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In vitro antioxidant activity of flower, seed and leaves of *Alcea hyrcana Grossh*

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Abstract. – *Objectives:* ***Alcea hyrcana Grossh*** **(*A. hyrcana Grossh*) (malvacea), is native to northern of Iran. Many of the plants belonging to the genus *Alcea* are known to possess ethnomedical and biological properties. In this study, antioxidant activities of methanolic extracts of flower, seed and leaves of *Alcea hyrcana Grossh* were evaluated by various antioxidant assays.**

Material and Methods: **1,1-diphenyl-2-picryl hydrazyl radical (DPPH), nitric oxide and hydrogen peroxide scavenging activities, Fe^{2+} chelating ability, reducing power and hemoglobin-induced linoleic acid peroxidation test were used to evaluate antioxidant activities. The total amount of phenolic compounds was determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve. Total phenolic and flavonoid contents also have been determined.**

Results: **All extracts showed good antioxidant activities. The *A. hyrcana Grossh* leaves extract exhibited strong ferrous chelating activity with IC_{50} = 0.11 ± 0.01 mg ml^{-1}, nitric oxide radical scavenging with IC_{50} = 0.45 ± 0.01 mg ml^{-1} and better reducing power activity than other extracts. The seeds extract showed high scavenging activity against free radicals, including both the hydrogen peroxide and DPPH radicals. Only leaves extract had good activity in linoleic acid model. Seeds extract had significant higher total phenol (68.9 ± 3.7 mg gallic acid equivalent/g of extract powder) and leaves had higher flavonoids contents (28.3 ± 2.6 mg quercetin equivalent/g of extract powder) than other parts.**

Conclusions: **The leaves, seed and flower extracts of *A. hyrcana Grossh* exhibited good but different levels of antioxidant activity in all the models studied. The extracts had good iron chelation, H_2O_2 and nitric oxide scavenging activities. Antioxidant activities may be attributed, at least in part, to the presence of phenols and flavonoids.**

Key Words:

Alcea hyrcana Grossh, Antioxidant activity, DPPH, Flavonoids, Phenols.

Introduction

It is well known that the consumption of dietary antioxidants of fruits and vegetables origin plays a positive role in the enhancement of health status in human[1]. Particularly, regulated production of reactive oxygen species (ROS) maintains the redox homeostasis that is essential for the physiological health of organisms[2]. However, during these metabolic processes, excessive production of ROS escapes from the protective shield of antioxidant mechanisms, causing oxidative damage to cellular components such as DNA, proteins, and lipids. Moreover, the oxidative stress caused from imbalance between the generation and the neutralization of ROS by antioxidant mechanism is responsible for many human diseases, including aging, cancer, and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease[3-5]. Therefore, it is very important that the intake of certain natural antioxidants is one of the reasonable strategies for the inhibition of the ROS-mediated cellular injuries and prevention against the above mentioned diseases[6]. This antioxidant capacity is widely used as a parameter to characterize nutritional health food or plants and their bioactive components. Recently, interest has considerably increased in finding naturally occurring antioxidant to replace synthetic antioxidants, which were restricted due to their side effects such as carcinogenesis[7].

Alcea genus has been used traditionally in Iran. Many of the plants belonging to the genus *Alcea* are known to possess ethnomedical and biological properties related to antiviral[8], anti-inflammatory, astringent, demulcent, diuretic, febrifuge, blood circulation[9] and antimicrobial activity[10]. *Alcea hyrcana Grossh* (*A. hyrcana Grossh*) (malvacea), is native to northern of Iran. Water-soluble polysaccharides have been isolated

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from the bark and stems of this plant including uronic acids, glucose, arabinose, and xylose[11]. To the best of our knowledge, no biological activities of the *A. hyrcana* flower, leaves and seed were reported. In the present study, we examined the antioxidant activity of methanol extracts of *A. hyrcana* flower, seed and leaves, employing various in vitro assay systems, i.e. DPPH and nitric oxide radical scavenging, reducing power, linoleic acid, iron ion chelating power and H_2O_2 scavenging ability, in order to understand the usefulness of this plant.

Materials and Methods

Chemicals

Ferrozine, linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide and hydrogen peroxide were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Gallic acid, quercetin, butylated hydroxyanisole (BMA), ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, EDTA and ferric chloride were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade or purer.

Plant Material and Preparation of Freeze-Dried Extract

Flower, seed and leaves of *A. hyrcana Grossh* were collected from Khazar-Abad area in north of Sari, Iran, in spring of 2009 and identified by Dr. Bahman Eslami. A voucher (No. 570-2) has been deposited in Sari School of Pharmacy Herbarium. The materials were dried at room temperature and coarsely ground (2-3 mm) before extraction. Each part (100 g) was extracted at room temperature by percolation using methanol for 24 h at room temperature. The extract was then separated from the sample residue by filtration through Whatman No.1 filter paper, repeated three times. The resultant extracts were concentrated in a rotary evaporator until a crude solid extracts were obtained.

Determination of Total Phenolic Compounds and Flavonoids Contents

Total phenol contents were determined by Folin Ciocalteau reagent[12,13]. The extract samples (0.5 ml of different dilutions) or gallic acid was 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. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg ml^{-1} solutions of gallic acid in methanol: water (50:50, v/v). Results were expressed in terms of gallic acid equivalents which is a common reference compound. Colorimetric aluminum chloride method was used for flavonoid determination[14]. 0.5 ml of sample in methanol was 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. The extract remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (UV – Visible EZ201, Perkin Elmer, Waltham, MA, USA). The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg ml^{-1} in methanol.

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[15]. Different concentrations of each extracts were added to methanol solution of DPPH (100 μM) at an equal volume. 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

The reducing power of flowers, seeds and leaves extracts of *A. hyrcana Grossh* was determined according to the method of Yen and Chen[16]. 2.5 ml 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 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 (UV – Visible EZ201, Perkin Elmer, Waltham, MA, USA). Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Assay of Nitric Oxide-Scavenging Activity

For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline (PBS), was mixed with different concentrations of each extracts dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without extract but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm (UV – Visible EZ201, Perkin Elmer, Waltham, MA, USA). Quercetin was used as positive control[17].

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 hydroperoxide decomposition reactions via Fenton chemistry[18]. These processes can be delayed by iron chelation and deactivation. The chelating of ferrous ions by *A. hyrcana Grossh* extracts was estimated by our recently published paper[19]. Briefly, the extract (0.05-3.2 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 (UV – Visible EZ201, Perkin Elmer: USA). The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0 - A_s)/A_s] \leftrightarrow 100$, 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

The inhibitory capacity of *A. hyrcana Grossh* extract against oxidation of linoleic acid by FTC method was tested. This method was adopted from Osawa and Namiki[20]. Twenty 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 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 (UV – Visible EZ201, Perkin Elmer, Waltham, MA, USA). 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[21]. A solution of hydrogen peroxide (40 mM) was prepared in PBS (pH 7.4). Extract (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 PBS 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

Total phenol compounds, as determined by Folin Ciocalteau method, are reported as gallic acid equivalents by reference to standard curve ($y = 0.0063x$, $r^2 = 0.987$). The total phenolic contents of flowers, seeds and leaves were 48.1 ± 3.2, 68.9 ± 3.7 and 14.7 ± 0.9 mg gallic acid equiva-

lent/g of extract powder, respectively. The total flavonoid contents of flowers, seeds and leaves were 24.3 ± 2.1, 24.7 ± 1.8 and 28.3 ± 2.6 mg quercetin equivalent/g of extract powder, respectively, by reference to standard curve ($y = 0.0067x + 0.0132$, $r^2 = 0.999$). IC_{50} for DPPH radical-scavenging activity was in the order: leaves (538 ± 17.3) > flower (450 ± 15.8) > seed (421 ± 12.3) μg ml^{-1}, respectively. The IC_{50} values for ascorbic acid, quercetin and BHA were 1.26 ± 0.11, 1.32 ± 0.07 and 13.49 ± 1.04 μg ml^{-1}, respectively. Figure 1 shows the dose-response curves for the reducing powers of the extracts. It was found that the reducing powers of extracts also increased with the increase of their concentrations. There were significant differences among the different extracts in reducing power. The leaves and flower extracts had shown better reducing power than seed (p <0.05). The activities were not comparable with vitamin C (p >0.01). The extracts also showed weak nitric oxide-scavenging activity between 0.2 and 3.2 mg ml^{-1}. The % inhibition was increased with increasing concentration of the extracts. The leaves extract had shown better reducing power than other parts. IC_{50} were 2.61 ± 0.15 for flowers, 0.97 ± 0.04 for seeds, 0.45 ± 0.01 for leaves and 0.21 ± 0.02 mg ml^{-1} for quercetin. In assay of chelating of ferrous ions, both extracts 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 0.05 to 3.2 mg ml^{-1}. *A. hyrcana Grossh* extracts showed good Fe^{2+} chelating ability. IC_{50} were 2.9 ± 0.18 for flowers, 0.42 ± 0.03 for seeds and 0.11± 0.01 mg ml^{-1} for leaves. EDTA showed very strong activity (IC_{50} = 18 μg ml^{-1}). Only leaves extract showed the time-course data for the antioxidant activity in the FTC method. Only this fraction showed good activity. The peroxidation inhibition (antioxidant activity) of extract exhibited values from 88.1 to 96.4% (during 48th - 72nd hours). None of other tested extracts exhibited any activity. The leaves extract manifested almost the same pattern of activity as vitamin C at different incubation times (p <0.05). The extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner. IC_{50} for H_2O_2 scavenging activity were in the order 160.6 ± 8.1 for seed, 554.7 ± 13.5 for leaves and 722.4 ± 27.1 μ ml^{-1} for flower. The IC_{50} values for ascorbic acid and BHA were 21.4 and 52.0 μ ml^{-1}, respectively.

Discussion

Total phenol compounds were determined by Folin Ciocalteu method and flavonoids contents by $AlCl_3$ methods. Seed extract had significant higher total phenol and leaves had higher flavonoids contents than other parts. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess

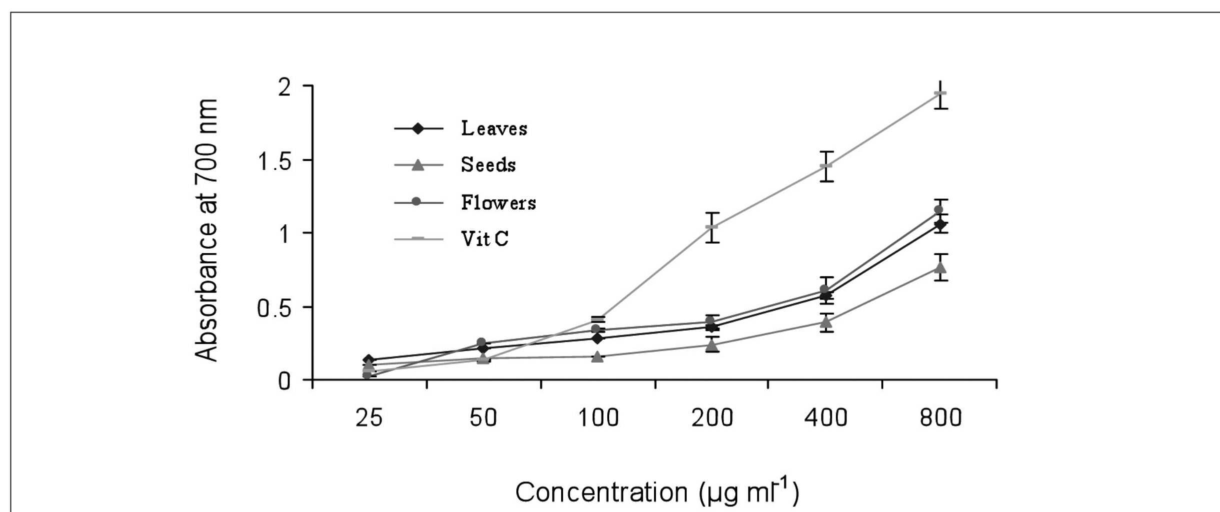

Figure 1. Reducing power of *Alcea hyrcana Grossh* flower, seed and leaves. Vitamin C used as control.

significant antioxidant activities[22]. Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases[23].

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples[24]. 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[25]. Based on the IC_{50} results, according to higher total phenol contents, it was shown that the seed extract of *A. hyrcana Grossh* had higher DPPH-scavenging activity than the others.

Fe (III) reduction is often used as an indicator of electron- donating activity, which is an important mechanism of phenolic antioxidant action [19]. 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. The leaves and flower extracts had shown better reducing power than seed (p <0.05) but were not comparable with vitamin C (p <0.01). Therefore, *A. hyrcana Grossh* shows reductive potential and can serve as electron donors, terminating the radical chain reaction.

The assay of nitric oxide-scavenging activity 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. The extracts also showed weak nitric oxide-scavenging activity between 0.2 and 3.2 mg ml^{-1}. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions[26]. 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[19]. 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[25].

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[27]. 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[28]. Foods are often contaminated with transition metal ions which may be introduced by processing methods. The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease[29]. 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 extract was estimated by the method of Dinis et al[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 color 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 on increasing concentration from 0.2 to 3.2 mg ml^{-1}. Metal chelating capacity was significant since the extract reduced the concentration of the catalyzing transition metal in lipid peroxidation[28]. 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[30]. *A. hyrcana Grossh* extracts showed good Fe^{2+} chelating ability.

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[31]. 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[30]. Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. The leaves extract manifested almost the same pattern of activity as vitamin C at different incubation times (p <0.05).

Scavenging of H_2O_2 by *A. hyrcana Grossh* extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water[32]. The differences in H_2O_2 scavenging capacities between the extracts may be attributed to the structural features of their active components, which determine their electron donating abilities[33,34]. The seeds had higher phenolic content and showed better scavenging activity than other parts. The IC_{50} values for vitamin C and BHA were 21.4 and 52.0 μ ml^{-1}, respectively. Although H_2O_2 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.

Conclusions

The leaves, seed and flower extracts of *A. hyrcana Grossh* exhibited good but different levels of antioxidant activity in all the models studied. The extracts had good iron chelation, H_2O_2 and nitric oxide scavenging activity. Further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed.

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