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Sambucus ebulus elburensis fruits: A good source for antioxidants

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ABSTRACT

The antioxidant activity of methanol and aqueous extract of Sambucus Ebulus (SE) was examined employing various 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 202.50 ± 1.38 for aqueous extract (SW) and 723.62 ± 3.36 µg ml^{-1} for methanol extract (SM). Reducing powers of all the extracts also increased with the increase of their concentrations. Both of them exhibited a weak reducing power at 25–800 µg ml^{-1}. The SW extract had shown better reducing power than SM. Tested extracts exhibited week Fe^{2+} chelating ability. Both extracts exhibited high antioxidant activity. There were no significant differences between them in peroxidation inhibition. SW extracts manifested better pattern of activity than Vitamin C and BHA at different incubation times. SE fruit extracts exhibited different levels of antioxidant activity in all the models studied. The SE extracts had good reductive capability for anti-lipid peroxidation and nitric oxide-scavenging activity. Aqueous extract had higher total phenol and flavonoid contents than methanol extract.

Keywords: Antioxidant activity, *Crataegus pentaegyna,* Radical scavenging, *Sambucus ebulus.*

INTRODUCTION

The importance of reactive oxygen and free radicals in cellular injury and the aging process, has attracted increased attention over the past 20 years (1). These molecules are considered to induce lipid peroxidation causing the deterioration of foods (2). Reactive oxygen species in the forms of super oxide anion, hydroxyl radical, and hydrogen peroxide are generated by normal metabolic processes, or from exogenous factors and agents. Antioxidant defenses in organisms against reactive oxygen species produced during normal cell aerobic respiration may be of endogenous or dietary origin (3). Therefore, synthetic antioxidants such as butyrate hydroxyanisole, butyrate hydroxytolune and tertiary butyl hydroquinone, have been used in the food industry as antioxidants. However, the uses of these synthetic antioxidants are restricted in some countries or states due to their toxic effects (4). Recently, the interest of finding natural antioxidants, especially those of plant origin, has increased greatly (5). Natural antioxidants derived from plants, especially phenolics, are of considerable interest as dietary supplements or food preservatives (6). In most cases, phenolics mediate their anti-carcinogenic effects by inhibiting all stages of chemical carcinogenesis, initiation, promotion and progression, as well as formation of carcinogens from dietary precursors (7). Four species of the genus *Sambucus* are growing in Iran. Of these species, *S. ebulus* extensively grows in the northern regions of Iran (8–9). Iranian traditional medicine uses, in various

occasions, the leaves and rhizomes of *Sambucus Ebulus* (*Caprifoliaceous*) (SE) in treating some inflammatory cases such as, bee and nettle bites, arthritis, and sore-throat (10). In addition, it has been reported to be an insect repellent, *anti*-hemorrhoid, anti bacterial toward *Helicobacter pylori*, useful in the treatment of burns and infectious wounds, edema, eczema, urticaria, the cold, inflammation and rheumatism (9, 11–12). Despite sporadic references on the activity of SE, there exist little or no systematic records on the use of SE as a widely accepted medicinal plant in Iran. Recently a significant anti-inflammatory activity was reported (8, 9). Flavonoids, steroids, tannins, glycosides, cardiac glycosides, caffeic acid derivatives, ebulitins, volatile substances, phenol and flavenoid content of this species was previously reported (8, 13). There is little information about antioxidant activity of *Sambucus* (14–15). Studies have established extracts of some plants which are rich in flavonoids (16) and many of these phenolic compounds have shown to be cytoprotective by reducing oxidative stress (17–18), thereby giving a solid basis to the proposal that the antioxidant content of plants could account for its cardio-protective properties (19). Assuming its therapeutic benefit, it is attributed to antioxidant activity, we decided to determine the antioxidant potential of fruit extracts of SE. For this purpose, antioxidant activity of SE fruits extracts were examined employing various invitro assay systems, i.e. DPPH and nitric oxide radical scavenging, reducing power, linoleic acid and iron ion chelating power, in order to understand the usefulness of this plant 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

SE fruits were collected from Mazandaran forest in the autumn of 2006, and confirmed by Dr Bahman Eslami. Fruits were dried at room temperature. Fruits were extracted at room temperature by percolation method using methanol (SM) or water (SW) separately. 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 content

Total phenols in each extract were determined by Folin Ciocalteu reagent (20–21). Briefly, 0.5 ml solution of each plant extracts in methanol were mixed with 5 ml of Folin Ciocalteu reagent (a 10 % v/ v in distilled water) and 4 ml of 1 M aqueous Na_2CO_3. The mixtures were kept for 15 minutes and the total phenol content were determined by colorimeter, at 765 nm with a double beam Perkin Elmer UV/ Visible spectrophotometer (UV- Visible EZ201, Perkin Elmer: USA). The standard curve was prepared using 25–300 μg ml^{-1} solutions of Gallic acid in methanol: water (50:50). Total phenol values are expressed in terms of Gallic acid, equivalent (mg/g of dry mass) which is a common reference compound. Flavonoid content of each extract was determined by following a colorimetric method (20–21). Briefly, 0.5 ml solution of each plant 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 30 minutes. The absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared by preparing quercetin solutions at concentrations of 12.5 to 100 μg ml^{-1} in methanol. Total flavonoid contents were calculated as quercetin equivalent (mg/g of dry mass) from a calibration curve.

DPPH radical-scavenging activity

The stable 1,1-diphenyl-2-picryl hydroxyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts (22–23). Different concentrations of each extracts were added, at an equal volume, to methanol solution of DPPH (100 μM). After 15 minutes at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated a total of three times. Vitamin C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration of the 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 (24). The reducing power of SE was determined according to the method of Yen and Chen (21). 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°C for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3,000 rpm for 10 minutes. 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 a positive control.

Assay of nitric oxide-scavenging activity

This 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 minutes. 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 a positive control (22–23).

Metal chelating activity

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 hydro peroxide decomposition reactions, via Fenton chemistry (27). The chelating of ferrous ions by SE was estimated by the method of Dinis et al., (13). 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 minutes. 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 a 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 (26). The inhibitory capacity of SE extracts was tested against oxidation of linoleic acid by FTC method. This method was adopted from Osawa and Namiki (20–21). 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 a screw cap container 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 minutes 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 hours, 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 a positive control.

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 EC_{50} values were calculated from linear regression analysis.

RESULTS

Total Phenol and Flavonoid contents

Total phenol compounds, as determined by Folin Ciocalteu method, are reported as Gallic acid equivalents, by reference to standard curve ($y = 0.0054x + 0.0628$). The total phenolic contents of SW and SM were 41.59 ± 0.25 and 27.37 ± 0.18 mg Gallic acid equivalent/g of extract powder, respectively. The total flavonoid contents of SW and SM were 23.80 ± 0.17 mg 14.70 ± 0.09 quercetin equivalent/g of extract powder, respectively, by reference to standard curve ($y = 0.0063\times$).

DPPH radical-scavenging activity

DPPH radical-scavenging activities based on IC_{50}, were 202.50 ± 1.38 for SW and 723.62 ± 3.36 μg ml^{-1} for SM. Based on total phenol contents, it was also shown that aqueous extracts had higher DPPH-scavenging activity than methanol one. 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

Fig. 1 shows the dose-response curves for the reducing powers of the SE fruits extracts. 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. All extracts exhibited a weak reducing power at 25 and 800 µg ml^{-1} that were not comparable with Vitamin C ($p < 0.001$).

Assay of nitric oxide-scavenging activity

Three extracts showed weak nitric oxide-scavenging activity between 50 and 800 µg ml^{-1}. The % inhibition was increased with increasing concentration of the extract. The SW extract showed very good reducing power with IC_{50}= 29 ± 1.3 µg ml^{-1}. SM showed weak activity with IC_{50} = 603 ± 3.8 µg ml^{-1}. There were no significant differences ($p > 0.05$) among the SW extract and quercetin. IC_{50} was 17 ± 1.1 µg ml^{-1} for quercetin.

Fe^{2+} Chelating ability

The chelating of ferrous ions by the extract was estimated by the method of Dinis et al., (13). The absorbance of Fe^{2+}-ferrozine complex was decreased dose-dependently, i.e. the activity was increased on increasing concentration from 0.2 to 3.2 mg ml^{-1}. It was reported that chelating agents are effective as secondary antioxidants, because they reduce the red ox potential, thereby stabilizing the oxidized form of the metal ion (27). All tested extracts exhibited week Fe^{2+} chelating ability. SM showed 48% and SW showed only 21 % at 3.2 mg ml^{-1}. EDTA showed very strong activity (IC_{50} = 0.018 mg ml^{-1}).

FTC Method

Figure 2 shows the time-course plots for the anti-oxidative activity of the different extracts of SE fruits, using the FTC method in 0.2 mg ml^{-1} concentration. The peroxidation inhibition of extracts exhibited values from 87 to 100% (at 24th hrs) up to 93 to 97% (at 72nd hrs). Both extracts exhibited high antioxidant activity. There were no significant differences ($p > 0.05$) among the different extract fractions in anti-oxidative activity. SW extracts manifested a better pattern of activity, than Vitamin C and BHA at different incubation times.

DISCUSSION

Total phenol compounds, as determined by folin Ciocalteu method, are reported as Gallic acid equivalents, by reference to standard curve. The total flavonoid contents of determined as mg quercetin equivalent/g of extract powder by reference to standard curve. Aqueous extract had significant higher total phenol contents than did methanol extract. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been

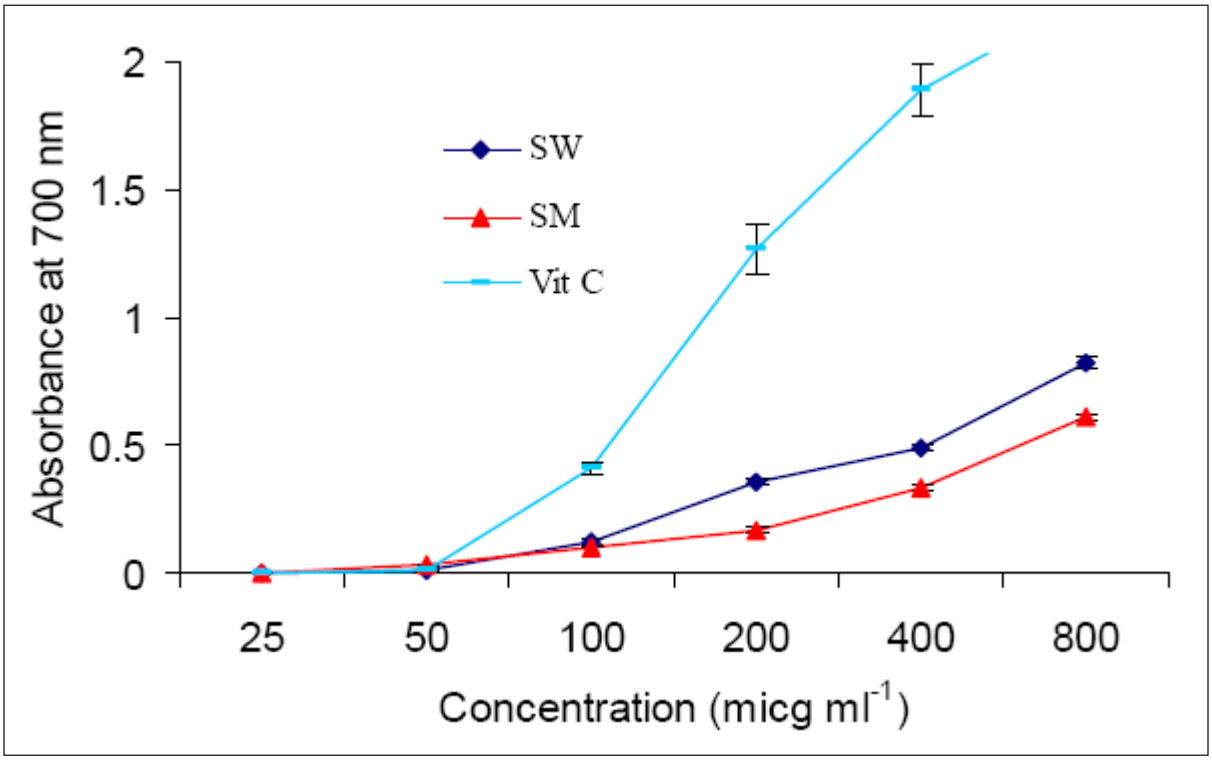

Figure 1: Reducing power of Sambucus ebulus fruit aqueous extract, SW and methanol extract, SM. Vit C used as control.

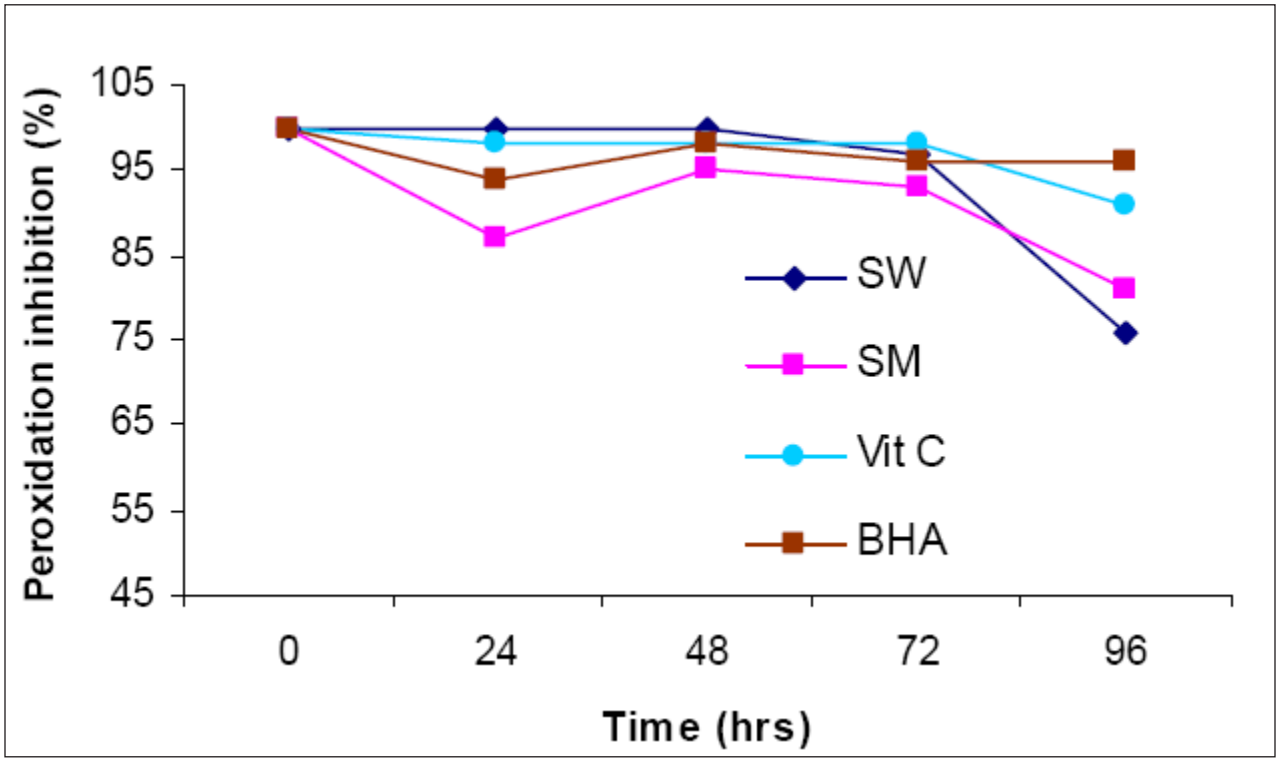

Figure. 2: Antioxidant activity of plants in FTC method at different incubation times. S. ebulus fruit aqueous, SW and methanol extract, SM (0.2 mg/ ml), Vitamin C and BHA used as control (0.1 mg/ml).

shown to possess significant antioxidant activities (28). High phenolic compounds may cause the anti-oxidative activities of this plant. The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (1). The activities of both extracts increased with increasing concentration. Based on total phenol contents, it was also shown that aqueous extracts had higher DPPH-scavenging activity than methanolic one. In the reducing power assay, the presence of reductants (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 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. It was found that the reducing powers of both extracts also increased with the increase of their concentrations (Fig. 1). There were no significant differences ($p > 0.05$) between extracts in reducing power. All extracts exhibited a weak reducing power at 25 and 800 µg ml^{-1} that were not comparable with Vitamin C ($p < 0.05$). It was evident that SE extracts did not show reductive potential and could not serve as electron donors. The SW extract showed the best reducing power. There were no significant differences ($p > 0.05$) among the SW extract and quercetin. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (29). This may explain good anti-inflammatory activity of SE fruit extracts (8–9). 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 (30). 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 (31). The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (32). Because Fe^{2+} causes the production of oxyradicals and lipid peroxidation, minimizing its concentration affords protection against oxidative damage. The chelating of ferrous ions by the extract was estimated by the method of Dinis et al., (13). In the presence of other chelating agents, the ferrozine complex formation is disrupted with demonstrating 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 3.2 mg ml^{-1}. It was reported that chelating agents are effective

as secondary antioxidants, because they reduce the red ox potential, thereby stabilizing the oxidized form of the metal ion (27). Nearly all tested extracts exhibited week Fe^{2+} chelating ability. Neither extracts showed good Fe^{2+} chelating ability. Results suggesting that their actions as an antioxidant may not be related to their iron binding capacity. Figure 2 shows activity of SE fruits, using the FTC method in 0.2 mg ml^{-1} concentration. Both of them exhibited high antioxidant activity. There were no significant differences ($p > 0.05$) among the different extract fractions in anti-oxidative activity. SW extracts manifested a better pattern of activity than Vitamin C and BHA at different incubation times.

The aqueous and methanol extracts of SE fruit exhibited different levels of anti-oxidant activity in all models studied. The SE extracts had good reductive capability for anti-lipid peroxidation and nitric oxide-scavenging activity. Further investigation of individual compounds, with their in-vivo anti-oxidant activities and different antioxidant mechanisms is needed.

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