FREE RADICAL SCAVENGING ACTIVITY AND ANTIOXIDANT CAPACITY OF *ERYNGIUM CAUCASICUM TRAUTV* AND *FRORIPIA SUBPINNATA*

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Summary

Eryngium caucasicum Trautv and *Froriepia subpinnata* were found in natively in the eastern parts of the south Caspian Sea coasts. Antioxidant activity of *E. caucasicum Trautv* and *F. subpinnata* were investigated employing six in vitro assay systems. IC_{50} for DPPH radical-scavenging activity was 0.27 ± 0.02 for *E. caucasicum* leaves and 0.42 ± 0.03 mg ml^{-1} for *F. subpinnata*, respectively. The extracts showed weak nitric oxide-scavenging and Fe^{2+} chelating ability activity. The IC_{50} of *E. caucasicum* was better than other one with IC_{50}= 0.21 mg ml^{-1} in NO scaveging test. The peroxidation inhibition of *E. caucasicum* extract exhibited values from 93 (at 24th) to 97% (at 72nd hrs). *F. subpinnata* exhibited very low antioxidant activity. The activity of *E. caucasicum* was comparable with Vit C (p> 0.05). No extract showed good scavenging activity of H_2O_2. The total amount of phenolic compounds in each extracts was determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve. Both of them had high total phenolic and flavonoid contents.

Key words: Antioxidant activity, DPPH, *Eryngium caucasicum*, Free radical scavenging activity, *Froriepia subpinnata*

Introduction

The role of free radicals in many disease conditions has been well established. Several biochemical reactions in our body generate reactive oxygen species. If they are not effectively scavenged by cellular constituents, they lead to disease conditions (1). Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers (2) and neurodegenerative diseases (3). Eryngium caucasicum (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 flavouring in the preparation of several local foods (4). Nothing was found in literature about this plant. Froriepia subpinnata (Umbelliferae) was found in the eastern parts of the south Caspian Sea coasts (5). Only Essential oil composition of this plant has been reported (6). No information is available about antioxidant activity of this plant. In this study, the antioxidant activity of these two native plants examined employing six various in vitro assay systems, i.e. DPPH and nitric oxide radical scavenging, reducing power, scavenging of hydrogen peroxide, 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

Chemicals: Ferrozine, Linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide were purchased from Sigma Chemicals Co. (USA). Gallic acid, quercetin, Butylated hydroxyanisole (BHA), ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, EDTA and ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

Plant Material and Preparation of Freeze-Dried Extract: *E. caucasicum* leaves and *F. subpinnata* aerial parts were collected from Mazandaran forest and identified by Dr. Bahman Eslami. A voucher (No. 971-974) 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, which was then freeze-dried for complete solvent removal.

Determination of Total Phenolic Compounds and Flavonoid Contents: Total phenolic compound contents were determined by the Folin-Ciocalteau reagent according to the recently published method (7). 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 the method of Ebrahimadeh et al. (8). 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 (7,8). 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. Vitamine 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 (9). The reducing power of extracts was determined according to the our recently publish paper (10). 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 [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50^oC 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 (7, 8).

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 (11). The chelating of ferrous ions by extracts was estimated by our recently published paper (12). 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 (13). The inhibitory capacity of extracts was tested against oxidation of linoleic acid by FTC method. This method was adopted from Osawa and Namiki (7,8). 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. Vit 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 (14). 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 *F. subpinnata* aeial parts were 62.3 ± 0.21 and 75.7 ± 0.24 mg gallic acid equivalent/g of extract powder, respectively. The total flavonoid contents of *E. caucasicum* leaves and *F. subpinnata* aeial parts were 25. 3 ± 0.19 and 35.2 ± 0.26 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 *F. subpinnata* aeial parts extract had significant higher total phenol and flavonoids contents than did *E. caucasicum* leaves extract. 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 (15).

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 (16). 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 (0.27 ± 0.02) > *F. subpinnata* (0.42 ± 0.03) 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. 1 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 no significant differences ($p > 0.05$) among the different extracts in reducing power. The reducing power of extracts were comparable with Vit C ($p > 0.05$).

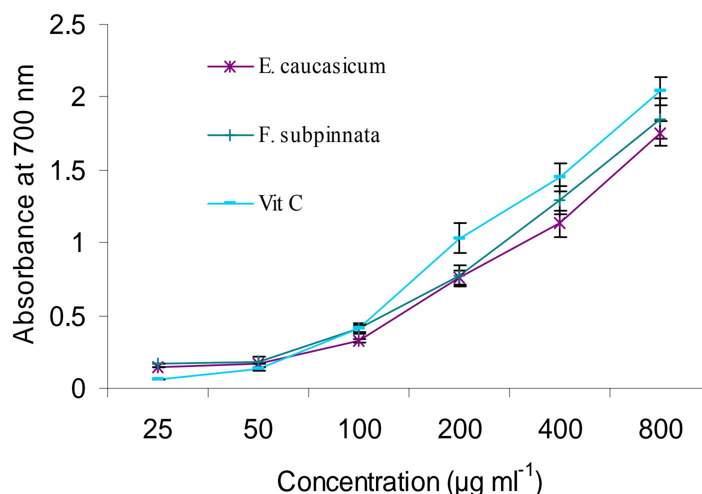

Fig.1. Reducing power of *E. caucasicum* leaves and *F. subpinnata* aerial parts. Vit C used as control.

Assay of Nitric Oxide-Scavenging Activity: The extracts showed moderately good nitric oxide-scavenging activity between 0.2 and 1.6 mg ml^{-1}. The percentage of inhibitions were increased with increasing concentration of the extracts. The *E. caucasicum* leaves extract had shown better scavenging activity with IC_{50}= 0.21 mg ml^{-1}. The IC_{50} for *F. subpinnata* aerial parts was 0.37 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 (17).

Fe^{2+} Chelating Ability: The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (18). 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 the redox potential, thereby stabilizing the oxidized form of the metal ion (19). Both of tested extracts exhibited very weak Fe^{2+} chelating ability. *E. caucasicum* and *F. subpinnata* extracts had shown only 23 and 42% inhibition at 0.8 mg ml^{-1}. EDTA showed very strong activity (IC50 = 0.018 mg ml^{-1}).

FTC Method: Figure 2 shows the time-course plots for the antioxidative activity of the plants extract using the FTC method. The peroxidation inhibition of *E. caucasicum* leaves extract exhibited values from 93 (at 24th) to 97% (at 72nd hrs). *F. subpinnata* extracts exhibited very low antioxidant activity (85% at 24th to 78% at 72nd hrs). There were significant differences (p> 0.001) among plants extracts. *E. caucasicum* leaves extract manifested almost the same pattern of activity as Vit C at different incubation times (at 72nd and 96th hrs, p> 0.05).

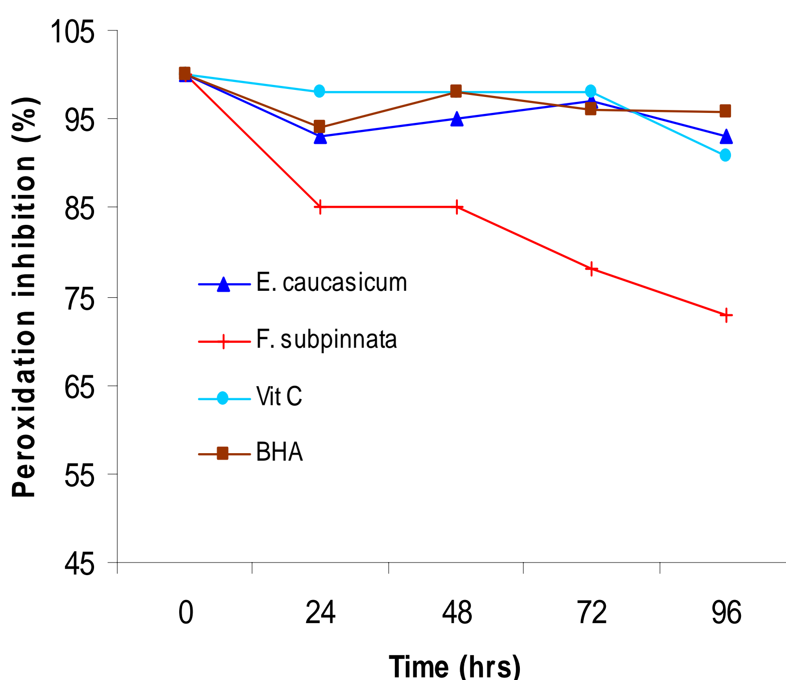

Fig. 2. Antioxidant activity of *E. caucasicum* leaves and *F. subpinnata* aerial parts in FTC method at different incubation times (0.4 mg/ ml). Vit C and BHA used as controls (0.1 mg/ ml).

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. No extract showed good scavenging activity. IC_{50} for scavenging of H_2O_2 was 0.81 ± 0.03 for *F. subpinnata* aerial parts and 1.30 ± 0.07 mg ml^{-1} for *E. caucasicum* leaves, 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. *E. caucasicum* leaves and *F. subpinnata* aerial parts methanolic extracts exhibited different levels of antioxidant activity in all the models studied. Further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed.

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