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In vitro Antioxidant and Free Radical Scavenging Activity of *Diospyros lotus* and *Pyrus boissieriana growing in Iran*

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ABSTARCT
Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of many diseases. The potential antioxidant activities of *Diospyros lotus* and *Pyrus boissieriana* fruits investigated employing six in vitro assay systems. IC_{50} for DPPH radical-scavenging activity was 1.45 ± 0.03 for *D. lotus* and 3.0 ± 0.04 mg ml^{-1} for *P. boissieriana*, respectively. The extracts showed weak nitric oxide-scavenging and Fe^{2+} chelating ability activity. The *D. lotus* extracts was better than *P. boissieriana*. The peroxidation inhibition of *D. lotus* and *P. boissieriana* extracts exhibited values from 89 (at 24th) to 94% (at 72nd hrs) and 91 to 95% respectively. Neither of them showed good scavenging activity of H_2O_2. The total amount of phenolic compounds in each extracts was determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve. Both of them had high total phenolic and flavonoid contents.
KEY WORDS - Antioxidant activity, *Diospyros lotus*, Free Radical Scavenging, *Pyrus boissieriana,*

INTRODUCTION
The role of free radicals in many disease conditions has been well established. Several biochemical reactions in our body generate reactive oxygen species. If they are not effectively scavenged by cellular constituents, they lead to disease conditions (1). Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers (2) and neurodegenerative diseases (3). *Pyrus boissieriana* (Rosaceae) is widely distributed in northern area of Iran. A phenyl glycoside in this plant has been reported (4). The fruits of *Diospyros lotus* L. (Ebenaceae) are febrifuge and used to promote secretions. The seed is regarded in China as being sedative (www.pfap.org/database). Chemical constituents of D. lotus has been published (5). Fatty acid compositional changes (6) and Changes in phenolic acid contents during fruit development of this plant were studied (7). Yet little information is available about antioxidative activity of these plants (8). In this study, we examined the antioxidant activity of these two native plants 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 understand the usefulness of this plant as a foodstuff as well as in medicine.

MATERIALS AND METHODS
Plant material and preparation of freeze-dried extract
D. lotus and *P. boissieriana* fruits were collected from Mazandaran forest and identified by Dr. Bahman Eslami. A voucher (No. 971-972) 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 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.

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Determination of total phenolic compounds and flavonoid content

Total phenolic compound contents were determined by the Folin-Ciocalteau method (9-10). The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 ml of 75 g l^{-1} 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 Ordonez et al. (9-10). Briefly, 0.5 mL solution of each plant extracts 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 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 (10-11). Different concentrations of each 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. Vitamine 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 (12). The reducing power of extracts was determined according to the method of Yen and Chen (13). 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 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 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 positive control (9-11).

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 (14). The chelating of ferrous ions by extracts was estimated by our recently published paper (15). 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 $[(A_0- A_s)/A_s]\times100$, 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 (16). The inhibitory capacity of extracts was tested against oxidation of linoleic acid by FTC method. This method was adopted from Osawa and Namiki (11). 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 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 all samples were run in triplicate and averaged. Vit 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 (17). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extracts (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 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 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 *D. lotus* and *P. boissieriana* fruits were 10.2 ± 0.9 and 15.8 ± 0.19 mg gallic acid equivalent/g of extract powder, respectively. The total flavonoid contents of *D. lotus* and *P. boissieriana* fruits were 2.1 ± 0.05 and 3.6 ± 0.07 mg quercetin equivalent g^{-1} of extract powder, respectively, by reference to standard curve ($y = 0.0067x + 0.0132$, $r^2 = 0.999$). *P. boissieriana* fruit extract had higher total phenol and flavonoids contents than did *D. lotus*. 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 (18).

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 (19). It was found that the radical-scavenging activities of all the extracts increased with increasing concentration. IC_{50} for DPPH radical-scavenging activity was 1.45 ± 0.03 for *D. lotus* and 3.0 ± 0.04 mg ml^{-1} for *P. boissieriana*, respectively. 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 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 (9). Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 1 shows the dose-response curves for the reducing powers of the extract. 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 two extracts in reducing power. The activity was not comparable with Vit C ($p < 0.001$).

Assay of nitric oxide-scavenging activity

The extracts showed weak nitric oxide-scavenging activity between 0.2 and 3.2 mg ml^{-1}. IC_{50} was 0.65 ± 0.05 mg ml^{-1} for *P. boissieriana*. The % inhibition was increased with increasing concentration of the extract. *D. lotus* showed only 12% inhibition at 1.6 mg ml^{-1}. However, activity of quercetin was very more pronounced than that of our extracts (17 ± 1.5 μg ml^{-1}). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (20).

Fe^{2+} chelating ability

The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (21). Because Fe^{2+} causes the production of oxyradicals and lipid peroxidation, minimizing its concentration affords protection against oxidative damage. In the presence of other chelating agents, the ferrozine complex formation is disrupted with the result that the red color of the complexes decreases (15). 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. It

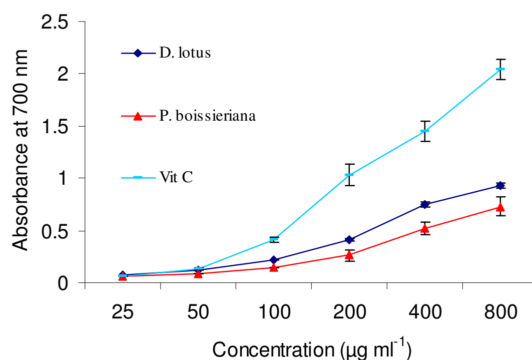

Figure 1: Reducing power of D. lotus and P. boissieriana fruits. Vit C used as control.

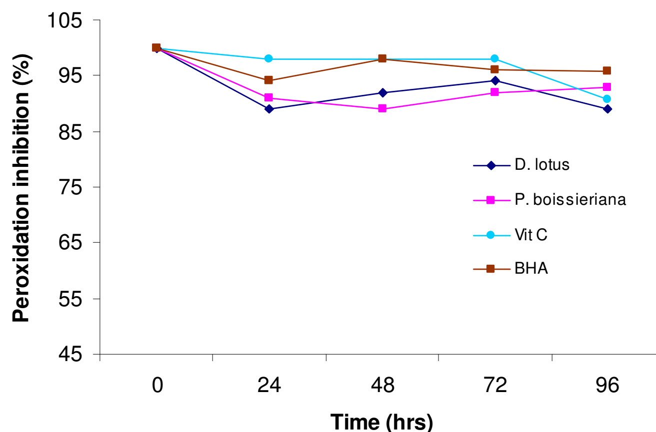

Figure 2: Antioxidant activity of D. lotus and P. boissieriana fruits in FTC method at different incubation times (0.4 mg/ ml), Vit C and BHA (0.1 mg/ ml) used as control.

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 (22). The extracts exhibited some Fe^{2+} chelating ability. IC_{50} for Fe^{2+} chelating ability were 0.93 and 0.38 mg ml^{-1} for *D. lotus* and *P. boissieriana*, respectively. EDTA showed very strong activity with IC50 = 0.018 mg ml^{-1}.

FTC Method

Figure 2 shows the time-course plots for the antioxidative activity of the plants extracts using the FTC method. Extracts exhibited good antioxidant activity. There were no significant differences ($p<$ 0.05) among extracts. They manifested almost the same pattern of activity as Vit C at different incubation times (at 72nd and 96th hrs, p> 0.05).

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. No extract showed good scavenging activity. IC_{50} for scavenging of H_2O_2 was in the order: 0.17 ± 0.01 and 1.01 ± 0.08 mg ml^{-1} for *P. boissieriana* and *D. lotus*, respectively. The IC_{50} values for Ascorbic acid and quercetin were 21.4 ± 0.12 and 52.0 ± 3.11 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. The plants extracts exhibited different levels of antioxidant activity in all the models studied. Further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed.

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