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Antioxidant Activity of *Crataegus pentaegyna* Subsp. *elburensis* Fruits Extracts Used in Traditional Medicine in Iran

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Abstract: In this study, the antioxidant activity of methanol and aqueous extracts of *Crataegus pentaegyna* subsp. *elburensis* (CP) were examined by 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 341.29±1.29 for methanol and 269.31±2.11 μg mL^{-1} for aqueous extract. Reducing powers of extracts also increased with the increase of their concentrations. Both extracts exhibited a weak reducing power at 25-800 μg mL^{-1}. Extracts exhibited weak nitric oxide radical scavenging and Fe^{2+} chelating ability. Methanol extract showed higher Fe^{2+} chelating ability (IC_{50} = 1.84±0.01 mg mL^{-1}). Both tested extracts exhibited high antioxidant activity. Extracts showed high total phenolic content.

Key words: 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 (Lee *et al*., 2004). These molecules are considered to induce lipid peroxidation causing the deterioration of foods (Duthie, 1993). 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 (Harman, 1995). 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 (Buxiang and Fukuhara, 1997). Recently, the interest of finding natural antioxidants, especially those of plant origin, has increased greatly (Jayaprakasha *et al*., 2000). Natural antioxidants derived from plants, especially phenolics, are of considerable interest as dietary supplements or food preservatives (Halliwell *et al*., 1995). 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 (Jang *et al*., 1997). In Iran, one of the hawthorn species, *Crataegus pentaegyna* subsp. *elburensis* (Rosaceae) (CP) populates the Northern states where its fruits are very popular. The medicinal use of extracts of plant species from the genus *Crataegus* dates back to ancient times (Bahorun *et al*., 2003). Today, the plant is mainly used for treating cardiovascular diseases and appears to be an anti-arrhythmic agent (Rigelsky and Sweet, 2002; Koch *et al*., 2005; Brixius *et al*., 2005; Long *et al*., 2006). Also, some protective effect on murine experimental colitis (Fujisawa *et al*., 2005), anti-inflammatory, gastroprotective and antimicrobial activities (Tadiæ *et al*., 2008; Kao *et al*., 2005), hypolipidemic effect (Zhang *et al*., 2002) and antioxidant activities from *C. monogyna* have been reported by Rakotoarison *et al*. (1997). It is well known that free radical-generated reperfusion injury occurs when oxygen is re-introduced to ischemic tissue (Ambrosio and Tritto, 1999). Studies have established extracts of *Crataegus* which are rich in proanthocyanidins and flavonoids (Bahorun *et al*., 1996) and many of these phenolic compounds have shown to be cytoprotective by reducing oxidative stress (Hertog *et al*., 1993; Zhang *et al*., 2001), thereby giving a solid basis to the proposal that the antioxidant content of *Crataegus* could account for its cardio-protective properties (Chatterjee *et al*., 1997). Assuming its therapeutic benefit, it is attributed to antioxidant activity, we decided to determine the antioxidant potential of fruit extracts of the indigenous hawthorn in a variety of *in vitro* assays. In

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spite of some reports pertaining to antioxidant activity of hawthorn species (Bahorun *et al*., 1994; Guo *et al*., 1999; Shahat *et al*., 2002; Ljubuncic *et al*., 2005), nothing was found regarding CP. In this study, we examined the antioxidant activity of CP fruits methanol and aqueous extracts, employing various *in vitro* assay systems, such as 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

This study was performed during June 2008 to October 2008 in Pharmaceutical Sciences Research Center, School of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran.

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: CP fruits were collected from Mazandaran forest in the autumn of 2007 and confirmed by Dr. Bahman Eslami. Fruits were dried at room temperature. Fruits were extracted at room temperature by percolation method using methanol (CM) or water (CW) separately. The resulting extracts were 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 (Ebrahimzadeh *et al*., 2008a, b). 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 min 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^{-1} of dry mass) which is a common reference compound. Flavonoid content of each extract was determined by following a colorimetric method (Ebrahimzadeh *et al*., 2008a, b). 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 min. 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^{-1} 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 (Ebrahimzadeh, 2008a, b). Different concentrations of each extracts were added, at an equal volume, to methanol solution of DPPH (100 μM). After 15 min 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 (Yildirim *et al*., 2001). The reducing power of CP extracts was determined according to the method of Ebrahimzadeh *et al*. (2008b). 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 min. 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 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 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 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 a positive control (Nabavi *et al*., 2008a, b).

Metal chelating activity: The chelating of ferrous ions by CP was estimated by the method of Ebrahimzadeh *et al*. (2008c). Briefly, the extract (0.2-3.2 mg mL^{-1}) 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:

$$\text{Inhibition (\%)} = [(A_0-A_s)/A_s]\times 100$$

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: The inhibitory capacity of CP extracts was tested against oxidation of linoleic acid by FTC method. This method was adopted from Osawa and Namiki cited by Ebrahimzadeh *et al*. (2008a, b). The 20 mg mL^{-1} 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 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:

$$\text{Inhibition (\%)} = 100-\frac{\text{Absorbance increase of the sample}}{\text{Absorbance increase of the control}}\times 100$$

All tests were run in duplicate and analyses of all samples were run in triplicate and averaged. Vitamin C and BHA used as a positive control.

Statistical analysis: Experimental results are expressed as Mean±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 ($y = 0.0063x$, $R^2 = 0.987$). The total phenolic contents of CW and CM were 92.12±0.47 and 85.15±0.66 mg gallic acid equivalent g^{-1} of extract powder, respectively. The total flavonoid contents of CW and CM were 10.56±0.01 and 23.98±1.02 mg quercetin equivalent g^{-1} of extract powder, respectively, by reference to standard curve ($y = 0.0067x+0.0132$, $R^2 = 0.999$).

DPPH radical-scavenging activity: DPPH radical-scavenging activities based on IC_{50}, were 269.31±2.11 for aqueous and 341.29±1.29 μg mL^{-1} for methanol extract. 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: Figure 1 shows the dose-response curves for the reducing powers of CP fruits extracts. It was found that the reducing powers of 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 ($p<0.01$).

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 CW extract showed the better reducing power activity with

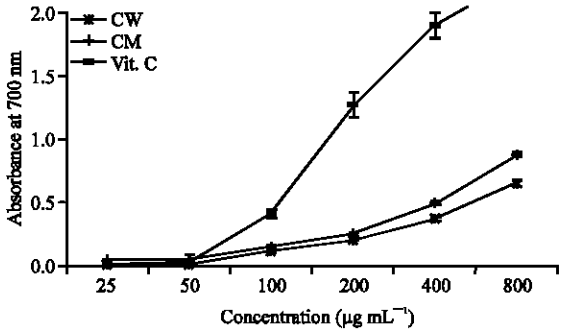

Fig. 1: Reducing power of *Crataegus pentaegyna* fruit aqueous extract, CW and methanolic extract, CM Vitamin C used as control

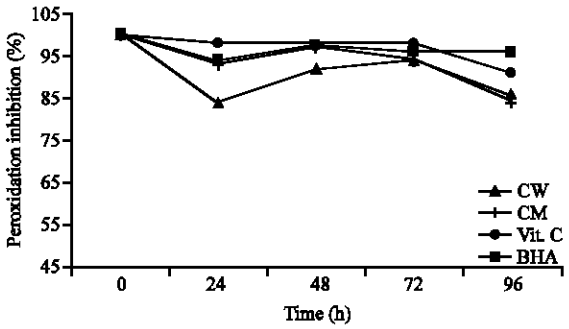

Fig. 2: Antioxidant activity of plants in FTC method at different incubation times. *Crataegus pentaegyna* fruit aqueous extract, CW and methanol extract, CM (0.2 mg mL^{-1}), vitamin C and BHA used as control (0.1 mg mL^{-1})

IC_{50} = 347±12 μg mL^{-1}. CM was a little weaker with IC_{50} = 369±15 μg mL^{-1}. Quercetin showed very significant activity (IC_{50} = 17±2 μg mL^{-1}).

Fe^{2+} chelating ability: The chelating of ferrous ions by the extract was estimated by the method of Dinis cited by Ebrahimzadeh *et al.* (2008c). 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}. Both extracts exhibited week Fe^{2+}, chelating ability. CW showed only 6% inhibition at 3.2 mg mL^{-1} but CM showed better Fe^{2+} chelating ability (IC_{50} = 1.84±0.01 mg mL^{-1}). EDTA showed very strong activity (IC_{50} = 18 μg mL^{-1}).

FTC method: Figure 2 shows the time-course plots for the anti-oxidative activity of the different extracts of CP fruits, using the FTC method. The peroxidation inhibition of extracts exhibited values from 84 to 93% (at 24th h) up to 94% (at 72 h). Both extracts exhibited high antioxidant activity. There were no significant differences ($p>0.05$) among two extract fractions in anti-oxidative activity. CM extracts manifested the same pattern of activity of 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 determined as mg quercetin equivalent g^{-1} of extract powder by reference to standard curve. Aqueous extracts had significant higher total phenol contents than methanol extract. Phenols and polyphenolic compounds are widely found in food products derived from plant sources and they have been shown to possess significant antioxidant activities (Saskai *et al.*, 1996). High total phenolic compounds of the extracts 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 (Lee *et al.*, 2003). 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 scavengers (Brand-Williams *et al.*, 1995). The activity of extracts increased with increasing concentration. Based on total phenol contents, it was also shown that aqueous extract had higher DPPH-scavenging activity than methanol 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 extracts also increased with the increase of their concentrations (Fig. 1). 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 ($p<0.01$). It was evident that the extracts from CP fruits did not show reductive potential and could not serve as electron donors. The 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 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 health. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada *et al.*, 1991). 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 (Hebbel *et al.*, 1990). 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 (Reznichenko *et al*., 2006). 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 (Halliwell, 1997). The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (Halliwell and Gutteridge, 1990). 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 Ebrahimzadeh *et al*. (2008c). 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 0.2-3.2 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 (Gordon, 1990). 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.

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 (Yu, 2001). Figure 2 shows activity of CP fruits, using the FTC method in 0.2 mg mL^{-1} concentration. Both of them exhibited high antioxidant activity. CM extracts manifested the same pattern of activity of vitamin C and BHA at different incubation times.

The aqueous and methanol extracts of CP fruit exhibited different levels of anti-oxidant activity in all models studied. Further investigation of individual compounds, with their *in vivo* anti-oxidant activities and different antioxidant mechanisms is needed.

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