL is villus length (Khajali *et al.,* 2014).

At 42 days of age, eight chickens from the control group and the group received the highest level of *K. odoratissima* (0.75%) were also selected, weighed and killed by decapitation. The lungs were harvested and immediately frozen in liquid nitrogen and stored at -70°C for subsequent RNA analysis. Total RNA from the

lungs was extracted using RNX-Plus reagent (Sinaclon Bioscience, Tehran, Iran). Tissues (100 mg) were homogenized in digestion buffer. The homogenate was then mixed with chloroform and centrifuged. Total RNA settled in the upper aqueous phase was then precipitated with isopropanol. RNA pellet was also rinsed with 75% ethanol. RNA samples were resuspended in DEPC-treated water. The RNA was treated by DNase (Sinaclon Bioscience, Tehran, Iran) to remove eventual residual DNA. RNA was then measured and qualified spectrophotometrically. Only RNA with an absorbance ratio (A260/A280) of >1.9 was used for the synthesis of cDNA. Total RNA was reverse transcribed into cDNA using PrimeScript™ RT Reagent Kit (Takara Bio Inc., Japan). The reverse transcription mixture was heated to 85°C for 5 s to inactivate reverse transcriptase and denature the RNA and then stored at -20°C.

The expression levels of SOD1, iNOS and β-actin transcripts were determined by real-time reverse transcriptase (RT)- polymerase chain reaction (PCR) using SYBR® Premix Ex Taq™ II (Tli Rnase H Plus) (Takara Bio Inc., Japan). In order to normalize the input load of cDNA between samples, β-actin was used as an endogenous standard. Authentic primers for SOD1, iNOS and β-actin were designed with Primer-Blast publically available in National Center for Biotechnology Information (NCBI). Details of the primers are depicted in Table 1. PCRs were conducted in a real-time PCR cycler (Rotor Gene Q 6000, Qiagen, USA) in triplicates for each ventricle sample. An aliquit of cDNA (1 µL) was added to the 10 µl of SYBR® Premix Ex Taq II Mix and 0.5 µM of each specific primer in a total volume of 20 µl. Thermal profile was 95°C for 30 s, 40 cycles of 94°C for 40 s, 64°C for 35 s and 72°C for 30 s. At the end of each phase, the fluorescence was measured and used for quantitative objectives. Data of gene expression were normalized to β-actin and analyzed using LinRegPCR software version 2012.0 (Amsterdam, Netherland), to give the threshold cycle number and reaction efficiency (Ruijter *et al.*, 2009). Relative transcript levels and fold changes in transcript abundance were computed using efficiency adjusted Pfaffl methodology (Dorak, 2006).

Statistical analysis

Results were compared by GLM using SAS (2007) software in a completely randomized design. When there was sampling within pens, data were subjected to a nested design. The statistical model used for growth performance data was $Y_{ij} = \mu + T_i + e_{ij}$. For other variables, the model was $Y_{ijk} = \mu + Ti + e_{ij} + \varepsilon_{ijk}$. In these models, Y_{ij} and Y_{ijk} are observations; µ is the general location parameter (i.e., the mean); T_i is the effect for being in treatment i; e_{ij} is random error; and ε_{ijk} is subsampling error. Means were separated by Duncan's multiple range test.

Table 1. Details of the primers used for quantitative real-time PCR analysis of chicken mRNAs

Target	Primers	PCR Product	Accession No.
β-Actin	5'-AGCGAACGCCCCCAAAGTTCT-3' 5'-AGCTGGGCTGTTGCCTTCACA-3'	139 bp[1]	NM-205518.1
SOD1[2]	5'-CACTGCATCATTGGCCGTACCA-3' 5'-GCTTGCACACGGAAGAGCAAGT-3'	223bp[1]	NM_205064.1
iNOS[3]	5'-AGGCCAAACATCCTGGAGGTC-3' 5'-TCATAGAGACGCTGCTGCCAG-3'	371bp[1]	U46504

[1]base pair; [2]Superoxide dismutase1; [3]inducible nitric oxide synthase.

Results and discussion

Body weight gain and feed conversion ratio throughout the trial were significantly (*P<0.05*) increased in relation to the control when *K. odoratissima* added at 0.75% (Table 2).

Table 2. Effects of *Kelussia odoratissima* on broiler's growth performance

	Dietary level of *Kelussia odoratissima* (%)				
	Control(0)	0.25	0.5	0.75	*SEM*
Feed intake (g/bird):					
5–21 days of age	1144.04[a]	1071.35[b]	1065.61[b]	1062.50[b]	17.31
21–42 days of age	3262.76	3252.77	3200.92	3294.69	46.47
5-42 days of age	4406.80	4324.12	4266.53	4357.19	46.66
Weight gain (g/bird):					
5–21 days of age	723.35	686.57	694.27	694.51	18.08
21–42 days of age	1651.72[b]	1705.81[ab]	1719.68[ab]	1793.59[a]	30.75
5-42 days of age	2375.07[b]	2392.38[ab]	2413.94[ab]	2488.1[a]	31.94
Feed conversion ratio:					
5–21 days of age	1.58	1.56	1.53	1.53	0.031
21–42 days of age	1.98[a]	1.9[ab]	1.86[b]	1.83[b]	0.027
5-42 days of age	1.85[a]	1.81[ab]	1.76[b]	1.75[b]	0.018

[a,b]Means in the same row with different letters are significantly different (*P<0.05*).

The villus height, villus width, lamina propria, and surface area in duodenum, jejunum, and ileum are presented in Table 3. *K. odoratissima* increased villus height and surface area significantly (*P<0.05*) at all dietary levels compared with the control. The villus width and lamina propria were also significantly (*P<0.05*) increased at 0.75% dietary level of *K. odoratissima* in all intestinal segments.

Table 3. Effects of *Kelussia odoratissima* on broiler's gut morphometry[1]

Item (mm, unless noted)	Dietary level of *Kelussia odoratissima* (%)				*SEM*
	Control(0)	0.25	0.5	0.75	
Duodenum:					
Villus height	1.15[b]	1.37[a]	1.40[a]	1.49[a]	0.048
Villus width	0.49[b]	0.51[ab]	0.54[a]	0.54[a]	0.019
Lamina propria	0.48[b]	0.50[b]	0.53[b]	0.61[a]	0.024
Surface area (mm^2)	1.74[b]	2.23[a]	2.40[a]	2.54[a]	0.137
Jejunum:					
Villus height	0.99[c]	1.17[b]	1.20[b]	1.34[a]	0.04
Villus width	0.39[c]	0.41[bc]	0.46[ab]	0.48[a]	0.022
Lamina propria	0.38[b]	0.47[a]	0.48[a]	0.51[a]	0.017
Surface area (mm^2)	1.24[c]	1.50[bc]	1.76[ab]	2.05[a]	0.111
Ileum:					
Villus height	0.62[c]	0.71[b]	0.75[b]	0.85[a]	0.027
Villus width	0.34[b]	0.35[ab]	0.38[ab]	0.40[a]	0.02
Lamina propria	0.29[b]	0.34[a]	0.36[a]	0.37[a]	0.016
Surface area (mm^2)	0.67[c]	0.79[bc]	0.92[ab]	1.08[a]	0.07

[1]Each mean represents values from 10 replicates.
[a-c]Means in the same row with different letters are significantly different ($P<0.05$).

Table 4 indicates serum variables of broilers received different levels of *K. odoratissima* in the feed. Broilers received *K. odoratissima* had significantly ($P<0.05$) lower concentrations of MDA than that of the birds fed on the control diet. Feeding *K. odoratissima* at 0.5 and 0.75% caused a significant ($P<0.05$) reduction in serum levels of triglycerides and cholesterol when compared to the control. In addition, abdominal fat deposition was significantly ($P<0.05$) reduced when *K. odoratissima* used at 0.75%.

Table 4. Effect of dietary *Kelussia odoratissima* on serum variables and abdominal fat deposition in broiler chickens measured at 42 days of age[1]

	Dietary level of *Kelussia odoratissima* (%)				*SEM*
	Control(0)	0.25	0.5	0.75	
Malondialdehyde (µmol)	4.05[a]	2.53[b]	2.14[b]	2.12[b]	0.355
Triglyceride (mg/dL)	196[a]	176[ab]	158[b]	151[b]	10.45
Cholesterol (mg/dL)	125[a]	113[ab]	105[b]	103[b]	3.98
Abdominal fat deposition (%)	1.41[a]	1.26[a]	1.14[ab]	0.88[b]	0.11

[1]Each mean represents values from 10 replicates.
[a,b]Means in the same row with different letters are significantly different ($P<0.05$).

The expression of SOD1 and iNOS genes in the lung of broiler chickens has been affected by feeding *K. odoratissima* to broiler chickens (Table 5). SOD1 gene has been highly overexpressed in broilers fed *K. odoratissima* at 0.75% (~24-fold increase relative to the control). Feeding *K. odoratissima* significantly ($P=0.004$) suppressed the expression of iNOS gene compared to the control.

Table 5. Effect of *Kelussia odoratissima* on expression of SOD1 and iNOS genes in lung tissue of broiler chickens measured at 42 d of age[1]

	SOD1[2]	iNOS[3]
Control (T1)	0.1391[b]	0.0498[a]
0.75% *Kelussia odoratissima* (T2)	3.3215[a]	0.0059[b]
SEM	0.237	0.0096

[1]Number of observation=15; [2]Superoxide dismutase 1; [3]inducible nitric oxide synthase.
[a,b]Means in the same column with different letters are significantly different (*P<0.05*).

Discussion

Significant improvements in body growth and feed conversion ratio due to the inclusion of *K. odoratissima* in broiler diets are in part consequence of significant improvement in gut morphometry. Increasing the villus height and villus surface area by feeding *K. odoratissima* suggests greater absorption of available nutrients, which has been reflected in significant improvement in body weight gain observed in the present study. A significant increase in the thickness of the lamina propria also explains the higher growth performance of broilers fed *K. odoratissima* in the present study. The lamina propria is regarded as the villus factory that influences intestinal secretory potency. The proliferative effect of *K. odoratissima* on intestinal villi can be explained by their potent antioxidant role, which is accounted for the naturally-occurring phthalides and polyphenols in this medicinal plant. Research has shown that the antioxidant potential of *K. odoratissima* is comparable to α-tocopherol when assayed by various methods (Ahmadi *et al.,* 2007). In line with our study, Miller *et al.* (2001) confirmed that dietary antioxidants protect gut epithelial cells from pro-apoptotic oxidant stress with subsequent increase in epithelial cell growth. Ronco *et al.* (2002) suggested that the modulation of lipid peroxidation attenuates cellular apoptosis and increases proliferation and regeneration processes in the liver. The histomorphological changes in the intestine of broiler chickens reported in the present study provide new information regarding the potential of *K. odoratissima* as a growth enhancer in broiler chicken's feed.

Feeding *K. odoratissima* resulted in significant (*P<0.05*) lower concentration of serum MDA when compared to the control. This finding is in accordance with overexpression of SOD1 as observed in the present study. These observations clearly suggest potent antioxidant activity of this medicinal plant. Ahmadi *et al.* (2007) indicated that the antioxidative effects of *K. odoratissima* were attributed to flavonoids and phthalides.

Reduction in expression of inducible iNOS in the lung of broilers fed *K. odoratissima* may reflect immunomodulatory and anti-inflammatory effects of this medicinal herb. The lung is a major target organ for numerous viral and bacterial diseases of birds in the absence of functional lymphoid system and therefore plays a crucial role in bird's immunity (Reese *et al.,* 2006). It has been shown that iNOS gene expression and subsequent nitric oxide (NO) production in whole lung of the

chicken are mainly originated from macrophage (Reese *et al*., 2006). Macrophages are distributed in considerable numbers throughout the bronchus-associated lymphoid tissue (BALT) nodules and constitute the immune system of bird's respiratory system. The number of macrophages is increased in bacterial and viral challenges (Reese *et al*., 2006). A review of literature shows an increase in concentration of NO produced by iNOS in macrophages can result in oxidative damage (Nijveldt *et al*., 2001). In these circumstances, activated macrophages greatly increase their simultaneous production of both NO and superoxide anions. NO reacts with free radicals, thereby producing the highly damaging peroxynitrite. NO injury takes place for the most part through the peroxynitrite route because peroxynitrite can directly oxidize low density lipoprotein (LDL), resulting in irreversible damage to the cell membrane. When flavonoids are used as antioxidants, free radicals are scavenged and therefore can no longer react with NO, resulting in less damage (Nijveldt *et al.,* 2001). Antibacterial and antiviral actions of *K. odoratissima* mediated through flavonoids and phthalides may account for significant decline in the number of macrophages in the lung of birds fed on this medicinal plant (Kosmider and Osiecka, 2004; Surai, 2014) and account for lower expression of iNOS in the present study. In line with our finding, Su *et al.* (2011) reported that ligustilide prevented lipopolysaccharide (LPS)-induced iNOS expression in macrophages by preventing reactive oxygen species (ROS) production and down-regulating the NF-κB.

Significant reductions in serum levels of triglycerides and cholesterol as well as reduced deposition of fat in the abdomen when *K. odoratissima* was added to broiler diets at 0.5 and 0.75% suggest anti-hyperlipidemic potential of this medicinal herb. The anti-hyperlipidemic effect of *K. odoratissima* is related to ferulic acid (Sudheer *et al*., 2008; Sajjadi *et al*., 2012) and flavonoids (Sudheesh *et al*., 1997). Sudheesh *et al*. (1997) indicated that flavonoids extracted from *Solanum melongena* Brinjal (Solanaceae) significantly increased the activity of HMG-CoA reductase with concomitant decrease in the activity of glucose-6-phosphate dehydrogenase. These changes in enzyme activities caused by flavonoids account for hypolipidemic action. As mentioned, reduced fat deposition in broiler chickens is beneficial to both producers and consumers.

Conclusion

In conclusion, *K. odoratissima* significantly improved growth performance and gut function with concomitant reduction in fat deposition. These findings might be attributed to strengthened antioxidant and immune systems and reflected in up-regulation of SOD1 gene and down-regulation of iNOS gene in the lung of chickens. *K. odoratissima*, therefore, showed the potential to be used as a suitable in-feed additive in broiler diets.

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