ANTIBACTERIAL ACTIVITIES OF EXTRACTS AND ESSENTIAL OILS OF THREE *ORIGANUM* SPECIES AGAINST PLANT PATHOGENIC BACTERIA AND THEIR POTENTIAL USE AS SEED DISINFECTANTS

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SUMMARY

The chemical compositions of hydrodistilled essential oils and *n*-hexane extracts from *Origanum acutidens*, *O. rotundifolium* and *O. vulgare* were analyzed by GC and GC-MS. Essential oils and hexane, chloroform, acetone and methanol extracts were tested against 25 plant pathogenic bacterial strains. Essential oils showed a wide spectrum of antibacterial activity, probably due to the phenolic components such as carvacrol and thymol. It was also shown that carvacrol, thymol and other main components such as terpinen-4-ol and linalool possess antimicrobial activity. Plant extracts, however, did not show any antibacterial activity, with the exception of the acetone and hexane extracts from *O. rotundifolium*. Our findings suggest that the essential oils may be valuable as potential antibacterial agents against plant pathogens, and show the potential value of *Origanum* oils as seed disinfectant.

Key words: antibacterial activity, plant pathogens, essential oil, oregano, seed disinfectant.

INTRODUCTION

Avoiding or mitigating crop losses due to plant diseases caused by pathogenic bacteria, fungi and viruses, and insects is one of the most important issues in plant production. Nowadays, rapid and effective control of fungal plant disease and microbial contamination in the crops is generally achieved using synthetic pesticides and, sometimes, antibiotics. Control of plant bacterial diseases remains difficult due to limited availability of commercial bactericides and prohibition to use antibiotics in many countries. Thus, chemical control of bacterial diseases is largely dependent on the use of copper compounds. However, such control methods prevent bacterial multiplication but are not adequate against seed-borne inocula. Furthermore, chemicals of this type evoke undesirable effects on the environment and leave residues toxic to mammalians in the products (Barnard *et al.*, 1997; Isman, 2000). The risk of developing resistance in microorganisms and the high cost-benefit ratio are other disadvantages of synthetic pesticides (Brent and Hollomon, 1993; Roy and Dureja, 1998).

Xanthomonas axonopodis pv. *vesicatoria* (*Xav*) the causal agent of bacterial leaf spot of pepper (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*), occurs commonly in the areas where these vegetable are grown (Vauterin *et al*., 1995; Jones *et al*., 2006). This pathogen causes serious economic losses to fruit yield and quality and its control with commercial disease management methods is extremely difficult (Swings and Civerolo, 1993). Recently, however, there has been a growing interest for research concerning alternative pesticides and antimicrobial active compounds, including plant extracts and essential oils (Basim *et al.*, 2000; Isman, 2000; Sokmen *et al.*, 2004; Kizil and Uyar, 2006; Sokovic and van Griensven, 2006; Kordali *et al.*, 2007, 2008; Kotan *et al.*, 2007a, 2007b; Altundag and Aslim, 2011).

The genus *Origanum* (oregano), family Lamiaceae, is represented in Turkey by 22 species, 21 being endemic to the country. *Origanum* species are aromatic and are used as condiment or herbal tea (Davis, 1982; Baser, 2002). Although there are numerous reports on the chemical composition and antibacterial activity of *Origanum vulgare* and *O. acutidens* against various bacteria of food, clinical and plant origin (Baser *et al.*, 1997; Pradhanang *et al.*, 2003; Sahin *et al.*, 2004; Sokmen *et al.*, 2004; Sokovic and van Griensven, 2006; Vasinauskiene *et al.*, 2006; Bozin *et al.*, 2006; Figueredo *et al.*, 2006; Esen *et al.*, 2007; Kordali *et al.*, 2008), there is only one report regarding the chemical composition of *O.*

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rotundifolium essential oil (Baser *et al.*, 1995). Furthermore, there is limited information on the chemical composition of hexane extracts and the activity of the essential oils, and of hexane, chloroform, acetone and methanol extracts of these Turkish *Origanum* species against phytopathogenic bacteria.

The objectives of this study were: (i) analyze the chemical compositions of the essential oils and hexane extracts of *O. acutidens*, *O. rotundifolium* and *O. vulgare* growing wild in eastern Anatolia and the fractions of the *n*-hexane extract of *O. acutidens* over silica gel column chromatography; (ii) evaluate the effects of *n*-hexane, $CHCl_3$, acetone and methanol extracts and hydrodistilled essential oils and/or their active components on 25 phytopathogenic bacteria, and (iii) determine whether *O. acutidens* essential oil can be used as potential seed disinfectant agent against *Xav.*

MATERIALS AND METHODS

General experimental procedures. NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer, operating at 400 MHz and 100 MHz for ^1H and ^{13}C, respectively, using $CDCl_3$ and dimethyl sulfoxide-d_6 (DMSO-d_6). Chemical shifts are expressed in δ (ppm) downfield from TMS as an internal standard and coupling constants reported in Hz. The IR spectra were determined with a FT-IR Perkin Elmer Model 1600 spectrophotometer. Column chromatography (CC) was carried out using silica gel 60 (70-230 and 200-400 mesh) thin layer chromatography (TLC) and preparative TLC on silica gel 60 precoated F-254 plates (Merck, Germany) (Cakir *et al.*, 2006; Kotan *et al.*, 2010). The spots on TLC were visualized by UV_{254}, UV_{366} and spraying with 1% vanillin-H_2SO_4 followed by heating at 105°C.

Plant materials. The aerial parts of *O. acutidens, O. rotundifolium* and *O. vulgare* subsp. *vulgare* were collected at Erzurum (Turkey) at the flowering stage in July-September 2008, and were air dried in the shade. Specimens of all three species have been deposited in the herbarium of Ataturk University, Erzurum.

Isolation of the essential oils. Dried plant samples (500 g) were subjected to hydrodistillation using a Clevenger-type apparatus for 4 h. Oils were extracted with $CHCl_3$ and then dried over anhydrous Na_2SO_4 and stored under N_2 atmosphere at 20°C in a sealed vial until use. Essential oil yields from *O. acutidens*, *O. rotundifolium* and *O. vulgare* were 1.23, 0.60 and 3.85% (w/w), respectively.

Extraction procedures. Dried and powdered plant samples (50 g from each plant) were extracted individually with *n*-hexane, chloroform, acetone and methanol at room temperature (500 ml × 4). After filtration, the organic solvents were evaporated under reduced pressure and temperature. For methanol extracts of the plant samples, the concentrated MeOH extracts were individually dissolved in distilled water (60°C) and filtered. The solutions were extracted with *n*-hexane for three times to remove lipophilic compounds. Then, water solutions were lyophilized in a Labconco 117 freeze-dryer at 5 μm-Hg and -50°C. The extract yields (w/w) of hexane, chloroform, acetone and methanol were 7.08, 7.70, 3.30% and 7.70% for *O. acutidens*; 2.82, 6.10, 4.94, and 5.22% for *O. rotundifolium*; 2.28, 2.68, 4.08 and 8.94% for *O. vulgare*, respectively.

To fractionate the *n*-hexane extract of *O. acutidens* and isolate the major component, a powdered plant sample (300 g) was extracted with hexane (1.5 litres x 5) at room temperature. The precipitate obtained was washed over *n*-hexane for five times resulting in a white amorphous powder (1.75 g) that was crystallized with $CHCl_3$. The purity of the crystals was controlled on GC-MS.

After precipitation, the extract (20.5 g) was fractioned on silica gel CC (150 g, 70-230 mesh) using the elution system $CHCl_3$-*n*-hexane (8:2), $CHCl_3$ and $CHCl_3$-acetone (8:2). A total of 55 fractions were collected, the fractions (50 ml each) were compared by TLC (silica gel) using $CHCl_3$-*n*-hexane (5:5, 6:4, 7:3), $CHCl_3$ and $CHCl_3$:Ethyl acetate (8:2) and those giving similar spots were combined. Six fractions (A-F) were finally obtained, their amounts being 4.73, 4.65, 2.25, 0.90, 3.76 and 2.60 g for A, B, C, D, E and F fractions, respectively.

GC-FID analysis. Analysis of the essential oil was performed using a Thermofinnigan Trace GC/A1300 (E.I) (San Jose, USA) equipped with a SGE/BPX5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm). Helium was the carrier gas, at a flow rate of 1 ml/min. Injector temperature was set at 220°C. The oven temperature range was 50-150°C at a rate of 3°C/min, then held isothermal for 10 min at 150°C and finally raised to 250°C at 10°C/min. Diluted samples (1/100, v/v, in methylene chloride) of 1.0 μl were injected manually and in the splitless mode. Quantitative data were obtained from FID area percentage data.

GC-MS analysis. Analysis of the essential oil was performed with a Thermofinnigan Trace GC/Trace DSQ /A1300 (San Jose, USA) equipped with a SGE-BPX5 MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Carrier gas was helium at a flow rate of 1 ml/min. Injector and MS transfer line temperatures were set at 220°C and 290°C, respectively. The oven

temperature was programmed from 50°C to 150°C at 3°C/min, held isothermal for 10 min and finally raised to 250°C at 10°C/min. Diluted samples (1/100, v/v, in methylene chloride) of 1.0 µl were injected manually in the splitless mode.

The identification of individual compounds was based on comparison of their relative retention indexes with those of authentic samples on SGE-BPX5 capillary column, and by matching of the mass spectra of the peaks with those obtained from authentic samples and/or the Wiley 7N and TRLIB libraries spectra and published data (Adams, 2007).

Phytopathogenic bacterial strains. Essential oils, plant extracts and six major monoterpenes were individually tested against a total of 25 phytopathogenic bacterial strains all of which were obtained from Dr. Recep Kotan (Department of Plant Protection, Faculty of Agriculture, Atatürk University, Erzurum). The bacterial strains were: *Alcaligenes piechaudii* RK-155, *Bacillus pumilus* RK-106, *Chromobacterium violaceum* RK-231, *Clavibacter michiganensis* subsp. *michiganensis* RK-Cmm, *Enterobacter intermedius* RK-90, *Erwinia carotovora* subsp. *atroseptica* RK-462, *Erwinia chrysanthemi* RK-421, *Erwinia rhapontici* RK-208, *Flavobacterium* sp. RK-299, *Pantoea agglomerans* RK-84, *Pseudomonas aeruginosa* RK-168, *Pseudomonas cichorii* RK-166, *Hherbaspirillum huttiensis* RK-260, *Pseudomonas putida* RK-249, *Pseudomonas syringae* pv. *syringae* RK-402, *Pseudomonas syringae* pv. *tomato* RK-Ps-tom, *Xanthomonas campestris* pv. *campestris* RK-Xa-cam, *Xanthomonas axanopodis* pv. *malvacearum* RK-Xa-mal, *Xanthomonas hortorum* pv. *pelargonii* RK-Xa-pel, *Xanthomonas axanopodis* pv. *vesicatoria* Xcv110c, *Xanthomonas axanopodis* pv. *vesicatoria* Xcv761, *Xanthomonas axanopodis* pv. *vitians* Xa-vit, *Xanthomonas campestris* pv. *raphani* RK-Xc-rap and *Xanthomonas campestris* pv. *zinniae* Xc-zin.

All these strains had been determined as pathogens of different host plants (Sahin and Kotan, 1999; Kotan and Sahin, 2002; Sahin *et al.*, 2002, 2003; Kotan *et al.*, 2005), and were stored at -80°C in 15% glycerol and Luria Broth (LB) until use.

Antibacterial activity assays. Antibacterial activity assays were carried out by disc diffusion method on tryptic soy agar (TSA, Merck, Germany) medium (Kotan *et al.*, 2010). The extracts, fractions of *O. acutidens* hexane extract and monoterpenes solutions were prepared by dissolving using suitable solvents (chloroform, acetone and water), and sterilized by filtration by 0.45 µm Millipore filters. Bacterial cultures were grown in tryptic soy broth (TSB, Merck, Germany) and their suspension (100 µl) containing 1×10^8 CFU ml^{-1} of bacteria was spread by a sterile swab on TSA medium. Oxoid blank disk (6 mm in diameter) were impregnated with 12.5 µl of the essential oils and liquid monoterpenes, carvacrol, linalol and terpinen-4-ol, and 1.25 mg of the extracts, fractions and solid monoterpenes (borneol and thymol) solutions were kept out for vaporization of organic solvents, then placed in the centre of the inoculated plates. Bacterial cultures were incubated at 27±2ºC for 48 h, and inhibition zones were measured in diameter (mm) around the discs. All the tests were made in triplicate.

Determination of minimal inhibition concentration (MIC). MIC values were determined using the modified agar-well diffusion method (Okeke *et al.*, 2001). Two-fold serial dilutions of the essential oils and carvacrol (500 µl/ml) were prepared by diluting 10% DMSO to achieve a decreasing concentration ranging from 500 µl/ml to 3.125 µl/ml. Solutions of the extracts, thymol and borneol were prepared at concentrations ranging from 10-100 mg/ml. Bacterial cultures were grown in triptic soy broth (TSB, Merck, Germany) and their suspension (100 µl) containing 1×10^8 CFU/ml of bacteria spread by a sterile swab on TSA medium. The Oxoid disks were impregnated with 12.5 µl of solutions of the essential oils, pure compounds and extracts, and placed in the middle of inoculated TSA agar plates. The bacterial cultures were incubated at 27±2ºC for 48 h. The least concentration of each the solution showing a clear zone of inhibition was taken as the MIC value. All tests were made in triplicate.

Seed disinfection assays. Tomato and pepper seeds (cvs Marmande and A36D1, respectively) purchased from a local market, were surface-disinfected by dipping for 3 min in 3% sodium hypochlorite and washing four times in sterile distilled water. Seeds were left to dry on sterile Whatman filter paper overnight in a laminar flow hood. Subsequently, *X. axanopodis* pv. *vesicatoria* (*Xav*) was grown in 50 ml Erlenmeyer flasks containing 20 ml of TSB medium on a rotary shaker at 28ºC for 24 h. Absorbance of the bacterial suspensions was measured spectrophotometrically at 600 nm and appropriately diluted to 1×10^8 CFU ml^{-1} in sterile distilled H_2O. Approximately 20 g of sucrose (10 g l^{-1}) were added to each Erlenmeyer flasks, and 90 g of the surface-sterilized seeds were soaked in this suspension and incubated under shaking at 80 rpm for two days at 28°C to coat their integument with the pathogen. After shaking, the seeds were removed and air-dried on sterile Whatman filter paper overnight in a laminar flow hood.

O. acutidens oil was applied at concentrations of 100, 40, 20, 10, 4 and 2 mg ml^{-1} based on MIC values. Prior to bioassays, solutions were prepared by dissolving in 10% DMSO in distilled water in 10 ml Erlenmeyer flasks. The seeds coated with *Xav* were soaked in the suspensions, and incubated while shaking at 80 rpm for one day at 28ºC until the seeds were uniformly coated with the suspensions. Seeds were left to dry on sterile

Table 1. Chemical composition of the essential oils and hexane extracts of *O. acutidens, O. rotundifolium* and *O. vulgare*.

RI[a]	Component	*O. acutidens*		*O. rotundifolium*		*O. vulgare*		Identification methods
		Oil (%)	Extract (%)	Oil (%)	Extract (%)	Oil (%)	Extract (%)	
994	Myrcene	0.20	-	-	-	-	-	GC, MS, RI
996	2-Octanone	-	-	-	-	0.73		GC, MS, RI
1023	α-Terpinene	0.13	-	-	-	-	-	GC, MS, RI
1034	*p*-Cymene	1.95	tr	tr	-	4.25	tr	GC, MS, RI
1042	1,8-Cineole	-	-	-	-	tr	-	GC, MS, RI
1067	α-Terpinene	0.71	tr	tr	-	0.59	tr	GC, MS, RI
1079	*cis*-Sabinene hydrate	0.31	tr	tr	tr	-	-	GC, MS, RI
1106	Linalol	-	-	10.34	tr	2.71	tr	GC, MS, RI
1117	*trans*-Sabinene hydrate	0.59	1.83	-	tr	-	-	GC, MS, RI
1153	Camphor	-	tr	tr	tr	0.48	-	GC, MS, RI
1172	Borneol	1.63	4.70	23.61	0.60	2.41	tr	GC, MS, RI
1178	Terpinen-4-ol	-	-	11.38	tr	2.30	tr	GC, MS, RI
1185	*p*-Cymen-8-ol	-	tr	-	tr	-	-	GC, MS, RI
1190	α-Terpineol	0.21	-	5.54	tr	2.96	tr	GC, MS, RI
1200	γ-Terpineol	0.30	-	-	-	-	-	MS, RI
1219	Thymol methyl ether	-	-	4.44	tr	tr	-	GC, MS, RI
1228	Carvacrol methyl ether	-	-	tr	0.96	tr	-	GC, MS, RI
1243	Carvone	-	-	-	-	0.91	-	GC, MS, RI
1251	Thymoquinone	tr	3.12	-	-	-	-	GC, MS, RI
1255	Linalol acetate	1.65	0.65	4.64	-	-	-	GC, MS, RI
1264	Geranial	0.22	-	-	-	-	-	GC, MS, RI
1278	Bornyl acetate	-	tr	2.94	tr	0.57	-	GC, MS, RI
1289	Thymol	tr	1.99	1.70	0.76	3.17	1.63	GC, MS, RI
1296	Carvacrol	86.99	6.87	22.81	46.00	27.17	1.34	GC, MS, RI
1337	*trans*-Carvyl acetate	-	1.29	-	-	-	-	GC, MS, RI
1356	Nerol acetate	0.10	-	tr	-	-	-	GC, MS, RI
1377	Geraniol acetate	0.23	-	-	-	-	-	GC, MS, RI
1383	β-Bourbonene	-	-	3.23	tr	2.37	tr	GC, MS, RI
1409	Methyl eugenol	-	tr	-	-	-	-	GC, MS, RI
1419	β-Caryophyllene	1.30	-	2.36	-	14.13	tr	GC, MS, RI
1433	β-Gurjunene	0.19	-	tr	-	0.46	-	GC, MS, RI
1460	α-Humulene	0.07	-	-	-	2.98	tr	GC, MS, RI
1478	γ-Muurolene	0.08	-	-	-	0.22	-	MS, RI
1486	Germacrene D	0.18	-	1.86	-	4.33	tr	GC, MS, RI
1494	Viridiflorene	0.19	-		-	-	-	GC, MS, RI
1501	Bicyclogermacrene	-	-	tr		0.67	-	MS, RI
1507	(*E,E*)-α-Farnesene	-	-	1.75	-	tr	-	MS, RI
1508	β-Bisabolene	-	tr	-	-	3.90	tr	MS, RI
1513	γ-Cadinene	0.08	-	-	-	0.98	-	MS, RI
1517	δ-Cadinene	0.29	-	-	-		-	GC, MS, RI
1574	Spathulenol	0.66	2.79	2.27	tr	3.98	2.44	GC, MS, RI
1579	Caryophyllene oxide	0.52	1.82	1.13	0.61	11.55	2.02	GC, MS, RI
1609	Humulene epoxide II	-	-	-	-	1.08	-	GC, MS, RI
1625	1-*epi*-Cubebol	-	-	-	-	0.35	-	MS, RI
1632	(*E*)-Sesquilavandulol	-	-	-	-	0.25	-	MS, RI
1659	α-Cadinol	-	-	-	-	1.33	-	GC, MS, RI
1694	Eudesma-4(15),7-dien-1β-ol	0.10	-	-	-	-	-	MS, RI
1747	Oplopanone	-	-	-	-	-	0.75	MS, RI
1908	Methyl hexadecanoate	-	1.14	tr	0.51	0.61	1.85	MS, RI
1923	*n*-Hexadecanoic acid	-	-		-	-	1.70	GC, MS, RI
1945	Abietatriene	-	0.70	-	tr	-	-	MS, RI
1951	(*E*)-Phytol	-	-	-	-	tr	-	GC, MS, RI
1955	Methyl linoleate	-	0.84		0.72		-	GC, MS, RI
1957	Isophytol	-	-	-	-	0.39	-	GC, MS, RI
2051	Ethyl linoleate	-	3.38	-	1.65	-	0.85	MS, RI
2100	*n*-Heneicosane	-	2.17	-	1.32	-	1.35	GC, MS, RI

2200	*n*-Docosane		-	-	-	-	0.98	GC, MS, RI
2300	*n*-Tricosane	-	tr	-	0.84	-	4.25	GC, MS, RI
2600	*n*-Hexacosane	-	tr	-	-	-	8.03	MS, RI
2624	*n*-Tetracosanol	-	65.04	-	42.97	-	68.88	MS, NMR
Grouped Components (%)								
Aromatic monoterpenes		88.94	11.98	28.95	47.72	34.59	2.97	
Monoterpene hydrocarbons		1.04	-	tr	-	0.59	-	
Oxygenated monoterpenes		5.24	8.47	58.45	0.60	12.34	-	
Sesquiterpene hydrocarbons		2.38	tr	9.20	-	30.04	-	
Oxygenated sesquiterpenes		1.28	5.75	3.40	1.12	19.15	7.00	
Others		-	72.13	-	47.50	1.12	85.19	
Total identified		98.88	98.33	100.00	96.94	97.83	96.07	

GC, identification based on retention times of authentic compounds on SGE-BPX5 capillary column; MS, tentatively identified based on computer matching of the mass spectra of peaks with Wiley 7N and TRLIB libraries and published data (Adams, 2007); RI, comparison of retention index of the compounds compared with published data (Adams, 2007); tr, traces (less than 0.07%).

[a] Retention index relative to *n*-alkanes on SGE-BPX5 capillary column.

Whatman filter paper overnight in the laminar flow hood. *Xav* suspensions in 10% DMSO and streptomycin sulfate (500 µg/ml) were used as controls. Petri dishes containing 20 seeds and 20 ml of TSA medium were incubated at 27±2ºC. After six days, the number of seeds infected or not with *Xav* or with saprophytic microorganisms was counted. Around seeds infected by *Xav*, round, convex, mucoid, yellow colonies grew on yeast dextrose agar (YDC). If no *Xav* or saprophytic microbial growth was detected on the plates, the seeds were considered as sterile. The treatments were arranged in completely randomized design with six replications for each treatment including controls.

Statistical analyses. To determine whether there was a statistically significant difference among the results of seed disinfectant assays, variance analyses were carried out using SPSS 10.0 software package. Differences between means were tested by Duncan Multiple Range Test and values with $p < 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

Chemical composition of the essential oils and hexane extracts. The essential oils and hexane extracts compositions of Turkish *O. acutidens*, *O. rotundifolium* and *O. vulgare* subsp. *vulgare* and the relative amounts of components are shown in Table 1. Essential oils of the three *Origanum* species differed in chemical composition. According to the analysis, *O. acutidens* oil contained a higher amount of carvacrol (86.99%) as compared with the essential oils of *O. rotundifolium* (22.81%) and *O. vulgare* (27.17%). Other main components of *O. rotundifolium* oil were borneol (23.61%), terpinen-4-ol (11.38%), linalol (10.34%), α-terpineol (5.54%), linalol acetate (4.64%) and thymol methyl ether (4.44%), whereas those of *O. vulgare* oil were β-caryophyllene (14.13%), caryophyllene oxide (11.55%), germacrene D (4.33%), *p*-cymene (4.25%), spathulenol (3.98%) and β-bisabolene (3.90%). Results also showed that *O. acutidens* oil is rich in aromatic monoterpenes, representing 88.94% of total oil as compared to other two essential oils (Table 1). *O. rotundifolium* oil was characterized by relatively high content of oxygenated monoterpenes (58.45%), whereas *O. vulgare* oil contained a relatively high amount of sesquiterpenes (49.19%) as compared with the essential oils of *O. acutidens* and *O. vulgare*.

Previous studies showed that essential oils isolated from *O. acutidens* and *O. rotundifolium* growing in various regions of the world had a different chemical composition (Baser *et al.*, 1995, 1997; Sokmen *et al.*, 2004; Figueredo *et al.*, 2006; Kordali *et al.*, 2008). For instance, it was shown that carvacrol (66.0-72.0%) and *p*-cymene (7.5-14.0%) were the major components of *O. acutidens* oils (Baser *et al.*, 1997; Sokmen *et al.*, 2004; Figueredo *et al.*, 2006). As shown in Table 1, our findings are generally in agreement with those previously reported. However, in contrast to our results, Baser *et al.* (1995) reported that *cis*-sabinene hydrate was the major component in the hydrodistilled essential oil of *O. rotundifolium* whereas we found that this component was in trace amount in the oil of this species, which contained mainly borneol (23.61%), carvacrol (22.81%), terpinen-4-ol (11.38%), linalol (10.34%) and α-terpineol (Table 1). As mentioned, the differences in chemical compositions of the *O. rotundifolium* oils from different locations of Turkey may be related to local, climatic and seasonal differences (Baser *et al.*, 1995).

Numerous reports on the essential oils of *Origanum* showed that those of some species are rich in carvacrol, thymol, γ-terpinene and *p*-cymene (Daferera *et al.*, 2003; Sokmen *et al.*, 2004; Bozin *et al.*, 2006; Figueredo *et al.*, 2006; Esen *et al.*, 2007; Kordali *et al.*, 2008; Al-

tundag and Aslim, 2011). Esen *et al.* (2007) found that the main constituents of wild *O. vulgare* subsp. *hirtum* collected from different localities of the Marmara region (Turkey) were carvacrol (82.9-7.5%), thymol (68.0-0.3%), *p*-cymene (28.1-7.8%) and γ-terpinene (7.8-0.1%). However, as shown in Table 1, the essential oil of *O. vulgare* subsp. *vulgare* from Erzurum district contains relatively low amounts of carvacrol (27.17%), thymol (3.17%), γ-terpinene (0.59%) and *p*-cymene (4.25%). Our results are also in agreement with the findings by Sahin *et al.* (2004), who documented that the essential oil of *O. vulgare* subsp. *vulgare* contained mainly β-caryophyllene (14.4%), spathulenol (11.6%), germacrene D (8.1%), α-terpineol (7.5%) and caryophyllene epoxide (5.8%), and low amounts of carvacrol (0.6%), thymol (0.8%) and *p*-cymene (0.7%).

Although there are numerous reports on the chemical composition of the studied *Origanum* species, this is the first report on the analyses of the hexane extracts isolated from the aerial parts of Turkish *Origanum* species. As shown in Table 1, in general, essential oils and hexane extracts were similar in terms of monoterpene constituents. However, the hexane extracts were characterized by relatively high amounts of less volatile components such as abietatriene, *n*-heneicosane, *n*-tricosane and *n*-hexacosane (Table 1), *n*-tetracosanol (68.88-42.97%) being the major component. This component was also isolated by silica gel column chromatography and its chemical structure was confirmed by IR, MS and NMR spectroscopic methods. Our results also showed that the hexane extracts contained lower amounts of terpenes components as compared to their hydrodistilled essential oils (Table 1).

Of the six fractions of *O. acutidens* hexane extract, fraction A contained generally terpenes hydrocarbons as well as *n*-alkanes and fatty acid ester derivatives, *p*-cymene (63.31%), γ-terpinene (9.77%), β-caryophyllene (6.14%), *n*-heneicosane (4.47%) and *n*-tricosane (4.04%). However, thymoquinone (39.79%), carvacrol (17.63%) and linalyl acetate (9.22%) were the main components of fraction B. As can be seen from Table 2, fractions C and D consisted of carvacrol (63.97 and 80.63%, respectively) as main component, whereas fraction E had mainly *n*-tetracosanol (86.70.%), as major component as well as carvacrol (9.27%). On the other hand, oxygenated monoterpenes such as borneol (38.39%), *trans*-sabinene hydrate (6.74%), carvacrol (6.69%), *cis*-sabinene hydrate (4.73%) and a steroid (8.29%) were detected to be the main components of fraction E (Table 2).

Antibacterial activities of the essential oils, crude plant extracts and pure compounds. As shown in Table 3, essential oils (12.5 μl ml^{-1}) were found to be very effective against all plant pathogenic bacterial strains producing 8-48 mm inhibition zones depending on the species. Essential oils showed also bactericidal effects against most of the bacteria tested (Table 3). The MIC values of the oils varied with the strains tested, ranging from 31.25 to 500 μl ml^{-1} confirming literature reports on the antimicrobial activity of *O. vulgare* and *O. acutidens* essential oils against various pathogens (Sahin *et al.*, 2004; Sokmen *et al.*, 2004; Bozin *et al.*, 2006; Sokovic and van Griensven, 2006; Esen *et al.*, 2007; Kordali *et al.*, 2008; Altundag and Aslim, 2011). Likewise, Altundag and Aslim (2011) documented that the essential oil of Turkish *O minutiflorum* possess strong antimicrobial activity against *X. vesicatoria*. This property of *Origanum* essential oils is apparently related to their high phenolic contents, particularly carvacrol and thymol (Sivropoulou *et al.*, 1996; Pradhanang *et al.*, 2003; Sahin *et al.*, 2004; Sokmen *et al.*, 2004; Bozin *et al.*, 2006; Sari *et al.*, 2006; Sokovic and van Griensven, 2006; Vasinauskiene *et al.*, 2006; Esen *et al.*, 2007; Kordali *et al.*, 2008; Altundag and Aslim, 2011). This claim is supported by our findings (Table 1), relative to the high content of carvacrol (86.99%) in *O. acutidens* oil. In further agreement with these findings, carvacrol and thymol were very effective against all bacteria tested (Table 4). However, although the oils of *O. rotundifolium* and *O. vulgare* were characterized by a relatively low content of carvacrol (22.81% and 27.17%, respectively) as compared to that of *O. acutidens*, they were very effective against the bacteria tested (Table 3). The same effect was shown by linalol and terpinen-4-ol, which are other major components of *O. rotundifolium* oil (Table 4). Thus, the potent antibacterial activity of *O. rotundifolium* oil can be attributed primarily to its major components, carvacrol, linalol and terpinen-4-ol. However, the major components of *O. vulgare* oil, caryophyllene oxide and β-caryophyllene, were not active against the tested bacterial strains. These results indicate that also the synergistic and/or antagonistic interactions between oil components could affect their antibacterial activity.

The present results show that, unlike essential oils, hexane, chloroform, acetone and methanol extracts were not active against the tested bacterial strains (Table 3). In fact, only the hexane and chloroform extracts of *O. rotundifolium* had a weak antibacterial activity against a limited number of bacteria. The results presented in Table 1 show that, in contrast to essential oils, the hexane extracts contain relatively low amounts of carvacrol and thymol and relatively high content of aliphatic components such as *n*-tetracosanol, *n*-hexacosane, *n*-tricosane, *n*-docosane, *n*-heneicosane. These compounds comprised 72.13, 47.50 and 85.19% of the hexane extracts of *O. acutidens*, *O. rotundifolium* and *O. vulgare*, respectively (Table 1). Therefore, a weak or no antibacterial activity can be attributed to hexane extracts despite the relatively high amount of these components which compares with low amounts of carvacrol and thymol. This likelihood is supported by the fact

Table 2. Chemical composition of the fractions obtained by silica gel column chromatography of hexane extract of *O. acutidens.*

Component	(%)	Component	(%)
Fraction A		Fraction D	
p-Cymene	63.31	Thymoquinone	0.60
γ-Terpinene	9.77	Thymol	4.03
β-Caryophyllene	6.14	Carvacrol	80.63
Methyl palmitate	3.66	Methyl eugenol	tr
Methyl linoleate	2.01	Citronelyl oxy acetaldehyde	tr
Ethyl linoleate	3.19	Caryophyllene oxide	2.24
n-Heneicosane	4.47	*n*-Tetracosanol	8.74
n-Tricosane	4.04	Total identified (%)	96.24
n-Hexacosane	3.41		
Total identified (%)	100.00		
Fraction B		Fraction E	
p-Cymene	6.86	Thymol	0.85
γ-Terpinene	tr	Carvacrol	9.27
Linalyl acetate	9.22	Dimethoxyacetophenone	1.12
Thymoquinone	39.79	*n*-Tetracosanol	86.70
2,5-Dimethyl, 3(2H)-Benzofuranone	2.68	Total identified (%)	97.94
Thymol	7.82		
Carvacrol	17.63		
Methyl eugenol	2.86		
Total identified (%)	86.86		
Fraction C		Fraction F	
2-Octanone	0.53	*cis*-sabinene hydrate	4.73
p-Cymene	0.87	*cis*-Linalol oxide	2.27
γ-Terpinene	0.74	*trans*-Sabinene hydrate	6.74
Dihydrocarvone	0.60	Borneol	38.39
Linalyl acetate	1.52	Carvacrol	6.69
Thymoquinone	6.94	(3*Z*)-Hexenyl tiglate	1.53
2,5-Dimethyl, 3(2H)-Benzofuranone	2.96	Spathulenol	1.69
Thymol	9.23	Squalene	7.58
Carvacrol	63.97	a Steroid (MW:463)	8.29
Methyl eugenol	4.44	Total identified (%)	70.35
Citronelyl oxy acetaldehyde	0.40		
Caryophyllene oxide	1.57		
(Z,Z)-Farnesyl acetone	0.20		
Total identified (%)	93.97		

tr = traces (less than 0.20%).

that *n*-tetracosanol, which is major component of hexane extracts, did not show any antibacterial activity against the tested bacteria (Table 4).

Among A-F fractions of hexane extract from *O. acutidens*, fraction D, which contains mainly carvacrol (80.63%), was active against some of the tested bacteria, whereas fraction C, characterized by a high content of carvacrol (63.97%), was effective only against *Flavobacter* sp. (Table 5). These results show that although the antibacterial effects of essential oils and hexane extracts from *Origanum* species are mainly due to their phenolic compounds, other major and/or minor components may also be responsible for the observed antibacterial activity. As can be seen from Table 5, A, B, E and F fractions, which contained no or low amounts of these components, did not show any activity against the tested bacteria.

Seed disinfection assays. As shown in Table 3, *O. acutidens* oil had a strong bactericidal effect against *Xav* which prompted its use in seed disinfection assays (Table 6). High concentrations (10, 20, 40 and 100 mg ml^{-1}) of *O. acutidens* oil were effective against *Xav* as a seed disinfectant. Furthermore, concentrations of 20, 40 and 100 mg ml^{-1} oil were as effective as streptomycin sulfate used as positive control. All this indicates that *O. acutidens* oil can be used as a seed disinfection reagent for the management of the tested bacterial species.

There have been many reports on the antimicrobial activity of essential oils and extracts from *Origanum* species against plant pathogens (Basim *et al.*, 2000; Kizil and Uyar, 2006; Kokoskova and Pavela, 2005; Vasinauskiene *et al.*, 2006; Soylu *et al.*, 2007). However, control of plant diseases though seed treatments with plant extracts and oils is a newly explored area whose impor-

Table 3. Antibacterial activity of the essential oil and extracts from *O. acutidens*, *O. rotundifolium* and *O. vulgare*.

Bacteria	Essential oils			Extracts												
				OA				OV				OR				
	OA	OV	OR	H	C	A	M	H	C	A	M	H	C	A	M
	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)	DD (MIC)
A. piechaudii RK-155	16 (62.5)	19 (125.0)	11[a] (250.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
B. pumilus RK-106	17[a] (250.0)	14[a] (250.0)	11[a] (125.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
C. violaceum RK-231	18 (62.5)	15[a] (125.0)	16[a] (250.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
C. m. subsp. *michiganensis* RK-Cmm	22 (62.5)	20 (125.0)	32 (125.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
E. intermedius RK-90	20 (125.0)	20 (125.0)	12[a] (250.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
E. amylovora RK-228	35 (62.5)	30 (62.5)	23 (125.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
E. c. subsp. *atroceptica* RK-462	36 (125.0)	37 (125.0)	12[a] (250.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
E. chrysanthemi RK-421	19 (62.5)	21[a] (31.25)	11[a] (125.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
E. rhapontici RK-208	36[a] (62.5)	40 (125.0)	16 (250.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
Flavobacter sp. RK-299	48 (62.5)	42 (125.0)	35 (125.0)	–	–	–	–	–	–	–	–	11[a] (90.0)	–	–	–	–
P. agglomerans RK-84	17 (62.5)	15 (125.0)	11[a] (62.5)	–	–	–	–	–	–	–	–	–	–	–	–	–
P. aeruginosa RK-168	15 (62.5)	22 (125.0)	9[a] (500.0)	–	–	–	–	–	–	–	–	–	8[a] (90.0)	–	–	–
P. cichorii RK-166	16[a] (125.0)	18[a] (62.5)	8[a] (500.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
H. huttiensis RK-260	31[a] (62.5)	26[a] (62.5)	21 (125.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
P. putida RK-249	10[a] (250.0)	14 (250.0)	16 (250.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
P. s. pv. *syringae* RK-204	29 (62.5)	32[a] (125.0)	20 (125.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
P. s. pv. *tomato* RK-Pst1	24 (125.0)	30[a] (125.0)	28 (125.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
X. a. pv. *malvacearum* RK-401	38[a] (62.5)	40[a] (62.5)	39 (125.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
X. a. pv. *vesicatoria* RK-399	38 (31.25)	15[a] (62.5)	30 (125.0)	–	–	–	–	–	–	–	–	–	9[a] (90.0)	–	–	–
X. a. pv. *campestris*RK-Xcc	40[a] (62.5)	40 (62.5)	33 (125.0)	–	–	–	–	–	–	–	–	–	–	–	–	–
X. c. pv. *raphani* RK-Xcr	38 (62.5)	32 (62.5)	45[a] (125.0)	–	–	–	–	–	–	–	–	–	9[a] (90.0)	–	–	–
X. a. pv. *vesicatoria* RK-Xcv1	40[a] (62.5)	36 (31.25)	42[a] (125.0)	–	–	–	–	–	–	–	–	–	8[a] (90.0)	–	–	–

X. a. pv. *vesicatoria* RK-Xcv 761	37 (62.5)	42 (62.5)	30 (125.0)	–	–	–	–	–	–	–	–	–	8[a] (90.0)	–	–
X. a. pv. *vitians* RK-Xcvi	42[a] (31.25)	34 (31.25)	38 (125.0)	–	–	–	–	–	–	–	–	–	–	–	–
X. c. pv. *zinniae* RK-Xcz	25 (62.5)	25[a] (125.0)	13[a] (125.0)	–	–	–	–	–	–	–	–	–	–	–	–
X. a. pv. *malvacearum* RK-397	38 (31.25)	37 (125.0)	35 (125.0)	–	–	–	–	–	–	–	–	–	–	–	–
X. a. pv. *pelargoni* RK-406	40 (31.25)	34 (125.0)	37 (125.0)	–	–	–	–	–	–	–	–	–	–	–	–

OA, *O. acutidens*; OV, *O. vulgare*; OR, *O. rotundifolium*; H, hexane extract; C, chloroform extract; A, acetone extract; M, methanol extract; –, not active; DD, Inhibition zone in diameter (mm) around the disks (6 mm) impregnated with 1.25 mg of the extracts, and 12.5 µl of the essential oil; MIC, minimal inhibitory concentration as µl/ml for the essential oil.
[a] Bactericidal effect was observed.

Table 4. Antibacterial activity of pure major components of essential oils and extracts.

Bacteria	Carvacrol		Thymol		Linalol		Terpinen-4-ol		*n*-Tetracosanol	Borneol	Caryophyllene oxide	β-Caryophyllene
	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	DD	DD	DD
A. piechaudii RK-155	16[a]	15.63	17[a]	20.0	12[a]	90.0	11[a]	90.0	–	–	–	–
B. pumilus RK-106	14[a]	15.63	22	20.0	10[a]	90.0	8[a]	90.0	–	–	–	–
C. violaceum RK-231	12[a]	31.25	17[a]	20.0	8[a]	110.0	11[a]	70.0	–	–	–	–
C. m. subsp. *michiganensis* RK-Cmm	23	31.25	41[a]	20.0	10[a]	110.0	9[a]	90.0	–	–	–	–
E. intermedius RK-90	16[a]	15.63	17[a]	20.0	14	90.0	17[a]	80.0	–	–	–	–
E. amylovora RK-228	28	31.25	26[a]	20.0	13[a]	80.0	20[a]	70.0	–	–	–	–
E. c. subsp. *atroceptica* RK-462	15[a]	15.63	21[a]	20.0	18[a]	80.0	10[a]	90.0	–	–	–	–
E. chrysanthemi RK-421	16[a]	15.63	20[a]	20.0	14[a]	90.0	15[a]	80.0	–	–	–	–
E. rhapontici RK-208	23	31.25	24[a]	10.0	13[a]	90.0	15[a]	70.0	–	–	–	–
Flavobacter sp. RK-299	39	15.63	49[a]	20.0	29	90.0	11[a]	90.0	–	–	–	–
P. agglomerans RK-84	19	15.63	15[a]	20.0	13[a]	80.0	12[a]	90.0	–	–	–	–
P. aeruginosa RK-168	13[a]	31.25	20[a]	20.0	–	ND	17[a]	70.0	–	–	–	–
P. cichorii RK-166	12[a]	31.25	21[a]	20.0	–	ND	16	70.0	–	–	–	–
H. huttiensis RK-260	23	15.63	31[a]	20.0	25[a]	80.0	27[a]	60.0	–	–	–	–
P. putida RK-249	17	62.50	9[a]	40.0	–	ND	9[a]	110.0	–	–	–	–
P. s. pv. *syringae* RK-204	26[a]	15.63	29[a]	20.0	26[a]	80.0	31[a]	60.0	–	–	–	–
P. s. pv. *tomato* RK-Pst1	25	15.63	26	20.0	34[a]	90.0	26	80.0	–	–	–	–
X. a. pv. *malvacearum* RK-401	38	15.63	34	20.0	33[a]	80.0	21[a]	70.0	–	–	–	–
X. a. pv. *vesicatoria* RK-399	28	15.63	28	20.0	23[a]	60.0	25[a]	70.0	–	–	–	–
X. a. pv. *campestris*RK-Xcc	25	15.63	29	20.0	24	80.0	20	70.0	–	–	–	–
X. c. pv. *raphani* RK-Xcr	30	15.63	26	20.0	14	80.0	22[a]	70.0	–	–	–	–
X. a. pv. *vesicatoria* RK-Xcv1	27	15.63	41[a]	20.0	21[a]	80.0	36[a]	70.0	–	–	–	–
X. a. pv. *vesicatoria* RK-Xcv 761	32	15.63	26	20.0	21[a]	60.0	22[a]	70.0	–	–	–	–
X. a. pv. *vitians* RK-Xcvi	28	15.63	38[a]	10.0	45[a]	50.0	43[a]	60.0	–	–	–	–
X. c. pv. *zinniae* RK-Xcz	20[a]	31.25	33	20.0	10[a]	110.0	9[a]	110.0	–	–	–	–
X. a. pv. *malvacearum* RK-397	23	15.63	29[a]	10.0	21	80.0	22	70.0	–	–	–	–
X. a. pv. *pelargoni* RK-406	29	15.63	30	20.0	33	80.0	21	70.0	–	–	–	–

DD, inhibition zone in diameter (mm) around the disks (6 mm) impregnated with 1.25 mg of thymol, borneol and caryophyllene oxide and 12.5 µl of carvacrol, linalol and terpinen-4-ol; MIC, minimal inhibitory concentration in µl/ml for carvacrol, β-caryophyllene and terpinen-4-ol and in mg/ml for thymol and caryophyllene oxide; – not active.
[a] Bactericidal effect was observed.

Table 5. Antibacterial activitiy of the fractions obtained by silica gel column chromatography of hexane extract from *O. acutidens*.

Bacteria	*O. acutidens* fractions					
	A	B	C	D		E
	DD	DD	DD	DD	MIC	DD
A. piechaudii RK-155	–	–	–	–	ND	–
B. pumilus RK-106	–	–	–	–	ND	–
C. violaceum RK-231	–	–	–	–	ND	–
C. m. subsp. *michiganensis* RK-Cmm	–	–	–	9[a]	90.0	–
E. intermedius RK-90	–	–	–	–	ND	–
E. amylovora RK-228	–	–	–	–	ND	–
E. c. subsp. *atroceptica* RK-462	–	–	–	8[a]	80.0	–
E. chrysanthemi RK-421	–	–	–	–	ND	–
E. rhapontici RK-208	–	–	–	–	ND	–
Flavobacter sp. RK-299	–	–	14	15	80.0	–
P. agglomerans RK-84	–	–	–	–	ND	–
P. aeruginosa RK-168	–	–	–	–	ND	–
P. cichorii RK-166	–	–	–	–	ND	–
H. huttiensis RK-260	–	–	–	10[a]	60.0	–
P. putida RK-249	–	–	–	–	ND	–
P. s. pv. *syringae* RK-204	–	–	–	–	ND	–
P. s. pv. *tomato* RK-Pst1	–	–	–	9[a]	70.0	–
X. a. pv. *malvacearum* RK-401	–	–	–	8[a]	70.0	–
X. a. pv. *vesicatoria* RK-399	–	–	–	–	ND	–
X. a. pv. *campestris*RK-Xcc	–	–	–	12	70.0	–
X. c. pv. *raphani* RK-Xcr	–	–	–	12	70.0	–
X. a. pv. *vesicatoria* RK-Xcv1	–	–	–	8[a]	70.0	–
X. a. pv. *vesicatoria* RK-Xcv 761	–	–	–	10[a]	70.0	–
X. a. pv. *vitians* RK-Xcvi	–	–	–	9[a]	90.0	–
X. c. pv. *zinniae* RK-Xcz	–	–	–	–	ND	–
X. a. pv. *malvacearum* RK-397	–	–	–	–	ND	–
X. a. pv. *pelargoni* RK-406	–	–	–	11[a]	70.0	–

DD, inhibition zone in diameter (mm) around the disks (6 mm) impregnated with 1.25 mg of the fractions; MIC, minimal inhibitory concentration in mg/ml; ND, MIC values not determined; –, not active.
[a]Bactericidal effect was observed.

Table 6. Number of tomato and pepper seeds infected with pathogenic (Pat.) and saprophytic (Sap.) bacteria treated with *O. acutidens* essential oil against *Xanthomonas anopodis.* pv. *vesicatoria.*

Treatments	Number of seeds infected with pathogenic and saprophytic bacteria			
	Pepper		Tomato	
	Sap.	Pat.	Sap.	Pat.
100 mg/ml oil	0.0[a]	0.0[a]	6.66[b]	0.0[a]
40 mg/ml oil	1.32[a]	0.0[a]	16.0[c]	0.0[a]
20 mg/ml oil	3.32[b]	0.0[a]	19.32[d]	0.0[a]
10 mg/ml oil	11.32[c]	11.32[b]	19.32[d]	0.0[a]
4 mg/ml oil	20.0[d]	20.0[c]	20.0[d]	2.66[b]
2 mg/ml oil	20.0[d]	20.0[c]	20.0[d]	4.66[c]
500 µg/ml Streptomycin	4.66[b]	0.0[a]	0.0[a]	0.0[a]
10% DMSO	20.0[d]	20.0[c]	20.0[d]	20.0[d]

[abcd] Means in the same column by the same letter are not significantly different according to Duncan Multiple Range Test (α= 0.05).

tance in organic farming is increasing, although data on its efficacy are still limited.

In conclusion, we have shown that carvacrol, thymol, linalol, terpinen-4-ol and the oils from *O. acutidens* and *O. rotundifolium* may be used as potential agents against some phytopathogenic bacteria. Insights gained from our study hold clues that will assist in directing future efforts toward developing novel and safe pesticides for controlling seed-borne bacteria in crop plants.

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