JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

In Vitro Antioxidant, Antimicrobial, and Antiviral Activities of the Essential Oil and Various Extracts from Herbal Parts and Callus Cultures of *Origanum acutidens*

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The essential oil and various extracts obtained from *Origanum acutidens* and methanol extracts (MeOH) from callus cultures have been evaluated for their antioxidative, antimicrobial, and antiviral properties. The essential oil exhibited strong antimicrobial activity with a significant inhibitory effect against 27 (77%) of the 35 bacteria, 12 (67%) of the 18 fungi, and a yeast tested and moderate antioxidative capacity in DPPH and β-carotene/linoleic acid assays. GC and GC-MS analyses of the oil resulted in the identification of 38 constituents, carvacrol being the main component. The MeOH extracts obtained from herbal parts showed better antioxidative effect than that of butylated hydroxytoluene (BHT), whereas callus cultures also exhibited interesting antioxidative patterns. Concerning antiviral activity, none of the extracts inhibited the reproduction of influenza A/Aichi virus in MDCK cells. The MeOH extracts from herbal parts inhibited the reproduction of HSV-1, and also callus cultures exerted slight antiherpetic effect.

KEYWORDS: ***Origanum acutidens*; Lamiaceae; essential oils; antioxidant activity; antimicrobial activity; anti-HSV activity; carvacrol; rosmarinic acid; callus cultures**

INTRODUCTION

Free radicals leading the oxidation of biomolecules are implicated in several diseases (*1*). Their deteriorative effects can be diminished by natural antioxidants available in foods. Also, oxidative reactions limit the shelf life of fresh and processed foodstuffs and are a serious concern in the food industry. Synthetic and/or natural antioxidants are very effective in the protection of unsaturated fats and oils and are, therefore, used as potential inhibitors of lipid peroxidation to stabilize fat-containing foodstuffs. However, the introduction of synthetic antioxidants has caused some problems due to their highly volatile nature, instability at high temperatures, and strict law restrictions, addressing consumer and thereby manufacturer preferences for natural antioxidant sources (*2*). Toxicity, increasing adverse drug reactions, and the appearance of drug resistance limit the use of the most common antibiotics and antiviral agents as therapeutics. Besides, foodborne diseases are still a major problem in the world, even in well-developed countries (*3*). A variety of microorganisms also lead to food spoilage, which is one of the most important matters concerning the food industry (*4*). Consequently, it is necessary to use safer and more reliable chemicals to prevent oxidation reactions and the growth of food-spoiling microbes and for the treatment of several diseases caused by pathogenic microorganisms and viruses. Recently, there has been a growing interest in plants as natural sources for the prevention of oxidation, controlling pathogens and/or toxin-producing microorganisms in foods, and the treatment of several diseases as therapeutics (*5*−*8*).

Many *Origanum* (Lamiaceae) plants are of great importance in this respect owing to their versatile uses as flavoring agents, fragrances, and therapeutics (*9*, *10*). *Origanum acutidens* is an endemic, herbaceous, and perennial plant growing mainly in calcareous and noncalcareous rocks, slopes, and screes (1000−3000 m) in Central Anatolia (*11*). As far as our literature survey could ascertain, the essential oil composition of *O. acutidens* was reported previously (*12*), but no information was available for its antimicrobial, antioxidant, and antiviral potential. There-

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10.1021/jf049859g CCC: $27.50
Published on Web 04/30/2004

Table 1. Chemical Composition of the Essential Oil of *O. acutidens*

no.	RRI[a]	components	composition %[e]
1	845	2-hexenal[d]	0.2
2	909	thujene[d]	1.1
3	917	α-pinene[c]	0.9
4	932	camphene[d]	0.4
5	960	sabinene[d]	0.1
6	963	β-pinene[c]	0.3
7	977	3-octanone[d]	0.5
8	981	β-myrcene[c]	1.7
9	995	α-phellandrene[d]	0.2
10	1009	α-terpinene[c]	1.0
11	1019	p-cymene[c]	7.5
12	1059	γ-terpinene[c]	5.3
13	1073	*cis*-sabinene hydrate[d]	0.4
14	1095	terpinolene[d]	0.1
15	1112	trans-sabinene hydrate[d]	0.2
16	1202	borneol[c]	0.9
17	1206	terpinen-4-ol[d]	0.4
18	1223	α-terpineol[c]	0.1
19	1227	*cis*-dihydrocarvone[d]	tr[b]
20	1230	methylchavicol[d]	0.3
21	1235	*trans*-dihydrocarvone[d]	0.1
22	1289	thymoquinone[d]	0.1
23	1295	*cis*-anethole[d]	0.3
24	1302	thymoquinone isomer[d]	0.2
25	1331	*trans*-anethole[d]	1.1
26	1349	thymol[c]	0.2
27	1358	carvacrol[c]	72.0
28	1388	thymol acetate[d]	0.1
29	1430	carvacrol acetate[d]	0.1
30	1476	β-caryophyllene[c]	1.0
31	1497	aromadendrene[d]	0.1
32	1513	α-caryophyllene[d]	tr
33	1540	γ-muurolene[d]	0.1
34	1555	bicyclogermacrene[d]	0.5
35	1567	β-bisabolene[d]	0.1
36	1599	α-bisabolene[d]	0.2
37	1637	spathulenol[d]	0.4
38	1919	n-hexadecane[d]	tr
		total	98.2

[a] Relative retention index on nonpolar HP-5ms column. [b] tr = trace, ≤0.06%. [c] Identification of compoments based on standards compounds. [d] Tentative identification. [e] Relative proportions of the essential oil components expressed as percentages obtained by GC-FID response.

fore, we report here the chemical analysis and the in vitro antimicrobial, antioxidant, and antiviral activities of the extracts and the essential oils from the herbal parts and callus cultures of *O. acutidens* (Lamiaceae).

MATERIALS AND METHODS

Plant Material. *O. acutidens* (Hand.-Mazz.) letswaart (Lamiaceae) was collected from the roadsides near the village of Beypinari (1200 m), Zara, Sıvas, Turkey, when flowering (July 2003). A voucher specimen has been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sıvas, Turkey (CUFH voucher AA 3105).

Essential Oil and Extracts. A dried and ground sample (100 g) from herbal parts of *O. acutidens* was submitted to hydrodistillation for 3 h using a British-type Clevenger apparatus (yield = 1.63%, v/w).

A portion (100 g) of dried plant material was placed in a Soxhlet extractor and extracted with hexane (HE) for 24 h (yield = 2.14%, w/w), followed by dichloromethane (DCM) for 18 h (yield = 1.34%, w/w) and methanol (MeOH) for 6 h (yield = 7.91%, w/w). All extracts obtained were lyophilized and kept in the dark at 4 °C until use.

Callus Cultures. Seeds were immersed in 70% aqueous EtOH for 1 min, followed by surface sterilization with a 10% NaOCl solution for 10 min and a rinse with sterile double-distilled water, and then laid on Gamborg's B5 basal medium (*13*), supplemented with naphthalene acetic acid (2.0 ppm), 6-benzylaminopurine (N^6-benzyladenine) (2.0 ppm), and sucrose (3.0%) as a carbon source. The initiated calli were subcultured using the aforesaid media by transferring small callus pieces at 28-day intervals. All cultures were maintained at 25 ± 2 °C in the dark. One-month-old calli (1 kg) were lyophilized, and the resulting powdered material (88 g) was extracted with MeOH, giving an extract in the yield of 6.20% (w/w).

Table 2. Amounts of Total Phenolic and Effects of the Essential Oil and Various Extracts of *O. acutidens* on the in Vitro Free Radical (DPPH) Scavenging and β-Carotene/Linoleic Acid Assays[a]

sample	total phenolics[b]	DPPH[c]	β-carotene/ linoleic acid[d]
essential oil		133.7 ± 0.5	90 ± 4.5
hexane (HE)	1.9 ± 0.4	not active	not active
dichloromethane (DCM)	154.0 ± 9.6	49.5 ± 3.5	95 ± 1.0
MeOH (herbal)	151.5 ± 12.0	18.0 ± 2.0	98 ± 1.0
MeOH (callus)	39.0 ± 3.6	71.5 ± 5.3	94 ± 1.5
BHT (positive control)		19.8 ± 0.5	96 ± 1.0
rosmarinic acid		2.9 ± 0.3	100

[a] Results are presented as mean ± standard deviation. [b] Amounts of total phenolics are given as gallic acid equivalent (μg mg $^{-1}$). [c] IC_{50} values of DPPH assay (as μg/mL). [d] Given as percentage of percent inhibition of the linoleic acid.

Essential Oil Analysis. GC and GC-MS analyses have been performed according to the methods given elsewhere (*5*). The oven temperature was kept at 50 °C for 3 min and then programmed to 240 °C at a rate of 3 °C/min. For the GC-MS analysis, the mass range was from *m*/*z* 35 to 400. The relative proportions of the essential oil components were expressed as percentages obtained by GC peak area normalization. The *n*-alkanes were used as reference points in the calculation of relative indices (RRI). The identification of the components was based on the comparison of their relative retention time and mass spectra with those of standards or NBS75K GC/MS library data and literature data (*14*). α-Pinene, β-pinene, and borneol were purchased from Sigma-Aldrich Co. (Steinheim, Germany); β-myrcene, α-terpinene, γ-terpinene, α-terpineol, carvacrol, and β-caryophyllene were purchased from Sigma Chemical Co. (St. Louis, MO); *p*-cymene was purchased from Aldrich Chemical Co. (Milwaukee, WI); and thymol was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Assay for Total Phenolics. Total phenolic constituents of the extracts were determined by employing the methods given in the literature (*15*) involving Folin−Ciocalteu reagent and gallic acid as standard.

Antioxidant Assay. The antioxidative capacity of the samples was evaluated by using DPPH and β-carotene/linoleic acid assays (*2*, *16*). In both assays, butylated hydroxytoluene (BHT) was used as positive control.

Antibacterial and Antifungal Assays. The essential oil and extracts were individually tested against a panel of 54 microorganisms including 35 bacteria, 18 fungi, and a yeast species. The microorganisms used and their sources are given in **Tables 3** and **4**. Disc-diffusion, microwell dilution, and MIC agar dilution were performed following the methodology given elsewhere (*5*). Standard antibiotics employed are given in **Tables 3** and **4**.

Antiviral Assay. *Viruses, Cells, and Media*. Human influenza virus A/Aichi/2/68 (H3N2) (A/Aichi) was grown in Madin−Darby canine kidney (MDCK) cells; the infectious titer was $10^{5.7}$ $TCID_{50}$/mL (50% tissue culture infectious doses/mL). Herpes simplex virus type 1 (HSV-1), strain DA, was grown in Madin−Darby bovine kidney (MDBK) cells; the infectious titer was 10^{10} $TCID_{50}$/mL. The virus stocks were stored at −70 °C and were from the collection of the Institute of Microbiology, Bulgarian Academy of Science, Sofia, Bulgaria. The cell lines were passaged as described before (*17*).

Compounds. Rimantadine hydrochloride was obtained from Hoffman-La Roche Inc., Nutley, NJ, and BVDU [*E*-5-(2-bromovinyl)-2′-deoxyuridine] from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany. The essential oil and the extracts were treated with dimethyl sulfoxide (DMSO), DCM, and HE accordingly and diluted in bidistilled sterile water to 10% solutions. For the experiments 2-fold dilutions were made in cell culture medium.

Cellular toxicity was determined as described before (*17*). The dose, causing 50% cytopathic effect (CPE) with respect to cell control (intact cells), was estimated (TC_{50}).

Table 3. Antimicrobial Activities of the Essential Oil of *O. acutidens*

test microorganism and source	DD[a]	MIC[b]	DD[a] (max)	MIC[b]
Acinetobacter baumanii, A8	12	250	18 (OFX)	31.25
Acinetobacter lwoffi, F1	18	62.50	24 (OFX)	62.50
Bacillus macerans, A199	22	62.50	19 (OFX)	15.62
Bacillus megaterium, A59	14	125	9 (SCF)	15.62
Bacillus subtilis, ATCC6633	12	250	28 (OFX)	62.50
Bacillus subtilis, A57	18	125	28 (OFX)	125
Brucella abortus, A77	16	250	12 (SCF)	62.50
Burkholdria cepacia, A225			22 (SCF)	125
Clavibacter michiganense, A227	24	31.25	25 (SCF)	16.62
Cedecea davisae, F2	16	125	14 (OFX)	62.50
Enterobacter cloacae, A135	14	125	20 (NET)	31.25
Enterococcus faecalis, ATCC29122	18	62.50	18 (SCF)	31.25
Escherichia coli, A1	24	62.50	– (OFX)	62.50
Klebsiella pneumoniae, F3	10	250	12 (OFX)	125
Klebsiella pneumoniae, A137	14	250	12 (OFX)	125
Morgonella morganii, F4	16	250	14 (OFX)	125
Proteus vulgaris, A161			12 (OFX)	125
Proteus vulgaris, KUKEM1329	22	62.50	13 (OFX)	125
Pseudomonas aeruginosa, ATCC9027	12	125	22 (NET)	31.25
Pseudomonas aeruginosa, ATCC27859	16	125	22 (NET)	15.62
Pseudomonas aeruginosa, F5			18 (NET)	125
Pseudomonas pseudoalkaligenes, F6	18	125	18 (NET)	125
Pseudomonas syringae pv.tomato, A35			24 (OFX)	125
Salmonella cholerasuis arizonae, F7	10	500	14 (NET)	250
Salmonella enteritidis, ATCC13076	22	62.50	27 (SCF)	62.50
Serratia plymuthica, F8	12	500	16 (NET)	125
Shigella sonnei, F9	8	500	24 (NET)	31.25
Staphylococcus aureus, A215	14	125	22 (SCF)	31.25
Staphylococcus aureus, ATCC29213	18	125	22 (SCF)	62.50
Staphylococcus epidermis, A233	18	125	– (SCF)	15.62
Staphylococcus hominis, F10			– (SCF)	15.62
Streptococcus pyogenes, ATCC176			10 (OFX)	62.50
Streptococcus pyogenes, KUKEM676			13 (OFX)	31.25
Xanthomonas campestris, A235	12	250	20 (SCF)	31.25
Yersinia enterocolitica, F11			16 (OFX)	62.50

[a] Inhibition zone in diameter (mm) around the disks impregnated with 10 μL of essential oil. OFX, ofloxacin (10 μg/disk); SCF, sulbactam (30 μg) + cefoperazona (75 μg) (105 μg/disk); and NET, netilmicin (30 μg/disk), were used as positive reference standard antibiotic disks (Oxoid). Maxipine (μg/mL) was used as reference antibiotic in microwell dilution assay (Sigma). [b] Minimal inhibitory concentrations as μg/mL.

Cytopathogenic Effect (CPE) Reduction Assay. The method was the same as described before (*17*). The concentration reducing CPE by 50% (EC_{50}) with respect to virus control was estimated from graphic plots. The selectivity index (SI) was determined from the ratio TC_{50}/EC_{50}. SI $\geq$ 4 was considered to stand for a significant selective inhibition.

Fifty Percent End Point Titration Technique (EPTT). The method was followed according to ref *7*. The antiviral effect was determined by the difference between the titers of control and treated viruses (δ log $TCID_{50}$/mL). The significance of the differences was estimated by Student's *t* test.

RESULTS AND DISCUSSION

Table 1 lists the chemical components of the essential oil. Thirty-eight components were identified representing 98.2% of the oil. A high content of monoterpenes was observed (95.4%); among them the oxygenated compounds are represented at 76.8%. Carvacrol was the predominant compound (72.0%), followed by the biogenetic precurcors *p*-cymene (7.5%) and γ-terpinene (5.3%). The oil studied showed a similar composition when compared to that used in a previous study (*12*). Also, the above-mentioned chemotype is characteristic for many *Origanum* species (*18, 19*). As expected, callus cultures were incapable of producing the essential oil in vitro.

The antioxidant activity of various *O. acutidents* extracts, callus culture, and the essential oil was studied in vitro. In both the extract and oil cases, the reactions followed a concentration-dependent pattern. **Table 2** shows antioxidative potentials of the samples tested. Both free radical scavenging and inhibition of linoleic acid oxidation of the methanol extract were comparable with those of the synthetic antioxidant BHT. Concerning the free radical scavenging activity, the superiority of the methanol extract could be attributed to the presence of polar phenolics comprising 15.1% of the extract (**Table 2**). Particularly, synergistic effects of phenolic acids, for example, rosmarinic acid and polyphenols, as well as other chemicals such as flavonoids could also be taken into account (*20*). The DPPH radical scavenging activity of the oil was also high, and this was obviously related to the high content of carvacrol (72%, v/v). This non-crystallizable phenol was found to be the main antioxidant constituents of the oils isolated from several *Origanum* species (*21, 22*).

Table 4. Anticandidal and Antifungal Activities of the Essential Oil of *O. acutidens*

	oil[a]		antibiotics[b]	
test yeast and fungi	DD	MIC	DD	MIC (amp)
yeast				
Candida albicans, A117	18	125	– (NET)	31.25
fungi				
Alternaria solani			– (NET)	NT
Aspergillus flavus	30	62.50	– (NET)	15.62
Aspergillus niger	35	62.50	– (NET)	15.62
Aspergillus variecolor	30	62.50	– (NET)	62.50
Fusarium acuminatum			– (NET)	62.50
Fusarium oxysporum	26	62.50	– (NET)	125
Fusarium solani	24	125	– (NET)	125
Fusarium tabacinum			– (NET)	62.50
Microsporum canis	32	31.25	– (NET)	62.50
Moniliania fructicola	35	31.25	– (NET)	62.50
Mortieraula alpina	24	62.50	– (NET)	125
Penicillum spp.	32	62.50	– (NET)	31.25
Rhizopus spp.	20	125	– (NET)	125
Rhizoctonia solani	38	15.62	– (NET)	31.25
Sclorotinia minor			– (NET)	125
Sclorotinia sclerotiorum			– (NET)	62.50
Trichophyton rubrum	40	15.62	– (NET)	15.62
Trichophyton mentagrophytes			– (NET)	31.25

[a] DD, inhibition zone in diameter (mm) around the disks impregnated with 10 μL of the essential oil; MIC, minimal inhibitory concentrations (μg/mL) of the essential oil. [b] DD, inhibition zone in diameter (mm) around the disks impregnated with extracts (300 μg/disk). NET (netilmicin, 30 μg/disk) was used as positive reference standard antibiotic disk (Oxoid). MIC, minimal inhibitory concentrations (μg/mL) of standard antibiotic; AmpB, amphotericin B (μg/mL) (Sigma).

The essential oil showed remarkable antimicrobial activity (**Tables 3** and **4**), exhibiting inhibitory effect against 27 (77%) of the 35 bacteria, 12 (67%) of the 18 fungi, and the yeast species tested, whereas the extracts from herbal parts (HE, DCM, and MeOH) and callus cultures (MeOH) remained inactive. This observation confirmed the findings in the previous studies reporting that the essential oils contain remarkable antimicrobial substances (carvacrol in particular) compared to plant extracts (*5*, *10*).

The evaluation of the antiviral activity indicated that none of the extracts inhibited the reproduction of influenza A/Aichi virus in MDCK (**Table 5**). It should be noted that most of the samples were toxic to the cell lines used; the MeOH extracts were less toxic. The MeOH extracts inhibited significantly in a dose-related manner the reproduction of HSV-1 in MDBK cells; the MeOH extract from callus culture showed the least toxicity, but its antiherpetic effect was diminished (**Table 5**; **Figure 1**). Our preliminary phytochemical investigations on callus cultures revealed the presence of rosmarinic acid and rutin.

Table 5. Cytotoxicity and Antiviral Effects of Extracts from *O. acutidens*

		virus-inhibitory effect			
	cytotoxicity[a]	A/Aichi		HSV-1	
sample	TC_{50}[b] (μg/mL)	EC_{50}[c] (μg/mL)	SI[d]	EC_{50}[c] (μg/mL)	SI[d]
HE extract	<0.08	>TC_{50}		>TC_{50}	
DCM extract	<0.3	>TC_{50}		>TC_{50}	
MeOH extract (herbal)	180.0	>TC_{50}		5.0	36
MeOH extract (callus)	1000	>TC_{50}		65.0	15.4
essential oil	<0.08	>TC_{50}		>TC_{50}	
rimantadine hydrochloride	>32	0.4	>90		
BVDU	>100			1	>100

[a] Toxicities for both cell lines were equal. [b] Fifty percent toxic concentration, [c] Fifty percent effective concentration, [d] Selectivity index (TC_{50}/EC_{50}).

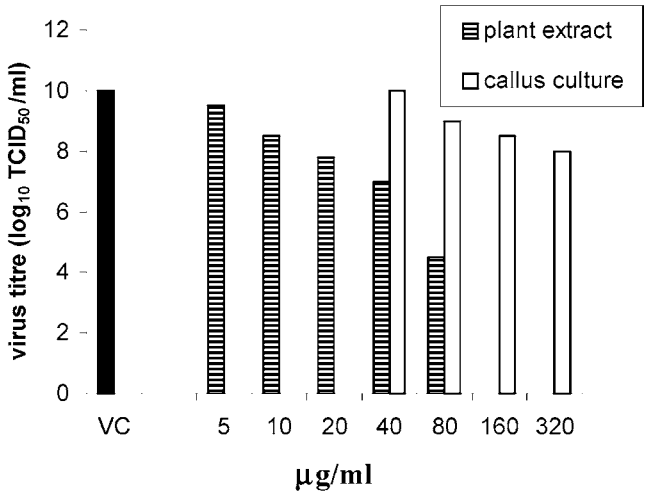

Figure 1. Dose dependence of the virus-inhibitory effect of the MeOH extracts of *O. acutidens* on the reproduction of HSV-1 in MDBK cells (EPTT assay). VC, virus control.

Thus, water-soluble substances, for example, phenolic acids, such as rosmarinic acid in particular, might be responsible for viral inhibition (*23*).

On the basis of these results, it can be concluded that the essential oil extracted from *O. acutidens* possesses compounds with antimicrobial and antioxidant properties, although no antiviral effect can be observed. MeOH extract, on the other hand, may be noteworthy to evaluate for its antioxidant and anti-HSV activities. Finally, special interest can be directed to callus cultures that may be of great importance in terms of producing antioxidant properties in vitro.

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Received for review January 27, 2004. Revised manuscript received March 22, 2004. Accepted March 23, 2004. We thank the Scientific and Technical Research Council of Turkey (TUBITAK) and Bulgarian Academy of Science (BAS) for financial support (Project TBAG-U/69, 103T001).

JF049859G