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Full Length Research Paper

Evaluation of Antioxidant Activity in *Origanum rotundifolium Boiss*. from Turkey: Essential Oil and Various Extract

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This study is designed to examine the chemical composition and *in vitro* antioxidant activity of the essential oil and various extracts of *Origanum rotundifolium* Boiss. from Turkey. The total of the 40 identified components accounted for 97.23% (GC) and 95.10% (FID) of the total oil. Major components of the oil were borneol, terpinen-4-ol, and spathulenol. To determine the presence of well known and widespread phenolic compounds, water-soluble extract of *O. rotundifolium* was analyzed by HPLC. In the methanol-soluble extract, caffeic acid, *p*-coumaric acid, (+)-catechin, and ferulic acid were found in several quantities. Antioxidant activity of the essential oil and extracts of *O. rotundifolium* has been determined by four different test systems namely DPPH, β-carotene/linoleic acid, chelating effect and reducing power. A strong correlation was observed between the radical scavenging capacity and polarity of the extracts. The methanol-soluble extract which contains the most polar phytochemicals showed the strongest antioxidant effect in all test systems. As expected, amount of the total phenolics and flavonoids was very high in methanol-soluble extract.

Key words: *Origanum rotundifolium*, extract, essential oil, antioxidant activity, HPLC.

INTRODUCTION

Oxidation processes are intrinsic in the energy manage-ment of all living organisms and are therefore kept under strict control by several cellular mechanisms (Halliwell and Gutteridge, 2007). However, the excessive produc-tion of free radicals and the unbalanced mechanisms of antioxidant protection result in the onset of numerous diseases and accelerate ageing. Small molecular weight antioxidants are considered as possible protection agents reducing oxidative damage of the human body, when the internal enzymatic mechanisms fail or are inadequately efficient (Halliwell, 1995). Oxidation, mediated by free radical reactions is also responsible for the rancidity of unpreserved food rich in unsaturated fatty acids and the natural antioxidants are suggested as a superior alternative for the synthetic ones such as BHA or BHT (Li et al., 2008).

Therefore, there is a growing interest in substances exhibiting antioxidant properties that are supplied to human and animal organisms as food components or as specific preventative pharmaceuticals. The plant kingdom offers a wide range of natural antioxidants. However, there is still not enough knowledge about the practical usefulness of most of them. In the group of secondary plant metabolites, antioxi-dant phenolics are commonly found in various fruits, vege-tables and herbs and they have been shown to provide a de-fense against oxidative stress from oxidizing agents and free radicals (Matkowski, 2006). Many herbal infusions, fre-quently used as home medicines have antioxidative and pharmacological properties related to the presence of phe-nolic compounds, especially phenolic acids and flavonoids. Polyphenols are also known for their ability to pre-vent fatty acids from oxidative decay, and provide an additional value to plants used as food ingredients, rich for example in ros-

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marinic acid (Fecka et al., 2007).

The genus *Origanum* consists of 38 species of annual, perennial and shrubby herbs; most of which are native or restricted to the Eastern part of the Mediterranean area, particularly in Italy, Greece and Turkey (Aligiannis et al., 2001). There are 24 species, and 27 taxa are available in the flora of Turkey and the East Aegean Islands, 16 of them are endemic (Guner et al., 2000).

Members of the genus *Origanum* comprise the most important aromatic plants throughout the world such as; sweet marjoram (*O. majorana* L.), the dittany of Crete (*O. dictamnus* L.), Italian oregano or pot marjoram (*O. onites* L.), Greek oregano or winter marjoram (*O. heracleoticum* L.) and Turkish wild oregano (*O. vulgare*) and bible hyssop or Syrian oregano (*O. syriacum*); all commercially available and exportable plants with appreciable market values (Kintzios, 2002). Many *Origanum* plants are widely used in the flavouring of food products and alcoholic beverages as well as perfumery for their spicy fragrance (Aligiannis et al., 2001) . Apart from their commercial importance, these plants have long been used as spices and condiments for foods such as sausages, soups, salads and meats (Novak et al., 2000).They have also been used in the treating of several ailments as anti-septic, stimulant, stomachic, expectorant, sudofiric and emmenagogic (Ryman, 1992). The importance of the essential oils from *Origanum* species is not limited only with their versatile uses, but they are also referred as potentially useful markers to characterise *Origanum* spe-cies (Kokkini, 1993).

Owing to their potential use in different purposes, composition and several biological properties of the essential oils from many *Origanum* species have often remained in the focus of several studies, leading to accumulate many informative data in the literature (Milos et al., 2000; Mockute et al., 2001; Gotsiou et al., 2002). In general, there are variations in the accumulation of phenolic metabolites among Origanum species like similar plants, indicating the chemical polymorphism that might result from their genetic background affected by environmental factors. Particularly, antioxidant properties of the extracts and particularly essential oils of *Origanum* species have recently been of great interest in both aca-demia and the food industry, since their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidants by natural ones (Ruberto et al., 2002; Puertas-Mejia et al., 2002). Antimicrobial activi-ties of several *Origanum* species have also been well documented (Tabanca et al., 2001; Sokovic et al., 2002; Daferera et al., 2003). Besides, biological properties of the various extracts obtained from cultivated or wild plants may also possess such properties and, as hap-pened in the case of rosmarinic acid, biologically active new substances may be introduced to food industry (Jun et al., 2001).

As far as our literature survey could ascertain, chemical composition and antioxidant activity of *O. rotundifolium* have not previously been reported except the study of Baser et al. (1995). In their study, only essential oils of *O. rotundifolium* were analyzed. Therefore, the general objective of this study was to screen the essential oil and various extracts obtained from *O. rotundifolium* for their possible antioxidant activities, to determine the chemical composition of the essential oil and quantitatively measuring the amounts of some phenolic compounds in the extracts. The antioxidant activities were determined by using four *in vitro* assays named as DPPH free radical scavenging, β-carotene-linoleic acid, chelating effect, and reducing power. Gallic acid equivalent total phenolic constituents and quercetin equivalent total flavonoids of the extracts were also determined.

MATERIALS AND METHODS

Chemicals

Potassium ferricyanide, ferrous chloride, ferric chloride, Folin–Ciocalteu's reagent (FCR), methanol, caffeic acid, and trichloroacetic acid (TCA) were obtained from E. Merck (Darmstadt, Germany) . 1.1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA), gallic acid, gentisic acid, *p*-coumaric acid, vanillic acid, ferulic acid, (+)-catechin, quercetin, apigenin, and naringenin were obtained from Sigma Chemical Co. (Sigma–Aldrich GmbH. Sternheim. Germany). Luteolin was from Roth (Karlsruhe, Germany). Rutin was from Alexis Biochemicals (Lausen, Switzerland). All other chemicals and solvents are of analytical grade.

Plant material

O. rotundifolium was collected from Bocka-Artvin, Turkey (A8), in July 2007. Aerial parts of the collected plants were dried in the shade. The voucher specimen has been deposited at the Her-barium of the Department of Biology, Cumhuriyet University, Sivas-Turkey.

Isolation of the essential oil

The air -dried *O. rotundifolium* were submitted for 5 h to water-distillation using a British-type Clevenger apparatus (ILDAM Ltd., Ankara-Turkey) (yield 2.13%). The obtained essential oil was dried over anhydrous sodium sulfate and after filtration, stored at +4°C until tested and analyzed.

Preparation of the extracts

A portion (100 g) of dried plant material was extracted with hexane (HE) (4.34%, w/w), followed by dichloromethane (DCM) (3.12%, w/w) and methanol (MeOH) (8.16%, w/w) in a Soxhlet apparatus (Isolab, Germany) for 6 h for each solvent. The latter extract was suspended in water and partitioned with chloroform to obtain water-soluble (6.38%, w/w) and water-insoluble (MC) (2.39%, w/w) sub-fractions (Sokmen et al., 1999). All extracts obtained were lyo-philised and kept in the dark at +4°C until use.

Gas Chromatography/Mass Spectrometry (GC/MS) analysis

GC–MS analyses were performed by using an Agilent-5973 Net-work System. A mass spectrometer with an ion trap detector in full scan mode under electron impact ionization (70 eV) was used. The chromatographic column used for the analysis was HP-5 capillary

column (30 m x 0.32 mm i.d., film thickness 0.25 lm). The carrier gas used was helium, at a flow rate of 1 mL/min. The injections were performed in splitless mode at 230°C. One microliter essential oil solution in hexane (HPLC grade) was injected and analyzed with the column held initially at 60°C for 2 min and then increased to 260°C with a 5°C/min heating ramp and subsequently kept at 260°C for 13 min. The relative percentage amounts of the separated com-pounds were calculated from total ion chromatograms by a com-puterized integrator.

HPLC analysis

The analytical HPLC system employed consisted of a JASCO high performance liquid chromatograph coupled with a UV–vis multi-wavelength detector (MD-910 JASCO). The analytical data were evaluated using a JASCO data processing system (DP-L910/V). The separation was achieved on a Waters Spherisorb® 5 μm ODS2 4.6 x 250 mm column at ambient temperature. The mobile phase consisted of water with 1% glacial acetic acid (solvent A), water with 6% glacial acetic acid (solvent B), and water–acetonitrile (65:30 v/v) with 5% glacial acetic acid (solvent C). The gradient used was similar to that used for the determination of phenolics in wine (Parrilla et al., 1999) with some modifications: 100% A 0 - 10 min, 100% B 10 - 30 min, 90% B/ 10% C 30 - 50 min, 80% B/ 20% C 50
- 60 min, 70% B/ 30% C 60 - 70 min, 100% C 70 - 105 min, 100% A 105 - 110 min; post-time 10 min before next injection. The flow rate was 0.5 ml/min and the injection volume was 20 μl. The monitoring wavelength was 280 nm. The identification of each compound was based on a combination of retention time and spectral matching.

Antioxidant activity

Scavenging effect on 1,1-Diphenyl-2-picrylhydrazyl

The hydrogen atoms or electrons donation ability of the corresponding extracts and some pure compounds were measured from the bleaching of purple colored methanol solution of DPPH. The effect of methanolic extracts on DPPH radical was estimated according to Hatano et al. (1988). 1 ml of various concentrations (0.2 - 1.0 mg ml^{-1}) of the extracts in methanol and water was added to a 1 ml of DPPH radical solution in methanol (final concentration of DPPH was 0.2 mM) . The mixture was shaken vigorously and allowed standing for 30 min; the absorbance of the resulting solu-tion was measured at 517 nm with a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). Inhibition of free radical DPPH in percent (I%) was calculated in following way:

$$I\% = 100 \times (A_{Control} - A_{Sample}) / A_{Control}$$

Where, $A_{Control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{Sample} is the absorbance of the test compound. BHT and BHA were used as a control.

Total antioxidant activity by β-carotene–linoleic acid method

In this assay antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius et al., 1998) . A stock solution of β-carotene–linoleic acid mixture was prepared as following: 0.5 mg β-carotene was dis-solved in 1 ml of chloroform (HPLC grade). 25 μl linoleic acid and 200 mg Tween 40 was added. Chloroform was completely eva-porated using a vacuum evaporator. Then 100 ml of oxygenated distilled water was added with vigorous shaking; 2.5 ml of this reac-tion mixture was dispersed to test tubes and 0.5 ml of various concentrations (0.4 - 2.0 mg ml^{-1}) of the extracts in methanol and water were added and the emulsion system was incubated for up to 2 h at 50°C. The same procedure was repeated with the positive control BHT, BHA and a blank. After this incubation period, absorbance of the mixtures was measured at 490 nm. Measurement of absor-bance was continued until the color of β-carotene disappeared. The bleaching rate (R) of β-carotene was calculated according to Equation (1).

$$R = \ln(a/b)/t \quad (1)$$

Where, ln = natural log, a = absorbance at time 0, b = absorbance at time t (30, 60, 90 and 120 min) (Cheung et al., 2003). The anti-oxidant activity (AA) was calculated in terms of percent inhibition relative to the control using Equation (2).

$$AA = [(R_{Control} - R_{Sample}) / R_{Control}] \times 100 \quad (2)$$

Antioxidative activities of the extracts were compared with those of BHT and BHA at 0.4 mg ml^{-1} and blank consisting of only 0.5 ml methanol and water.

Reducing power

The reducing power was determined according to the method of Oyaizu (1986). Each extract (0.2-1.0 mg ml^{-1}) in methanol and water (2.5 ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricynide and the mixture was incubated at 50°C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid were added, and the mixture was centrifuged at 200 g (MSE Mistral 2000, London, UK) for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml of deionized water and 0.5 ml of 0.1% ferric chloride. Finally the absorbance was measured at 700 nm against a blank. BHT and BHA were used as a control.

Chelating effects on ferrous ions

The chelating effect was determined according to the method of Dinis et al. (1994). Briefly, 2 mL of various concentrations (0.05 - 0. 25 mg/mL) of the extracts in methanol 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). Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured spectrophotometricaly at 562 nm. The inhibition percentage of ferrozine–Fe^{2+} complex formation was calculated by using the formula given below:

$$\text{Metal chelating effect (\%)} = [(A_{Control} - A_{Sample})/A_{Control}] \times 100$$

Where $A_{Control}$ is the absorbance of control (The control contains $FeCl_2$ and ferrozine, complex formation molecules) and A_{Sample} is the absorbance of the test compound. EDTA was used as a control.

Assay for total phenolics and flavonoids

Although some phenolic acids and flavonoids have been quantitatively determined in the extracts, we tried to define the total amounts of these compound classes qualitatively. By this way, presence of the compounds apart from the standard samples has been taken into the consideration for a healthy discussion.

Total phenolic constituent of the methanol extracts were determined by employing the methods given in the literature (Chandler and Dodds, 1983; Slinkard and Singleton, 1977) involving Folin-Ciocalteu reagent and gallic acid as standard. 1 ml of extract solution containing 2000 μg extract was added to a volumetric flask. 45 ml distilled water and 1 ml Folin-Ciocalteu reagent was added and

flask was shaken vigorously. After 3 min, a 3 ml of Na_2CO_3 (2%) solution was added and the mixture was allowed to stand for 2 h by intermittent shaking. Absorbance was measured at 760 nm. The concentrations of phenolic compounds were calculated according to the following equation that was obtained from the standard gallic acid graph:

A = 0.00201 gallic acid (μg) – 0.0074 (R^2: 0.9908)

Total flavonoid content was determined using the Dowd method as adapted by Arvouet-Grand et al. (1994). Briefly, 1 ml of 2% aluminum trichloride ($AlCl_3$) in methanol was mixed with the same volume of the solvent extracts (2000 μg). Absorption readings at 415 nm were taken after 10 min against a blank sample consisting of a 1 ml extract solution with 1 ml methanol without $AlCl_3$. The concentrations of flavonoid compounds were calculated according to the following equation that was obtained from the standard quercetin graph:

Absorbance = 0.0258 quercetin (μg) – 0.0060 (R^2 = 0.9987)

RESULTS AND DISCUSSION

Chemical composition of the essential oil and phenolic compounds determined in the extracts

GC analysis of the chemical composition of the essential oil of *O. rotundifolium* is confirmed by FID and the results are presented in Table 1. The total of the 40 identified components accounted for 97.23% (GC) and 95.10% (FID) of the total oil. Major components of the oil were borneol (15.99% -GC and 28.21%-FID), terpinen-4-ol (18.74% -GC and 8.64%-FID), and spathulenol (9.88%-GC and 8.89%-FID). According to the results obtained from the both systems, monoterpenoids consist of 58% of the total oil approximately. The second large compound class was monoterpenes (approximately 15%). While sesquiterpenes and sesquiterpenoids were represented in small quantities in the oil, only one diterpene identified.

To determine the presence of well known and widespread phenolic compounds, water-soluble extract of *O. rotundifolium* was analyzed by HPLC since the polar characteristics of these compounds. Chromatogram of this extract is presented as Figure 1. Among the com-pounds tested for their presence, caffeic acid was the most abundant one. This is followed by *p*-coumaric acid, (+)-catechin, and ferulic acid. On the other hand, rosmarinic acid, gallic acid, gentisic acid, vanillic acid, quercetin, apigenin, naringenin, luteolin, and rutin could not be determined in the extract.

Baser et al. (1995) examined the composition of essential oils *O. rotundifolium*. There is no concordance between the essential oils identified by our study and Baser et al. (1995) and the compounds identified and found in high ratio in their study were as follows: cis-sabinene hydrate (21.5%), lynalyl acetate (7.5%), alpha-terpineol (6.2%), beta-caryophyllene (6.1%), and trans-sabinene hydrate (4.2%). Essential oils can change year to year because of weather conditions.

In the literature, no report is available on the composition of extracts of *O. rotundifolium*. According to a study carried out by Proestos et al. (2006), (+)-catechin and ferulic acid have been determined in aqueous methanol extract of *O. dictamnus*. The chemical variations in the content of compounds found in *Origanum* species may be attributed to the influence of different conditions of sampling locations.

Antioxidant activity

Antioxidant activity of the essential oil and extracts of *O. rotundifolium* has been determined by four different test systems namely DPPH, β-carotene/linoleic acid, chelating effect and reducing power. In essence, the antioxidants react with the stable free radical, that is, 1,1-diphenyl-2-picrylhydrazyl (deep violet color) and convert it to 1,1-diphenyl-2-picrylhydrazine with discoloration. The degree of discoloration indicates the free radical scavenging potentials of the sample/antioxidant and it has been found that known antioxidant such as cysteine, gluta-thione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (hydroquinone, pyrogallol etc.) reduce and decolorize 1,1-diphenyl-2- picrylhydrazyl by their hydrogen donating ability (Blois, 1958).

As can be seen from the Table 2, free radical scavenging effect of the samples exhibited a dose-depen-dent increase. Radical scavenging activity of the essen-tial oil was measured as 15.30% ± 0.64 at 0.8 mg.ml $^{-1}$ concentration. It is extremely important to point out that, a strong correlation was observed between the radical scavenging capacity and polarity of the extracts. The methanol-soluble extract which contains the most polar phytochemicals showed the strongest effect (75.40% ± 0.26). This extract was followed by dichloromethane (47.61% ± 0.30). Scavenging capacities of the hexane and methanol-insoluble extracts have been found almost equal. None of the samples evaluated here showed activity as strong as the synthetic antioxidants BHT (92.68% ± 0.12) and BHA (96.27% ± 0.06).

In β-carotene/linoleic acid model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of β -carotene and linoleic acid, which generates free radicals.

The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene group attacks the highly unsaturated β-carotene molecules. As a result, β-carotene is oxidized and broken down in part; subsequently the system loses its chromo-phore and characteristic orange color, which is monitored spectrophotometrically.

In the case of β-carotene/linoleic acid test system, the most active extract, as expected, was the methanol-soluble one (76.91% ± 0.13 at 2.0 mg.ml^{-1} concentration) (Table 3). This activity was followed by methanol-insoluble extract which contains the more non-polar phytochemicals when compared to the methanol-soluble extract (37.64% ± 2.26). The weakest activity was exhibited

Table 1. Chemical composition of the essential oil of *O. rotundifolium*.

No.	Compounds	GC			FID		
		% Area	Exp. RI[a]	Exp. RT[b]	% Area	Exp. RI	Ident. LRI[c]
1	α- Thujene	0.07	932	4.96	-	-	930
2	α-Pinene	0.32	939	5.13	0.40	938	939
3	Camphene	0.95	954	5.53	1.07	951	956
4	Sabinene	0.81	978	6.23	1.32	975	975
5	Myrcene	0.42	996	6.77	0.64	986	991
6	Unknown	0.23	1009	7.19	-	-	-
7	α-Terpinene	0.15	1023	7.61	-	-	1020
8	*o*-Cymene	9.93	1034	8.00	9.55	1027	1029
9	δ-Terpinene	0.25	1063	9.14	0.23	1063	1060
10	Terpinolene	0.24	1093	10.24	1.20	1086	1089
11	Linalool	1.21	1101	11.04	1.88	1099	1097
12	*cis*-*p*-Menth-2-en-1-ol	0.87	1126	11.89	1.19	1118	1122
13	Camphor	2.22	1142	12.51	1.88	1141	1146
14	Borneol	15.99	1173	14.22	28.21	1171	1169
15	Terpinen-4-ol	18.74	1179	14.56	8.64	1178	1177
16	α-Terpineol	5.60	1187	15.08	4.50	1189	1189
17	*cis*-Piperitol	0.62	1198	15.75	0.72	1199	1196
18	Unknown	0.49	1209	16.09	0.46	1218	
19	Thymol methyl ether	3.93	1231	16.57	2.59	1232	1235
20	Carvacrol methyl ether	2.16	1248	16.94	1.65	1238	1245
21	Linalyl acetate	3.34	1258	17.40	3.47	1252	1257
22	Bornyl acetate	2.19	1291	18.53	1.59	1284	1289
23	Thymol	1.75	1293	20.34	-	-	1290
24	Unknown	0.82	1355	21.15	1.92	1351	
25	β-Bourbonene	2.34	1389	22.64	1.89	1391	1388
26	Unknown	0.42	1398	23.15	0.53	1401	
27	*E*-Caryophyllene	1.37	1421	24.05	1.083	142	1419
28	β-Copaene	0.42	1432	24.46	0.28	1435	1432
29	Unknown	0.24	1445	25.05	0.19	1449	
30	Unknown	0.29	1459	25.45	0.22	1459	
31	Unknown	0.40	1465	25.71	0.29	1465	
32	Germacrene D	0.22	1486	26.61	-	-	1485
33	β-Bisabolene	1.24	1508	27.82	1.16	1504	1506
34	Δ-Cadinene	0.17	1526	28.38	1.81	1532	1523
35	Unknown	0.67	1545	29.15	0.62	1547	
36	Spathulenol	9.88	1576	30.78	8.89	1571	1578
37	Unknown	1.40	1608	32.22	-	-	
38	Unknown	2.74	1662	33.73	2.88	1666	
39	Unknown	1.89	1703	34.95	1.24	1698	
40	Abietatriene	0.24	2062	46.84	0.91	2065	2057
	Total identification	97.23			95.10		
	Compound classes		% Area (GC)			% Area (FID)	
	Monoterpenes		14.01			15.60	
	Monoterpenoids		58.57			57.05	
	Sesquiterpenes		5.76			6.22	
	Sesquiterpenoids		9.88			8.89	
	Diterpenes		0.24			0.91	

[a]Exp. RT, Experimental Retention Time (as minutes) [b]Exp. RI, Experimental Retention Indices

[c]Ident. LRI, Linear Retention Indices. Identification based on comparison of retention index with those of published data (Adams, 2007).

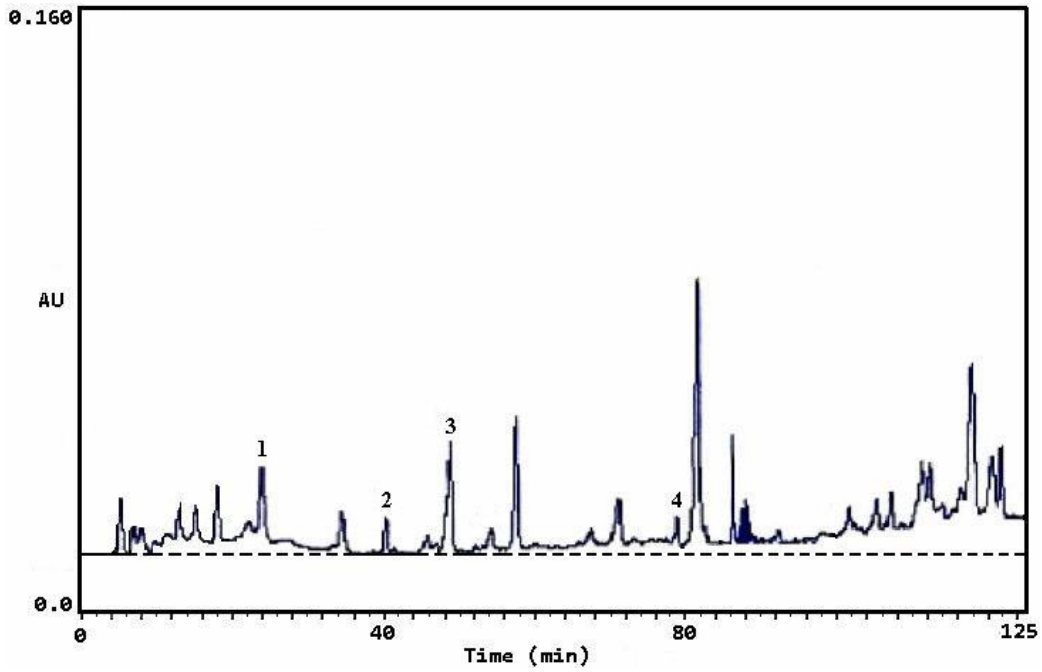

Figure 1. HPLC chromatogram of *O. rotundifolium* water-soluble extract: (1) *p*-coumaric acid, (2) (+)-catechin, (3) caffeic acid, (4) ferulic acid.

Table 2. Scavenging effect (%) of *O. rotundifolium* essential oil and extracts on DPPH free radical at different concentrations[a].

Sample	Sample concentration (mg ml^{-1})		
	0.2	0.4	0.8
Essential oil	4.87 ± 0.20	8.26 ± 0.18	15.30 ± 0.64
Hexane extract	10.13 ± 0.24	20.79 ± 0.26	36.79 ± 0.17
Dichloromethane extract	16.40 ± 0.19	23.29 ± 0.24	47.61 ± 0.30
Methanol-insoluble extract	18.09 ± 0.63	31.90 ± 0.16	36.20 ± 0.37
Methanol-soluble extract	44.13 ± 0.32	55.95 ± 0.11	75.40 ± 0.26
BHT	92.68 ± 0.12	-	-
BHA	96.27 ± 0.06	-	-

[a]Values expressed are means ± S.D. of three parallel measurements.

by hexane extract with a value of 13.50% ± 0.81. Antioxidant activity of the essential oil of *O. rotundifolium* was determined as 34.46% ± 1.82. In the concentration of 2.0 mg.ml^{-1}, activities of the synthetic antioxidants BHT and BHA were determined as 94.68% ± 0.04 and 99.27% ± 0.39, respectively.

Metal ions can initiate lipid peroxidation and start a chain reaction that leads to the deterioration of food (Gordon, 1990). The catalysis of metal ions also corre-lates with incidents of cancer and arthritis (Halliwell et al., 1995). Ferrous ions, the most effective pro-oxidants, are commonly found in food systems (Yamaguchi et al., 1998). In the present study, the chelating ability of the essential oil and extracts of *O. rotundifolium* toward ferrous ions was investigated. Table 4 shows the chelating effects of the samples compared with EDTA as standard on ferrous ions. As can be seen from the table, chelating capacity of the extracts was increased with the increasing concentration. As expected, the most powerful activity was exhibited by methanol-soluble extract. Chelating effect of this extract was measured as 89.85% ± 0.31 at 1.0 mg.ml^{-1} concentration. Essential oil did not show any activity. EDTA which is used as a positive control agent showed strong activity (99.08% ± 0.08 at 0.25 mg.ml^{-1} concentration).

One another way of defense mechanisms in preventing body against the hazardous effects of free radicals is reducing of these molecules by the antioxidant substances

Table 3. Antioxidant activity (%) of *O. rotundifolium* essential oil and extracts in β-carotene–linoleic acid system[a].

Sample	Sample concentration (mg ml^{-1})		
	0.4	1.0	2.0
Essential oil	29.20 ± 0.12	33.44 ± 3.35	34.46 ± 1.82
Hexane extract	10.78 ± 0.52	12.08 ± 2.43	13.50 ± 0.81
Dichloromethane extract	12.50 ± 0.34	20.23 ± 2.32	25.80 ± 1.89
Methanol-insoluble extract	26.22 ± 0.85	34.24 ± 0.74	37.64 ± 2.26
Methanol-soluble extract	59.47 ± 0.82	71.71 ± 0.71	76.91 ± 0.13
BHT	94.38 ± 0.60	95.18 ± 0.17	94.68 ± 0.04
BHA	93.55 ± 0.07	94.29 ± 0.01	99.27 ± 0.39

[a]Values expressed are means ± S.D. of three parallel measurements.

Table 4. Chelating effect (%) of *O. rotundifolium* essential oil and extracts[a].

Sample	Sample concentration (mg ml^{-1})		
	0.25	0.50	1.0
Essential oil	NA[b]	NA	NA
Hexane extract	1.80 ± 0.52	5.69 ± 1.34	7.06 ± 1.18
Dichloromethane extract	6.43 ± 0.43	7.28 ± 1.23	9.74 ± 0.98
Methanol-insoluble extract	9.28 ± 0.58	28.13 ± 0.47	36.74 ± 1.61
Methanol-soluble extract	72.43 ± 0.28	79.78 ± 0.17	89.85 ± 0.31
EDTA	99.08 ± 0.08	-	-

[a]Values expressed are means ± S.D. of three parallel measurements
[b]NA: Not Active

In the present study, assay of reducing activity was based on the reduction of Fe^{3+}/ferricyanide complex to the ferrous form in presence of reductants (antioxidants) in the tested samples. The Fe^{2+} was then monitored by measuring the formation of Perl's Prussian blue at 700 nm (Oyaizu, 1986). Table 5 shows the reducing power of the essential oil and extracts of *O. rotundifolium* as a function of their concentration. The reducing power of the samples increased with concentration. According to the results, the most active sample was methanol-soluble again with an absorbance value of 0.654 ± 0.007 at 1.0 mg.ml^{-1} concentration. At this concentration value, this activity was followed by dichloromethane extract (0.181 ± 0.002). Reducing power of BHT and BHA at 1.0 mg.ml^{-1} were 0.751 ± 0.010 and >3.500, respectively.

Plant phenolics constitute one of the major groups of compounds acting as primary antioxidant free radical terminators (Chen et al., 2008). Phenolic acids such as *p*-hydroxy benzoic acid, tyrosol, caffeic acid, *p*-coumaric acid, rutin, and ferulic acid have been proven as the strong antioxidant phytochemicals. As can be seen from the figure 1, three well known phenolic acids (*p*-coumaric acid, caffeic acid and ferulic acid) and one catechin [(+)-catechin] was determined in the methanol-soluble extract of *O. rotundifolium* in several quantities. Among them, the most abundant was caffeic acid which known as an excellent antioxidant substance.

Flavonoids, as one of the most diverse and widespread groups of natural compounds, are also probably the most natural phenolics (Shimoi et al., 1996). These com-pounds possess a wide spectrum of chemical and bio-logical activities including radical scavenging properties. Shimoi et al. (1996) concluded that plant flavonoids which show antioxidant activity *in vitro* also function as anti-oxidants *in vivo*. A strong relationship between total phe-nolic content and antioxidant activity in fruits, vegetables, grain products, and plants subject of ethnopharma-cological treatments has also been reported (Dorman et al., 2003).

The basic structure of flavonoids comprises two ben-zene rings (A and B) linked through a heterocyclic pyran or pyrone (with a double bond) ring in the middle. The antioxidant activity of flavonoids depends on the structure and the substituents of the heterocyclic and B-rings, in particular the presence of an *o*-di-OH structure on the B-ring, a 2,3-double bond in conjugation with a 4-oxo func-tion and the additional presence of 3- and 5-OH groups on the heterocyclic ring, as observed in quercetin (Heijnen et al., 2002; Silva et al., 2002).

Assays for total phenolics and flavonoids

Total phenolic assay was carried out based on the absor-bance values of the various extract solutions, reacted

Table 5. Reducing power (absorbance at 700 nm) of *O. rotundifolium* essential oil and at different concentrations[a].

Sample	Sample concentration (mg ml^{-1})		
	0.2	0.4	1.0
Essential oil	0.041 ± 0.002	0.069 ± 0.003	0.172 ± 0.009
Hexane extract	0.008 ± 0.001	0.021 ± 0.002	0.074 ± 0.002
Dichloromethane extract	0.027 ± 0.002	0.053 ± 0.003	0.181 ± 0.002
Methanol-insoluble extract	0.018 ± 0.001	0.033 ± 0.003	0.114 ± 0.003
Methanol-soluble extract	0.112 ± 0.008	0.256 ± 0.013	0.654 ± 0.007
BHT	0.203 ± 0.004	0.341 ± 0.005	0.751 ± 0.010
BHA	2.151 ± 0.043	>3.500	>3.500

[a] Values expressed are means ± S.D. of three parallel measurements

Table 6. Total phenolics and flavonoids of *O. rotundifolium* extracts.

Sample	Phenolic content (µg GAEs/mg extract)[b]	Flavonoid content (µg QEs/mg extract)[c]
Hexane extract	8.69 ± 1.29	7.19 ± 0.42
Dichloromethane extract	18.35 ± 1.72	9.72 ± 0.62
Methanol-insoluble extract	10.66 ± 0.25	2.84 ± 0.06
Methanol-soluble extract	41.85 ± 1.26	17.40 ± 0.84

[a] Values expressed are means ± S.D. of three parallel measurements
[b] GAEs. gallic acid equivalents.
[c] QEs. quercetin equivalents

with Folin-Ciocalteu reagent and compared with the standard solutions of pyrocatechol equivalents as described above. Data obtained from the total phenolic assay supports the key role of phenolic compounds in free radical scavenging and/or reducing systems. As expected, amount of the total phenolics was very high in methanol-soluble extract (41.85 ± 1.26 µg GAEs.mg^{-1} extract). It was followed by dichloromethane extract with a value of 18.35 ± 1.72 µg GAEs/mg (Table 6) . It is extremely im-portant to point out that; there is a positive correlation between antioxidant activity potential and amount of phe-nolic compounds of the extracts. Amount of total flavor-noids was also found in the highest value in methanol-soluble extract (17.40 ± 0.84 µg QEs.mg^{-1} extract).

Conclusion

This study demonstrates that there is *in vitro* antioxidant activity of the essential oil and various extracts of *O. rotundifolium* Boiss. from Turkey. In order to prolong the storage stability of foods and to reduce the damage to human body, synthetic antioxidants are used for industrial processing. But according to toxicologists and nutriationists, the side effects of some synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have already been documented. For example these substances can show carcinogenic effects in living organisms. From this point of view, governmental authorities and consumers are concerned about the safety of their food and about the potential effects of synthetic additives on health. Therefore, there is a growing interest into the investigation of potentially active natural phytochemicals. This is the first report on the antioxidant potential of the essential oils and several extracts having different polarities of *O. rotundifolium*. On the other hand, further detailed studies of essential oils and extracts of *O. rotundifolium* is required to determine which of their components are more responsible for its antioxidant effect and to clarify their cytotoxicity and other biological properties.

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