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Effect of *Securigera Securidaca* Seed on the Gene Expression of Pulmonary Hypertensive Broiler Chickens Induced by Cool Temperature and High-Altitude

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Abstract

Securigera securidaca (Fabaceae) or goat pea is growing wild in West Asia, Europe and Africa as an annual herb. In Persian, it is called "Gandeh Talkheh". The seeds of goat pea are applied in the traditional medicine to treat different dysfunctions e.g., hyperlipidemia and hypertension. This study was done at altitude of 2100 m above sea level. The effects of dietary *Securigera securidaca* seed (*S. securidaca*) were studied on hematological parameters, ascites incidence, and gene expression in the liver in chickens under cold temperature condition. Number of 200 broiler chicks (1-day-old male, Ross 308 breed) were randomly divided into 20 floor pens and reared for 42 days. Experimental groups were defined by the inclusion of 0 (control), 1, 2, 3, and 4 g/kg *S. securidaca* in the diets. The results of the experiment showed that broiler chickens fed a diet containing 2 to 4 g/kg *S. securidaca* seed had lower abdominal fat and relative liver weight as well as right ventricular ratio to total ventricles (RV/TV) compared to control, while nitric oxide and hematocrit were higher (P<0.05). The transcript of glutathione peroxidase (GPX) and superoxide dismutase (SOD) genes were influenced in the liver by feeding *S. securidaca* Seed. Liver NOS and GPX mRNAs were considerably increased in the groups supplemented *S. securidaca* seed (3 and 4 g/kg) compared to the control birds (P < 0.05). In conclusion, *S. securidaca* medicinal herb effectively improves pulmonary hypertensive response in broiler chickens.

Introduction

Pulmonary hypertension syndrome (PHS) is a common disease in modern strains of broiler chickens, due to the imbalance between demand of muscles for oxygen and supply of oxygen by pulmo-cardiovascular system. In susceptible birds, PHS is started by an elevation of either cardiac output due to high metabolic rate or an increase of vascular resistance to blood flow in hypobaric hypoxia (Julian, 2007). When broilers are reared at high altitude with environmental hypoxia, pulmonary vasoconstriction occurs, then vascular resistance and pulmonary arterial pressure were increased (Burton *et al.,* 1968; Julian, 2007). Erythropoiesis and angiogenesis are further physiological responses to chronic hypoxia for reoxygenation of hypoxic tissue (Walshe and D'Amore, 2008). It is proposed that increased blood pressure in the pulmonary circulation (pulmonary hypertension) can cause enlargement of the right ventricle (RV) and eventually congestive heart failure (Franco., 2012). It has been proposed that ascites and endothelial function disorder is probably dependent to reactive oxygen species (ROS) which make oxidative stress. Oxidative stress occurs when tissue depleted of antioxidants (Ruiz-Feria., 2009). On the other hand, inability of antioxidant system during PHS development causes the tissue injury due to increased markers of reactive oxygen, displaying a state of oxidative stress in this syndrome (Balog, 2003). There are exogenous and endogenous compounds of antioxidants that both responsible for scavenging ROS, or their precursors and inhibition of their

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formation (Uttara *et al*., 2009). Endogenous antioxidants include catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD) while exogenous antioxidants, derived from diet, consist of ascorbic and lipoic acid, polyphenols and carotenoids (Kohen and Nyska, 2002). In recent years, it has been found that medicinal plants could be the best natural resources for antioxidants to prevent cellular oxidative damage. Medicinal herbs are rich of active antioxidant components e.g. flavonoids, polyphenols, tannins, and alkaloids, which can justify their application in the treatment of different diseases (Sidhu and Tanu, 2013).

Securigera securidaca (Fabaceae) or goat pea, as an annual wild herb is widespread in West Asia, Europe and Africa. It is popularly called "Gandeh Talkheh" in Persian. Phytochemical analysis of goat pea seed extract has revealed the existence of flavonoids, steroidal and pentacyclic triterpenoid-type saponins, cardenolides, and tannins (Hosseinzadeh *et al*., 2002) which confirms its antioxidant characteristics. However, the usefulness of this medicinal plant has been demonstrated in the treatment of hyperlipidemia, hypertension, diabetes, epilepsy, gastric disturbances, and chronotropic, diuretic, and hypokalemic disorders (Garjani *et al*., 2009).

Materials and Methods

Plant

S. securidaca (L.) seeds were prepared from local shops (Shahrekord-Iran) and identified by Shahrekord University (Faculty of Agriculture). The milled seeds of this plant were supplemented in the diets of this study.

Table 1. Basal diet composition

Item (% unless noted)	Starter (1–21days)	Grower (22– 42 days)
Corn	50.7	58.3
Soybean meal (44% CP)	41.2	34.1
Soy oil	4	3.9
Dicalcium phosphate	1.7	1.3
Oyster shell	1.5	1.5
Salt	0.3	0.3
DL-Methionine	0.1	0.1
L-Lysine	-	-
Mineral supplement	0.25	0.25
Vitamin supplement	0.25	0.25
Securigera securidaca seed*		
Calculated composition		
AME (kcal/kg)	3000	3100
CP	21.5	19.5
Met	0.55	0.45
Met+Cys	0.95	0.75
Lys	1.25	1.04
Thr	0.93	0.9
Arg	1.39	1.16
Ca	0.95	0.87
Available P	0.45	0.35
Na	0.18	0.15
Cl	0.28	0.30
K	0.90	0.92

[a] Supplied per kilogram of diet: vitamin A, 3600IU; vitamin D3, 850 IU; vitamin E, 7.3 mg; vitamin K3, 1.6 mg; thiamine, 0.72 mg; riboflavin, 3.3 mg; niacin, 0.4 mg; pyridoxin, 1.2 mg; cobalamin, 0.6 mg; folic acid, 0.5 mg; choline chloride, 200 mg.
[b] Supplied per kilogram of diet: Mn, 50mg; Zn, 45mg; Fe, 25mg; Cu, 4 mg; I, 0.65 mg; Se, 0.15 mg.
* Treatments 1, 2, 3, 4 and 5 contained 0 (control) 1, 2, 3 and 4 g *S. securidaca* /kg diets, respectively.

Animals, Experimental Treatments and Diet

This study was performed in the poultry research site of Shahrekord University, Iran (altitude = 2100 m). Number of 200 broiler chicks (1-day-old male, Ross 308 breed) were randomly divided into 20 floor pens (10 birds per pen, average weight of chicks in each pen 48.5 g). The size of each pen was 1.5 m^2 and was equipped with facilities such as bell drinker and feed trough. All birds were behaved based on the admitted standards for animal ethics. Birds were maintained at environmental decreasing temperatures (1-7 days, 32±1°C; 8-14 days, 25±1°C; 15-21 days, 20±1°C; 22-42 days, 15±1°C) (Ahmadipour, 2018a,d). Birds were exposed to 23 hrs of light per day during rearing. Accessibility to feed and water was unlimited. Standard diets were provided as mash form the starting (1 to 21 days of age) and growing (22 to 42 days of age) stages according to the NRC (1994) recommendations. The diets in experimental groups were supplemented by 0 (control), 1, 2, 3, and 4 g/Kg *S. securidaca.*, All five diets had equal levels of metabolizable energy (Table 1).

Measurements

At the end of trial, 8 chickens from each group (2 birds / pen) were randomly selected and weighed, then blood samples (3mL) were collected from the brachial vein by a 5 ml syringe with needle gauge 20. The samples were immediately transferred to a tube containing EDTA as anticoagulant. Hematocrit of blood samples was measured according to Jain (1993). Briefly, the samples were collected into a microhematocrit tube. After sealing of one end of the tubes with a sealant, they were centrifuged using microhematocrit centrifuge (9000g, 5 min) and, then the value of hematocrit measured by a microhematocrit ruler. The plasma was also separated from bloods following centrifuge at 2500g for 10 min, then were used to determine nitric oxide (NO) and malondialdehyde (MDA) amounts. The measurement of serum NO was done according to Ahmadipour (2018a). The method of Nair and Turner (1984) was also used to assay serum MDA. This substance is the product of cellular lipid peroxidation, and then could be partially an index of oxidative stress. To analyze aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) as hepatic enzymes, commercial enzymatic kits were prepared (Pars Azmoon, Tehran, Iran).

Following the removal of hearts, the ventricles were separated and weighed to determine the ratio of right ventricular weight to total ventricular weight (RV/ TV). This is as ascites index (%) which indicates the progression of pulmonary hypertension and ascites. If the ratio was increased more than 0.25 it was indicated as pulmonary hypertension (Ahmadipour *et al*., 2018b,c,d).

Quantitative real-time PCR

Birds were slaughtered at the last day of experiment; the liver was removed and frozen in liquid nitrogen and then stored at -70°C until RNA extraction. To extract total RNA, briefly, the liver tissues (100 mg) were homogenised in RNX-Plus reagent (as digestion buffer) (Sinaclon Bioscience, Karaj, Iran). After adding of chloroform, the mixture was centrifuged. Total RNA was settled in the upper liquid that was precipitated by isopropanol and rinsed with ethanol. The extracted RNA was resolved in DEPC-treated water. Immediately, the RNA samples were treated by DNase (Sinaclon Bioscience, Karaj, Iran) to clean contamination of DNA. The amounts of RNA were then analyzed by spectrophotometry in absorbance ratio of A260/A280. Only the samples with the ratio more than 1.9 were used to produce cDNA. To synthesize cDNA, PrimeScript ™ RT Reagent Kit (Takara Bio Inc., Japan) were applied (Ahmadipour *et al*., 2018b,c).

SOD1, GPX and β-actin mRNAs were amplified in the cDNA samples by real-time PCR using SYBR® Premix Ex Taq™ II (Tli Rnase H Plus) (Takara Bio Inc., Japan). For this kit, 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. In this method, β-actin was as an internal standard. Primers of the mentioned genes were designed with Primer-Blast publically available in NCBI. Details of the primers are presented in Table 2. Each sample were amplified in triplicate using a real-time thermocycler (Rotor Gene Q 6000, Qiagen, USA). The PCR program 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. The threshold cycle of samples was determined and offered available in LinRegPCR software version 2012.0 (Amsterdam, Netherland) for analyzing reaction efficiency. Pfaffl method was used to count the relative gene expression (target / β-actin) and fold changes (Hassanpour *et al*., 2014).

Table 2. Details of the primers used for quantitative real time PCR analysis of chicken mRNAs

Target	Primers	PCR product (bp)	Accession no.
β-Actin	5′-AGCGAACGCCCCCAAAGTTCT-3′ 5′-AGCTGGGCTGTTGCCTTCACA-3′	139	NM_205518.1
GPX1	F:5′-GCTGTTCGCCTTCCTGAGAG-3′ R:5′-GTTCCAGGAGACGTCGTTGC-3′	118	NM_001277853.1
SOD1	5′-CACTGCATCATTGGCCGTACCA-3′ 5′-GCTTGCACACGGAAGAGCAAGT-3′	223	NM_205064.1

SOD1: superoxide dismutase 1; GPX: glutathione peroxidase; bp: base pair

Statistical analysis

GLM procedure of SAS software (SAS, 2007) were used to analyze data in a completely randomized design. The statistical model used Yij = µ + Ti + eij. In this model, Yij is observation; µ is the mean; Ti is the effect for being in treatment i and eij is random error, Means were analyzed by the Duncan's multiple range tests.

Result

The effect of different levels of *S. securidaca* seed on blood parameters is depicted in Table 3. Broilers supplemented *S. securidaca* at levels more than 2 g/kg diet had more amounts of NO and less amounts of MDA compared to control ($P < 0.05$). Supplementing *S. securidaca* seed to broiler diets decreased AST, ALT, ALP, and hematocrit compared

to the control ($P < 0.05$). However, there is in significant difference between treatments when *S. securidaca* seed was supplemented to broiler diets at the levels more than 1 g/kg ($P > 0.05$).

Table 3. Effect of *S. securidaca* on broiler blood parameters

Item	Dietary levels of *S. securidaca* (g/kg)						
	Control (0)	1	2	3	4	*SEM*	*p*-value
Alanine amino transaminase (u/L)	3.67^{a}	3.1^{b}	2.78^{c}	2.67^{c}	2.63^{c}	0.09	0.0001
Spartat amino transaminase (u/L)	190.6^{a}	181.8^{ab}	158.3^{bc}	151.9^{c}	156.9^{bc}	9.05	0.015
Alkalin phosphatas (u/L)	1857.8^{a}	1616.7^{b}	1393.2^{c}	1349.8^{c}	1360.7^{c}	50.2	0.0001
Malonedialdehyde (µmol/L)	3.72^{a}	3.24^{ab}	2.52^{bc}	2.16^{c}	2.14^{c}	0.275	0.0005
Plasma nitric oxide (µmol/L)	9.91^{b}	11.4^{b}	14.68^{a}	16.2^{a}	16.84^{a}	1.07	0.0001
Hematocrit (%)	44.7^{a}	42.2^{a}	38^{b}	36.8^{b}	36.5^{b}	1.21	0.0001

Superscripts in the same row with different letters are significantly different ($P < 0.05$).

Table 4 shows the carcass characteristics of broilers fed with different levels of *S. securidaca* seed at 42 days of slaughtering age. Use of *S. securidaca* seed in broiler diets at levels 2 to 4 g/kg decreased liver and heart proportions as well as RV/TV ratio in comparison with the control ($P < 0.05$). The effect of dietary treatments on liver gene expression of broilers is shown in Table 5. SOD1 and GPX1 transcripts increased in liver of broilers when *S. securidaca* seed was supplemented at levels of 3 and 4 g/kg compared to control ($P < 0.05$). However, the expression of these genes was not observed among the control group and those groups that received less than 3 g/kg of *S. securidaca* seed.

Table 4. Effect *Securigera securidaca* on broiler carcass characteristics at 42 days of age

Item	Dietary levels of *Securigera securidaca* (g/kg)						
	Control (0)	1	2	3	4	*SEM*	*p*-value
Heart (% of BW)	0.63^{a}	0.59^{ab}	0.56^{bc}	0.53^{c}	0.53^{c}	0.016	0.0007
Liver (% of BW)	2.28^{a}	2.17^{ab}	2.1^{ab}	1.96^{b}	1.96^{b}	0.073	0.015
Abdominal fat (% of BW)	1.43^{a}	1.26^{ab}	1.09^{ab}	0.85^{b}	0.91^{b}	0.15	0.027
RV:TV ratio (g/g)	0.29^{a}	0.27^{a}	0.23^{b}	0.22^{c}	0.22^{c}	0.014	0.001

Superscripts in the same row with different letters are significantly different ($P < 0.05$).
RV:TV – right ventricle to total ventricle weight ratio.

Table 5. Effect of *Securigera securidaca* on liver gene expression of broilers at 42 days of age

Item	Gene	Dietary levels of *Securigera securidaca* (g/kg)						
		Control (0)	1	2	3	4	*SEM*	*p*-value
Liver	GPX	0.0034^{b}	0.0134^{b}	0.0304^{ab}	0.077^{a}	0.0813^{a}	0.019	0.019
	SOD1	0.0031^{b}	0.051^{b}	0.484^{ab}	0.66^{a}	0.67^{a}	0.16	0.012

Superscripts in the same raw with different letters are significantly different ($P < 0.05$).

Discussion

Todays, natural products as a main source of new bioactive agents have been noticed. In this regard, medicinal plants have been widely used for control and treatment of many diseases. In fact, these plants have enormous capacity for providing novel drugs with a new mechanism of action (Singh *et al*., 2012). Earlier study demonstrated that there are bioactive compounds including the sterols (3.23%), Fatty acids (42.8%), Phenols (17.9%), and Polyols (24.05%) in carbon tetrachloride extract of *S. securidaca* (Ahmadi *et al*., 2016). Ibrahim *et al*, (2015) also reported that total phenolics and flavonoids are the main bioactive compounds of this plant. These compounds are antioxidant in terms of pharmacological activity. Flavonoids are able to scavenge ROS, activate antioxidant enzymes, elevate metal chelating activity, reduce α-tocopheryl radicals, and inhibit oxidases (Surai, 2014). ROS is one of the most important factors in PHS pathogenesis that could produce MDA in many affected tissues due to lipid peroxidation in mitochondrial and cell membranes and could also defect antioxidant enzymes and their synthesis. It has been confirmed the elevation of free radicals and MDA in blood and other tissues of ascites chickens, but the activity of SOD1 and GPX was considerably diminished (Ahmadipour *et al*., 2018b).

The results of the present study demonstrated that supplementing *S. securidaca* to broiler diets at levels more than 2 g/kg lead to significant reductions of MDA in serum which as a biomarker of lipid oxidation could be a main index of oxidative stress. There are many factors that make a critical condition for chicken body to produce greater amounts of ROS (Braun and Sweazea, 2008) and subsequently to be very susceptible to oxidative stress compared to mammals. They are including higher metabolic rate (2–2.5 times), higher body temperature (about 3°C), and higher blood sugar concentration (at least 2 times) (Lindstedt and Calder, 1976). In the present

study, supplementing *S. securidaca* seed at the levels of at least 2 g/kg decreased MDA level and stimulated the gene expression of SOD1 and GPX which indicates lower lipid peroxidation in the chickens with PHS. The RV/TV was considerably decreased in birds fed by the *S. securidaca* seed. This index was more than 0.25 in the control group that is the indication of PHS incidence in the birds of this group. Flavonoids and phenolic compounds, due to vasodilatation, reduce the pulmonary arterial pressure and the supply of right ventricular hypertrophy (Ahmadipour *et al*., 2017, 2018c). Flavonoids probably protect endogenous vasodilators such as NO (endothelium-derived factor) from superoxide-induced inactivation by scavenging ROS (Rice-Evans and Packer, 2003). According to the results of this experiment, the *S.securidaca* seeds are able to increase nitric oxide concentration in the serum and decreased PHS. Similar to our results, Ahmadipour (2018a,d) showed that feeding *Securigera securidaca* seed (3 g/kg diet) to broiler chickens reared at high altitudes from sea level and cold conditions increased the expression of inducible nitric oxide synthase (iNOS) and serum nitric oxide concentration and reduced hematocrit). These data may confirm that *S. securidaca* inhibits the progress of heart hypertrophy and especially right ventricular hypertrophy.

A reliable approach to diagnose pathological conditions is to assay blood enzyme activity (Senanayake *et al*., 2015). In the liver damage, enzymes such as ALT, AST, and ALP are released into the blood stream. In the hypoxic state (before ascites incidence), oxygen deficiency causes more lipid peroxidation and subsequent liver, heart and lung damage in chickens that leading to increased serum levels of ALT, AST, and ALP (Janeway, 2001). It has been reported previously that medicinal plants, for their phenolic and flavonoids compounds prevent the destruction of liver cells due to their antioxidant properties (Fathi *et al*., 2015). Our study in line with Ahmadipour *et al* (2017) showed that serum ALT, AST, and ALP levels in birds with ascites were increased, and *S. securidaca* could considerably reduce these enzymes.). It has been reported that Hawthorn extract reduced the serum ALT, AST, and ALP in broilers susceptible to PHS. This plant through its flavonoids and oligometric proanthocyanidins compounds preserves hepatocytes against free radicals in the body by scavenging these adverse agents, and then reduces the releasing of mentioned enzymes (Choi *et al*., 2007). Lipolytic effect of *S. securidaca* has reflected in reduced deposition of fat in abdominal cavity. Lipolytic effect of *S. securidaca* is attributed to flavonoids (Ahmadipour *et al*., 2018b). Liver is the main place for lipogenesis in birds, although the involvement of bone marrow, adipose tissue and skin is not negligible (Leveille, 1969). Several studies have proved antidiabetic and antilipidemic properties of *S. securidaca* (Porchezhian and Ansari, 2001; Azarmiy *et al*., 2009). Jadhav and Puchchakayala (2012) were reported that this plant enhances peripheral glucose usage by skeletal muscles and stimulates β-cells of pancreas that these two effects may justify its hypoglycemic and hypolipidemic activities. Garjani *et al*. (2009) determined that hydroalcoholic extract of *S. securidaca* seeds in hypercholestrolemic rats could reduce serum cholesterol level. They recommended that flavonoids and saponins of *S. securidaca* are the principal factors in the decrease of serum lipid levels and lipid peroxidation.

Conclusion

It is concluded that broiler chickens fed a diet containing 2 to 4 g/kg S. securidaca seed had lower hematocrit, abdominal fat and right ventricular ratio to total ventricles, while nitric oxide was higher. The transcript of GPX and SOD genes were also influenced in the liver by feeding of this plant. Therefore, the inclusion of *S. securidaca* seed as a herbal medicine in broiler chicken diets at the levels more than 2 g/kg could help to prevent ascites in susceptible broilers rearing in the altitude 2100 m. This effect could be due to antioxidative properties of *S. securidaca*.

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