

Antihemolytic and antioxidant activities of *Biebersteinia multifida*

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Abstract. – Objectives: *Biebersteinia multifida* is a common herb known in Iran. Its roots have been used locally in folk medicine of western region of Iran in the treatment of many diseases. The antioxidant activity and its inhibition of erythrocyte hemolysis were investigated.

Material and Methods: 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), nitric oxide and hydrogen peroxide scavenging activities, Fe²⁺ chelating ability, reducing power and hemoglobin-induced linoleic acid peroxidation were used to evaluate antioxidant activities. Antihemolytic activity was evaluated by H₂O₂ induced hemolysis in rat erythrocyte. The total amount of phenolic compounds was determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve.

Results: Root had higher phenol contents (80.1 ± 3.1 mg ml⁻¹) and showed highest activity in DPPH radical-scavenging activity (95.9 ± 3.2 µg ml⁻¹). It also showed better reducing power than other parts. In Fe²⁺ chelating, leaf extract was the most potent (789 ± 33 µg ml⁻¹). Extracts exhibited good H₂O₂ scavenging in a concentration dependent manner. All extracts exhibited good protection against hemoglobin-catalyzed peroxidation linoleic acid system. In nitric oxide scavenging model, root extract showed the best activity (696 ± 2.7 µg ml⁻¹). Root and leaf extracts contained total phenol and flavonoids contents than other extracts. Tested extracts show weak activity in H₂O₂ induced hemolysis in rat erythrocyte which was not comparable with vitamin C.

Conclusions: *Biebersteinia multifida* extracts exhibited different levels of antioxidant and antihemolytic activities in all tested models. Biological effects may be attributed, at least in part, to the presence of phenols and flavonoids in the extracts.

Key Words:

Antihemolytic activity, Antioxidant activity, *Biebersteinia multifida*, DPPH, Flavonoid content.

Introduction

The pathology of numerous chronic diseases, including cancer and heart disease, involves oxidative damage to cellular components. Reactive oxygen species (ROS), capable of causing damage to DNA, have been associated with carcinogenesis, coronary heart disease, and many other health problems related to advancing age^{1,2}. Minimizing oxidative damage may well be one of the most important approaches to the primary prevention of these aging-associated diseases and health problems, since antioxidants terminate direct ROS attacks and radical-mediated oxidative reactions, and appear to be of primary importance in the prevention of these diseases and health problems. Antioxidants have been detected in a large number of food and agricultural products, including cereal grains, vegetables, fruits, and plant extracts^{3,4}. Among the various medicinal plants, some endemic and edible species are of particular interest because they may be used for producing raw materials or preparations containing phytochemicals with significant antioxidant capacities and high content of mineral with health benefits. *Biebersteinia multifida* DC (*Geraniaceae*) is a common herb known in Iran as Adamak⁵. Its roots are thick and dense with a light to dark brown color and have been used locally in folk medicine of western re-

gion of Iran for the treatment of musculoskeletal disorders and restoring bone fractures. Isolation of an alkaloid (vasicinone) and some polysaccharides and peptic substances from this species have been reported⁶. Seven compounds from *Biebersteinia heterostemon* with hypotensive, analgesic and immunity stimulating effects have been isolated⁷. The anti-inflammatory and antinociceptive effects of *Biebersteinia multifida* root have been reported previously⁶. To the best of our knowledge, there is no scientific report on antioxidant activity of different parts of *Biebersteinia multifida*. In this study, the antioxidant activity of flower, stem, root and leaf of *Biebersteinia multifida* at flowering stage were examined employing seven various *in vitro* assay systems in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

Materials and Methods

Chemicals

Ferrozine, linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl, Potassium ferricyanide and hydrogen peroxide were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Gallic acid, quercetin, butylated hydroxyanisole, ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, EDTA and ferric chloride were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade or purer.

Plant Material and Preparation of Freeze-Dried Extract

Flower, stem, root and leaf of *Biebersteinia multifida* (at flowering stage) were collected from Sari forest (northern of Iran) and identified by Dr. Bahman Eslami. A voucher (No. A423-A426) has been deposited in the Sari School of Pharmacy herbarium. The materials were dried at room temperature for 2 weeks and then oven dried at 35°C, for 2 days. Dried materials were coarsely ground (2-3 mm) before extraction. Each part (100 g) was extracted by percolation using ethanol/water (80/20 w/w) for 24 h at room temperature. The extract was then separated from the sample residue by filtration through Whatman No.1 filter paper, repeated three times. The resultant extracts were concentrated in a rotary evaporator until a crude solid extracts were obtained which were then freeze-dried for complete solvent removal.

Determination of Total Phenolic and Flavonoid Contents

Total phenolic contents were determined by the Folin-Ciocalteu reagent according to the our recently published papers^{8,9}. The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagent for 5 min and 2.0 ml of 75 g/l sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoids were estimated using our recently published paper^{8,9}. Briefly, 0.5 ml solution of each plant extracts in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (UV- Visible EZ201, Perkin Elmer: Norwalk, CA, USA). Total flavonoid contents were calculated as quercetin from a calibration curve.

DPPH Radical-Scavenging Activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts^{10,11}. Different concentrations of each extracts were added, at an equal volume, to methanolic 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 radicals.

Reducing Power Determination

Ferric ion (Fe⁺³) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action¹¹. The reducing power of extracts was determined according to our recently published paper^{12,13}. Different amounts of each extracts (25-800 µg ml⁻¹) in water were 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 3000 rpm 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 (UV-Visible EZ201, Perkin Elmer: Norwalk, CA, USA). Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Assay of Nitric Oxide-Scavenging Activity

The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of each extracts dissolved in water and incubated at room temperature for 150 min. After the incubation period, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control^{14,15}.

Metal Chelating Activity

Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry¹⁶. The chelating of ferrous ions by extracts was estimated by our recently published paper^{17,18}. Briefly, the extract (0.2-3.2 mg/ml) was added to a solution of 2 mM FeCl₂ (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 (UV-Visible EZ201, Perkin Elmer: Norwalk, CA, USA). The percentage inhibition of ferrozine-Fe²⁺ complex formation was calculated as $[(A_0 - A_s)/A_s] \times 100$, where A₀ was the absorbance of the control, and A_s was the absorbance of the extract/standard. Na₂EDTA was used as positive control.

Antioxidant Activity in Hemoglobin Induced Linoleic Acid Peroxidation Test

The antioxidant activity of extracts was determined by a modified photometry assay^{19,20}. Reaction mixtures (200 ml) containing 10 ml extracts (10-400 mg), 1 mmol/l of linoleic acid emulsion, 40 mmol/l of phosphate buffer (pH 6.5), and 0.0016% hemoglobin, were 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 of FeCl₂ and 50 ml of ammonium thiocyanate (30 g/100 ml) (UV-Visible EZ201, Perkin Elmer: Norwalk, CA, USA). Vitamin C was used as positive control.

Scavenging of Hydrogen Peroxide

Briefly, a solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extracts (0.1-1 mg ml⁻¹) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide (UV-Visible EZ201, Perkin Elmer: Norwalk, CA, USA). The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows: % Scavenged (H₂O₂) = $[(A_0 - A_1)/A_0] \times 100$ where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the sample of extract and standard^{4,21}.

Antihemolytic activity

Preparation of Rat Erythrocytes

All the animal experiments were carried out with the approval of institutional animal Ethical Committee. Male Wistar rats in the body weight 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 anesthesia and blood was collected by heart puncture in heparinized tubes. Erythrocytes were isolated and stored according to the method described by Yuan et al²². Briefly blood samples collected were centrifuged (1500 × g, 10 min) at 4°C, erythrocytes were separated from the plasma and buffy coat and were washed three times by centrifugation (1500 × g, 5 min) in 10 volumes of 10 mM phosphate buffered saline (pH 7.4; PBS). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes stored at 4°C and used within 6 h for further studies.

***In Vitro* Assay for Inhibition of Rat Erythrocyte Hemolysis**

The rat erythrocyte hemolysis was performed with H₂O₂ as free radical initiator. To 100 µl of 5% (v/v) suspension of erythrocytes in PBS, 50 µl of extract with different concentrations (5-25 µg in PBS pH 7.4), which corresponds to 100-3200 µg of extract, was added. To this, 100 µl of H₂O₂ (1 M in PBS, pH 7.4) was added. The reaction mixture was shaken gently while being incubated at 37°C for 3 h. The reaction mixture was diluted with 8 ml of PBS and centrifuged at 2000 × g for 10 min. The absorbance of the resulting supernatant was measured at 540 nm by spectrophotometer (UV – Visible EZ201, Perkin Elmer: Norwalk, CA, USA) to determine the hemolysis. Likewise, the erythrocytes were treated with 100 µM H₂O₂ and without inhibitors (extracts) to obtain a complete hemolysis. The absorbance of the supernatant was measured at the same condition. The inhibitory effect of the extract was compared with vitamin C. To evaluate the hemolysis induced by leaves extract, erythrocytes were preincubated with 50 µl of extracts corresponding to 25 µg extract for 1 h and the hemolysis was determined. Percentage of hemolysis was calculated by taking hemolysis caused by 100 µM H₂O₂ as 100%. The IC₅₀ values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% hemolysis¹²³.

Statistical Analysis

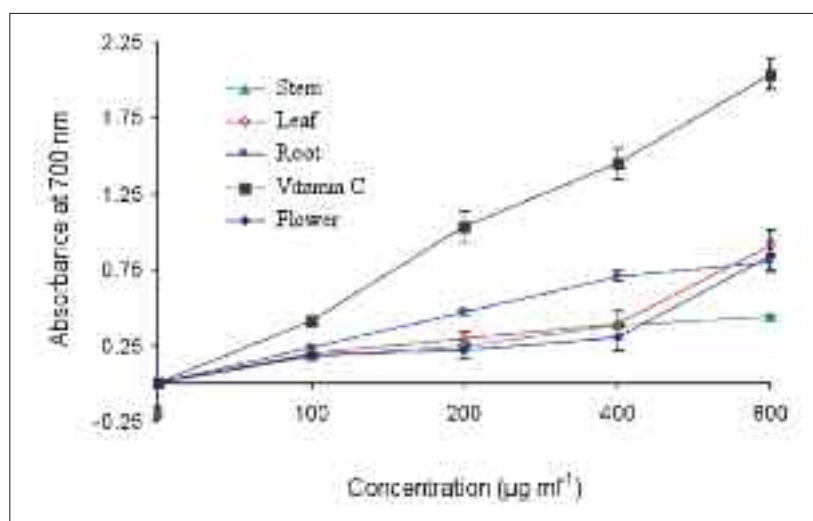
Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p < 0.05$) and the means separated by Duncan's multiple range test. The IC₅₀ values were calculated from linear regression analysis.

Results and Discussion

Total phenol compounds, as determined by Folin Ciocalteu method, are reported as gallic acid equivalents by reference to standard curve ($y = 0.0054x + 0.0628$, $r^2 = 0.987$). The total phenolic contents of *Biebersteinia multifida* extracts were in order of: root > leaf > stem > flower, respectively. The total flavonoid contents of different part of *Biebersteinia. multifida* were in order of: leaf > flower > stem > root, respectively, by reference to standard curve ($y = 0.0063x$, $r^2 =$

0.999). 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 activities. Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases⁴. The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples^{8,24}. DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers⁴. IC₅₀ for DPPH radical-scavenging activity were in order of: root > leaf > stem > flowers, respectively. Because of higher total phenol in root extract, it showed more potent activity than other parts. 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. In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reducing of Fe³⁺ to Fe²⁺ by donating an electron. Amount of Fe²⁺ complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 1 shows the dose– response curves for the reducing powers of the extracts from *Biebersteinia multifida*. It was found that the reducing powers of extracts also increased with the increase of their concentrations. The root extract had shown better reducing power than other parts ($p < 0.05$) that was not comparable with vitamin C ($p < 0.01$). The extracts also showed weak NO scavenging activity between 0.2 and 3.2 mg ml⁻¹. The % inhibition was increased with increasing concentration of extract. Results exist in Table I. The root extract had shown better scavenging activity than other extracts (696 ± 2.7 µg ml⁻¹ vs IC₅₀ = 5.28 ± 0.2 µg ml⁻¹ for quercetin). In addition to reactive oxygen species, NO is also implicated in inflammation, cancer and other pathological conditions²⁵. The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human

Figure 1. Reducing power of *Biebersteinia multifida* extracts. Vitamin C used as control. Results are means \pm SD.



health⁴. The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease²⁶. Because Fe²⁺ also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe²⁺ concentration affords protection against oxidative damage. The chelating of ferrous ions by extract was estimated by our recently published papers^{27,28}. Ferrozine can quantitatively form complexes with Fe²⁺. In the presence of other chelating agents, the complex formation is disrupted with the result that red color of the complexes decreases. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has

chelating activity and captures ferrous ions before ferrozine. The absorbance of Fe²⁺-ferrozine complex was decreased dose-dependently, *i.e.* the activity was increased on increasing concentration from 0.4 to 3.2 mg ml⁻¹. Metal chelating capacity was significant since the extract reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion¹⁵. *Biebersteinia multifida* leaf extract showed better Fe²⁺ chelating ability than other parts (Table I). The extracts showed good inhibitory ability on lipid oxidation (Figure 2). Among extracts The root extract showed better

Figure 2. Antioxidant activities of *Biebersteinia multifida* extracts against in hemoglobin induced linoleic acid peroxidation test. Results are means \pm SD.

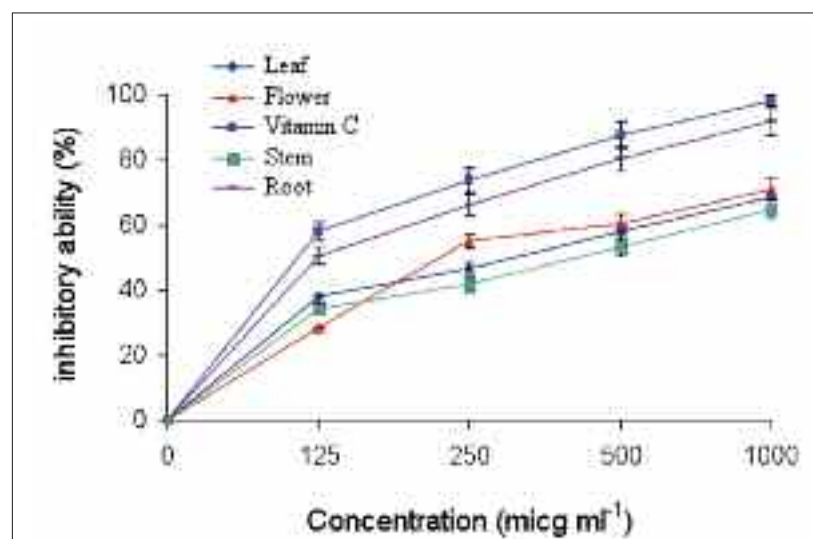


Table 1. Phenol and flavonoids contents, antioxidant and antihemolytic activities of *Biebersteinia multifida* extracts. Results are means \pm SD.

Extract	Total phenol contents (mg g ⁻¹)	Total flavonoid contents (mg g ⁻¹)	DPPH free radical scavenging, IC ₅₀ (μg ml ⁻¹) ^a	Nitric oxide scavenging, IC ₅₀ (μg ml ⁻¹) ^b	H ₂ O ₂ scavenging activity, IC ₅₀ (μg ml ⁻¹) ^c	Fe ²⁺ chelating ability (%) ^d or IC ₅₀ (μg ml ⁻¹) ^e	Antihemolytic activity ^e IC ₅₀ (μg ml ⁻¹)
Root	80.1 \pm 3.1	7.4 \pm 0.3	95.9 \pm 3.2	696 \pm 2.7	921.9 \pm 42	12%	998
Leaf	61.4 \pm 2.2	39.9 \pm 2.1	466 \pm 21	1140.7 \pm 53	1364 \pm 54	789 \pm 33	1022
Flower	39.5 \pm 1.5	20.8 \pm 0.9	685 \pm 31	913 \pm 39	723.9 \pm 25	1465 \pm 61	1070
Stem	44.1 \pm 1.9	19.8 \pm 0.8	607.6 \pm 2.8	1215 \pm 58	1031.2 \pm 46	993.4 \pm 47	1096

^aIC₅₀ of BHA was 53.96 \pm 3.1, vitamin C, 5.05 \pm 0.1 and quercetin 5.28 \pm 0.2 μg ml⁻¹, respectively.

^bInhibition at 1.6 mg ml⁻¹. IC₅₀ of quercetin was 5.28 \pm 0.2 μg ml⁻¹.

^cIC₅₀ for vitamin C and quercetin were 21.4 \pm 1.1 and 52 \pm 2.6 mg ml⁻¹, respectively.

^dInhibition at 1.6 mg ml⁻¹. EDTA used as control (IC₅₀ = 18 \pm 1.5 μg ml⁻¹).

^eIC₅₀ for vitamin C was 235 \pm 9 μg ml⁻¹.

activity than other extracts ($p < 0.01$) in protection of hemoglobin-induced peroxidation linoleic acid system that was comparable with vitamin C ($p > 0.05$) (Figure 2). Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2 , thus neutralizing it to water¹³. The differences in H_2O_2 scavenging capacities between the extracts may be attributed to the structural features of their active components, which determine their electron donating abilities. Extracts were capable of scavenging H_2O_2 in a concentration dependent manner. IC_{50} for H_2O_2 scavenging activity were in the order: flower > root > stem > leaf. The IC_{50} values for ascorbic acid and quercetin were 21.4 ± 1.1 and $52.0 \pm 2.6 \mu\text{g ml}^{-1}$, respectively. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems¹⁴. Effect of extracts was tested and found that it did not show any harmful effect on erythrocytes. The extracts showed 50% hemolysis inhibition at concentrations ranging from 0.9 to 1.1 mg ml^{-1} . The vitamin C exhibited an IC_{50} value of $235 \pm 9 \mu\text{g ml}^{-1}$ (Table I).

Conclusion

Biebersteinia multifida extracts exhibited different levels of antioxidant activity in all tested models. Further investigation of individual compounds, their *in vivo* antioxidant activities is needed. Such identified potential and natural constituents could be exploited as cost effective food/feed additives for human health. As regards this last sentence we would like to underline that in humans the herbal remedies may be cause of toxicity in various organs, mainly in the liver, which is responsible for their metabolism. In fact, many herbal remedies are complex mixture of various components, many of those until now uncertain, whose mechanisms of action are almost unknown.

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