ANTIOXIDANT ACTIVITY OF *HYOSCYAMUS SQUARROSUS* FRUITS

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Summary

Antioxidant activity of Fruits of *Hyoscyamus squarrosus* was investigated employing six in vitro assay systems. Extract showed good antioxidant activity. IC_{50} for DPPH radical-scavenging activity was 0.325± 0.012 μg ml^{-1}. *Hyoscyamus squarrosus* fruits showed good nitric oxide-scavenging activity between 0.1 and 1.6 mg ml^{-1}(IC_{50} = 0.972 ± 0.07 mg ml^{-1}). The extract had show good reducing power that was not comparable with Vit C ($p< 0.01$). also, Extract showed very good Fe^{2+} chelating ability(IC_{50} =104 ± 3.8 μg ml^{-1}). The total amount of phenolic compounds in extract was determined as gallic acid equivalents and total flavonoid content was calculated as quercetin equivalents from a calibration curve.

Key words: Key words: Antioxidant activity, *Hyoscyamus squarrosus*, DPPH, phenol

Introduction

Plants are rich sources of natural antioxidants. Among the various medicinal and culinary plants, some endemic species are of particular interest because they may be used for producing raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits (1, 2). 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 (3). Thus, recent studies have investigated the potential of plant products as antioxidants against various diseases induced by free radicals (4). Additionally, it has been determined that the antioxidant effect of plant products is mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins, and phenolic diterpenes (5). *Hyoscyamus* genus is member of *solanaceae* family and has 18 species in Iran (6). This genus known to possess medicinal properties related to high content of tropane alkaloids such as Hyoscyamine, scopolamine and to lesser extent atropine(7). *Hyoscyamus squarrosus* grow naturally in Iran, Pakistan and Afghanistan (6) and To the best of the author's Knowledge just antimicrobial activity of this species previously reported (8).

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: *Hyoscyamus squarrosus* fruits (summer 2007) was collected from Golestanak area (Central Elburz, Iran) and identified by Dr. Bahman Eslami A voucher (No.1142) has been deposited in the Sari School of Pharmacy herbarium. Materials dried at room temperature and coarsely ground before extraction. Then, it 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 content was determined by the Folin-Ciocalteau reagent according to the recently published method (9). The extract sample (0.5 ml) 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. Result was expressed as gallic acid equivalents. Total flavonoids was estimated using the method of Dehpour et al. (9). Briefly, 0.5 mL solution of extract in methanol was 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 content was 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 extract (9). Different concentrations of extract 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 (10). The reducing power of extract was determined according to the our recently publish paper (11). Different amounts of extract (50-1600 $\mu g\ ml^{-1}$) 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. 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 extract 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 (12).

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 (3). The chelating of ferrous ions by extract was estimated by our recently published paper (13). Briefly, 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 (14). The inhibitory capacity of extract was tested against oxidation of linoleic acid by FTC method. This method was adopted from our recently published paper (15). 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 sample was run in triplicate and averaged. Vit C and BHA used as positive control.

Scavenging of hydrogen peroxide: The ability of the extract to scavenge hydrogen peroxide was determined according to the method of Ruch (15). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extract (0.1-1 mg ml^{-1}) in distilled water was 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 extract 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 (16).

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 *Hyoscyamus squarrosus* fruit was 178.9 ± 7.8 mg gallic acid equivalent/g of extract powder. The total flavonoid contents of *H.squarrosus* was 16.4 ± 1.06 mg quercetin equivalent/g of extract powder, by reference to standard curve ($y = 0.0067x + 0.0132$, $r^2 = 0.999$). 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 (16).

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 (18). It was found that the radical- scavenging activities of all the extract increased with increasing concentration. IC_{50} for DPPH radical-scavenging activity was 0.325± 0.012 μg 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 sample 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. The extract had show good reducing power but it was not comparable with Vit C ($p < 0.01$). The reductive abilities of the fruit extract of *H. squarrosus* was evident that did show reductive potential and could serve as electron donors, terminating the radical chain reaction.

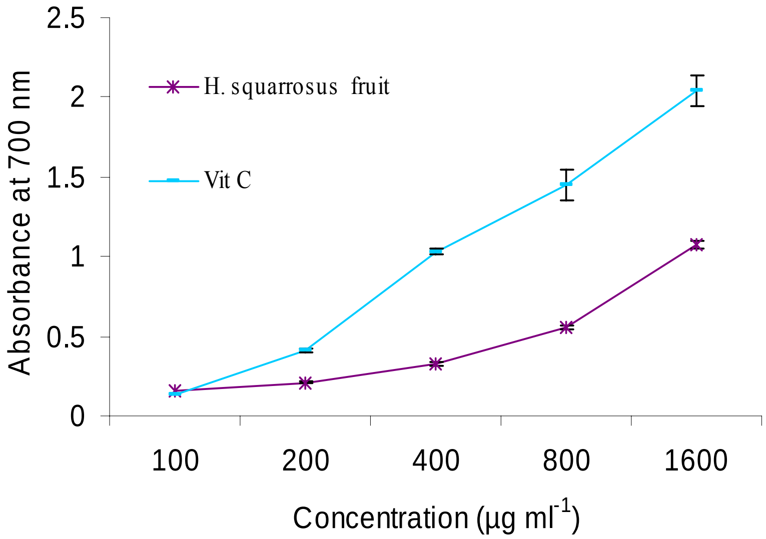

Fig.1. Reducing power of methanolic extract of *H. squarrosus* fruits.

Assay of nitric oxide-scavenging activity: The % inhibition was increased with increasing concentration of the extract. The extract had show good nitric oxide scavenging (0.972 ± 0.07 mg ml^{-1} for fruits vs 0.20 ± 0.01 mg ml^{-1} for quercetin). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (16). 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.

Fe^{2+} chelating activity of *H. squarrosus* fruit extract

The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (17,20). 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. 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 50 to 800 μg ml^{-1}. Metal chelating capacity was significant since the extract reduced the concentration of the catalyzing transition metal in lipid peroxidation. 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 (13,21). *H. squarrosus* Fruit extract showed good Fe^{2+} chelating ability(IC_{50} = 104 ± 3.8 μg ml^{-1}). EDTA showed very strong activity (IC_{50} = 18 μg ml^{-1}).

Scavenging H_2O_2

Scavenging of H_2O_2 by *H. squarrosus* extract may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water (9,22). The differences in H_2O_2 scavenging capacities between the extract may be attributed to the structural features of their active components, which determine their electron donating abilities (9). The *H. squarrosus* fruit extract was capable of scavenging hydrogen peroxide in a concentration dependent manner (IC_{50} = 178.87 ± 11.3 μg ml^{-1}). The IC_{50} values for Ascorbic acid and BHA were 21.4 and 52.0 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

FTC Method

The extract exhibited low antioxidant activity in the FTC method. The peroxidation inhibition (antioxidant activity) of extract exhibited values from 33% (at 24th hrs) and from 66.8% (at 72nd hrs).

The extract of *H. squarrosus* exhibited good but different levels of antioxidant activity in some models studied. Further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed.

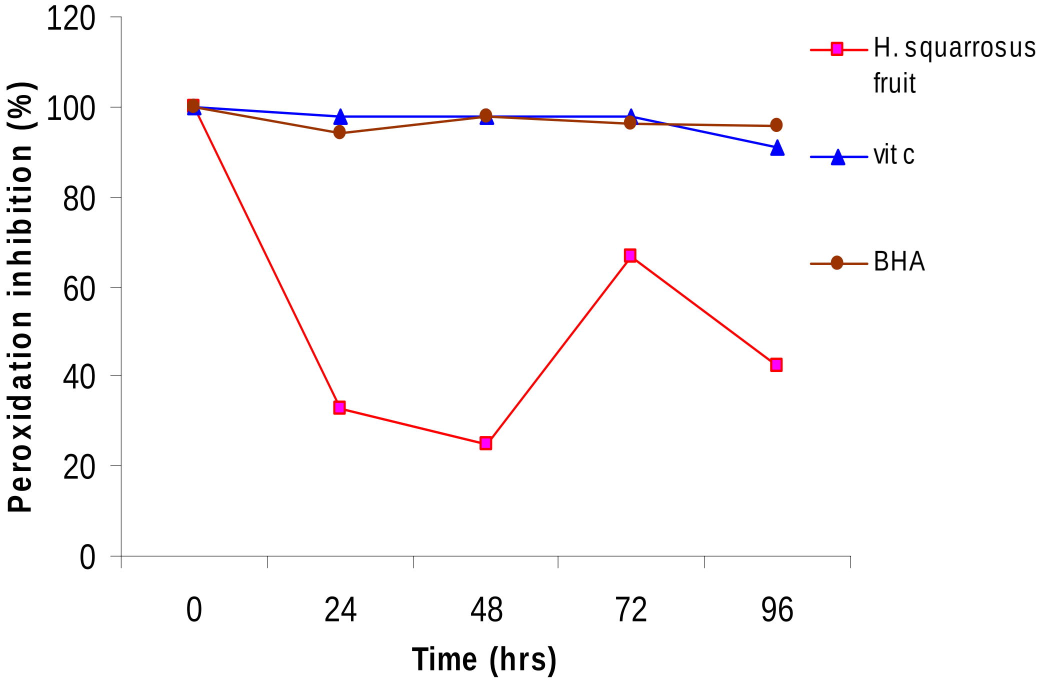

Fig. 2. Antioxidant activity of methanolic extract of *H. squarrosus* fruits in FTC method at different incubation times. *H. squarrosus* (0.2 mg ml^{-1}), Vit C and BHA (0.1 mg ml^{-1}).

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