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Antioxidant activity of leaves and inflorescence of *Eryngium Caucasicum Trautv* at flowering stage

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ABSTRACT

Methanol extracts of leaves and inflorescence of *Eryngium Caucasicum Trautv* at flowering stage were investigated for their antioxidant activities employing six in vitro assay systems, i.e. DPPH and nitric oxide radical scavenging, reducing power, linoleic acid and iron ion chelating power. IC_{50} for DPPH radical-scavenging activity was 0.15 ± 0.01 for leaves and 0.39 ± 0.02 mg ml^{-1} for inflorescence. Reducing powers of both extracts increased with the increase of their concentrations. Leaves extract showed better activity than Vitamin C ($p< 0.05$). Extracts showed weak nitric oxide-scavenging activity. Leaves extract exhibited better Fe^{2+} chelating ability (IC_{50}=0.25 mg ml^{-1}) that was comparable with EDTA. (IC_{50}=18 μg ml^{-1}). Inflorescence extracts had shown a very weak activity. Extracts showed very good scavenging activity of H_2O_2. IC_{50} was 25.5 ± 1.3 for leaves and 177.2 ± 11.6 mg ml^{-1} for inflorescence, respectively. No antioxidant activity exhibited in linoleic acid test. Extracts exhibited different levels of antioxidant activity in all the models studied.

Keywords: Antioxidant activity, *DPPH, Eryngium caucasicum,* Free radical scavenging activity, Folin-Ciocalteau.

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 (1–3). Minimizing oxidative damage may well be one of the most important approaches to the primary prevention of these aging-associated diseases ad health problems, since antioxidants terminate direct ROS attacks and radical-mediated oxidative reactions, and appear to be or 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 (4, 5). In the family Umbelliferae (Apiaceae) 117 cultivated species excluding ornamentals have been recorded now, primarily used as medicinal plants (41%); vegetables, salad plants and tuberous starch crops (23.1%); spice plants (19.7%), as well as fodder plants (11.1%); essential oil plants (4.3%) and hedge plants (0.8%) (6) including numerous neglected and underutilized crops with great potential for prospective evaluation. A new umbelliferous crop Eryngium caucasicum Trautv (Caucasian Eryngo, Subfam, Saniculoideae) founds in cultivation in Northern Iran and has reported recently (7). This taxon was not included in the last edition of the most comprehensive catalogue in the subject, the Mansfeld's Encyclopedia (8).

Figure. 1 Flowering plant of Eryngium caucasicum (cf. ref. 6).

E. caucasicum Trautv (Apiaceae) was found as a new cultivated vegetable plant in home gardens in northern Iran. Young leaves are used as a cooked vegetable and for flavoring in the preparation of several local foods (7). A good antioxidant activity of *E. Caucasicum Trautv* leaves at non-flowering stage has been reported recently by our group (9). Nothing was found in literature about the leaves and inflorescence of this native plant at flowering stage (Fig. 1) that is so . different from non-flowering stages. In this study, the antioxidant activity of leaves and inflorescence of *E. Caucasicum Trautv* at flowering stage examined employing six various in vitro assay systems, i.e. DPPH and nitric oxide radical scavenging, scavenging of hydrogen peroxide, reducing power, linoleic acid and iron ion chelating power, in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

MATERIALS AND METHODS

Plant material and preparation of freeze-dried extract

E. caucasicum leaves (at flowering stage) and inflorescence were collected from khazar abad area and identified by Dr. Bahman Eslami. A voucher (No. 987–988) has been deposited in the Sari School of Pharmacy herbarium. Materials dried at room temperature and coarsely ground before extraction. Each part was extracted by percolation method using methanol. The resulting extract was concentrated over a rotary vacuum until a crude solid extract was obtained.

Determination of total phenolic compounds and flavonoid content

Total phenolic compound contents were determined by the Folin-Ciocalteau reagent according to the recently published method (10). The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteau 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 (10). 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 (Perkin Elmer). 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 (11–13). 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. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Reducing power determination

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action (14). The reducing power of extracts was determined according to our recently publish papers (9, 15). Different amounts of each extracts (25–800 $\mu g\ ml^{-1}$) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (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 $FeCl_3$ (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.

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 (10, 16).

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 chemistry (17). The chelating of ferrous ions by extracts was estimated by our recently published paper (16, 18). Briefly, the extract (0.2–3.2 mg/ml) was added to a solution of 2 mM $FeCl_2$ (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 inhibition of ferrozine- Fe^{2+} complex formation was calculated as $[(A_0 - A_s)/A_s] \times 100$, where A_0 was the absorbance of the control, and A_s was the absorbance of the extract/ standard. Na_2EDTA was used as positive control.

Determination of Antioxidant Activity by the FTC Method

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation (19). The inhibitory capacity of extracts was tested against oxidation of linoleic acid by FTC method. This method was adopted from Osawa and Namiki (18, 20). Twenty mg/ml of samples dissolved in 4 ml of 95% (w/v) ethanol were mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 ml), 0.05 M phosphate buffer pH 7.0 (8 ml), and distilled water (3.9 ml) and kept in screwcap containers at 40°C in the dark. To 0.1 ml of this solution was then added 9.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured, and it was measured again every 24 h until the day when the absorbance of the control reached the maximum value. The percent inhibition of linoleic acid peroxidation was calculated as: (%) inhibition=100 – [(absorbance increase of the sample/absorbance increase of the control) × 100]. All tests were run in duplicate, and analyses of all samples were run in triplicate and averaged. Vitamin C and BHA used as positive control.

Scavenging of Hydrogen Peroxide

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch (9, 12, 20). 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. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows: % Scavenged $(H_2O_2)=[(A_o - A_1)/A_o] \times 100$ where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract and 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 test. The EC_{50} values were calculated from linear regression analysis.

RESULTS AND DISCUSSION

Total phenol and flavonoid contents

Total phenol compounds are reported as gallic acid equivalents by reference to standard curve ($y = 0.0063x$, $r^2 = 0.987$). The total phenolic contents of *E. caucasicum* leaves and inflorescence were 37.6 ± 1.5 and 63.1 ± 1.44 mg gallic acid equivalent/g of extract powder, respectively. The total flavonoid contents of *E. caucasicum* leaves and inflorescence were 60.0 ± 2.8 and 18.3 ± 0.9 mg quercetin equivalent/g of extract powder, respectively, by reference to standard curve ($y=0.0067x + 0.0132$, $r^2=0.999$). It was noted that leaves extract had significant higher flavonoids contents than did inflorescence. The latter had higher total phenol 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 activities (9,12).

DPPH radical-scavenging activity

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (21). It was found that the radical- scavenging activities of all the extracts increased with increasing concentration. IC_{50} for DPPH radical-scavenging activity was in the order: *E. caucasicum* leaves (0.15 ± 0.01) > *E. caucasicum* inflorescence (0.39 ± 0.02) mg ml^{-1}. The IC_{50} values for Ascorbic acid, quercetin and BHA were 5.05 ± 0.12, 5.28 ± 0.43 and 53.96 ± 2.13 µg ml^{-1}, respectively.

Reducing power

In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} 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. Fig. 2 shows the dose- response curves for the reducing powers of the extract. It was found that the reducing powers of all the extracts also increased with the increase of their concentrations. There were significant differences ($p< 0.05$) among the leaves with Vit C. Leaves showed very better activity than inflorescence.

Assay of nitric oxide-scavenging activity

The extracts showed very weak nitric oxide-scavenging activity between 0.2 and 3.2 mg ml^{-1}. The percentage of inhibitions was increased with increasing concentration of the extracts. The *E. caucasicum* leaves extract had shown better scavenging activity with IC_{50}= 1.26 ± 0.07mg ml^{-1}. The IC_{50} for *E. caucasicum* inflorescence was 2.37± 0.11 mg ml^{-1}. However, activity of quercetin was very more pronounced than that of our extracts (IC_{50}=17 µg ml^{-1}). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (22).

Fe^{2+} chelating ability

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 (23). In addition, brain iron dysregulation and its association with amyloid precursor protein plaque formation are implicated in Alzheimer's disease (AD) pathology and so iron chelation could be considered a rational therapeutic strategy for AD (24). The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (25). Because Fe^{2+} causes the production of oxyradicals and lipid peroxidation, minimizing its concentration affords protection against oxidative damage. In the presence of other chelating agents, the ferrozine complex formation is disrupted with the result that the red color of the complexes decreases. The absorbance of Fe^{2+}-ferrozine complex was decreased dose-dependently, i.e. the activity was increased on increasing concentration from 0.2 to 0.8 mg ml^{-1}. It was reported that chelating agents are effective as secondary antioxidants because they reduce

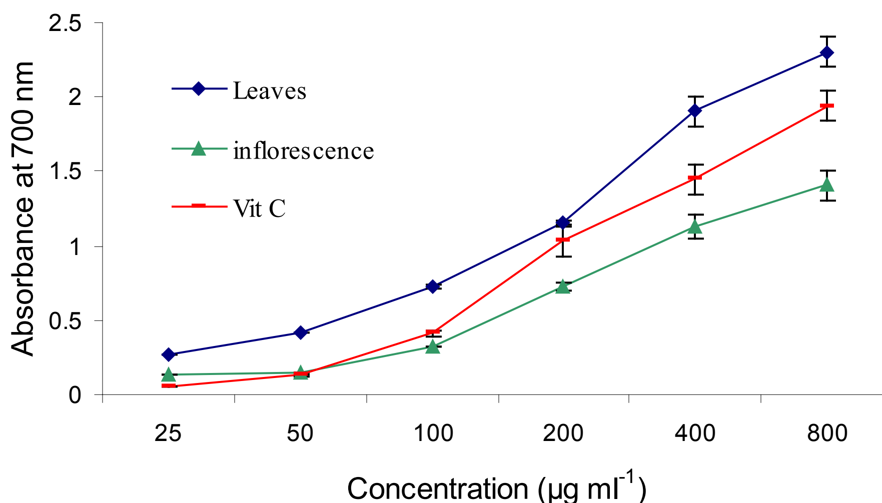

Figure 2: Reducing power of *E. caucasicum* leaves and inflorescence (flowering stage). Vit C used as control.

the redox potential, thereby stabilizing the oxidized form of the metal ion (26). Leaves extract exhibited good Fe^{2+} chelating ability (IC_{50}=0.25 mg ml^{-1}) that was comparable with EDTA (IC_{50}=18 μg ml^{-1}). Inflorescence extracts had shown a very weak activity, IC_{50}=1.46 ± 0.07 mg ml^{-1}.

FTC Method

No extracts exhibited any good antioxidant activity in linoleic acid model. The peroxidation inhibition of *E. caucasicum* leaves extract exhibited values from 59% (at 24th) to 73% (at 72nd hrs). The inflorescence extract exhibited very low antioxidant activity (87% at 24th to 15% at 72nd hrs). There were significant differences (p> 0.001) among two parts and Vitamin C at different incubation times.

Hydrogen Peroxide Scavenging

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 water. The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. Both of them showed very good scavenging activity. IC_{50} for scavenging of H_2O_2 was 25.5 ± 1.3 for leaves and 177.2 ± 11.6 mg ml^{-1} for inflorescence, respectively. The IC_{50} values for Ascorbic acid and quercetin were 21.4 ± 0.12 and 52.0 ± 3.11 μg 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 systems (12).

Leaves and inflorescence extracts (at flowering stage) showed very better scavenging activity of H_2O_2, reducing powers and Fe^{2+} chelating ability than leaves (at non-flowering stages) according to our recently published paper (9). The latter showed stronger NO scavenging and peroxidation inhibition (9).

E. caucasicum leaves (at flowering stage) methanolic extracts exhibited different levels of antioxidant activity in all the models studied. It showed good scavenging of H_2O_2, Fe^{2+} chelating ability, DPPH radical-scavenging activity and reducing power. Further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed.

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