Inhibitory Effects of Various Essential Oils and Individual Components against Extended-Spectrum Beta-Lactamase (ESBL) Produced by *Klebsiella pneumoniae* and Their Chemical Compositions

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Abstract: In the current study, *in vitro* inhibitory activity of several essential oils obtained from the cultivated plants, *Foeniculum vulgare*, *Mentha piperita* and *M. spicata*, *Ocimum basilicum*, *Origanum majorana*, *O. onites*, *O. vulgare*, *Satureja cuneifolia*, and a number of individual essential oil components of terpene and aromatic types were screened against 10 isolated strains of *Klebsiella pneumoniae* producing extended-spectrum beta-lactamase (ESBL) enzyme, which makes this microorganism quite resistant against the antibiotics: trimetoprime-sulfametoksazol, sulbactam-ampicilin, clavulonate-amoxicilin, ceftriaxon, cefepime, imipenem, ceftazidime, tobramicine, gentamisine, ofloxacin, and ciprofloksasin. All of the essential oils and the components exerted a remarkable inhibition ranging between 32 and 64 μg/mL against all of these strains as strong as the references (ampicilin and oflaxocin) inhibiting at 32 μg/mL. Besides, chemical compositions of the essential oils were elucidated by gas chromatography-mass spectrometry (GC-MS). The essential oils and the pure components widely found in essential oils screened herein have shown remarkable inhibition against ESBL-producing *K. pneumoniae* strains, which leads to the suggestion that they may be used as food preservatives for this purpose.

Keywords: aromatic plants, ESBL, essential oil, GC-MS, *Klebsiella pneumoniae*

Practical Application: The essential oils obtained from *Foeniculum vulgare*, *Mentha piperita* and *M. spicata*, *O.cimum basilicum*, *Origanum majorana*, *O. onites*, *O. vulgare*, and *Satureja cuneifolia* as well as common essential oil components have shown notable inhibitory effects against 10 isolated strains of *Klebsiella pneumoniae* producing extended-spectrum beta-lactamase (ESBL) enzyme and they might be used as food preservative or ingredient.

Introduction

One of the problems of contemporary medicine is an escalating number of bacterial strains with hazardous phenotypes of resistance. The feared bacterial pathogens include *Klebsiella pneumoniae* strains producing extended-spectrums beta-lactamase (EBSL) enzymes. Outbreaks caused by multiresistant *K. pneumoniae* strains, especially having ESBL-producing ability, are a rising severe predicament (Bush 2002). *K. pneumoniae*, a gram-negative microorganism, is of a high prevalence in hospital infections and causes bacterial pneumonia (about 3%) (Marra and others 2006). It is an opportunistic human pathogen causing urinary tract infections and also known to be present in the soil, water, and vegetables (Soriano and others 2000). It was also reported that the food prepared for intensive care patients in the hospital kitchens was frequently contaminated with *Klebsiella* species (Casewell and Phillips 1978; Cooke and others 1980).

Multiple drug resistance is common and under the control of transmissible plasmids. Thereby, the increasing resistance against agents has been a major concern in recent years. Because of the existence of enzymes of ESBL-producing organisms, *K. pneumoniae* has been resistant to virtually all β-lactam antibiotics (Philippon and others 1989). Also, use of variety of other antibiotic classes has been found to be associated with subsequent infections due to ESBL-producing microorganisms (Livermore 1995).

Aromatic plants, which contain essential oil in favorable amount, are commonly used in world cousins as spice for their desirable flavor as well as food preservative due to their high antimicrobial properties. Actually many essential oils including the ones tested in this study have been reported to possess strong antibacterial activity against various bacterial strains. For instance, *Foeniculum vulgare* and *Origanum munitiflorum* showed antibacterial activity against some common foodborne pathogens: *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurinum*, and *Staphylococcus*

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Table 1–MIC values μg/mL of the essential oils and individual components against the ESBL-producing *K. pneumoniae* strains.

Essential oils (in alphabetical order)	ESBL-producing *K. pneumoniae* strains										
	K_1 R (SXT; AMC; SAM; CRO; CPM; IMP; TOB)	K_2 R (SXT; AMC; CRO; IMP; TOB)	K_3 R (AMC; SAM; CRO; CPM)	K_4 R (AMC; CRO; CAZ; CPM; TOB; GM)	K_5 R (AMC; CRO; CPM; GM)	K_6 R (AMC; SAM; CRO; CPM)	K_7 R (SXT; AMC; SAM; CRO; CPM; OFX)	K_8 R (SXT; AMC, SAM; CRO; CPM; OFX; CIP)	K_9 R (SXT; AMC, SAM; CRO; CPM; OFX; CIP)	K_{10} R (AMC; SAM; CRO; OFX)	*K. pneumoniae* RSKK 574
Foeniculum vulgare	64	64	32	64	32	32	64	64	64	32	8
Mentha piperita	64	64	32	64	32	32	64	64	64	32	8
Mentha spicata	64	64	32	64	32	32	64	64	64	32	8
Ocimum basilicum	64	64	32	64	32	32	64	64	64	32	8
Origanum majorana	64	64	32	64	32	32	64	64	64	32	8
Origanum onites	64	64	32	64	32	32	64	64	64	32	8
Origanum vulgaris	64	64	32	64	32	32	64	64	64	32	8
Satureja cuneifolia	64	64	32	64	32	32	64	64	64	32	8
Essential oil components (in alphabetical order)											
4-Allylanisol	64	64	32	64	32	32	64	64	64	32	8
trans-Anethol	64	64	32	64	32	32	64	64	64	32	8
(−)-Borneol	64	64	32	64	32	32	64	64	64	32	8
L-Bornylacetate	64	64	32	64	32	32	64	64	64	32	8
Camphen	64	64	32	64	32	32	64	64	64	32	8
Carvacrol	64	64	32	64	32	32	64	64	64	32	8
(−)-Carvone	64	64	32	64	32	32	64	64	64	32	8
Citral	64	64	32	64	32	32	64	64	64	32	8
Citronellol	64	64	32	64	32	32	64	64	64	32	8
Citronellal	64	64	32	64	32	32	64	64	64	32	8
Cuminyl alcohol	64	64	32	64	32	32	64	64	64	32	8
Cuminyl aldehyde	64	64	32	64	32	32	64	64	64	32	8
Cumol	64	64	32	64	32	32	64	64	64	32	8
Dihydrocarvone	64	64	32	64	32	32	64	64	64	32	8
Eugenol	64	64	32	64	32	32	64	64	64	32	8
Farnesol	64	64	32	64	32	32	64	64	64	32	8
Geranol	64	64	32	64	32	32	64	64	64	32	8
Geranyl ester	64	64	32	64	32	32	64	64	64	32	8
Isoborneol	64	64	32	64	32	32	64	64	64	32	8
Isomenthone	64	64	32	64	32	32	64	64	64	32	8
Isopulegol	64	64	32	64	32	32	64	64	64	32	8
d-Limonene	64	64	32	64	32	32	64	64	64	32	8
Linalool	64	64	32	64	32	32	64	64	64	32	8
Linalyl ester	64	64	32	64	32	32	64	64	64	32	8
Linalyl oxide	64	64	32	64	32	32	64	64	64	32	8
(−)-Menthol	64	64	32	64	32	32	64	64	64	32	8
Nerol	64	64	32	64	32	32	64	64	64	32	8
(−)-Phencone	64	64	32	64	32	32	64	64	64	32	8
α-Pinene	64	64	32	64	32	32	64	64	64	32	8
β-Pinene	64	64	32	64	32	32	64	64	64	32	8
Piperitone	64	64	32	64	32	32	64	64	64	32	8
γ-Terpinene	64	64	32	64	32	32	64	64	64	32	8
Thymol	64	64	32	64	32	32	64	64	64	32	8

Table 1–Continued

Essential oils (in alphabetical order)	ESBL-producing *K. pneumoniae* strains										
	K_1 R (SXT; AMC; SAM; CRO; CPM; IMP; TOB)	K_2 R (SXT; AMC; CRO; IMP; TOB)	K_3 R (AMC; SAM; CRO; CPM)	K_4 R (AMC; CRO; CAZ; CPM; TOB; GM)	K_5 R (AMC; CRO; CPM; GM)	K_6 R (AMC; SAM; CRO; CPM)	K_7 R (SXT; AMC; SAM; CRO; CPM; OFX)	K_8 R (SXT; AMC, SAM; CRO; CPM; OFX; CIP)	K_9 R (SXT; AMC, SAM; CRO; CPM; OFX; CIP)	K_{10} R (AMC; SAM; CRO; OFX)	*K. pneumoniae* RSKK 574
Vanillin	64	64	32	64	32	32	64	64	64	32	8
References											
AMP	32	32	32	32	32	32	32	32	32	32	2
OFX	32	32	32	32	32	32	32	32	32	32	<0.12

R = resistant; SXT = trimetoprime-sulphametoxazole; SAM = sulbactam-ampicillin; AMC = clavulonate-amoxicilin; CRO = ceftriaxon; CPM = cefepime; IMP = imipenem; TOB = tobramicine; CAZ = ceftazidime; GM = gentamicine; CIP = ciprofloxacin; AMP = ampicilin; OFX = ofloxacin.

aureus (Dadalioglu and Evrendilek 1994). In another study (Rafii and Shahverdi 2007), the essential oils from *Anethum graveolens*, *Mentha piperita*, and *Mentha spicata* were tested against *Enterobacter cloacae* and piperitone found in these essential oils enhanced the antibacterial activity. Antimicrobial activity of the essential oils of *Ocimum basilicum* and *Origanum vulgare* was found to have a remarkable antimicrobial activity toward 13 bacteria (Bozin and others 2006). The essential oil of *Rosmarinus officinalis* was reported to exhibit a potent antibacterial effect against several oral pathogens including *Streptococcus mutans*, *S. mitis*, *S. sanguinis*, *S. salivarius*, *S. sobrinus*, and *Enterococcus faecalis* (Bernardes and others 2010). Pereira and others (2004) reported 100% antibacterial efficiency of the essential oil of *Salvia officinalis* against *Klebsiella* and *Enterobacter* species, 96% against *E. coli*, 83% against *Proteus mirabilis*, and 75% against *Morganella morganii* isolated from subjects diagnosed with urinary tract infection. Considering sturdy antibacterial activity of the above-mentioned plant species and the increase in ESBL-producing microorganisms that will be sure to create significant therapeutic problems in the future, we have planned the current study in order to find out new effective alternatives from bioresources against ESBL-containing *K. pneumoniae* strains.

Therefore, inhibitory activity of a number of essential oils obtained from the cultivated plants, *F. vulgare* Mill., *M. piperita* L. and *M. spicata* L., *O. basilicum* L., *Origanum onites* L., *O. vulgare* L., and *Origanum majorana* L., and *Satureja cuneifolia* Ten., and several terpene and aromatic types of components widely found in essential oils was evaluated against the isolated strains of *K. pneumoniae* that are resistant against some antibiotics in the disk diffusion test such as trimetoprime-sulphamethoxazole, sulbactam-ampicilin, clavulonat-amoxicilin, ceftriaxon, cefepime, imipenem, ceftazidime, tobramicine, gentamisine, and ofloxacin using the *in vitro* broth microdilution test method. In addition, chemical compositions of the studied essential oils were analyzed by gas chromatography-mass spectrometry (GC-MS).

Materials and Methods

Plant materials

F. vulgare, *M. piperita, M. spicata*, *O. basilicum*, *O, onites*, *O. vulgare*, *O. majorana*, and *S. cuneifolia* Ten. were cultivated in the experimental farm belonging to Faculty of Agriculture, Selçuk University, in ecological conditions of Konya province (Turkey) in 2008. The samples were harvested at fully mature stages of the plants during the harvest season in 2008 and used in the experiments.

Tested individual compounds

The individual components tested in this study (4-allylanisol, *trans*-anethol, (–)-borneol, L-bornyl acetate, camphen, carvacrol, (–)-carvone, citral, citronellol, citronellal, cuminyl alcohol, cuminyl aldehyde, cumol, dihydrocarvone, eugenol, farnesol, geranol, geranyl ester, isoborneol, isomenthone, isopulegol, d-limonene, linalool, linalyl ester, linalyl oxide, (–)-menthol, nerol, (–)-phencone, α- and β-pinene, piperitone, γ-terpinene, thymol, and vanillin) were purchased from Carl Roth Chemical Company (Karlsruhe, Germany).

Distillation of the essential oils

The aerial parts (100 g for each) of each plant species were subjected to hydrodistillation for 3 h using Clevenger-type apparatus to obtain the essential oils. The essential oil yields (v/w%) were 1.2% for *F. vulgare*, 3.2% for *M. piperita*, 1.8% for *M. spicata*, and 0.9% for *O. basilicum*, 3.5% for *Origanum majorona*, 2.8% for

O. vulgare, 2.6% for *O. onites*, and 1.0% for *S. cuneifolia*. The essential oils were stored at −20 °C until analysis and microbiological studies.

GC-MS conditions and identification

GC analysis was performed on an Agilent 6890N Network GC system combined with an Agilent 5975C VL MSD Network Mass Selective Detector (Agilent, Santa Clara, Calif., U.S.A.). The GC conditions were regulated as follows: column: DB Waxetr (60 m × 0.25 mm × 0.25 μm); oven temperature program: the column held initially at 60 °C for 10 min after injection, then increased to 220 °C with 4 °C/min heating ramp for 10 min and increased to 240 °C with 10 °C/min heating ramp without hold; injector temperature 250 °C; carrier gas: helium; inlet pressure: 9.60 psi; linear gas velocity: 7 cm/s; initial flow: 0.3 mL/min; split ratio: 65.0:1; injection volume: 1.0 μL.

The essential oil components were identified by comparison of their retention times by comparison of their retention index (RI) to series of *n*-alkanes. Computer matching against commercial libraries including Wiley GC-MS Library, Adams Library, and MassFinder 3 Library as well as literature data reported previously for the essential oils studied herein were also used for the identification.

Microbiological studies

Preparation of the test materials. All of the essential oils and the components were prepared in dimethylsulfoxide (DMSO) at a final concentration of 256 μg/mL and sterilized by filtration using 0.22 μm Millipore (Bedford, Mass., U.S.A.) and used as the

Table 2–Chemical compositions of the essential oils of *Foeniculum vulgare* and *Ocimum basilicum*.

	Foeniculum vulgare				*Ocimum basilicum*			
Nr	Retention time (min)	Relative retention indices[a]	Compound	Composition (%)	Retention time (min)	Relative[a] retention[a] indices[a]	Compound	Composition (%)
1	10.30	1022	α-Pinene	0.57	10.31	1022	α-Pinene	0.32
2	14.00	1111	β-Phellandrene	0.05	14.02	1111	β-Pinene	0.71
3	14.61	1123	Sabinene	0.23	14.63	1124	Sabinene	0.29
4	16.56	**1163**	Myrcene	0.10	16.58	1164	Myrcene	0.49
5	16.66	**1138**	α-Phellandrene	0.04	17.36	1180	α-Terpinene	0.07
6	18.29	1165	Limonene	8.03	18.24	1197	Limonene	0.25
7	18.60	1200	1,8-Cineole	0.34	18.67	1203	1,8-Cineole	7.73
8	18.67	1203	γ-Terpinene	0.04	19.23	1210	2-*E*-Hexanal	0.05
9	19.81	1217	(*Z*)-β-Ocimene	0.45	19.83	1217	(*Z*)-β-Ocimene	0.05
10	20.33	1223	Tricyclene	0.10	20.35	1224	γ-Terpinene	0.15
11	20.56	1226	3-Methyl-brendene	0.04	20.58	1226	(*E*)-β-Ocimene	0.28
12	21.46	1237	*o*-Cymene	0.26	21.97	1243	Terpinolene	0.11
13	21.63	1239	Isoamyl-2-methyl-butyrate	0.03	28.09	1439	*cis*-Linalool oxide	0.10
14	21.95	1243	α-Terpinolene	0.03	29.1	1468	*trans*-Linalool oxide	0.16
15	22.36	1248	Isopentyl isopentanoate	0.03	29.69	1485	*Z*-Myroxide	0.04
16	26.62	1298	Fenchone	1.96	30.03	1495	α-Copaene	0.09
17	28.35	1447	*cis*-Limonene oxide	0.03	30.95	1524	Camphor	0.30
18	28.79	1459	*Trans*-Limonene oxide	0.09	**31.68**	**1547**	**Linalool**	**75.94**
19	29.96	1493	Fencyl acetate	0.21	32.85	1584	Elemene	0.07
20	30.93	1523	Camphor	0.06	37.97	1762	α-Fenchyl acetate	0.42
21	34.22	1630	*p*-Mentha-1,5-dien-8-ol	0.05	33.20	1595	β-Elemene	1.22
22	35.50	1674	*cis*-*p*-Mentha-2,8-dien-1-ol	0.09	33.51	1605	Terpinen-4-ol	0.86
23	35.70	1681	Estragole	5.18	34.77	1648	*trans*-Muurola-3,5-diene	0.10
24	36.02	1692	1,8-Menthadien-4-ol	0.03	35.26	1665	(*E*)-β-Farnesene	0.04
25	36.29	1701	α-Terpineol	0.04	35.57	1676	δ-Terpineol	0.18
26	36.91	1723	Germacrene D	0.05	35.69	1680	Estragole	0.09
27	37.64	1750	Carvone	0.15	35.78	1683	*trans*-Muurola-1,5-diene	0.45
28	38.19	1770	*cis*-Anethole	0.11	35.95	1689	Neral	0.09
29	**40.36**	**1849**	***trans*-Anethol**	**80.73**	36.31	1702	α-Terpineol	0.82
30	45.49	2049	*p*-Anisaldehyde	0.88	36.63	1713	β-Chamigrene	0.08
31					36.92	1724	Germacrene D	1.45
32					37.02	1727	δ-Guagine	0.51
33					37.24	1735	Allo-Aromadendrene	0.09
34					37.39	1741	Geranial	0.12
35					37.61	1749	Bicyclogermacrene	0.20
36					37.89	1759	Geranyl propanotate	0.20
37					38.18	1769	δ-Amorphene	0.11
38					38.32	1775	γ-Cadinene	0.74
39					39.63	1822	Nerol	0.10
40					40.33	1848	*cis*-Calamenene	0.05
41					40.77	1864	Geraniol	2.40
42					44.69	2016	α-Himachalene	0.07
43					45.23	2038	*E*-Nerolidol	0.08
44					46.11	2074	1,10-Di-*epi*-cubenol	0.24
45					48.63	2182	Eugenol	0.61
46					48.72	2186	*Tau*-Cadinol	1.37
47					50.11	2246	α-Cadinol	0.09
				Total	**99.99**		Total	**99.99**

[a]Relative retention indices calculated against *n*-alkanes (c8 to c26).

stock solutions. Reference antibacterial agents of ampicilin (AMP; Fako, Istanbul, Turkey) and ofloxacin (OFX; Hoechst Marion Roussel) were obtained from their respective manufacturers and dissolved in a phosphate buffer solution (ampicilin; pH: 8.0, 0.1 mol/L), and in water (ofloxacin). The stock solutions of these agents were prepared in medium according to CLSI (Clinical and Laboratory Standards Institute; formerly National Committee for Clinical Laboratory Standards) recommendations.

Microorganisms and inoculum preparation. Isolated strains of *K. pneumoniae* that are resistant to trimetoprime-sulphametoxazole (SXT; Oxoid, Hoechst Marion Roussel, Kansas City, Mo., U.S.A.; 25 μg; $\leq$ 10 mm), sulbactam-ampicilin (SAM; Oxoid; 20 μg; $\leq$ 11 mm), clavulonate-amoxicilin (AMC; Oxoid; 20 μg; $\leq$ 13 mm), ceftriaxon (CRO; Oxoid; 30 μg; $\leq$ 25 mm), cefepime (CPM; Oxoid; 30 μg; $\leq$ 14 mm), imipenem (IMP; Oxoid;10 μg; $\leq$ 13 mm), ceftazidime (CAZ; Oxoid; 30 μg; $\leq$ 14 mm), tobramicine (TOB; Oxoid; 10 μg; $\leq$ 12 mm), gentamicine (GM; Oxoid; 10 μg; $\leq$ 12 mm), ofloxacin (OFX; Oxoid 5 μg; $\leq$ 12 mm), and ciprofloxacin (CIP; Oxoid 5 μg; $\leq$ 13 mm) in the disk diffusion test were used for determination of antibacterial activity as MIC (minimum inhibition concentration). *K. pneumoniae* RSKK 574 (Refik Saydam Central Hygiene Institute-Culture Collection, The Ministry of Health of Republic of Turkey, Ankara) was used as the control strain.

Mueller Hinton Broth (MHB; Oxoid) and Mueller Hinton Agar (MHA; Oxoid) were applied for growing and diluting of the bacteria suspensions. The microorganism suspensions used for inoculation were prepared at 10^7 cfu (colony forming units)/mL by diluting fresh cultures at McFarland 0.5 density (10^8 cfu/mL). Suspensions of all bacteria were added in each well of the diluted extracts density of 10^5 cfu/mL (Clinical and Laboratory Standards Institute, CLSI 2008).

Antibacterial activity test. The microdilution method was employed for the antibacterial tests. Media were placed into each 96 wells of the microplates. Solutions of the essential oils and components at 256 μg/mL were added into first rows of microplates and 2-fold dilutions of the compounds (128 to 0.0312 μg/mL) were made by dispensing the solutions to the remaining wells. Culture suspensions (10 μL) were inoculated into all the wells. The sealed microplates were incubated at 35 °C for 18 h. The lowest concentration of the test samples that completely inhibited macroscopic growth was determined and MICs were reported (Ozcelik and others 2006).

Table 3–Chemical compositions of the essential oils of *Origanum majorana* and *Origanum onites*.

	Origanum majorana				*Origanum onites*			
Nr	Retention time (min)	Relative retention indices[a]	Compound	Composition (%)	Retention time (min)	Relative retention indices[a]	Compound	Composition (%)
1	10.38	1024	α-Pinene	0.63	10.30	1022	α-Pinene	0.27
2	10.57	1028	α-Thujene	1.48	10.46	1026	α-Thujene	0.66
3	12.19	1069	Camphene	0.09	12.16	1068	Camphene	0.18
4	14.05	1112	β-Pinene	0.16	14.00	1111	β-Pinene	0.10
5	14.67	1125	Sabinene	0.17	15.89	1150	δ-3-Carene	0.03
6	15.94	1151	γ-Terpinene	0.08	16.58	1164	β-Myrcene	1.86
7	16.72	1166	Myrcene	2.01	16.68	1166	α-Phellandrene	0.25
8	16.81	1168	α-Phellandrene	0.17	17.37	1180	α-Terpinene	2.07
9	16.94	1171	Pseudo-Limonene	0.01	18.22	1198	Limonene	0.29
10	17.48	1182	δ-2-Carene	1.36	18.67	1203	Sabinene	0.15
11	18.27	1198	Limonene	0.16	19.21	1210	2(*E*)-Hexanal	0.02
12	18.65	1203	1,8-Cineole	0.14	19.83	1217	Tricyclene	1.22
13	18.73	1204	β-Phellandrene	0.22	20.45	1225	γ-Terpinene	14.98
14	19.89	1218	(*Z*)-β-Ocimene	0.12	20.59	1226	(*E*)-β-Ocimene	0.09
15	20.62	1227	γ-Terpinene	6.90	21.51	1237	*p*-Cymene	4.13
16	21.65	1239	*o*-Cymene	2.52	21.96	1243	Terpinolene	0.05
17	22.01	1243	Terpinolene	0.05	28.83	1461	Sabinene hydrate	0.73
18	28.36	1447	1-Octen-3-ol	0.18	31.48	1540	Linalool	0.39
19	28.89	1462	*cis*-Sabinenehydrate	1.08	31.70	1547	*trans*-Sabinene	0.31
20	29.53	1481	Bicycloelemene	0.02	31.88	1553	Linalool butanoate	0.21
21	31.53	1542	Linalool	0.03	32.18	1563	*cis*-Sabinene hydrate	0.02
22	31.72	1548	*trans*-Sabinene hydrate	0.09	32.35	1568	Fenchone	0.04
23	33.53	1606	(*E*)-Caryopylene	0.77	33.51	1605	*trans*-Caryophyllene	1.77
24	35.74	1682	α-Humulene	0.03	35.75	1682	α-Humulene	0.04
25	36.34	1714	α-Terpineol	0.43	36.30	1702	Linalyl propionate	0.16
26	36.89	1723	Borneol	0.20	36.90	1723	Germacrene D	0.08
27	37.62	1749	Bicyclogermacrene	0.56	37.05	1729	Borneol	0.78
28	38.06	1766	Carvone	0.06	37.18	1733	β-Bisabolone	0.56
29	40.83	1866	Geraniol	0.01	37.62	1749	Bicyclogermacrene	0.19
30	41.74	1900	Spiro-(4,4)-nonene	0.04	37.90	1759	Geranyl acetate	0.05
31	45.04	2030	Caryophylene oxide	0.03	41.28	1883	Carvacrol acetate	0.19
32	45.53	2050	(*E*)-Nerolidol	0.02	44.49	2007	Caryophyllene oxide	0.15
33	46.20	2078	Endo-1-bourbonanol	0.03	47.70	2142	Spathulenol	0.08
34	46.82	2104	Elemol	0.05	48.38	2172	Thymol acetate	0.07
35	46.08	2073	Spathulenol	0.09	48.63	2182	(*E*)-Isoeugenol	0.13
36	48.89	2193	Tymol	0.43	48.81	2190	Thymol	0.28
37	**49.90**	**2237**	**Carvacrol**	**79.58**	49.25	2209	3-Methyl-4-isopropylphenol	0.04
38	50.39	2259	β-Eudesmol	0.02	**49.61**	**2225**	**Carvacrol**	**67.35**
			Total	**99.70**			Total	**99.96**

[a]Relative retention indices calculated against *n*-alkanes (c8 to c26).

Results and Discussion

Inhibitory effects of the essential oils and the essential oil components

Inhibitory effects of the essential oils and the individual components on tested strains of ESBL-producing *K. pneumoniae* are presented in Table 1. When compared with the references (ampicilin and ofloxacin), all of the test samples showed a remarkable activity at the equal level against the isolated strains of all *K. pneumoniae* at 32 and 64 μg/mL concentrations, which are close to the inhibitory concentrations exhibited by references.

Table 4–Chemical compositions of the essential oils of *Origanum vulgare* and *Satureja cuneifolia*.

	Origanum vulgare				*Satureja cuneifolia*			
Nr	Retention time (min)	Relative retention indices[a]	Compound	Composition (%)	Retention time (min)	Relative retention indices[a]	Compound	Composition (%)
1	10.36	1023	α-Pinene	0.37	10.31	1022	α-Pinene	0.74
2	10.54	1028	α-Thujene	0.72	10.46	1026	α-Thujene	0.69
3	12.19	1069	Camphene	0.13	12.16	1068	Camphene	0.63
4	12.98	1088	Caproaldehyde	0.02	14.00	1111	β-Pinene	0.14
5	14.05	1112	β-Pinene	0.10	14.61	1123	Sabinene	0.03
6	14.66	1124	Sabinene	0.05	15.09	1133	2,4(10)-Thujadien	0.09
7	15.16	1135	Sabinyl acetate	0.02	15.89	1150	γ-Terpinene	0.05
8	15.94	1151	Tricyclene	0.06	16.57	1163	Myrcene	0.89
9	16.22	1156	3-Heptanone	0.01	16.67	1165	γ-Phellandrene	0.09
10	16.69	1166	β-myrcene	1.33	17.35	1179	δ-2-Carene	1.05
11	16.78	1168	α-Phellandrene	0.10	18.22	1197	Limonene	0.72
12	16.93	1171	Pseudolimonene	0.001	18.59	1203	1,8-Cineole	0.46
13	17.45	1181	δ-2-Carene	0.87	19.22	1210	2*E*-Hexanal	0.12
14	18.27	1198	Limonene	0.14	19.83	1217	(*Z*)-β-Ocimene	0.99
15	18.72	1204	β-Phellandrene	0.16	20.38	1224	γ-Terpinene	4.67
16	19.3	1211	2*E*-Hexanal	0.20	20.59	1226	(*E*)-β-Ocimene	0.61
17	20.61	1227	γ-Terpinene	6.03	21.59	1238	*o*-Cymene	22.27
18	20.66	1228	β-Ocimene	0.05	21.96	1243	Terpinolene	0.09
19	20.80	1229	3-Octanone	0.11	26.34	1295	Nonanal	0.03
20	21.65	1239	*o*-Cymene	2.81	27.24	1415	Thujol	0.09
21	22.02	1244	Terpinolene	0.05	28.06	1438	*p*-Cymene	0.25
22	26.37	1295	3-Octanol	0.07	28.42	1449	1-Octen-3-ol	0.31
23	28.36	1447	1-Octen-3-ol	0.26	28.84	1461	*trans*-Sabinene hydrate	0.42
24	28.88	1462	*cis*-Sabinene hydrate	0.68	29.09	1463	Furanoid	0.11
25	31.54	1542	Linalool	0.10	30.06	1496	Thujol	0.06
26	31.72	1548	*trans*-Sabinene hydrate	0.18	30.94	1523	Camphor	0.02
27	32.89	1585	Bornyl Acetate	0.08	31.49	1541	Linalool	3.03
28	33.60	1608	Caryophyllene	2.20	31.70	1548	*cis*-Sabinene hydrate	0.12
29	33.90	1619	*cis*-Dihydro carvone	0.01	32.18	1563	*cis*-*p*-Menth-2-en-1-ol	0.09
30	34.74	1648	*trans*-Dihydro carvone	0.08	33.50	1605	Caryophyllene	2.03
31	35.75	1682	α-Humulene	0.09	33.86	1617	*cis*-Dihydrocarvone	0.19
32	36.33	1702	α-Terpineol	0.16	34.20	1629	*trans*-Ocimene	0.03
33	36.86	1722	Borneol	0.41	34.47	1638	*trans*-Dihdrocarvone	0.15
34	37.19	1734	β-Bisabolone	0.22	35.49	1673	Tricyclo(4,4,0,0(2,8))dec-4-ene	0.13
35	37.67	1751	Bicyclogermacrene	0.13	35.74	1682	α-Humulene	0.04
36	38.03	1764	Carvone	0.09	36.29	1701	α-Terpineol	0.27
37	38.97	1798	Methyl salicylate	0.04	36.45	1707	Viridiflorene	0.04
38	39.56	1820	*cis*-Epoxy-Ocimene	0.05	37.02	1727	Borneol	2.40
39	40.62	1859	*p*-Cymen-8-ol	0.03	37.19	1734	β-Bisabolone	1.44
40	41.46	1890	Carvacrol acetate	0.06	37.62	1749	Bicyclogermacrene	0.28
41	41.73	1900	2,5-Dimethyl-3-vinyl-hexa-1,4-diene	0.04	38.17	1769	*trans*-Cadina-1(6),4-diene	0.10
42	42.91	1946	*o*-Isopropylphenetole	0.03	38.31	1774	δ-Cadinene	0.05
43	43.31	1961	*Z*-Jasmone	0.01	38.48	1780	*Z*(α)Bisabolone	0.07
44	43.55	1970	7-Amino-3-methylpyrimido(4,5) pyridozin-5(6H)-one	0.01	39.03	1800	Cumin aldehyde	0.19
45	43.74	1978	(*E*)-Jasmone	0.02	40.54	1856	*p*-Cymene-8-ol	0.76
46	45.01	2029	Caryophyllene oxide	0.08	40.66	1860	Thymol acetate	0.08
47	46.48	2090	1,10-Di-*epi*-cubenol	0.02	42.84	1943	α-Thujaplicin	0.12
48	47.36	2127	Cumin alcohol	0.02	44.20	1996	Caryophyllene oxide	0.05
49	48.06	2157	Spathulenol	0.04	44.35	2002	Shisofuran	0.13
50	48.89	2193	Thymol	0.24	44.48	2007	Caryophyllene oxide	0.89
51	**49.89**	**2237**	**Carvacrol**	**81.25**	47.40	2129	Cumin alcohol	0.30
52	52.37	2340	Pyrazine-2,3-diethyl-5-methyl	0.01	47.68	2141	Spathulenol	0.41
53	56.65	2462	Heneicosone	0.01	**48.85**	**2191**	**Thymol**	**41.66**
54					49.54	2221	Carvacrol	9.34
			Total	**99.90**			Total	**99.20**

[a]Relative retention indices calculated against *n*-alkanes (c8 to c26).

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Table 5–Chemical compositions of the essential oils of *Mentha piperita* and *Mentha spicata*.

	Mentha piperita				*Mentha spicata*			
Nr	Retention time) (min)	Relative retention indices[a]	Compound	Composition (%)	Retention time (min)	Relative retention indices[a]	Compound	Composition (%)
1	10.36	1023	α-Pinene	0.54	10.38	1024	α-Pinene	0.64
2	10.45	1025	α-Thujene	0.07	10.51	1027	α-Thujene	0.02
3	11.12	1042	2,5-Diethyletrahydrofuran	0.01	14.10	1113	β-Pinene	0.08
4	12.21	1069	Camphene	0.02	14.70	1125	Sabinene	0.46
5	12.98	1088	Hexanal	0.004	16.67	1165	Myrcene	0.69
6	14.09	1113	β-Pinene	0.83	16.94	1171	Pseudo-limonene	0.02
7	14.68	1125	Sabinene	0.42	17.41	1181	δ-2-Carene	0.04
8	16.63	1165	Myrcene	0.27	18.54	1202	Limonene	7.84
9	16.92	1171	Pseudo-limonene	0.004	18.83	1205	1,8-Cineole	2.07
10	17.40	1180	δ-2-Carene	0.13	19.14	1209	1,3,8-*p*-Menthatriene	0.04
11	18.34	1200	1,8-Cineole	3.83	19.34	1212	2*E*-Hexanal	0.37
12	19.31	1211	2*E*-Hexanal	0.16	19.90	1218	(*Z*)-β-Ocimene	0.31
13	19.88	1218	(*Z*)-β-Ocimene	0.24	20.40	1224	γ-Terpinene	0.06
14	20.39	1224	γ-Terpinolene	0.26	20.63	1227	(*E*)-β-Ocimene	0.11
15	20.62	1227	(*E*)-β-Ocimene	0.06	20.77	1229	3-Octanone	0.02
16	21.52	1238	*o*-Cymene	0.02	21.53	1238	*o*-Cymene	0.005
17	22.00	1243	Terpinolene	0.08	22.00	1243	Terpinolene	0.04
18	22.46	1249	*n*-Amyl isovalerate	0.08	24.07	1268	3-Octanol acetate	0.14
19	22.79	1253	Vinyl amyl ketone	0.08	26.38	1296	3-Octanol	1.16
20	26.60	1298	3-Octanol	0.12	27.93	1435	1,3,8-*p*-Menthatriene	0.009
21	28.60	1454	1-Octen-3-ol	0.08	28.13	1440	*p*-Cymene	0.02
22	**29.71**	**1486**	**Isomenthone**	**50.08**	28.88	1462	*trans*-Sabinene hydrate	0.16
23	29.84	1489	*cis*-3-Hexenyl isovalerate	0.05	29.08	1468	Isomenthone	0.16
24	30.08	1496	Menthofuran	5.06	29.69	1485	*cis*-3-Hexenyl isovalerate	0.09
25	30.40	1506	*p*-Menthone	4.19	30.05	1495	(*S*)-3-Nonanol	0.11
26	30.99	1525	β-Bourbonene	0.06	30.98	1525	β-Bourbonene	0.66
27	31.22	1532	3-*p*-Menthone	0.02	31.56	1543	Linalool	0.37
28	31.58	1543	Linalool	0.19	32.84	1583	Isopulegone	0.12
29	31.76	1549	*trans*-Sabinene hydrate	0.09	33.80	1615	Caryophyllene	1.08
30	32.04	1558	Bicyclo (3,1,1)hepta-3-one-2,6,6-trimethyl	0.04	34.05	1624	*cis*-Hydrocarvone	4.08
31	32.32	1567	Menthyl acetate	0.66	34.31	1633	Dihydrocarvone	0.02
32	32.86	1584	Isopulegone	0.25	34.56	1641	*trans*-Dihydro carvone	0.48
33	33.19	1594	*cis*-Isopulegone	0.07	35.07	1659	*trans*-Muurola-3,5-diene	0.24
34	33.59	1608	*trans*-Caryophyllene	1.81	35.70	1681	(*E*)-2,6-Dimethyl-5, 7-octodien-2-ol	0.23
35	34.45	1637	Isomenthol	0.11	35.89	1687	Isodihydrocarveol acetate	0.23
36	34.99	1656	Menthol	21.77	36.18	1697	*trans*-Muurola-4(14),5-diene	0.27
37	35.20	1663	Pulegone	2.19	36.68	1715	α-Terpineol	0.16
38	35.40	1670	*trans*-β-Farnesene	0.28	**38.34**	**1775**	**Carvone**	**75.07**
39	35.70	1681	δ-Terpineol	0.20	38.53	1782	α-Copaene	0.11
40	35.98	1690	α-Caryophyllene	0.06	39.10	1803	*cis*-Dihydroagorofuran	0.02
41	36.41	1705	Linalyl propionate	0.17	39.28	1809	α-Muurolene	0.06
42	36.60	1712	*trans*-Sabinol	0.08	39.50	1818	*cis*-Carvone oxide	0.08
43	37.09	1730	Germacrene D	1.40	39.69	1825	2,6-Dimethyl-3,5,7-octa-triene-2-ol	0.02
44	37.62	1749	Piperitone	0.99	40.36	1849	*cis*-Calamene	0.26
45	38.20	1770	δ-Cadiene	0.03	40.53	1855	*trans*-Carvone oxide	0.14
46	39.34	1812	Mrytenol	0.06	40.71	1863	Neryl acetone	0.02
47	39.89	1832	*p*-Ment-1-en-9-ol acetate	0.01	41.20	1880	*cis*-Carveol	0.20
48	40.01	1836	1(7),5,8-*o*-Menthadien	0.01	42.84	1943	*E*-Jasmone	0.02
49	40.69	1861	Isopiperitenone	0.02	43.07	1952	5-Dodecen-7-en	0.01
50	40.89	1869	Limonen-10-yl acetate	0.05	43.32	1961	*cis*-Jasmone	0.47
51	43.29	1960	*cis*-Jasmone	0.06	44.47	2007	Caryophyllene oxide	0.03
52	43.72	1977	Mentha-1,4,8-triene	0.07	45.64	2055	3-Ethenyl cyclooctene	0.03
53	45.85	2064	Germacrene-D-4-ol	0.01	45.85	2064	Germacrene D-4-ol	0.03
54	46.76	2101	Viridiflorol	0.33	46.12	2075	1,10-Di-epi-cubenol	0.10
55	48.70	2185	(*E*)-Isoeugenol	0.03	46.75	2101	Viridiflorol	0.01
56	49.07	2201	α-Cadinol	0.01	47.64	2139	Spathulenol	0.03
57	50.11	2246	*trans*-Muurdol	0.02	50.11	2247	α-Cadinol	0.07
58					51.46	2305	β-Copaen-4α-ol	0.01
			Total	**97.86**			Total	**99.38**

[a]Relative retention indices calculated against *n*-alkanes (c8 to c26).

Chemical compositions of the essential oils analyzed by GC-MS

Chemical compositions of the essential oils studied herein were identified by GC-MS (Table 2 to 5). The major components of the essential oils *F. vulgare*, *M. piperita*, *M. spicata*, *O. basilicum*, *O. majorana*, *O. onites*, and *O. vulgare* were found to be *trans*-anethol (80.73%), isomenthone (50.08%), carvone (75.05%), linalool (75.94%), and carvacrol (79.58%, 67.35%, and 81.25%), respectively. Thymol was dominant in the essential oil of *S. cuneifolia*.

Penicillins as well as cephalosporins are called "β-lactam antibiotics" and are characterized by 3 fundamental structural requirements: the fused β-lactam structure, a free carboxyl acid group, and one or more substituted amino acid side chains. An increase of bacterial strains resistant to β-lactams, especially to the 3rd-generation cephalosporins, has been observed in many countries because of the production of β-lactamases, the enzymes responsible for the destruction of the cyclic amide bond of β-lactams. Essential oils are employed in many products, for instance in foodstuffs, pharmaceuticals, and cosmetics and also well known to display strong antimicrobial activity. The essential oils screened in our study had the same level of inhibition against K_3, K_5, K_6, and K_{10} at 32 μg/mL as ampicilin and oflaxocin, while they were also comparatively active toward rest of the strains at 64 μg/mL. In contrast, the essential oils and the common essential oil components showed a greater inhibition against the reference strain of *K. pneumoniae* (RSKK 574) at 8 μg/mL.

Although there have been abundant studies on anti-*Klebsiella* activity of essential oils and plant extracts (Hammer and others 1999; Rasooli and Mirmostafa 2003; Marques and others 2004; Mirzana and Nada 2004; Pereira and others 2004; Prabuseenivasan and others 2006; Rafii and Shahverdi 2007), we have encountered only a few studies on the inhibitory activity of essential oils against ESBL-producing *K. pneumoniae* strains. Si and others (2008) studied the antibacterial effect of the oregano essential oil ESBL-producing *E. coli* and their data indicated that multiple drug-resistant *E. coli* was very sensitive to the oregano essential oil and polymycin having MIC values at 0.5 and 0.8 μg/mL, respectively. This finding showed that the oregano essential oil was found to be more effective than the reference (polymycin).

The plant species studied herein have been reported to contain many flavonoid derivatives (Hawas and others 2008; Mimika-Dukic and Bozin 2008; Kaur and Arora 2009; Dorman and Hiltunen 2010). It should also be mentioned about a previous study on the antibacterial activity of several flavonoid derivatives along with some antibiotics against 20 ESBL-producing *K. pneumoniae* isolates, which were susceptible to imipenem and cefmetazole, but were resistant to ampicillin, ampicillin/sulbactam, aztreonam, cefazolin, cefoperazone, cefotaxime, ceftazidime, ceftriaxone, cefuroxime, piperacillin, and ticarcillin (Lin and others 2005). Among the tested flavonoids, myricetin, a flavonol derivative, was found to inhibit ESBL-producing *K. pneumoniae* isolates at a high MIC at 256 μg/mL, but exhibited a significant synergic activity against ESBL-producing *K. pneumoniae* in separate combination with amoxicillin/clavulanate, ampicillin/sulbactam, and cefoxitin. Accordingly, it might be considered that their flavonoid contents might be contributing to their remarkable inhibitory effects partly toward ESBL-producing *K. pneumoniae* strains.

Among the essential oil components, citral, geraniol, and menthol were found to inhibit Gram-negative bacteria well, in particular (Pauli 2003). According to the same report, microbiological inhibitory data of substances mainly found in essential oils revealed that only a small number of compounds have ability to inhibit bacteria and monoterpenes, typical constituents of essential oils. Our *in vitro* results demonstrated that the essential oils and components widely found in essential oils as given in Table 1 possess a notable inhibition equivalent to each other. It is well known that, since the herbal remedies usually consist of a multicomponent mixture, this situation also represents a precondition for interactions such as synergism or antagonism (Iten and others 2009). Therefore, this could be the case for the essential oils screened here which is the fact that synergistic interaction(s) among essential oil components may be taking place as none of them are dominantly active.

Conclusion

In conclusion, this study describes the inhibitory effects of a number of essential oils and their common components against ESBL-producing *K. pneumoniae* strains. In the literature survey, only a few reports performed on inhibitory activity of any natural products or plant extract against ESBL-producing *K. pneumoniae* strains were available. Therefore, we describe the first study on ESBL-producing *K. pneumoniae* inhibitory activity of the above-mentioned essential oils and the common essential oil components. Our results illustrated that natural products could be the notable inhibitors of ESBL-producing Gram-negative strains and more attention should be paid to natural products to discover lead compounds in combat to ESBL-producing microorganism strains.

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