Iranian Journal of Pharmaceutical Research (2003) 235-239
Received: August 2003
Accepted: November 2003

Original Article

Antioxidant Activity of *Otostegia persica* (Labiatae) and its constituents

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Abstract

The antioxidant activity of the different extracts and fractions of aerial parts of *Otostegia persica* (Burm.) Boiss. were evaluated using beta-carotene bleaching and lipid peroxidation methods. The inhibitory activity of the plant extracts on the peroxidation of linoleic acid were measured by ferric thiocyanate method in comparison to methanolic extracts of Green tee, *Ginkgo biloba*, Vit.E and BHA as positive controls. Methanolic extract of the plant exhibited strong antioxidant activity .Two compounds of methanolic extract which were separated by column and paper chromatography showed significant antioxidant activity comparable to butylated hydroxy anisole (BHA) and alpha tocopherol. These active compounds were identified as morin and quercetin by using UV, IR, MS, ^{1}H and ^{13}C NMR.

Keywords: *Otostegia persica*; Labiateae; Antioxidant activity; Lipid peroxidation; Free radicals; Morin; Quercetin.

Introduction

Recent developments in biomedicals point to the involvement of free radicals in many diseases (1). Free radicals attack the unsaturated fatty acids in the biomembranes resulting in membrane lipid peroxidation, a decrease in membrane fluidity, loss of enzymes and receptor activity and damage to membrane proteins leading to cell inactivation (2). Free radicals also attack DNA and cause mutation leading to cancer (3). For these reasons antioxidants are of interest for the treatment of many kinds of cellular degeneration (4). Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions. There are two basic categories of antioxidant namely synthetic and natural ones. Restriction on the use of synthetic anti-oxidants is being imposed because of their carcinogenicity (5, 6). Thus the interest in natural antioxidants has been increased considerably. As resources of natural antioxidants much attention has been paid to plants (7, 8). Especially, the antioxidants present in edible plants have recently been considered as food additives (9, 10).

The antioxidant activity of several Iranian plants has been reported (11). In this paper the antioxidant activity of *Otostegia persica* (Labiateae) known as "Goldar" which is an endemic plant in Kerman province in Iran is reported.

The aqueous extract of the aerial parts of the plant has been used as antispasmodic, antihistaminic and anti arthritis (12). The antioxidant activity of the methanolic extract of the plant was compared to methanolic extract of Green tea and *Ginkgo biloba.*

In spite of the many uses of the plant, no study on its biological activities has so far been reported with exception of several articles about

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another species such as diterpenens and volatile oil of *O.fruticosa* (13,14) and flavonoids of *O.limbata* (15). Several extracts of plant and fractions were subjected to a preliminary antioxidant-screening test.

Experimental

Materials

The 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 40, beta carotene and BHA were purchased from Sigma Chemical Co. Ammonium thiocyanate and the other chemicals were purchased from Merck.

UV spectra were obtained with a Shimadzu UV-160A spectrophotometer, ^1H and ^{13}C NMR spectra were recorded on Varian 400 Unity plus spectrophotometer, for ^1H-NMR, TMS was used as internal standard .For mass spectroscopy, Finnigan Mat TSQ-70 spectrophotometer was used.

Analytical TLC, column and paper chromatography were carried out on Schleicher&Schuell F_{254} plates, silica gel G_{60} (35-70 µm, Merck) and Watman No.1 paper chromatography respectively.

Methods

Extraction

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

Rapid evaluation of antioxidant activity by beta-carotene bleaching method

The rapid evaluation of antioxidant activity of hexane, chloroform and methanolic extracts were determined according to the beta-carotene bleaching method (16,17). In this procedure the plant extracts, Vit.E and BHA were applied on TLC plates and after developing with a suitable solvent system, plates were sprayed with a beta-carotene solution and exposed to daylight until discoloring of the background (6h.) The active compounds were seen as orange color on the plate. Methanolic extracts of Green tea, *Ginkgo biloba*, Vit.E and BHA were used as positive controls. Extracts which showed strong antioxidant activity were subjected to further tests. The same experiment was performed for the isolated fractions and compounds.

Antioxidant activity evaluation by ferric thiocyanate method

The antioxidant activity of hexane, chloroform and methanolic extracts were determined using ferric thiocyanate method (FTC) (18). In this method, 500µg of each sample was dissolved in EtOH and added to a reaction mixture containing 2.88 ml of 2.5% linoleic acid and 9 ml of 40mM phosphate buffer in a vial. The vials were incubated at 40°C for 96 hours. During incubation (each 12 h), 0.1 ml of each vial was diluted with 9.7 ml of 75% EtOH, 0.1 ml ammonium thiocyanate and 0.1 ml FeCl2. The absorbance of samples was measured at 500 nm and the percent of inhibition was determined. Methanolic extracts of *Green tea* and *Ginkgo* were used as positive controls with the same concentration. Ethanol with sample and without reagents was used as negative control. This experiment was performedfor isolated fractions and isolated compounds, separately. BHA and alpha 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 methanolic extract of *Otostegia persica*, and paper chromatography was used for the purification of compounds. The methanolic extract of *Otostegia persica* was fractionated by a column (5 cm diameter.100 cm Length) packed with silica gel G_{60}. The column was eluted consecutively with chloroform %100, %10 ethyl acetate in chloroform to ethyl acetate%100. Elution was continued with %10 methanol in ethyl acetate to methanol %100. A quantity of 100 ml was collected for each

Table 1-Antioxidant activity of different extracts from *O.Persica* as measured by the ferric thiocyanate method after 60h incubation.

Sample	Absorbance at 500 nm	Percent of inhibition [a]
Control	1.050 ± 0.026 [b]	0.00
Methanolic ex. of O.persica	0.085 ± 0.024	95.87 ± 1.1*
Hexane ex.of O.persica	1.98 ± 0.21	2.5 ± 0.013
Chloroform ex. of O.persica	2.010 ± 0.012	1.9 ± 0.021
Methanolic ex. of Green tea	0.046 ± 0.017	96.03 ± 0.018*
Methanolic ex. of Ginkgo biloba	0.431 ± 0.028	77.20 ± 1.38*
alpha Tocopherol	0.257 ± 0.016	87.45 ± 0.76*
Butylated hydroxy anizole (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] x 100.
A high inhibition percent indicates a high antioxidant activity.
[b]Results are presented as mean ± standard deviation(n=5).
* statistically significant ($p < 0.05$).

fraction. The solvent was removed under reduced pressure. The chromatography was monitored by TLC using two different solvent systems: A, ethyl acetate-chloroform (40:60) and B, ethyl acetate-acetic acid - formic acid - water (100:11:11:26). Eight major fractions were obtained (F_1-F_8).

Paper chromatography

Fraction F5 which showed significant antioxidant activity was further purified by paper chromatography (Watman No.1). The paper was developed descending with t-butanol- acetic acid - water (3:1:1v/v/v) (TBA). The paper was dried and examined under short (254nm) and long (365nm) ultraviolet (UV) wavelength 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 (19). Four compounds were isolated from fraction F_5 which were designated as F_{5A} to F_{5D}.

Statistical analysis

Statistical analysis was performed according to student t-test. Analysis of variance was performed by Anova procedure.

Results and Discussion

Antioxidant activity by beta-carotene bleaching method:

The developed TLC plate after spraying with the reagent of beta-carotene showed dis-coloring of the background after 6 hours, while the methanolic extract of *Otostegia persica* had orange band, and hexane and chloroform ex-tracts had no color bands. Among the isolated fractions, F_5 showed two orange bands which were established to be morin and quercetin.

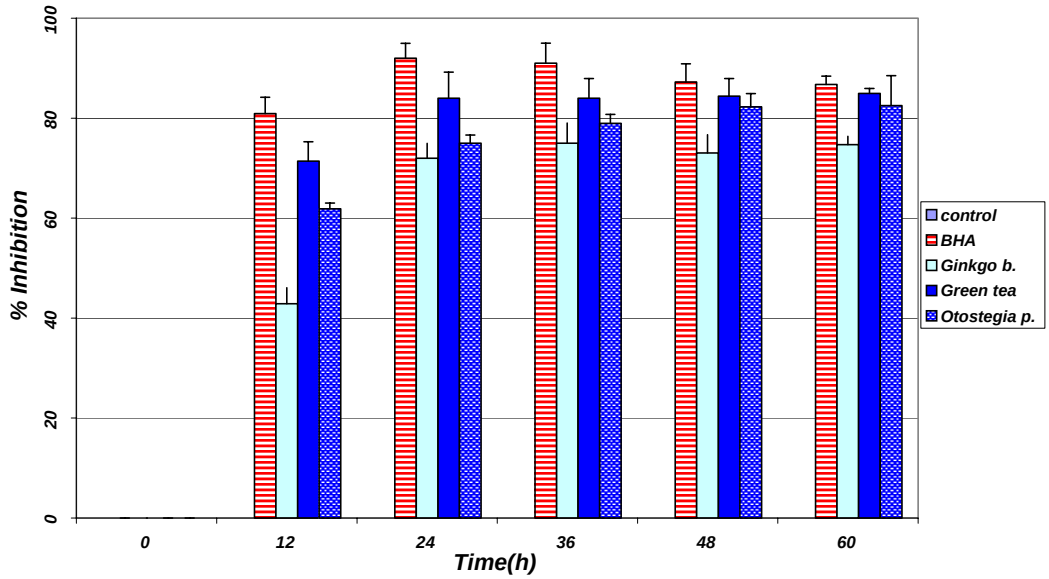

Figure 1. Antioxidant activity of methanolic extract of O.persica in comparison to negative and positive controls.

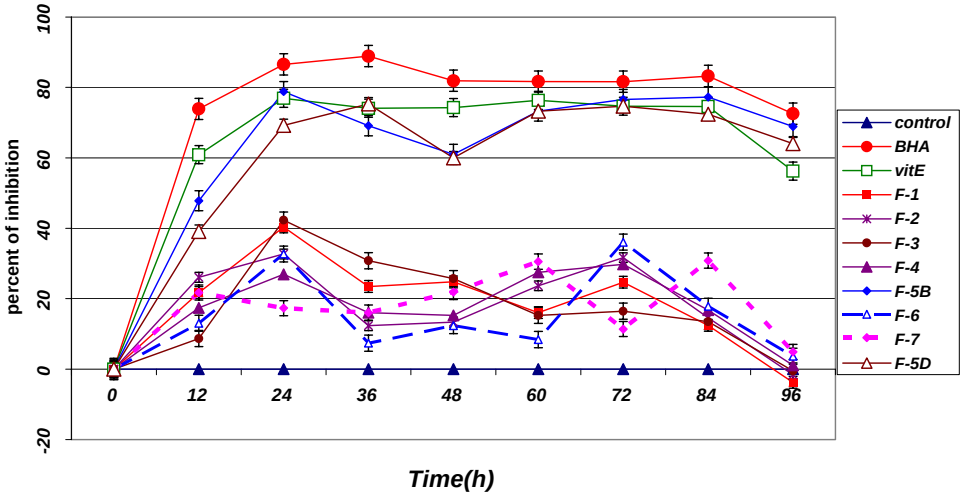

Figure 2. Antioxidant activity of different fractions of O.persica in comparison to negative and positivecontrols

Antioxidant activity by ferric thiocyanate method

Table 1- lists the antioxidant activity of the various extracts from *Otostegia persica* of the three sorts of extracts, of which methanloic one displayed strong antioxidant activity, and the others showed no activity. These observations are in agreement with the reports that methanol is an ideal and effective solvent for extraction of antioxidants. (20, 21, 22)

Methanolic extract of *Otostegia persica* was shown to be more active than *Ginkgo biloba* and almost equal to Green tea. Because of these results, this extract further was studied.

Figure 1 shows the antioxidant activity of methanolic extract of the plant in the linoleic acid peroxidation system (ferric thiocyanate method). The results indicate that methanolic extract of *Otostegia persica* significantly ($p<0.05$) inhibits the linoleic acid peroxidation compared to the negative control.

Figure 2 illustrates antioxidant activity of 200 µg of isolated fractions, determined according to the ferric thiocyanate method (18). From 8 major fractions, F_5 displayed the strongest antioxidant activity. Among F_5 subfractions, only F_{5B} and F_{5D} exerted antioxidant activity. Therefore, our investigation focused on the purification of these two compounds.

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

		Morin	Quercetin
R_f Value	**TBA**	0.75	0.56
	15%AcOH	0.2	0.04
Color	**UV**	Yellow	Yellow
	UV/NH3	Yellow	Yellow
Natural product reagent		Orange	Yellow / Orange

Identification of the active compounds of F_5

Table 2- shows the chromatographic data of compounds F_{5B} and F_{5D}. UV spectrum of these compounds exhibited two absorption peaks in the range of 240-280nm and 300-350nm which indicating that these compounds are flavones or flavonols and from the position of absorption peaks in the presence of shift reagents $AlCl_3$, NaOMe, NaOAc and H_3BO_3 in comparison to those in literature (23), it was concluded that both these compounds are flavonols. The ^{1}H-NMR and ^{13}C –NMR data of F_{5B} and F_{5D} were identical with those of morin and quercetin, respectively.

Table 3- lists the ^{1}H and ^{13}C NMR data of compounds F_{5B} and F_{5D}. These values were similar to those of morin and quercetin (20, 16).

In this experiment, we investigated antioxidant activity of hexane, chloroform, and methanolic extracts of *Otostegia persica*. Results showed that methanolic extract an has activity comparable to Green tea and *Ginkgo biloba*.

Fraction F_5 (400 mg) of methanolic extract gave 2 compounds F_{5B} (80 mg) and F_{5D} (67 mg) by paper chromatography which showed strong antioxidant activity. These two compounds were identified as morin and quercetin by spectroscopic methods.

F_{5B} and F_{5D} showed antioxidant activity equal to BHA and were stronger than alpha tocopherol. Many of flavonoids such as morin

Table 3. ^{1}H and ^{13}C NMR spectral data of compounds of F_{5B} and F_{5D} (in DMSO-d6)

Position	F_{5B}		F_{5D}	
	^{1}H	^{13}C	^{1}H	^{13}C
2	-	149.6	-	146.9
3	-	136.2	-	135.5
4	-	176.2	-	175.8
5	-	160.9	-	160.7
6	6.17,d[1],J = 2.4 Hz	98.0	5.2,d,J=2 Hz	98.2
7	-	163.7	-	163.9
8	6.28,d,J = 2.4 Hz	93.4	5.4,d,J=2 Hz	93.3
9	-	156.8	-	156.2
10	-	102.9	-	103.1
1´	-	109.2	-	122.1
2´	-	156.7	7.68,d,J=2 Hz	115.3
3´	6.39,d,J = 2.4 Hz	103.6	-	145.0
4´	-	160.4	-	147.6
5´	6.33,dd,J = 2.4,8.4 Hz	106.8	6.9,d,J=8.4 Hz	115.6
6´	7.22,d,J = 8.4 Hz	131.7	7.56,dd,J=2,8.4 Hz	120.0

[1]d:doublet

and quercetin have been reported to have anti-inflammatory activity (24, 25). In arthritis, oxidation damage leading to lipid peroxidation and formation of low mass oligosaccharids causing damage to bone and cartilage. Antioxidants are able to inhibit this process (26) and suppress the inflammation. The composition of the methanolic extract of *Otostegia persica* and the results of our work warrant its use in folk medicine as anti arthritis.

Aknowledgement

This research was supported by a grant from the National Research Center of Medical Sciences.

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