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Antimicrobial activities of methanol extracts and essential oils of *Rosmarinus officinalis*, depending on location and seasonal variations

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Abstract

Rosmarinus officinalis is widely found in the lands of Aegean and Mediterranean regions of Turkey. The goal of this work was to test the antimicrobial activity of the essential oils and methanolic extracts of *R. officinalis* collected from three different regions at four different time intervals of the year against *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Enterococcus feacalis*, *Escherichia coli*, *Staphylococcus epidermidis*, *Bacillus subtilis* and *Candida albicans*. Essential oils were obtained from the aerial parts of the plant by using a Clevenger apparatus, for 4 h. After distillation, the distillates were filtered, air-dried and then extracted by using a Soxhlet apparatus for 9 h to obtain the methanolic extracts. The antimicrobial activities of the methanolic extracts were tested by the disc diffusion technique. The antimicrobial activities of the essential oils obtained from *R. officinalis* were determined by minimum inhibitory concentration (MIC).The results indicated that the tested bacteria were sensitive to the essential oils and partially to the methanolic extracts. The antimicrobial activities of the essential oils against the tested bacteria differed, depending on location and seasonal variations.

Keywords: *Rosmarinus officinalis*; Antimicrobial activity; Essential oil; GC–MS

1. Introduction

The presence and growth of microorganisms in food may cause spoilage and result in a reduction in quality and quantity (Soliman & Badeaa, 2002). One of the two mechanisms determining how food-borne diseases are primarily caused, is by infection as a consequence of consuming foods contaminated with the growth of pathogenic microorganisms, such as bacteria, mould, viruses and parasites (Vattem, Lin, Labbe, & Shetty, 2004). In addition to passive transfer of pathogens to food, active growth of a pathogen may also occur in foods, for instance because of improper storage, which leads to marked increases in microbial load (Madigan, Martinko, & Parker, 1997). For these reasons, microbial contamination of food still poses important public health and economic concerns for the human society. Plant secondary metabolites, such as essential oils and plant extracts (Tepe et al., 2004), are studied for their antimicrobial activities and most essential oils derived from plants are known to possess insecticidal, antifungal, acaricidal, antibacterial and cytotoxic activities (Faleiro, Miguel, Guerrero, & Brito, 1999). Therefore, they are intensely screened and applied in the fields of pharmacology, pharmaceutical botany, medical and clinical microbiology, phytopathology and food preservation (Daferera, Ziogas, & Polissiou, 2000). Recently, many studies have focussed on the biological and antimicrobial properties of the essential oils derived from *R. officinalis* species and their main constituents (Daferera et al., 2000; Faleiro et al., 1999; Koschier & Sedy, 2003; Ohno et al., 2003). In an

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attempt to identify biologically active components in the essential oils of *R. officinalis* (known as rosemary), widely used in folk medicine, cosmetics, phytocosmetics (Pintore et al., 2002) and the flavouring of food products, we carried out a study of the antimicrobial activities of the essential oils and methanolic extracts. To our knowledge, antimicrobial activities of essential oils, together with methanolic extracts of *R. officinalis*, have not been reported to date.

2. Materials and methods

2.1. Plant material

R. officinalis specimens were collected from three different locations, namely, Canakkale (south part of Marmara region), Izmir (Aegean region) and Mersin (east part of Mediterranean region) at four different time intervals, namely, December 2003, March, June and September 2004. The specimens were dried at 30 °C in a conventional oven and stored in the cold room of Ege University Science and Technology Center.

2.2. Distillation of essential oil

The dried aerial parts were ground prior to the operation and then 100 g of ground rosemary were submitted to water distillation for 4 h using a Clevenger apparatus. The distilled essential oils were dried over anhydrous sodium sulfate, filtered and stored at +4 °C.

2.3. Analysis conditions

2.3.1. GC analysis conditions

The GC analysis was carried out with a Hewlett–Packard HP6890, equipped with a HP-Innowax silica capillary column (60 m × 0.25 mm ∅, film thickness 0.25 μm) and a flame ionisation detector. Nitrogen was used as the carrier gas with a step flow programme from 1.2 to 0.9 ml/min. Injector and detector temperatures were both set at 250 °C. Column temperature was programmed to 60 °C for 10 min, gradually increased to 220 °C at 4 °C/min, held for 10 min and then increased to 240 °C at 1 °C/min. Split ratio was 10:1 whereas split flow was 12 ml/min. One microliter of sample (dissolved in hexane as 20% v/v) was injected into the system.

2.3.2. GC/MS analysis conditions

For GC/MS analysis, a Hewlett–Packard G 1800A GCD System, equipped with a HP-Innowax silica capillary column (60 m × 0.25 mm ∅, film thickness 0.25 μm) was used. Helium was the carrier gas at a flow rate of 0.7 ml/min and the split ratio was 50:1. Mass units were monitored from 35 to 425 *m*/*z* at 70 eV. The same column temperature programme was applied as in GC analysis. The components were identified by comparing their relative retention times and mass spectra with the data from the Baser library of essential oil constituents, Wiley, MassFinder and Adams GC/MS libraries. GC and GC/MS analysis were both conducted at the Department of Pharmacy at Anadolu University.

2.3.3. Preparation of methanolic extracts

After distillation, the distillate was filtered, air-dried and then extracted by using a Soxhlet apparatus for 9 h (adapted from Chang, Ostric-Matijasevic, Hsieh, & Huang, 1977) to obtain the methanolic extracts from which the solvents were evaporated using a rotary vacuum evaporator and stored at +4 °C.

2.4. Antimicrobial activity

2.4.1. Microbial strains

The antimicrobial activity of methanolic extracts and the essential oils were individually tested against a panel of microorganisms, including *S. aureus* ATCC 6538 P, *P. vulgaris* ATCC 6897, *P. aeruginosa* ATCC 27853, *K. pneumonia* CCM 2318, *E. feacalis* ATCC 29212, *E. coli* ATCC 11230, *S. epidermidis* ATCC 12228, *B. subtilis* ATCC 6633 and *C. albicans* ATCC 10239. Bacterial strains were cultured overnight at 37 °C in Mueller Hinton agar (MHA, Oxoid). Yeast was cultured overnight at 30 °C in Sabouraud dextrose agar (SDA, Oxoid).

2.4.2. Antimicrobial activity assays

2.4.2.1. General. Two different methods were employed for the determination of antimicrobial activities: disc diffusion method for the methanol extracts and minimum inhibitory concentrations (MICs) method for the essential oils (NCCLS, 2000a, 2002). The MICs of the essential oils against the test microorganisms were determined by the broth microdilution method (NCCLS, 2000b, 2002). All tests were performed in duplicate.

2.4.2.2. Disc diffusion method of methanol extracts. The disc diffusion method was applied for the determination of antimicrobial activities of the methanol extracts (NCCLS, 2000a). Methanol extracts were dissolved in dimethyl sulfoxide (DMSO). Then geometric dilutions ranging from 125 to 15.6 mg/ml of the extracts were prepared. All the fractions were filter-sterilised using a 0.22 μm membrane filter. A suspension of the tested microorganism (0.1 ml of 10^8 cells/ml) was spread over the surface of agar plates (MHA and SDA). Filter papers having a diameter of 6 mm, soaked with 20 μl of methanol extracts were placed on the inoculated agar plates. Before incubation, all Petri dishes were kept in the refrigerator (4 °C) for 2 h. Then they were incubated at 37 °C for 24 h for bacteria and at 30 °C for 48 h for the yeast. The diameters of the inhibition zones were measured in millimeters.

2.4.2.3. Determination of minimum inhibitory concentration (MIC) of essential oils. A broth microdilution susceptibility assay was performed using NCCLS methods for the determination of the MIC (NCCLS, 2000b). All tests were

performed in Mueller Hinton broth (MHB, Oxoid) supplemented with Tween 80 detergent (0.5% (v/v)) but the test for yeast was performed in Sabouraud dextrose broth (SDB, Oxoid) again supplemented with Tween 80. Bacterial strains were cultured overnight at 37 °C in MHB and the yeast was cultured overnight at 30 °C in SDB. Geometric dilutions, ranging from 20 to 0.31 mg/ml of the essential oil, were prepared in a 96-well microtitre plate, volume being 20 μl. Then 160 μl of MHB, and the same amount of SDB for the yeast, were added onto microplates. Finally, 20 μl of 10^6 colony forming units (cfu/ml) (according to Mc Farland turbidity standards) of standardised microorganism suspensions were inoculated onto microplates and the test was performed in a volume of 200 μl. Plates were incubated at 37 °C for 24 h for bacteria and at 30 °C for 48 h for the yeast. The same tests were performed simultaneously for growth control (MHB + Tween 80 + m.o.) and sterility control (MHB + Tween 80 + test oil). Gentamycin was used as reference compound for antibacterial activities. The MIC was calculated as the highest dilution showing complete inhibition of the tested strain.

2.4.2.4. Determination of minimum bactericidal concentration (MBC) of essential oils. Referring to the results of the MIC assay, the wells showing complete absence of growth were identified and 5 μl of each well were transferred to agar plates (MBA and SDA) and incubated at previously-mentioned times and temperatures. The complete absence of growth was considered as the minimum bactericidal concentration.

3. Results and discussions

3.1. Chemical compositions and antimicrobial activities of essential oils

The compositions of essential oils, representing different locations and time intervals, were studied and it was calculated that the content of 1,8-cineole of the essential oils from Mersin (61.4%, 60.9%, 50.7% and 58.1% from December to September, respectively), were the highest among other constituents and the other two oils (Fig. 1). Pintore et al. (2002) also reported that two major types of rosemary oil can be distinguished with respect to some major constituents and that oils with over 40% of 1,8-cineole were characteristically from Morocco, Tunisia, Turkey, Greece, Yugoslavia, Italy and France. 1,8-Cineole contents of Izmir oils (27.9%, 34.3%, 14.9% and 15.5%) are higher than those of Canakkale oils (12.7, 22.7, 12.1 and 12.3). This can be explained by the climate: a very hot climate in Mersin, moderately hot in Izmir and a cool climate in Canakkale. Oils from Canakkale were richer in terms of camphor (17.0%, 24.1%, 16.1% and 16.0%) and verbenone (45.2%, 5.5%, 11.1% and 12.2%) compared to Izmir oils (camphor; 10.2%, 14.9%, 9.9% and 13.7%) (verbenone; 8.3%, 4.4%, 43.5% and 11.8%) (Fig. 1). On the

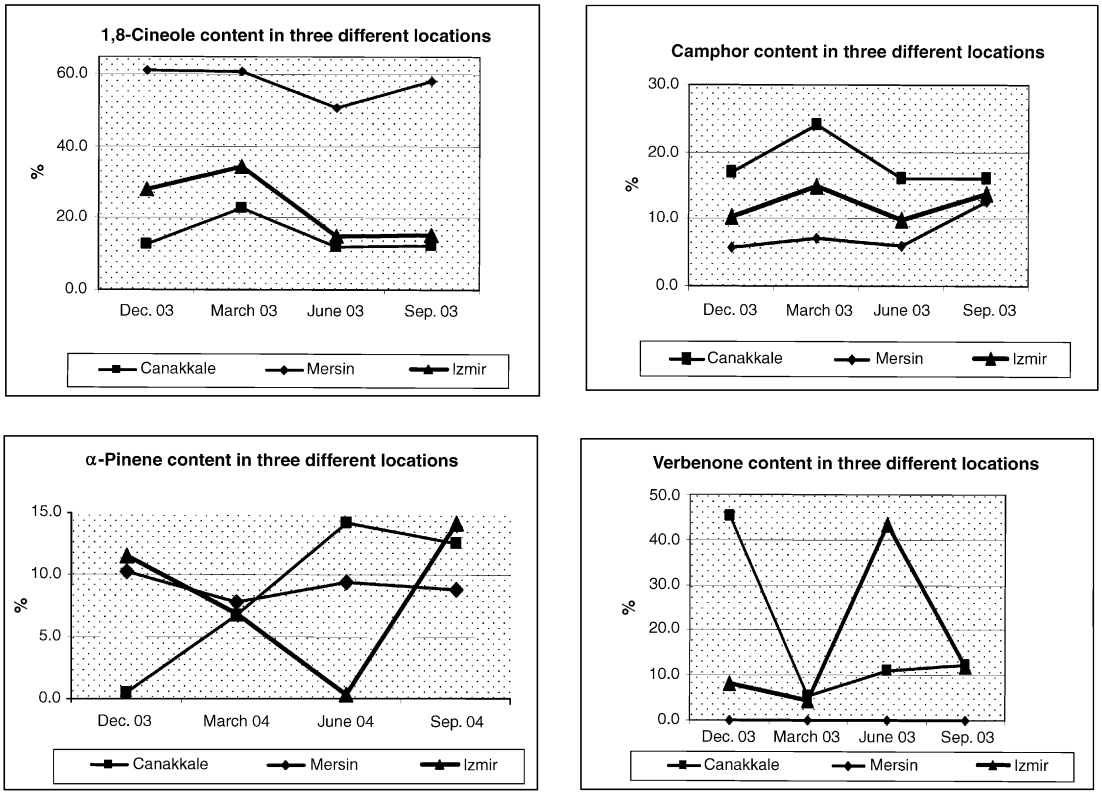

Fig. 1. Seasonal variations of 1,8-cineole, camphor, α-pinene and verbenone in the essential oils.

Table 1
Compositions of the essential oils of *Rosmarinus officinalis*[a,b]

RRI[c]	Compounds[d]	Mersin				Izmir				Canakkale			
		M1 December (%)	M2 March (%)	M3 June (%)	M4 September (%)	I1 December (%)	I2 March (%)	I3 June (%)	I4 September (%)	C1 December (%)	C2 March (%)	C3 June (%)	C4 September (%)
1014	Tricyclene	0.1	0.1	0.1	0.1	0.1	0.1	–	0.1	–	0.1	0.2	0.2
1032	α-Pinene	10.2	7.8	9.4	8.8	11.5	6.9	0.4	14.2	0.5	6.7	14.2	12.6
1072	α-Fenchene	0.2	0.1	0.1	0.1	tr	tr	0.1	0.1	–	tr	0.1	0.1
1076	Camphene	3.4	2.9	3.4	4.2	2.6	1.6	–	2.8	0.2	2.0	3.4	3.0
1118	β-Pinene	1.1	2.5	1.6	1.7	0.7	1.1	–	0.8	–	0.5	0.8	0.9
1140	Thuja-2,4(10)-diene	tr	tr	tr	tr	0.7	0.5	–	1.1	–	0.5	1.0	0.8
1159	δ-3-Carene	0.1	0.1	0.1	tr	0.8	0.6	0.1	1.3	–	0.7	1.5	0.9
1174	Myrcene	1.5	2.1	1.9	3.9	1.3	0.7	0.2	1.5	–	0.8	1.5	1.4
1187	*o*-Cymene	–	–	–	–	0.1	0.1	–	0.1	–	0.2	0.1	–
1188	α-Terpinene	0.4	0.3	0.7	0.3	0.3	–	–	0.3	–	–	0.5	0.4
1195	Dehydro-1,8-cineole	–	–	–	0.8	–	–	–	–	–	–	–	tr
1203	Limonene	2.4	tr	tr	–	2.6	tr	–	3.8	tr	tr	3.6	3.4
1213	1,8-Cineole	61.4	60.9	50.7	58.1	27.9	34.3	14.9	15.5	12.7	22.7	12.1	12.3
1246	(*Z*)-β-Ocimene	tr	tr	tr	0.4	tr	tr	tr	0.1	tr	tr	tr	0.1
1255	γ-Terpinene	0.3	0.4	1.0	0.4	0.2	0.1	–	0.4	–	0.2	0.6	0.5
1265	5-Methyl-3-heptanone	0.1	0.1	0.1	0.1	0.3	–	–	0.3	–	0.2	0.2	0.2
1266	(*E*)-β-Ocimene	–	tr	tr	tr	tr	tr	–	tr	–	–	tr	tr
1280	*p*-Cymene	2.3	1.9	1.5	2.3	2.4	1.9	–	1.6	0.4	1.6	1.1	1.0
1290	Terpinolene	0.1	0.1	0.4	0.2	0.1	0.1	–	0.6	–	0.1	0.9	0.8
1391	(*Z*)-3-Hexenol	–	tr	0.1	tr	–	tr	–	0.1	–	tr	–	0.1
1400	Nonanal	–	tr	tr	–	tr	0.1	–	0.1	–	0.1	0.2	0.1
1445	Filifolone	–	–	–	–	0.4	0.9	–	0.3	–	0.8	0.3	0.3
1450	*trans*-Linalool oxide (Furanoid)	–	–	–	–	–	–	0.3	–	0.6	–	–	–
1452	α ,*p*-Dimethylstyrene	0.1	tr	tr	0.1	0.2	tr	–	0.1	–	tr	–	0.1
1452	1-Octen-3-ol	0.2	0.4	0.3	0.4	0.6	tr	0.3	0.6	0.4	0.7	0.4	0.5
1478	*cis*-Linalool oxide (Furanoid)	–	tr	tr	tr	0.1	tr	0.4	–	0.8	tr	–	tr
1497	α-Copaene	–	tr	–	tr	0.1	0.1	–	–	–	tr	0.2	tr
1499	α-Campholene aldehyde	0.1	–	tr	tr	0.1	–	–	0.1	–	tr	–	–
1522	Chrysanthenone	–	–	–	–	0.2	tr	–	–	–	tr	0.2	tr
1532	Camphor	5.8	7.1	5.9	12.6	10.2	14.9	9.9	13.7	17.0	24.1	16.1	16.0
1553	Linalool	0.6	0.5	0.9	0.8	2.9	5.1	1.5	4.0	1.8	6.5	3.4	4.2
1562	Isopinocamphone	–	tr	–	–	1.0	2.3	0.9	1.8	1.0	3.5	2.8	2.2
1586	Pinocarvone	tr	tr	tr	tr	0.2	0.3	–	0.3	–	0.5	–	0.4
1591	Fenchyl alcohol	–	tr	–	–	–	0.1	–	–	–	tr	–	–
1597	Bornyl acetate	0.2	1.6	4.2	0.6	0.6	1.4	0.2	1.0	0.5	0.6	1.5	2.3

1611	Terpinen-4-ol	0.6	1.4	1.0	0.6	1.2	1.5	0.6	1.0	0.9	1.0	1.5	1.0
1612	β-Caryophyllene	0.2	0.6	0.8	0.3	0.6	0.3	0.2	1.0	0.1	0.8	0.8	1.5
1648	Myrtenal	tr	tr	–	–	tr	tr	–	–	–	0.1	–	–
1663	Phenylacetaldehyde	0.1	0.1	0.1	tr	tr	–	0.3	–	–	tr	–	–
1670	*trans*-Pinocarveol	tr	tr	tr	tr	0.2	0.4	–	0.1	0.3	tr	–	–
1682	δ-Terpineol	0.5	0.7	0.6	0.2	0.5	0.4	0.3	0.5	0.3	0.2	–	0.8
1683	*trans*-Verbenol	–	tr	–	–	0.3	0.5	0.7	0.3	0.4	0.4	0.6	0.3
1687	α-Humulene	tr	tr	0.1	–	–	tr	–	tr	–	0.1	–	tr
1700	*p*-Mentha-1,8-dien-4-ol (= Limonen-4-ol)	–	tr	tr	–	0.1	0.1	–	tr	–	0.1	–	tr
1706	α-Terpineol	2.6	3.0	6.8	0.9	3.3	4.0	1.9	2.2	2.0	2.9	1.8	2.0
1719	Borneol	3.9	3.7	6.8	1.6	8.7	8.3	5.3	8.1	4.5	8.3	7.8	7.4
1725	Verbenone	–	–	–	tr	8.3	4.4	43.5	11.8	45.2	5.5	11.1	12.2
1738	*p*-Mentha-1,5-dien-8-ol	–	tr	tr	–	–	0.1	0.2	–	–	–	–	–
1751	Carvone	–	tr	–	tr	0.1	0.1	–	0.2	–	0.1	–	0.3
1773	δ-Cadinene	tr	tr	tr	tr	0.2	0.1	–	0.2	–	0.2	–	0.3
1776	γ-Cadinene	tr	tr	tr	tr	0.2	0.1	–	0.1	–	0.1	–	0.1
1786	*ar*-Curcumene	–	–	tr	–	0.1	0.2	–	–	–	–	–	–
1804	Myrtenol	–	tr	tr	–	–	0.9	–	–	–	–	–	–
1805	α-Campholene alcohol	–	–	–	–	tr	0.1	–	1.1	0.1	1.2	–	1.4
1845	*trans*-Carveol	tr	–	–	–	0.6	0.1	–	0.6	–	tr	–	1.0
1853	*cis*-Calamenene	tr	tr	–	–	1.1	tr	–	0.2	–	0.1	0.9	1.7
1856	*m*-Cymen-8-ol	–	–	–	–	–	0.5	–	–	–	–	–	–
1864	*p*-Cymen-8-ol	tr	tr	tr	tr	0.2	1.0	0.9	1.2	2.0	1.6	1.7	0.4
1941	α-Calacorene	tr	tr	tr	–	0.1	tr	–	0.2	–	tr	–	0.1
1949	Piperitenone	–	–	–	–	0.3	0.1	–	tr	0.4	–	–	tr
2008	Caryophyllene oxide	0.1	0.2	0.1	tr	0.1	0.3	6.0	0.3	1.3	0.2	0.2	0.5
2030	Methyl eugenol	0.1	0.1	0.1	tr	0.2	0.2	3.8	0.2		0.2		0.3
2186	Eugenol	–	tr	tr	tr	0.1	tr	–	0.1	–	tr	–	0.2
2232	Clovenol	–	tr	–	–	0.2	tr	–	tr	–	tr	–	0.1
2324	Caryophylla-2(12),6(13)-dien-5α-ol (= Caryophylladienol II)	–	tr	–	–	0.1	tr	–	–	–	tr	0.1	–
2389	Caryophylla-2(12),6-dien-5α-ol (= Caryophyllenol I)	–	0.1	0.1	–	0.5	0.1	–	tr	0.2	0.1	0.2	0.1
2392	Caryophylla-2(12),6-dien-5β-ol	–	tr	tr	–	0.2	0.2	–	tr	–	tr	0.1	–
Total		98.7	98.8	98.9	99.1	95.7	97.2	92.9	96.2	93.3	96.3	93.7	96.2

[a] % values were calculated from GC FID data alkane series.
[b] tr; trace(<0.1%).
[c] Relative retention index calculated against.
[d] Compounds listed in the order of elution from HP6890 MS column.

Table 2
Antimicrobial activity of the essential oils of *Rosmarinus officinalis*

Microorganisms	Minimum inhibitory concentration MIC/minimum bactericidal concentration MBC (mg/ml)[a]																			
	I1		I2		I3		I4		C1		C2		C3		C4		M1		M2		M3		M4		A
Staphylococcus aureus	10	20	10	>20	10	10	10	10	5	20	10	20	20	>20	10	20	20	>20	10	20	20	>20	>20	>20	4
Proteus vulgaris	10	10	2.5	5	2.5	5	10	20	5	5	5	5	5	5	10	20	5	5	5	10	10	10	20	>20	nt
Pseudomonas aureginosa	10	10	5	10	10	10	20	20	5	5	5	5	20	20	20	20	5	10	10	10	20	20	>20	>20	8
Klebsiella pneumonia	20	20	10	20	10	10	>20	>20	10	10	10	20	20	20	20	20	20	20	20	20	20	20	>20	>20	nt
Enterococcus feacalis	10	10	5	10	2.5	2.5	>20	>20	5	5	5	5	5	5	20	>20	5	5	5	5	10	10	>20	>20	128
Escherichia coli	20	20	10	10	10	10	20	20	10	10	10	10	20	20	20	20	20	20	10	20	20	20	>20	>20	nt
Staphylococcus epidermidis	20	20	10	10	20	10	20	20	10	10	10	10	20	20	20	>20	20	20	20	20	20	>20	20	20	nt
Bacillus subtilis	20	20	5	5	5	2.5	5	20	20	2.5	5	2.5	5	10	10	20	10	20	5	5	5	10	5	>20	2
Candida albicans	5	10	2.5	5	5	2.5	5	10	10	2.5	5	2.5	5	5	5	10	10	5	20	2.5	5	5	10	10	nt

[a] I: Izmir, C: Çanakkale, M: Mersin, 1, December; 2, April; 3, June; 4: September; A: gentamycine; nt, not tested.

other hand, camphor contents of oils from Mersin were remarkably low (5.8%, 7.1%, 5.9% and 12.6%) whereas no verbenone was detected except in the September samples with a trace amount. α-Pinene contents were moderately high in Mersin oils (10.2%, 7.8%, 9.4% and 8.8%) and very similar in Izmir oils (11.5%, 6.5%, 0.4% and 14.2%) and Canakkale oils (0.5%, 6.7%, 14.2% and 12.6%) (Fig. 1) (Table 1).

Table 2 shows that the essential oils from *R. officinalis* possess a moderate antibacterial activities. *E. feacalis* and *P. vulgaris* were the most sensitive microorganisms to the essential oils from three different locations. Considering the average antimicrobial activities of essential oils from Mersin (M), Canakkale (C) and Izmir (I), it is observed that the highest activities were attained in spring, represented by samples collected in March. In respect to the locations, MIC and MBC values of Canakkale and Izmir oils were higher than the values obtained for Mersin. Although 1,8-cineole contents of Mersin oils (50–60%) are very high, the low MIC and MBC values may be related to the low content of camphor and lack of verbenone.

3.2. Antimicrobial activities of methanol extracts

In general, methanol extracts exhibited very low antimicrobial activities compared to the essential oils. The results of the antimicrobial screening showed low activity against *S. aureus* whereas the rest of the extracts were inactive against other microorganisms (data not shown).

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