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Mineral Elements and Antioxidant Activity of Three Locally Edible and Medicinal Plants in Iran

MOHAMMAD ALI EBRAHIMZADEH†, SEYED MOHAMMAD NABAVI†‡, SEYED FAZEL NABAVI†¶, SHAHRAM ESLAMI† and AHMAD REZA BEKHRADNIA*
Department of Medicinal Chemistry, School of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran
Fax: (98)(151)3543084; Tel: (98)(151)3543081-3
E-mail: reza_bnia@yahoo.com; abekhradnia@mazums.ac.ir

Certain inorganic trace elements play an important role in biochemical process in cell. In the present study, the elemental composition (Zn, Fe, Cu, Mn, Ni and Cr) in three locally edible traditional medicinal plants (*Diospyros lotus* fruit, *Froriepia subpinnata* and *Eryngium caucasicum* leaves) widely used in some disorders has been studied using atomic absorption spectroscopy. In addition, total antioxidant capacity and their total phenol and flavonoid contents were investigated by various assay systems. Iron, zinc and manganese were ranked the highest amounts in three plants. *F. subpinnata* and *E. caucasicum* leaves are better sources for minerals than *D. lotus* fruit. Extracts also showed good antioxidant activity in tested models. Specially, *F. sabpinnata* aqueous extract with the amount of phenol and flavonoids showed high activity.

Key Words: Antioxidant, Elements, Atomic absorption, Minerals, *Diospyros lotus*, *Eryngium caucasicum*, *Froriepia subpinnata*.

INTRODUCTION

In recent years, there has been a growing interest in trace element concentrations in the environment and they are considered a factor indispensable for its proper functioning. These elements are present in enzymes and activate them, thereby in an essential way influencing the biochemical process in cells[1,2]. Research during the past three decades has added chromium, nickel and iron to the list of essential elements[3]. Plants are an important link in the transfer of trace elements from soil to humans[4]. Reactive oxygen species (ROS) have been found to play an important role in the initiation and/or progression of various diseases such as atherosclerosis, inflammatory injury, cancer and cardiovascular disease[5,6]. Thus, recent studies have investigated the potential of plant products as antioxidants against various diseases induced by free radicals[7]. Additionally, it has been determined that the antioxidant

†Pharmaceutical Sciences Research Center, School of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran.
‡Young Researchers Club, Islamic Azad University, Ghaemshahr Branch, Ghaemshahr, Iran.
¶Student Research Development Committee, Mazandaran University of Medical Sciences, Sari, Iran.

effect of plant products is mainly attributed to phenolic compounds, such as flavonoids, phenolic acids and tannins[8]. Many current human health problems relate to diets. Micronutrients are involved in numerous biochemical processes and an adequate intake of certain micronutrients relates to the prevention of deficiency diseases. Malnutrition is of major concern for many tropical developing countries especially in third world. Iron deficiency anemia, for example, affects one third of the world population[9,10]. Fruits and vegetables are safe and valuable sources of minerals[10,11]. 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[12]. The fruits of *Diospyros lotus* L. (*Ebenaceae*) are febrifuge and used to promote secretions. The seed is regarded in China as being sedative[6]. Chemical constituents of *D. lotus* has been published previously[13]. Fatty acid compositional changes and changes in phenolic acid contents during fruit development and antioxidant activity of methanol extract of this species were studied recently[6,14]. *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 flavoring in the preparation of several local foods[15]. Antioxidant activity of methanol extract of *E. cacasicum* has been reported recently[7]. *Froriepia subpinnata* (*Umbelliferae*) was found in the eastern parts of the south Caspian Sea coasts[16]. Only Essential oil composition and antioxidant activity of methanol extract of aerial part of this plant has been reported recently[7,17]. To the best of our knowledge there is no scientific report on mineral contents and antioxidant activity of aqueous extracts of *F. subpinnata* leaves, *E. caucasicum* leaves and *D. lotus* fruit. In the present study, the elemental composition of *D. lotus* fruit, *F. subpinnata* and *E. caucasicum* leaves has been studied using atomic absorption spectroscopy. The level of Zn, Fe, Cu, Mn, Ni and Cr were found. In addition, their antioxidant capacity were investigated 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 check the usefulness of these plants as a foodstuff as well as in medicine.

EXPERIMENTAL

Ferrozine, linoleic acid, trichloroacetic acid (TCA), folin-ciocalteau reagent, DPPH, potassium ferricyanide were purchased from Sigma-Aldrich (St. Louis, MO, 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 extract: *D. lotus* fruit (at the end of fall 2008) *F. subpinnata* and *E. caucasicum* leaves (in spring 2008) were collected from Panbeh Chooleh, Sari, Iran and identified by Eslami. A voucher (No. 1012-

14) 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 water. The resulting extracts were concentrated over a rotary vacuum until a crude solid extracts were obtained, which were then freeze-dried for complete solvent removal.

The properly dried and ground plant samples were ash-dried overnight at 400-420 °C in a vitreosil crucible. Care was taken for temperature not to exceed 450 °C to avoid losses of zinc. The procedure also destroys all organic matter, leaving an inorganic residue that was kept in a desiccator until needed for analysis. Two grams of ash were dissolved in a 1:3 mixture of hydrochloric and nitric acids diluted to 50 mL with distilled water and used for analysis by means of an atomic absorption spectrometer Perkin-Elmer AAS 100 (Wellesley, MA)[18]. Table-1 gives the operating conditions for the AAS. Sixteen blank control solutions were used to estimate the detection limits of the investigated elements following the same analytical procedures. Three times the standard deviation was used as detection limit (Table-1).

TABLE-1
OPERATING CONDITIONS OF THE ATOMIC ABSORPTION SPECTROMETER* (AAS 100)

Elements	Detection wave length (nm)	Drying temperature (ºC)	m.p. (ºC)	Detection limits (µg/g)
Cu	324.8	120	1085	0.033
Fe	248.3	120	1535	0.047
Zn	213.9	120	450	0.035
Mn	279.5	120	1246	0.009
Ni	232.0	120	1455	0.056
Cr	357.9	120	1900	0.018

*For all elements, the slit width were 0.2 nm. The air and acetylene flow rates were 4.0 and 0.5 L/min, respectively.

Determination of total phenolic compounds and flavonoid contents: Total phenolic compound content was determined by the Folin-Ciocalteau reagent according to the recently published method[19,20]. The extracts samples (0.5 mL) were mixed with 2.5 mL of 0.2 N Folin-Ciocalteau reagent for 5 min and 2 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 our recently published paper[21,22]. Briefly, 0.5 mL solution of extract 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 0.5 h. 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[23,24]. Different concentrations of 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[25]. The reducing power of extracts were determined according to publish papers[26,27]. Different amounts of extracts (50-1600 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 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[28,29].

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[5]. The chelating of ferrous ions by extracts were estimated by published papers[30,31]. Briefly, the extracts (0.2-3.2 mg/mL) were 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 ferric thiocyanate (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[7]. The inhibitory capacities of extracts were tested against oxidation of linoleic acid by FTC method. This method was performed according to published papers[32-34]. 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 (8 mL) and distilled water (3.9 mL) and kept in screw cap 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 per cent 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 published papers[35,36]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (0.1-1.0 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 10 min 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 IC_{50} values were calculated from linear regression analysis.

RESULTS AND DISCUSSION

The yield, total phenol and flavonod contents of extracts have been showed in Table-2. Total phenol compounds were reported as gallic acid equivalents by reference to standard curve ($y = 0.0063x$, $r^2 = 0.987$). The total flavonoid contents were reported as mg quercetin equivalent/g of extract powder, by reference to standard curve ($y = 0.0067x + 0.0132$, $r^2 = 0.999$). The maximum of extractable polyphenolic content and flavonoid content record in *F. subpinnata*. 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[20].

TABLE-2
YIELD OF EXTRACTION, PHENOL AND FLAVONOID CONTENTS AND ANTIOXIDANT ACTIVITIES OF *D. lotus* FRUIT, *F. subpinnata* AND *E. caucasicum* LEAVES AQUEOUS EXTRACTS

Sample name	Yield (%)	Total phenol contents (mg g^{-1})	Total flavonoid contents (mg g^{-1})	DPPH Free radical scavenging, IC_{50} (mg mL^{-1})*	Nitric oxide scavenging, IC_{50} (mg mL^{-1})**	H_2O_2 scavenging activity, IC_{50} (mg mL^{-1})***	Fe^{2+} chelating ability (%)**** or IC_{50} (µg mL^{-1})
D. lotus	15	11.9 ± 0.9	14.30 ± 0.6	1.17 ± 0.02	38 %	1.08 ± 0.05	39 %
F. subpinnata	35	132.6 ± 5.8	39.62 ± 2.4	0.14 ± 0.06	0.490 ± 0.02	0.98 ± 0.04	286 ± 12
E. caucasicum	29	80.2 ± 3.6	34.92 ± 1.1	7.99 ± 0.30	0.846 ± 0.03	0.80 ± 0.03	166 ± 7

*IC_{50} of BHA was 53.96 ± 3.1, vitamin C, 5.05 ± 0.1 and Quercetin 5.28 ± 0.2 µg mL^{-1}, respectively. **Inhibition at 1.6 mg mL^{-1}. IC_{50} of Quercetin was 5.28 ± 0.2 µg mL^{-1}. ***IC_{50} for vitamin C and Quercetin were 21.4 ± 1.1 and 52 ± 2.6 mg mL^{-1}, respectively. ****Inhibition at 3.2 mg mL^{-1}. EDTA used as control (IC_{50} = 18 ± 1.5 µg mL^{-1}).

Elemental composition: The yield of the samples from aqueous extract and ash have been shown in Table-2. Table-3 presents the elemental analysis in ash of the above-mentioned plants by AAS technique. The concentration of various elements analyzed in the present work decreases in the order: *D. lotus*: Fe > Mn, Zn; *F. subpinnata*: Fe > Zn, Cu > Mn and *E. caucasicum*: Fe > Zn > Mn > Cr > Cu. This study confirmed the well-known fact that *F. subpinnata* and *E. caucasicum* leaves are better sources of minerals than *D. lotus* fruit. The daily requirements of an adult man are as follows: 10-15 Fe, 12-15 Zn and 2-3 Cu (mg/d)[10]. On the contrary, the foods consumed in third world countries population are poor in minerals such as Fe and the consumption of this vegetable could bring the amount required to meet the requirements either. Although, these vegetables come from the Mazandaran forest and supply is limited because they are native to northern of Iran, but they has a good potential for human nutrition. This study shows the trace metals content in these plants. The knowledge of the chemical form of the elements in plants of economic interest might be crucial because actions can be taken to reduce or minimize the toxic effects of the environment pollutant heavy metals[1].

TABLE-3
AMOUNTS OF TRACE ELEMENTS IN THE PLANTS BY AAS ANALYSIS (µg/g)

Sample	Zn	Mn	Fe	Cu	Cr
D. lotus	0.10	0.10	0.82	0.00	0.00
F. subpinnata	0.69	0.38	15.91	0.69	0.00
E. caucasicum	0.83	0.50	17.18	0.08	0.41

Values are averages of three independent measurements having a percision of approx ± 4-6 %.

DPPH Radical-scavenging activity: IC_{50} for DPPH radical-scavenging activity exist in Table-2. The model of scavenging the stable DPPH radical is widely used method to evaluate the free radical scavenging ability of various samples[20,37]. DPPH is a stable nitrogen-centered free radical the colour 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 scavengers[21]. It was found that the radical-scavenging activities of all the extracts increased with increasing concentration. *F. sabpinnata* aqueous extract with highest amount of phenol and flavonoids showed the highest DPPH-scavenging activity.

Reducing power: Fe(III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action[16]. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+}-Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm[26]. Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 1 shows the dose response curves for the reducing powers of the extracts. It was found that the reducing powers of all the extracts also increased with the increase of their concentrations. *F. subpinnata* extract showed remarkable reducing power. There were no significant differences among the *F. subpinnata* extract and vitamin C ($p > 0.05$). Polyphenolic contents of all the sample extracts appear to function as good electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. Similar observation between the polyphenolic constituents in terms of dose dependent and reducing power activity have been reported for several plant extracts[18]. It was evident that this extract could serve as electron donors for terminating the radical chain reaction.

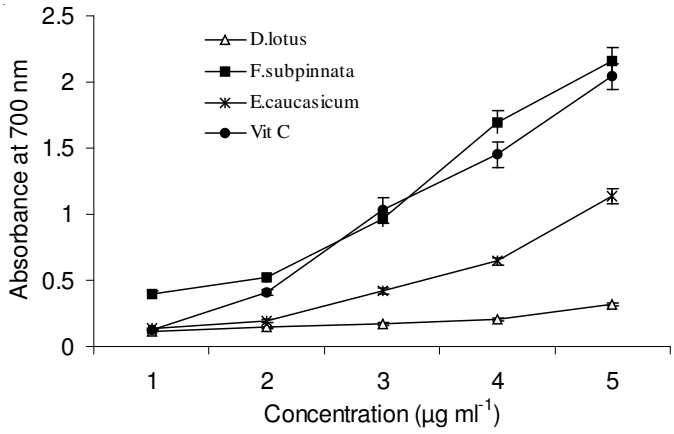

Fig. 1. Reducing power of *D. lotus* fruit, *F. subpinnata* and *E. caucasicum* leaves aqueous extracts. Vitamin C used as control

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[17]. Results presented in Table-2. *F. subpinnata* extract exhibit better activity than others (IC_{50} = 0.49 ± 0.02 mg mL^{-1}). However the activity of quercetin was more pronounced than that of present extracts (18.0 ± 1.5 μg mL^{-1}). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions[2].

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[29]. 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 Alzheimer's disease[26]. Foods are often contaminated with transition metal ions which may be introduced by processing methods. 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[1]. These processes can be delayed by iron chelation and deactivation. Iron is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease[4]. Because Fe^{2+} also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe^{2+} concentration in Fenton reactions affords protection against oxidative damage. The chelating of ferrous ions by the extracts was estimated by recently published paper[19]. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red colour 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 ion before ferrozine. The absorbance of Fe^{2+}-ferrozine complex was decreased dose-dependently, *i.e.*, the activity was increased on increasing concentration from 0.2-3.2 mg mL^{-1}. Metal chelating capacity was significant since the extract reduced the concentration of the catalyzing transition metal in lipid peroxidation[2]. 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[26]. Results presented in Table-2. *E. caucasicum* leaves extract showed good chelating activity but it was not comparable with EDTA ($p < 0.01$).

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 (Table-2). Methanol extracts showed weak activity that was not comparable with vitamin C and quercetin ($p < 0.001$). 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.

Ferric thiocyanate 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[2,29]. The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical[4]. Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Plant extracts did not show any activity in peroxidation inhibition in FTC method. Vitamin C and BHA used as controls. They showed 91-98 % inhibition at different incubation times (24-96 h).

Conclusion

The plants extracts exhibited different levels of antioxidant activity and mineral content in studied models. Further investigation of individual compounds, their *in vivo* antioxidant activities and in different antioxidant mechanisms is needed. Such identified potential and natural constituents could be exploited as cost effective food additives for human health.

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