ANTIOXIDANT AND ANTIHEMOLYTIC ACTIVITIES OF LEONTODON HISPIDUS

M.A. Ebrahimzadeh1, S. Eslami1, S.M. Nabavi1, S.F. Nabavi1, B. Eslami1
1Mazandaran University of Medical Sciences, School of Pharmacy, Pharmaceutical Sciences Research Center, Sari, Iran
2University of Mazandaran, Department of Biology, Babolsar, Iran
3Islamic Azad University, Department of Biology, Branch of Ghaemshahr, Iran
Correspondence to: Seyed Mohammad Nabavi
E-mail: Nabavi208@gmail.com

ABSTRACT
In this study, antioxidant and antihemolytic activities of aerial parts of Leontodon hispidus hydroalcoholic extract were investigated with different in vitro assay systems. Extracts showed good antioxidant activity. IC50 for DPPH radical-scavenging activity was 241.3±7.91 µg/ml. Extracts showed moderate Fe²⁺ chelating ability (IC50=683.1±23.22 µg/ml). Extracts exhibited good antioxidant activity in hemoglobin induced linoleic acid model and also it was capable to scavenge hydrogen peroxide in a concentration dependent manner. Extracts showed good antihemolytic activity against H2O2 induced hemolysis (IC50=364.1±10.55 µg/ml). The total amount of phenolic compounds in the extract was determined as gallic acid equivalents and total flavonoid content was calculated as quercetin equivalents from a calibration curve. Also cumarin was determined by HPLC/DAD.


Keywords: antioxidant activity, Leontodon hispidus, flavonoid, antihemolytic activity

Introduction
Oxidative stress is well known as an inducer of cellular and tissue pathogenesis and contributor to the pathophysiology of a variety of diseases including cancer and inflammatory disorders, atherosclerosis, carcinogenesis, drug toxicity, reperfusion injury and neurodegenerative diseases (11). Antioxidants can protect living organism from damage caused by the excessive production of free radicals and the concomitant lipid peroxidation, protein damage and DNA strand breaking (9). Thus antioxidants can play a preventive role for the above mention diseases. Human body has multiple mechanisms and antioxidant systems which protect the cellular molecules against damage induced by free radicals (1). However, generally these systems do not exercise sufficient protection against oxidative stress. Hence, certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order that the reactive oxygen species (ROS) in human body are balanced. On the other hand synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are very effective and are used for industrial processing but they have frequently brought up some questions about their safety and efficiency ever since their initial introduction into the food industry (17). For example, pneumotoxicity, hepatotoxicity and nephrotoxicity of Butylated hydroxytoluene were previously reported (13). Also carcinogenic effect of BHT and BHA was reported (17). Hence, compounds especially from natural sources that are capable to provide protection against ROS mediated damage may have potential application in prevention and/or for curing of diseases.

The genus of Leontodon is member of Compositae and has five species that are found in Iran. Leontodon hispidus with persian name “shir dandan-e-moy zebar” is one of the members of this genus which is grown wild in Elburz Mountains (16). Previously, cytotoxicity and anti-inflammatory activities of hypocretenolides extracted from this species have been reported (22, 23). To the best of our knowledge, there are no reports concerning antioxidant and antihemolytic activities of this particular species.

Materials and Methods
Plant materials
Leontodon hispidus was collected from Veresk area, Elburz Mountains, Mazandaran, Iran and identified by Dr. Bahman Eslami, assistance professor of plant systematic and ecology, Department of Biology, Islamic Azad University, branch of Ghaemshahr, Iran, where a voucher specimen (No 448) was deposited.

Chemicals
Ferrozine, Linoleic acid, Trichloroacetic acid (TCA), 1,1-Diphenyl-2-picryl hydrazyl (DPPH), Potassium ferricyanide and Hydrogen peroxide were purchased from Sigma Chemicals Co. (USA). Gallic acid, Quercetin, Butylated hydroxyanisole (BHA), Vitamin C, EDTA and Ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

Freeze-dried extract
Aerial parts of plant were dried at room temperature and coarsely ground before extraction. A known amount of aerial
parts of plant was extracted at room temperature by percolation method using ethanol-water (70-30 v/v). The resulting extract was concentrated over a rotary vacuum until a crude solid extract was obtained (13.4%), which was then freeze-dried for complete solvent removal.

**Determination of total phenolic and flavonoid contents**

Total phenolic content was determined by the Folin-Ciocalteau method (6). The extract sample (0.5 ml of 1.6 mg ml⁻¹) was mixed with 2.5 ml of 0.2N Folin-Ciocalteau reagent for 5 min and 2.0 ml of 75 g l⁻¹ sodium carbonate were then added. Absorbance was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoid content was estimated using the method of Nabavi et al. (17). Briefly, 0.5 ml solution of plant extract was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate, and 2.8 ml of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (Perkin Elmer). Total flavonoid content was calculated as quercetin from a calibration curve.

**Antioxidant activity**

1. **DPPH radical-scavenging activity**

The stable 1, 1-diphenyl-2-picylhydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extract (6). Different concentrations of sample were added, at an equal volume, to ethanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamin C, BHA and Quercetin were used as standard controls. IC₅₀ values denote the concentration of sample, which is required to scavenge 50% of DPPH free radical.

2. **Reducing power determination**

The reducing power of extract was determined according to the method of Dehpour et al. (4). 2.5 ml of sample (25-800 μg ml⁻¹) in water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 1000 g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

3. **Metal chelating activity**

The chelation of ferrous ions by the extract was estimated by the method of Dinis et al. (5). Briefly, 1 ml of sample (0.2–3.2 mg ml⁻¹) was added to a solution of 2 mM FeCl₃ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe²⁺ complex formation was calculated as \[\{(A_o-A_1)/A_o\} \times 100\], where \(A_o\) was the absorbance of the control, and \(A_1\) was the absorbance of the extract/standard. EDTA was used as positive control.

4. **Scavenging of hydrogen peroxide**

Briefly, a solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). 2 ml of sample (0.1-1 mg ml⁻¹) in distilled water was added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of the sample at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide and extract. The percentage of hydrogen peroxide scavenging by the extract and standard was calculated as follows: % scavenged \[\text{[H}_2\text{O}_2\text{]}=\{(A_o-A_1)/A_o\} \times 100\], where \(A_o\) was the absorbance of the control and \(A_1\) was the absorbance in the presence of the samples of extract and standard (17).

5. **Antioxidant activity in a hemoglobin-induced linoleic acid system**

The antioxidant activity of extract was determined by a modified photometry assay (6). Reaction mixture (200 ml) containing 10 ml extract (10–400 mg ml⁻¹), 1 mmol l⁻¹ of linoleic acid emulsion, 40 mmol l⁻¹ of phosphate buffer (pH 6.5), and 0.0016% hemoglobin suspension, was incubated at 37°C for 45 min. After the incubation, 2.5 ml of 0.6% HCl in ethanol was added to stop the lipid peroxidation. The amount of peroxide value was measured in triplicate using the thiocyanate method by reading the absorbance at 480 nm after coloring with 100 ml of 0.02 mol l⁻¹ of FeCl₃ and 50 ml of ammonium thiocyanate (0.3 g ml⁻¹). Vitamin C was used as positive control.

**Antihemolytic activity of extract**

1. **Preparation of rat erythrocytes**

All the animal experiments were carried out with the approval of institutional animal ethical committee of University of Mazandaran, Babolsar. Male rats with a body weight in the range of 180-220 g were housed in individual polypropylene cages and had free access to food and water. The animals were fed with standard diet. The animals were sacrificed under anaesthesia and blood was collected by heart puncture in heparinized tubes. Erythrocytes were isolated and stored according to the method described by Yuan et al. (20). Briefly, the collected blood samples were centrifuged (1000 g, 10 min) at 4°C, erythrocytes were separated from the plasma anduffy coat and were washed three times by centrifugation (1000 g, 5 min) in 10 volumes of 10 mM phosphate buffered saline (pH 7.4). The supernatant anduffy coats of white cells were carefully removed with each wash. Washed erythrocytes were stored at 4°C and used within 6 h for further studies.

2. **Antihemolytic activity of extract against _H_2O_2_ induced hemolysis**

Antihemolytic activity of the extract was assessed as described by Nabavi et al. (17). Erythrocytes from male rat blood were separated by centrifugation and washed with phosphate buffer (pH 7.4). Erythrocytes were then diluted with phosphate buffered saline to give 4% suspension. 0.5 ml
of different concentrations of the extract was added to 2 ml of erythrocyte suspension and the volume was made up to 5 ml with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 ml of H₂O₂ solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H₂O₂ in the reaction mixture was adjusted to bring about 90% hemolysis of blood cells after 240 min. After incubation the reaction mixture was centrifuged at 250 g for 10 min and the extent of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation.

**Assay of putative Biological active components: coumarin**
A Knauer series liquid chromatography system comprising of degasser, pump, auto-sampler, thermostatted column compartment, and diode array detector (DAD) was used. The used column was a C₁₈ reversed phase Kingsorb 5 mm (250 × 4.6 mm) employed at 30°C. Separations were done in the isocratic mode, using acetonitrile-water (40-60; v/v) at a flow rate of 1 ml min⁻¹; with an injection volume (“loop”) of 20 μl; UV detection was at 274 nm (3). Determination of the content of the coumarin in plant extract was performed by the external standard method, using pure coumarin (Sigma) as standard.

**Statistical analysis**
Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance (p < 0.05) and the means separated by Duncan’s multiple range tests. The IC₅₀ values were calculated from linear regression analysis.

**Results and Discussion**
Total phenolic content, as determined by Folin Ciocalteu method, is reported as gallic acid equivalents by reference to standard curve (y=0.0054x+0.0628, r²=0.987). The total phenolic content of *Leontodon hispidus* aerial parts was 47.81±1.19 mg gallic acid equivalent g⁻¹ of extract. The total flavonoid content of *Leontodon hispidus* was 19.57±0.61 mg quercetin equivalent g⁻¹ of extract powder, by reference to standard curve (y=0.0063x, r²=0.999). The extract showed high level of total phenol and flavonoid contents. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activity (19). The amount of cumarin determined by HPLC/DAD obtained from extract was 31.25 μg gr⁻¹ of extract. IC₅₀ for DPPH radical-scavenging activity was 241.3±7.91 μg/ml. The IC₅₀ values for Ascorbic acid, quercetin and BHA were 5.05±0.1, 5.28±0.2 and 53.96±3.1 μg ml⁻¹, respectively. High Phenol and flavonoid content in of this plant may lead to its potent DPPH radical scavenging activity. Phenol and flavonoid can reduce DPPH radicals by either the process of hydrogen- or electron- donation and changes its colour from violet to yellow. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (7). Another assay that is mechanism related to electron donating ability of the extract is reduction power assay. In this assay, the presence of electron donor in the sample would result in the reducing of Fe³⁺ to Fe²⁺. Then the amount of Fe²⁺ complex can be monitored by measuring the formation of Perl’s Prussian blue at 700 nm. An increasing absorbance at 700 nm indicates an increase in the reductive ability. **Fig. 1** shows the dose-response curves for the reducing power of the extract. It was found that the reducing power of the extract also increased with the increase of its concentration. Vitamin C was used as a standard antioxidant (4, 6).

![Fig. 1. Reducing power of *Leontodon hispidus* extract (aerial parts). Vitamin C used as control](image)

*Leontodon hispidus* showed moderate iron chelating activity (IC₅₀=683.1±23.22 μg ml⁻¹). EDTA showed very powerful activity (IC₅₀=18±0.5 μg ml⁻¹). Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications in human and thereby improves quality of life and overall survival in some diseases such as Thalassemia major (12). The usage of iron chelators can ameliorate the symptoms of iron overload and improve the quality of life and overall survival rate for sufferers. Deferoxamine is used as an iron chelator for treatment of iron overload in Thalassemia major. Deferoxamine overdose may result in some adverse effects (18). Deferiprone is an orally absorbed bidentate iron chelator that can induce urinary iron excretion, promote negative iron balance and reduce hepatic iron levels in some transfusion-dependent individuals. A number of adverse effects include arthrosis and nausea. Agranulocytosis may also occur in 0.6 to 4% and requires cessation of therapy in up to 30% of patients. Thus, an urgent need to identify an orally active chelator regimen that is as effective as deferoxamine and has an acceptable degree of tolerability remains (18). So, in recent years search for natural iron chelators with lower side effects has been intensified (10). On the other hand previous studies showed that iron chelators and hydroxyl-radical scavengers have a protective role against acute renal failure and especially for aminoglycoside antibiotic-mediated nephrotoxicity (15). These processes can be suppressed by iron chelation and deactivation. The transition metal, iron, is capable of generating
free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (8). So, extra Fe\(^{2+}\) removal would provide protection against oxidative damage. The chelating of ferrous ions by the extract was estimated according to our recent paper (8). Ferrozine can quantitatively form complexes with Fe\(^{2+}\). In the presence of other chelating agents, the complex formation is disrupted which results in a change of the red colour as the complexes decrease. In this assay, both the extract and EDTA interfere with the formation of ferrous and ferrozine complexes, suggesting that the extract has chelating activity and captures ferrous ions before ferrozine is produced.

The extract was capable of scavenging hydrogen peroxide in a concentration dependent manner (IC\(_{50}\) was 148.4±5.34 µg ml\(^{-1}\)). The IC\(_{50}\) values for vitamin C and quercetin were 21.4±1.1 and 52±2.6 µg ml\(^{-1}\), respectively. Scavenging of H\(_2\)O\(_2\) by the extract may be attributed to its phenolics, and other active components which can donate electrons to H\(_2\)O\(_2\), thus neutralizing it to water (14). Hydrogen peroxide itself is not very reactive, but sometimes it can cause cytotoxicity by giving rise to hydroxyl radicals in the cells (4). Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. The tested extract showed good activity in hemoglobin-induced linoleic acid system but there were significant differences in the results for the extract and vitamin C (p<0.01) (Fig. 2).

![Fig. 2. Antioxidant activity of *Leontodon hispidus* extract against hemoglobin-induced lipid peroxidation. Each value is expressed as mean from 3 measurements ± standard deviation; positive control (Vitamin C)](image)

Erythrocytes are considered as prime targets for free radical attack owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the O\(_2\) transport associated with redox active hemoglobin molecules, which are potent promoters of reactive O\(_2\) species. Specially, linoleic and arachidonic acids are targets of lipid peroxidation (21). The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activity. Superoxide indirectly initiates lipid peroxidation because superoxide anions act as a precursor of singlet oxygen and hydroxyl radicals (6). Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. The extract showed good activity in hemoglobin-induced linoleic acid peroxidation. The effect of *Leontodon hispidus* extract was tested and it was found that they did not show any harmful effect on erythrocytes (IC\(_{50}\) was 364.1±10.55 µg ml\(^{-1}\) vs. Vitamin C- 235±9 µg ml\(^{-1}\)). Antihemolytic activity of quercetin and other flavonoid have previously been reported and the good activity of the extract maybe result of high flavonoid content especially quercetin (2).

Conclusions

Our study detected remarkable antioxidant and antihemolytic activities in hydroalcoholic extract of *Leontodon hispidus*. These effects may be result of high phenol and flavonoid contents. Therefore further biochemical experiments which will be focused on evaluating the mechanism of this activity are very promising.

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REFERENCES