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Otostegia persica as a Source of Natural Antioxidants

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Abstract

The antioxidant activity of different extracts of aerial parts of *Otostegia persica* (Burm.) Boiss., Labiateae were evaluated using β-carotene bleaching and lipid peroxidation models. The inhibitory activity of these extracts on the peroxidation of linoleic acid was measured by ammonium thiocyanate in comparison to green tea [*Camellia Sinensis* (L.) Kuntze] *Ginkgo biloba* L., and butylated hydroxyanisole (BHA). A methanol extract of the plant exhibited the highest antioxidant activity. Five compounds were separated and purified from the methanol extract by column and paper chromatography, respectively. Three isolated flavonols showed significant antioxidant activity comparable to BHA and vitamin E in both methods. These active compounds were identified by UV, IR, ^{1}H and ^{13}C NMR, and MS spectroscopies as morin, kaempferol, and quercetin. The only identified *C*-glucoflavone (isovitexin) also exhibited interesting antioxidant activity, but weaker than the flavonols. *trans*-Cinnamic acid showed no activity in these methods.

Keywords: Antioxidant, flavonoid, goldar, Labiateae, lipid peroxidation, *Otostegia persica*.

Introduction

Lipid peroxidation that is initiated by reactive oxygen radicals such as the hydroxyl radicals defines the biological damage caused by free radicals that are formed under oxidative stress (Gazzani et al., 1998). Lipid peroxidation is involved in the pathogenesis of diseases such as atherosclerosis, liver disease, diabetes, aging, and cancer (Takashi & Tkayuki, 1997). Moreover, the formation of lipid peroxides and their secondary products such as reactive carbonyl compounds also causes various kinds of biological damage (Chaudhary et al., 1994). For these reasons, antioxidants are of interest for the treatment of many kinds of cellular degeneration (Tutour, 1990). Restrictions on the use of synthetic antioxidants are being imposed because of their carcinogenicity (Botherweck et al., 2000; Bauer et al., 2001). Thus, the interest in natural antioxidants has increased considerably. Among naturally occurring phenol compounds, flavonoids have gained a particular interest because of their broad pharmacological activity. They are frequently compounds of human diet, and, during the past decade, evidence has accumulated indicating that flavonoids such as quercetin, kaempferol, and morin are an important class of antioxidants (Yang et al., 2001). As resources of natural antioxidants, much attention has been paid to plants (Zhang et al., 1990). Especially, the antioxidants present in edible plants have been considered as food additives (Igarashi et al., 1990). Because of the existence of other compounds with antioxidant properties in addition to flavonoids in plants, the aqueous or hydro-alcoholic extracts of medicinal plants have been used for treatment of diseases in traditional medicine.

Otostegia persica (Burm.) Boiss., Labiateae, known as "goldar," is an endemic plant that can be found in its natural habitant in Kerman province in Iran. The aqueous extract of the aerial parts of the plant has been used as antispasmodic, antihistaminic, and antiarthritic in folk medicine (Ghahraman, 1996). In spite of the many uses of the plant, only one study on its biological activities has so far been reported (Sharififar et al., 2003). In this research, various extracts of the plant and fractions were subjected to a preliminary antioxidant-screening test. The antioxidant activity of the methanol extract of the plant was compared to methanol extract of green tea, *Ginkgo biloba*, and BHA.

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Materials and Methods

Plant materials were collected in July 1999 from Dehbakri in Kerman province. The plant was identified by the Department of Botany of the Research Institute of Forests and Rangelands (TARI), Tehran. A voucher specimen has been deposited at the Herbarium of TARI. Linoleic acid, ferrous chloride ($FeCl_2$), Tween 20, β-carotene, and BHA were purchased from Sigma Chemical Co. (Taufkirchen, Germany). Ammonium thiocyanate and the other chemicals were purchased from Merck Co. (Darmstadt, Germany). UV spectra were obtained with a Shimadzu UV-160A spectrophotometer (Tokyo, Japan), IR spectra were acquired on a Perkin-Elmer 1420 recording spectrometer (Buckinghamshire, UK). ^1H and ^{13}C NMR spectra were recorded on Varian 400 Unity plus spectrophotometer (California, USA). For ^1H NMR, tetramethylsilane (TMS) was used as internal standard. For mass spectrophotometry (MS), a Finnigan Mat TSQ-70 spectrophotometer (California, USA) was used. Analytical thin-layer chromatography (TLC), column and paper chromatographies were carried out on Schleicher & Schuell F_{254} plates (Dassel, Germany), silica gel G_{60} (35–70 μm, Merck), and Whatman nos. 1 and 3 paper chromatography, respectively.

Extraction

Aerial parts of *O. persica* were finely powdered in a mill, and a 500 g sample was extracted with methanol. Most of the solvent was removed under reduced pressure. The residue was re-extracted with hexane and chloroform, respectively. The solvents were evaporated under the reduced pressure. All extracts were tested for antioxidant activity.

Antioxidant activity

Rapid evaluation of antioxidant activity by β-carotene bleaching method

The rapid evaluation of antioxidant activity of hexane, chloroform, and methanol extracts were determined according to the β-carotene bleaching method (Mehta et al., 1994). In this procedure, after activation at 100°C for 1 h, TLC plates (0.25 mm) precoated with silica gel G were streaked with 200 μl of samples and developed by a mixture of ethyl acetate/water/formic acid/acetic acid (100:26:11:11). Plates were sprayed with a solution of 9 mg of β-carotene dissolved in 30 ml chloroform to which 2 drops of linoleic acid were added and exposed to daylight for 6 h. The intensity of the resulting orange color corresponded to the relative antioxidant activity of the extracts. Methanol extracts of green tea, *Ginkgo biloba*, and BHA were used as positive controls. Extracts that showed strong antioxidant activity were subjected to further tests. The same experiment was done for isolated fractions and compounds. In two latter tests, BHA and α-tocopherol were used as positive controls.

Antioxidant activity evaluation by ammonium thiocyanate method

The antioxidant activity of hexane, chloroform, and methanol extracts were determined using the ammonium thiocyanate method (Masude et al., 1992). Each sample (500 μl) in 0.5 ml of EtOH was mixed with linoleic acid emulsion (2.5 ml) and phosphate buffer (2 ml, 0.02 M, pH = 7.0). The linoleic acid emulsion was prepared by mixing 0.284 g of linoleic acid, 0.284 g of Tween 20 as emulsifier, and 50 ml phosphate buffer, and then the mixture was homogenized. The reaction mixture was incubated at 37°C. Aliquots of 0.1 ml were taken at different intervals during incubation. The degree of oxidation was measured by sequentially adding ethanol (4.7 ml, 75%), ammonium thiocyanate (0.1 ml, 30%), sample solution (0.1 ml), and ferrous chloride (0.1 ml, 0.02 M in 3.5% HCl). After 3 min, the peroxide values was determined by reading the absorbance at 500 nm using a spectrophotometer. A control was performed with linoleic acid but without the extracts. Methanol extracts of green tea, *Ginkgo biloba*, and BHA were used as positive controls, and their solvents were used as negative controls. This experiment was done for isolated fractions and compounds separately. In two latter tests, BHA and α-tocopherol were used as positive controls.

Isolation of the antioxidant fractions from Otostegia persica

Column chromatography was used as a primary method for the fractionation of the methanol extract of *O. persica*, and paper chromatography was used to isolate the purified compounds. The methanol extract of *O. persica* was chromatographed on a column (2.5 cm, 80 cm) containing silica gel G_{60}. The elution was carried out by different solvents in the following sequence: 100% hexane followed by 10% chloroform in hexane to 100% chloroform, 10% ethyl acetate in chloroform to 100% ethyl acetate. Elution was continued with 10% methanol in ethyl acetate to 100% methanol. A quantity of 100 ml was collected for each fraction. The solvent was removed under reduced pressure. The chromatography was monitored by TLC using solvent systems of ethyl acetate/chloroform (40:60) and ethyl acetate/acetic acid/formic acid/water (100:11:11:26). Eight major fractions were obtained.

The fractions that showed antioxidant activity (fraction 5, 8 named F_5, F_8) obtained from ethyl acetate-methanol (40:60) were repeatedly streaked on Whatman no. 3 MM chromatography paper until a total of 20 mg

Table 1. Antioxidative activity of different extracts from *O. persica* as measured by the thiocyanate method after 60 h incubation.

Sample	Absorbance at 500 nm	Percent of inhibition[a]
Control	1.050 ± 0.026[b]	0.00
Methanol extract of *O. persica*	0.085 ± 0.024	95.87 ± 1.1*
Hexane extract of *O. persica*	1.98 ± 0.21	2.5 ± 0.013
Chloroform extract of *O. persica*	2.010 ± 0.012	1.9 ± 0.021
Methanol extract of green tea	0.046 ± 0.017	96.03 ± 0.018*
Methanol extract of *Ginkgo biloba*	0.431 ± 0.028	77.20 ± 1.38*
α-Tocopherol	0.257 ± 0.016	87.45 ± 0.76*
Butylated hydroxyanisole (BHA)	0.002 ± 0.001	99.92 ± 0.02*

[a]Percent of inhibition (capacity to inhibit the peroxide formation in linoleic acid) = [1− (absorbance of sample at 500 nm)/(absorbance of control at 500 nm] × 100. A high inhibition percentage indicates a high antioxidant activity.
[b]Results are presented as mean ± standard deviation ($n = 5$).
*Statistically significant ($p < 0.05$).

had been applied. The papers were equilibrated for 16 h and developed descendingly in *t*-butanol- acetic acid-water (3:1:1 v/v/v) (TBA). The chromatograms were dried and examined under short (250 nm) and long (360 nm) ultraviolet (UV) wavelengths before and after exposure to ammonia fumes. The major fractions were eluted with 80% aqueous methanol. The eluted compounds were further purified by paper chromatography using 15% acetic acid as the solvent (Markham, 1982). Four major compounds have been isolated from fraction 5 (F_5), which were designated F_{5A} to F_{5D}, and only one compound from F_8, designated as F_8. These five compounds were subjected to two antioxidant experiments.

Statistical analysis

Analyses were carried out five times ($n = 5$). Statistical analysis was performed according to Student's *t*-test. Analysis of variance was performed by ANOVA procedure.

Results

Antioxidant activity by β-carotene bleaching method

Observation of TLC plates after spraying the reagent of β-carotene showed that after discoloring the background of plates, the methanol extract of the *Otostegia persica*

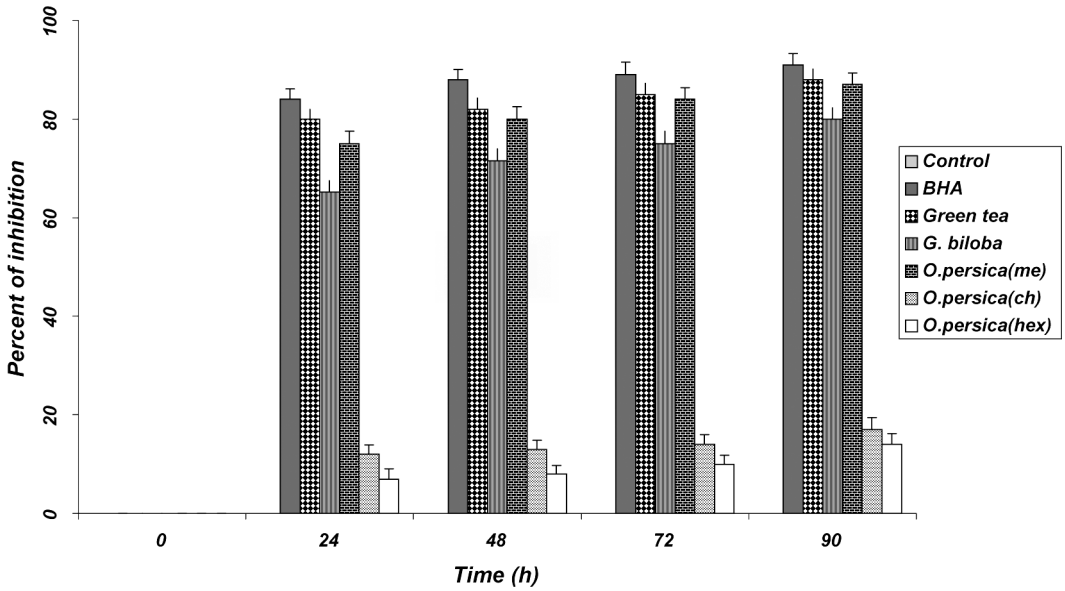

Figure 1. Antioxidant activity of various extracts of *O. persica* in comparison to positive controls by ammonium thiocyanate method.

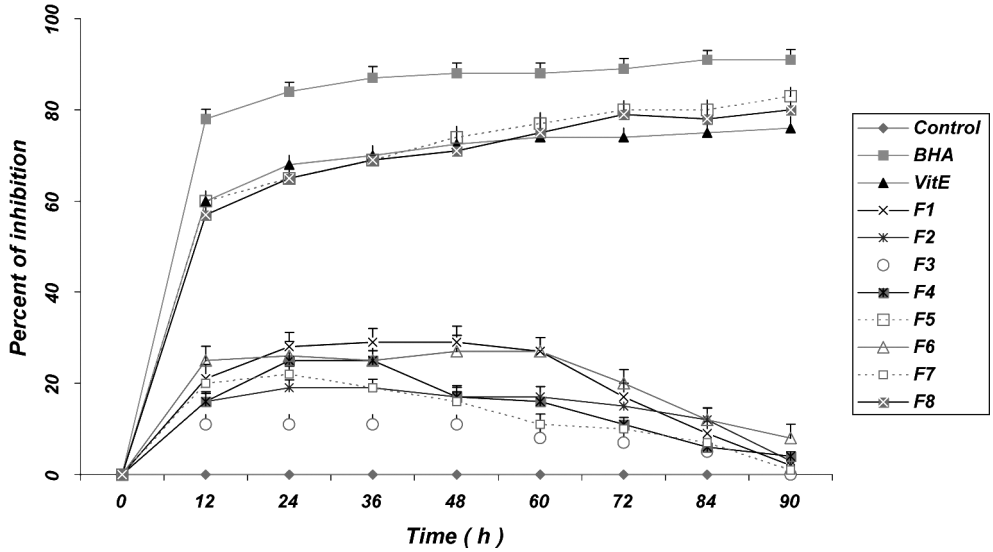

Figure 2. Antioxidant activity of different fractions in comparison to positive controls by ammonium thiocyanate method.

has orange bands, and the color persisted after 6 h. Hexane and chloroform extracts showed no color bands. Among the isolated fractions, F_5 showed three orange bands, and F_8 exhibited one color band.

Antioxidant activity by ammonium thiocyanate method

Table 1 lists the antioxidant activity of the various extracts from *O. persica*. Of the three types of extracts, the methanol one displayed a strong antioxidant activity, whereas the others showed no activity. These observations are in agreement with reports (Wang, 1997; Meyer et al., 1997) that methanol is an ideal and effective solvent for extraction of antioxidants.

The methanol extract of *O. persica* was more active than *Ginkgo biloba* and almost equal to green tea. Because of these results, this extract was further studied. Figure 1 shows the antioxidant activity of various

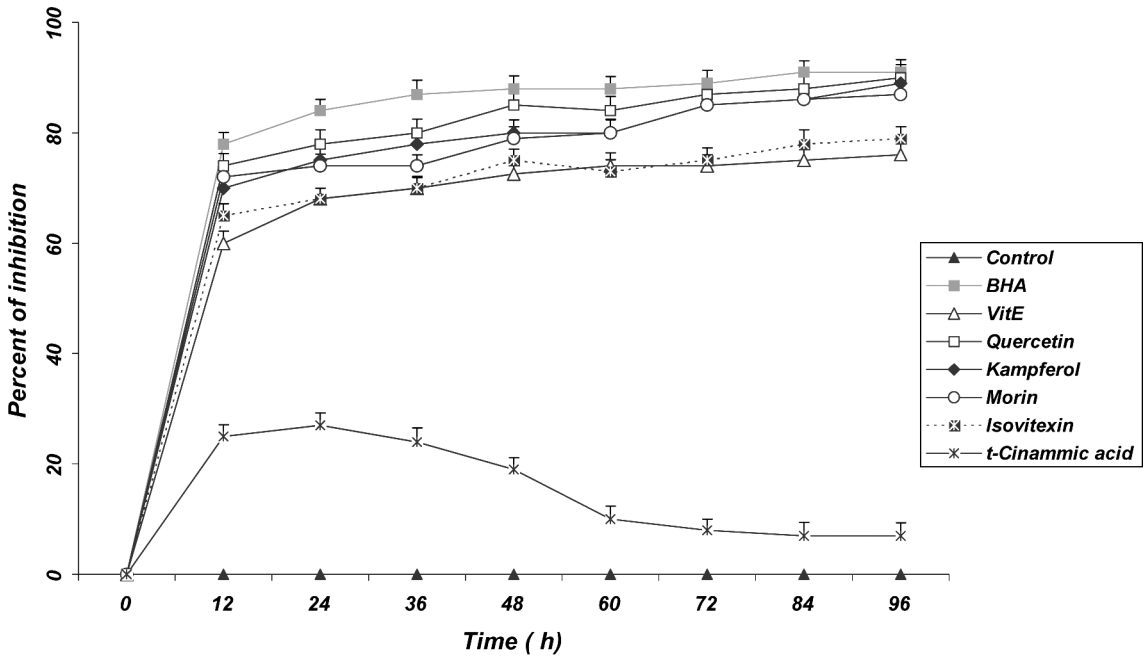

Figure 3. Antioxidant activity of major compounds of *O. persica* in comparison to positive controls by ammonium thiocyanate method.

Table 2. Paper chromatographic data of isolated subfractions of F_{5A}, F_{5B}, F_{5C}, F_{5D}, and F_8.

	Color		$R_f \times 100$	
Compound	UV	UV/NH_3	TBA[a]	15% Acetic acid
F_{5A}	Blue	Blue/green	82	69
F_{5B}	Yellow	Yellow	76	22
F_{5C}	Dull yellow	Dull yellow	78	4
F_{5D}	Yellow	Yellow	70	58
F_8	Deep purple	Yellow green	54	57

[a]TBA = *T*-butanol-acetic acid-water (3:1:1).

extracts of the plant in the linoleic acid peroxidation system (ammonium thiocyanate method). The results obtained indicate that the methanol extract of *O. persica* significantly ($p < 0.05$) inhibits the linoleic acid peroxidation compared to the negative control.

Figure 2 illustrates antioxidant activity of 200 ppm of isolated fractions, determined according to the ammonium thiocyanate method. Of eight major fractions, F_5 and F_8 displayed the strongest antioxidant activity. These two fractions significantly ($p < 0.05$) inhibit the linoleic acid peroxidation relative to control. Figure 3 shows that among F_5 subfractions, F_{5B}, F_{5C}, and F_{5D}, exerted strong antioxidant activity. F_{5A} showed no activity, and F_8 also inhibited oxidation of linoleic acid considerably. Therefore, our investigation focused on the identification of active compounds.

Structural identification of the active compounds of the plant

The identification of the active compounds was made by UV, IR, nuclear magnetic resonance (^1H and ^{13}C NMR), and MS. Table 2 shows the chromatographic data of compounds of F_{5A}, F_{5B}, F_{5C}, F_{5D}, and F_8. Structural confirmation of these compounds was accomplished by comparing the melting point and spectral data obtained with authentic compounds in the literature (Mabery et al., 1970; Markham, 1982). All data of identified compounds matched well with the published data. On the basis of these findings, the structure of compounds of F_{5A}, F_{5B}, F_{5C}, F_{5D}, and F_8 were established to be *trans*-cinnamic acid, kampferol, morin, quercetin, and isovitexin, respectively.

Discussion

In this experiment, we investigated antioxidant activity of hexane, chloroform, and methanol extracts of *Otostegia persica*. Results showed that methanol extract has activity comparable to green tea and *Ginkgo biloba*. Fractions F_5 and F_8 of methanol extract by paper chromatography gave five compounds. Four of them showed strong antioxidant activity. These four compounds were flavonoids and identified as kampferol, morin, quercetin, and isovitexin from their spectral data. The results of both methods correlate. Flavonoids occur throughout the entire plant kingdom. The most widely distributed flavonoids, flavones and flavonols, are mainly hydroxylated in the B-ring at the 3′ and 4′ positions followed by the 4′ position only. Many of the flavonoids and related compounds are known to possess strong antioxidant characteristic (Plumb et al., 1999; Hu & Diang, 1996). The major mode of action of these compounds is related to their ability to scavenge free radicals driving from the phenolic moiety of the structure (Pincemail et al., 1989). Hydroxylation of the B-ring is an important factor of the antioxidant activity of flavonoids, although it is not a prerequisite for manifesting the activity (Jovanovic et al., 1994). In the current study, flavonols such as kampferol, morin, and quercetin that have hydroxyl groups at 3′ and 4′ positions exhibited the highest antioxidant activity among the flavonoids tested. Their activity was equal to BHA and was stronger than α-tocopherol, but isovitexin, which does not possess a hydroxyl at 3′ position, exhibited lower activity. On the basis of the results obtained we can conclude that the high antioxidant activity of F_5, close to BHA, probably is caused by a large amount of flavonols. On the other hand, flavonols seemed to be absent in the hexane and chloroform fractions. Many flavonoids such as morin and quercetin have been reported to have anti-inflammatory activity (DeWhalley et al., 1990; Masuda et al., 1993). In arthritis, oxidation leads to lipid peroxidation and formation of low-mass oligosaccharides, causing damage to bone and cartilage. Antioxidants are able to inhibit this process (Bors, 1996; Bohm, 1998) and suppress inflammation. The composition of the methanol extract of *Otostegia persica* and the results of our work warrant its use in folk medicine as an antiarthritis agent.

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