Pak. j. sci. ind. res. Ser. B: biol. sci. 2016 **59**(1) 6-11

Variation in *Myrtus communis* L. Essential Oil Composition and its Antibacterial Activities Components

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(received January 29, 2015; revised May, 28, 2015; accepted June 5, 2015)

Abstract. The *Myrtus communis* L. leaves samples were collected from five locations of its native grown areas in Lattakia, Syria, during their blooming seasons (June, 2009). Essential oil (EO) extraction was carried out by hydro-distillation in a Clevenger apparatus. The EO was analysed by both gas chromatography -Flame Ionization Detector (GC-FID) and gas chromatography/mass (GC/MS) techniques. The EO yield of the dry samples was found to be around 1.88%. The main identified components of EO were: ∝-pinene 30.40%, 1,8-cineole 17.66%, limonene 8.96%, myrtenol 5.78%, and β-caryophyllene 5.00%. The bulk EO and the separated components were tested for their antibacterial activities against *Escherichia coli* O157, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Yersinia enterocolitica* O9, *Brucella melitensis*, *Proteus* spp., and *Pseudomonas aeruginosa* by using broth micro-dilution method. It was found that citronellal and nerol were the most effective components against all pathogens.

Keywords: essential oil, bacteria, minimum inhibitory concentration, susceptibility, *Myrtus communis*

Introduction

The development of microbial resistance to antibiotics is a global concern. Isolation of microorganisms less susceptible to regular antibiotics and the recovery of increased resistant isolates during antibacterial therapy is rising throughout the world which highlights the need for new principles (Khan and Rauf, 2014).

Several plant species have shown promising microbiostatic and microbiocidal activities against a range of enteric pathogenic microbionta. These have been attributed to the presence of minute doses of bioactive principles referred to as phytochemicals and terpenes which are among the most widespread and chemically diverse groups of natural products (Omojate *et al*., 2014). Since ancient ages, several diseases have been treated with plant extracts (Miyata, 2007). *Myrtus communis* (commonly known as myrtle), is one of the most important aromatic and medicinal species in the Myrtaceae family which includes more than 5650 species that are rich in essential oils (Mulas *et al*., 2002). It is widespread in the Mediterranean regions (Chalcat *et al*., 1998), typically a sub-shrub (height: 1-3 m), with white flowers (blossoming time: June to July), wildly growing in the Syrian coastal region (altitude: 0-300 m). Myrtle is known as ''Al Ass'' or ''Al rihan'' in Arabic. This species is a very aromatic plant due to the high content of essential oils in its leaves, flowers, and fruit glands (Bonjar, 2004). Several studies have focused on the antiviral (Sokmen *et al*., 2004), antifungal (Curini *et al*., 2003), antioxidant (Messaoud and Boussaid, 2011), and antimicrobial (De Laurentis *et al*., 2005) properties of myrtle extracts. The leaves contain tannins, flavonoids such as quercetin, catechin and myricetin derivatives and volatile oils (Yoshimura *et al*., 2008). The essential oil obtained from the leaves by steam distillation is also important in perfumery.

The first studies on the essential oil from aerial parts of *M. communis* were undertaken by Lawrence (1996; 1993; 1990). The large variability in the chemical composition of its essential oils has attracted many researchers and has been the subject of many studies (Wannes *et al*., 2007; Jamoussi *et al*., 2005). Chemical composition (essential oil, volatile fraction) of *M. communis* aerial parts was studied in this study from five localities in Syria. This is the first report that has been undertaken on the essential oil compositions of Syrian myrtle leaves in order to determine its effect on some locally gram negative bacteria.

Materials and Methods

Study area. The fresh young leaves samples of *M. communis* L. were collected from five locations of the

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plant's native growing areas in Lattakia city located in the coastal regions of Syria. Harvesting period was stretched for 30 days after flowering in June, 2009. The five collection locations differ in the climates and altitude conditions and details of plant collection data are given in Table 1. Young leaves samples of *M. communis* L. were cut into small pieces, shade dried for one week, then ground by special electric mill and stored separately in polyethylene bags until analysis.

Bacterial samples. Local isolates of *Escherichia coli* O157, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Yersinia enterocolitica* O9, *Brucella melitensis*, *Proteus* spp. and *Pseudomonas aeruginosa*, were freshly grown in 2YT broth (peptone, 16 g/L; yeast extract, 10 g/L; NaCl, 5 g/L; [Difco, BD, Spars, MD]) and incubated for 24-48 h. Prior to antimicrobial sensitivity test, 0.2 mL of overnight culture of each organism was dispensed into 20 mL of sterile Mueller Hinton Broth (Hi-media Laboratory Pvt. Ltd., Mumbai, India) and then incubated for about 16-24 h to standardise the cultures to approximately 10^8 CFU/mL. The bacteria were suspended in a sterile phosphate-buffered saline (PBS). Bacteria abundance in PBS was monitored by recording the optical density (OD) at 590 nm. The exact counts were assessed retrospectively by viable counts on 2YT agar plates.

Chemicals. All solvents used in the experiments (acetonitril, tetrahydrofuran, acetone) were purchased from Merck (Germany). Sodium chloride, sulphuric acid, and anhydrous sodium sulphate were obtained from Sigma–Aldrich (Germany).

Standards. Thujone (trans), α-pinene, β-myrcene, terpinen, limonene, 1.8-cineole, linalool, bornyl acetate, citronellal, eugenol, geranyl acetate, nerol, β-caryophyllene were purchased from Aldrich Company, USA.

Essential oil extraction. Essential oil was extracted by hydro-distillation over 180 min using 50 g of dried leaves in 500 mL double distilled water using a Clevenger-type apparatus. After that the essential oil was dried by filtration through anhydrous sodium sulphate, collected in tightened vials and stored at 4 °C until needed. Results were expressed on the basis of dry matter weight.

Gas chromatography (GC-FID) and mass spectrometer. Identification of the essential oil components were done using GC-FID and confirmation of the results was carried out using GC-MS. GC analysis was conducted using a 30 m column HP-5 (0.25 mm i.d 0.25 μm film thickness) with helium as the carrier gas. Fragment energy of 70 eV mass spectra were acquired by using an 70 eV ionisation voltage. Oven temperature was kept at 50 °C for 2 min, programmed to 110 °C at a rate of 2 °C/min and held isothermally for 3 min; then programmed to 175 °C at a rate of 4 °C/min and held isothermally for 2 min; programmed to 250 °C at a rate of 5 °C/min and held isothermally for 5 min. Injection mode was splitless, the injector temperature was 250 °C, and the detector temperature was 275 °C.

GC-MS conditions were (Mass range: 50–500 mass unit, source temperature 260 °C, EI 70-ev). Essential oils have been analysed using GC-FID Agilent technologies 6890 N network GC system, supported with Agilent technologies 5973 inert Mass Selective Detector (Agilent, USA) by the following analysis conditions: the column (HP-5 ms (30 m × 0.25 mm) ID 0.25 μm), carrier gas 0.9 mL/min (constant flow of Helium gas). The GC-MS system was operated by the same conditions like GC-FID. The same conditions of temperature programming were used for oil samples in order to calculate the retention index (RI). Identification of components in the oil was based on RI. Individual components were identified by comparison of both mass spectra and their GC retention data; other identifications were made by comparison of mass spectra with those in the data system libraries. The quantitative analysis of yields were determined according to reference materials and standards obtained from Aldrich. Calculations were made with the use of gas chromatography, Chem Station software, and the injection volume (1 μL). The identity of each essential oil component was determined by comparing retention time values of gas chromatography on polar columns and by comparing mass spectrum and library database (Nist and Willy).

Chromatograms were computed by the normalisation method from the GC peak areas, calculated as mean values of two injections. Essential oil components were identified by comparison of their retention times with those of pure reference standards. Quantitative data were obtained from the electronic integration of the FID peak areas.

Microdilution test. The minimum inhibitory concentrations (MICs) were determined using 96-well microtitre plates in order to determine the antibacterial activity of oils and their components against the human pathogenic bacteria. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL. Compounds to be investigated were added into Muller-Hinton Agar (MHA, Merck) with bacterial inoculum (1.0×10^4 CFU/100 μL per well). Positive

control was done with the same conditions but without essential oils. Negative control was also done with the same conditions but without adding the bacteria. The microplates were incubated for 24 h at 37 °C. The lowest concentrations without visible growth were defined as the concentrations that completely inhibited bacterial growth (MICs). The optical density of each well was measured at a wavelength of 590 nm by microplate reader (Thermo-lab Systems Reader, Finland) and compared with a blank and the positive control. Three replicates were done for each oil and for each component.

Statistical analysis. All data were reported as means ± standard deviation of three samples. Statistical analysis was performed with ANOVA.

Results and Discussion

The average essential oil in all samples was 1.88% (w/w) of the dry matter (Table 1). The results of the chemical analyses of essential oils investigated are presented in Table 2. The average of yield of *M. communis* L. oil is 1.88% (w/w) for each collection site, the main components are ∝-pinene (30.40%), 1,8-cineole (17.66%) and limonene (8.96%).

The results of antibacterial activity of essential oils are presented in Table 3. Bulk oil and all the components of *M. communis* L. oil were tested in the microdilution method. Bulk oil showed bacteriostatic activity at the concentration of (25-50 µL/mL) against *Y. enterocolitica* O9, *B. melitensis*, *Proteus* spp., *P. aeruginosa* and *S. typhimurium*; but no effect was observed against *E. coli* O157 and *K. Pneumoniae.*Tujene, γ-terpinene, nerol and myrtenol showed antibacterial activity against all bacterial strains tested in the microdilution method, MIC at 0.3-50.0 µL/mL. In contrast, α-pinene, β-myrcene, limonene, bornyl acetate, geranyl acetate, and β-caryophyllene were inactive against *Y. enterocolitica* O9, *B. melitensis*, *Proteus* spp., *P. aeruginosa*, *S. typhimurium*, *E. coli* O157 and *K. pneumoniae*.

The chemical composition of the myrtle leaf essential oils (belonging to different regions and harvested at different periods) has been widely studied (Tuberoso *et al*., 2007; De Laurentis *et al*., 2005). However, this is the first report on the essential oil composition of Syrian myrtle leaves. Thereafter, the leaf and berry essential oil compositions from various mediterranean origins have also been investigated such as in Turkey (Ozek *et al*., 2000), France (Bradesi *et al*., 1997), Italy (Pirisino *et al*., 1996), Portugal (Boelens and Jimenez, 1991), Spain (Boelens and Jimenez, 1992), 1,8-cineole (18.3%), linalool (16.3%) and myrtenyl acetate (14.5%), limonene (5.7-43.4% and 6.2-44.2%), 1, 8-cineole (5.9-26.6% and 8.7-30.40%), respectively. The present results demonstrated that Syrian *M. communis* L. consists of 14 components, which form (90.1-94.5)% of the bulk essential oil. While, the Tunisian *M. communis* L. essential oil combined of 24 components (Jamoussi *et al*., 2005) and other study carried out by Mahboubi and Ghazian Bidgoli (2010) revealed the presence of 70 components, representing 99.23% of the total myrtle essential oil. Tunisian and Algerian *M. communis* L. essential oil combined of 23 identified compounds (Ben Ghnaya *et al.,* 2013) representing 93.73% of total oil, which was found to be rich in monoterpenes hydrocarbons (53.38%) particularly α-pinene (35.30%) and α-limonènes (14.76%). Whereas, Brada *et al*. (2012) demonstrated that Algerian *M. communis* L. leaf oil was composed of 28 compounds representing 95.4% of the total composition of the oil. α-pinene was the major constituent of leaf oil at a concentration of (46.9%), followed by 1,8-cineole (25.2%). The Syrian *M. communis* L. composition characterised by its richness of monoterpene hydrocarbons in leaves, largely due to

Table 1. Collection sites, climatic conditions (year 2009), and yield of essential oils in dry matter of the collected samples

Collection sites	Altitude (m)	Latitude (°)	Longitude (°)	Climatic conditions			EO% (w/w)
				Minimum temperature a year (°C)	Maximum temperature a year (°C)	Rain fall in average/ year (mL)	
Alkirdaha	300	3536	3602	5.5	32.2	1055	1.85±0.13
Shaditi	305	3536	3603	5.3	31.7	1058	1.79±0.11
Salah Aldin	350	3536	3604	6.3	29.3	1089	1.95±0.12
Kismin	120	3541	3559	8.2	29.3	1120	1.89±0.12
Wata Alkhan	120	3541	3559	4	34.6	1135	1.92±0.14

Table 2. The essential oil constituents yields of *M. communis* L. samples for each collection site

RI	Class	Component name	The isolated component yield % (w/w)						Identification*
			Alkirdaha	Shiditi	Salah Aldin	Kismin	Wata Al khan	Average	
933	MH	α-pinene	29.50	28.70	31.40	30.30	32.10	30.40	FID-Std /MS
988	MH	β-myrcene	1.60	1.40	1.70	1.60	2.10	1.68	FID-Std /MS
1034	MH	Limonene	8.70	6.70	9.80	9.20	10.40	8.96	FID-Std /MS
1043	OM	1,8-cineole	19.20	22.20	18.40	16.30	12.20	17.66	FID-Std /MS
1063	MH	γ-Terpinen	2.80	3.30	2.50	2.40	2.30	2.66	FID-Std /MS
973	MH	Sabinene	0.80	1.20	0.70	1.10	0.90	0.94	GC-MS
1225	OM	Geraniol	1.10	0.90	0.70	1.30	0.90	0.98	GC-MS
1342	SH	β-elemene	1.30	1.10	0.90	1.20	0.70	1.04	GC-MS
1375	SH	α-humulene	1.20	0.90	0.70	1.10	0.80	0.94	GC-MS
1479	SH	Germacrene	0.90	0.70	0.60	0.80	0.60	0.72	GC-MS
1097	OM	Linalool	3.40	2.60	5.80	4.70	5.10	4.32	FID-Std /MS
1115	MH	Thujone (*trans*)	2.10	1.90	2.70	2.20	2.90	2.36	FID-Std /MS
1233	OM	Citronellal	1.80	2.10	2.30	2.20	2.70	2.22	FID-Std /MS
1124	OM	Nerol	1.90	2.40	2.60	2.30	3.10	2.46	FID-Std /MS
1289	OM	Bornyl acetate	2.20	2.10	2.70	2.60	2.90	2.50	FID-Std /MS
1273	OM	Geranyl acetate	3.10	2.40	3.40	3.60	3.80	3.26	FID-Std /MS
1143	OM	Eugenol	3.30	3.50	3.70	3.90	4.10	3.70	FID-Std /MS
1119	SH	α- caryophyllene	5.20	4.70	5.40	4.80	4.90	5.00	FID-Std /MS
1324	MH	Myrtenol	5.30	6.40	5.80	5.50	5.90	5.78	GC-MS
Total	**-**	**-**	96.90	97.10	99.70	98.10	98.38	98.53	-

RI = a retention indices; The identification of the compounds (∝-Humulene- Germacrene- β-Elemene- ∝-Phellandrene; Geraniol-Sabinene) are in consistent with library search (Willy-Nist) between 80-85%; Identification* FID-Std /MS =Flame Ionization detector /Mass Spectroscopy; GC-MS = Gas chromatography-mass spectrometry; Monoterpene hydrocarbons (MH)= 53.73%; Oxygenated monoterpenes (OM) = 37.1%; Total Monoterpenes % 90.83; Sesquiterpene hydrocarbons (SH)= 7.7%.

α-pinene (30.40 %), while the Tunisian myrtle essential oil contains 19.20% α-pinene (Messaoud and Boussaid, 2011). Two studies have been published on the antimicrobial activities of plant compounds against different types of microbes, including food-borne pathogens (Gunduz *et al*., 2009; Burt, 2004).

Akalu *et al*. (2007) determined the antimicrobial activity of the essential oils obtained from *M. communis* L. *in vitro* using agar well diffusion method against *S. aureus*, *Bacillus cerus*, *E. coli*, *P. aeruginosa*, *S. pyogens*, *Aspergillus niger* and *Candida albicans*, at different concentrations. The oil showed activity against all tested bacteria and fungi with minimal activity against *P. aeruginosa*. Also Syrian *M. communis* L. essential oil characterised by remarkable yield of γ-terpinene (2.66%) which has a high MIC value (2.3-3.3 μL/mL) as compared to other components' MICs. The yields of all components varied among the populations according to their growing conditions and climate deviation. These variations were not remarkable when compared to the significant deviation between two localities of the Montenegro coastline myrtles. Mimica-Dukic *et al*. (2010) observed that significant differences between the two samples of *M. communis* L. were found in the ranges of α-pinene (14.7%-35.9%) and myrtenyl acetate (5.4%-21.6%). In the present study, it was found that the essential oil of local *M. communis* L. did not contain α-terpineol, β-pinene, p-cymene, linalyl acetate, β-caryophyllene, phellandrene and myrtenyl acetate, while other studies showed that they were among the main components of *M. communis* L. essential oils (Mahboubi and Ghazian Bidgoli, 2010; Mimica-Dukic *et al*., 2010; Wannes *et al*., 2007). The bulk essential oil inhibited 5 of the 7 bacteria in high concentration (25-50 μL/mL), while the essential oil components differed in its influences against the tested bacterial strains. Thujone (*trans*), γ-terpinene, nerol and myrtenol inhibited all tested bacteria in wide range of MICs (1-50 μL/mL). But β-myrcene, α-pinene, limonene, bornyl acetate, geranyl acetate and β-caryophyllene expressed no antibacterial effects. The other components showed inhibition effects against some but not all tested bacterial strains. *E. coli* O157 was the most resistant isolate that was inhibited by 5 of 15 substances (the bulk essential

Table 3. The minimum inhibitory concentrations for the bulk essential oil and for the constituents (MIC µL/mL)

	K. pneumoniae	*S. typhimurium*	*P. aeruginosa*	*Proteus* spp.	*B. melitensis*	*Y. enterocolitica* O9	*E. coli* O157
Bulk oil	NIE	25.00±9.32	50.00±9.62	50.00±20.97	50.00±9.62	25.00±5.78	NIE
α-pinene	NIE	NIE	NIE	NIE	NIE	NIE	NIE
β-myrcene	NIE	NIE	NIE	NIE	NIE	NIE	NIE
Limonene	NIE	NIE	NIE	NIE	NIE	NIE	NIE
1,8-cineole	NIE	50.00±9.62	50.00±20.97	50.00±9.62	50.00±0.00	50.00±9.62	NIE
γ- terpinene	6.25±2.79	12.50±1.33	25.00±1.33	25.00±0.00	12.50±2.88	25.00±1.33	12.50±1.33
Linalool	NIE	50.00±0.00	50.00±0.00	50.00±9.62	NIE	NIE	NIE
Thujone (*trans*)	25±4.8	25.00±5.78	50.00±9.62	50.00±9.62	50.00±9.62	50.00±20.97	50.00±20.97
Citronellal	50±20.97	6.25±2.78	<0.30	3.10±0.00	3.10±0.00	6.25±2.78	NIE
Nerol	3.1±0.86	6.25±0.00	6.25±0.00	6.25±2.79	6.25±0.00	12.50±0.00	6.25±2.78
Bornyl acetate	NIE	NIE	NIE	NIE	NIE	NIE	NIE
Geranyl acetate	NIE	NIE	NIE	NIE	NIE	NIE	NIE
Eugenol	12.5±2.88	50.00±9.62	NIE	6.25±2.79	NIE	NIE	6.25±0.00
β-caryophyllene	NIE	NIE	NIE	NIE	NIE	NIE	NIE
Myrtenol	25±9.32	50.00±9.62	50.00±0.00	25.00±4.80	50.00±20.97	25.00±0.00	25.00±9.32

NIE = No inhibitory effects.

oil and 14 components), while *S. typhimurium* and *Proteus* spp. were the most sensitive isolates that were inhibited by 9 of the 15 of previous substances. Present results confirmed the bioactive properties of *M. communis* L. essential oil and some of its components and reinforced other studies results (Gunduz, 2009; Deriu *et al*., 2007; Takeuchi and Frank, 2000). Previous studies have reported the antimicrobial effects of myrtle leaves oil *in vitro* conditions on *E.coli* and *Y. enterocolitica* (Bouzouita *et al*., 2003; Sagdie *et al*., 2003).

Conclusion

Myrtus communis L. essential oil and its components have exhibited some inhibitory effect against some Syrian gram negative isolates. The EO main components were α-pinene, 1,8-cineole, limonene, myrtenol, and β-caryophyllene. The most effective components were citronellal and nerol against all pathogens used in our study. Synergistic and antagonistic effects and more research are needed for evaluation of these components and it will be tested in a future study.

Acknowledgement

The authors would like to thank the Director General and the head of the Molecular Biology and Biotechnology Department, Atomic Energy Commission (AECS) of Syria for their support.

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