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Chemical Composition and Antimicrobial Activities of the Essential Oil from *Myrtus communis* Leaves

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Abstract: Nosocomial pathogens are associated with increased hospital stay lengths and mortality rates. Increasing resistance to antibiotics makes the treatment of these infections more difficult. Novel antimicrobial compounds derived from natural sources may be useful for addressing antiobiotic resistance. The objective of this study is to determine the chemical composition and antimicrobial activities of essential oils from *Myrtus communis* L. (Myrtaceae) leaves against pathogens causing nosocomial infections. The chemical composition of essential oil from *M. communis* leaves was analysed by gas chromatography-mass spectrometry (GC-MS). The antimicrobial activity of the essential oil against bacteria and fungi was evaluated by broth micro-dilution as per the Clinical and Laboratory Standards Institute (CLSI) methods. GC-MS analysis revealed that the major constituents of the essential oil were α-pinene (39.2 %), 1,8-cineole (22.0 %), and linalool (18.4 %). The essential oil exhibited antimicrobial activity against all Gram positive and Gram negative bacteria with MICs in the range of 0.5-32 µL/mL and 8-64 µL/mL, respectively. MICs for the tested clinical and standard fungi were in the range of 0.03-16 µL/mL. The essential oil exhibited strong antibacterial and antifungal activities against all the causative agents of nosocomial infections examined, particularly against strains with antibiotic resistance. The essential oil from *M. communis* leaves is a potential source of novel antimicrobial agents for the treatment of nosocomial infections.

Key words: Antifungal; antibacterial; minimum inhibitory concentration; *Myrtus communis*; *Candida,* Myrtle.

Introduction

In recent years, pathogens causing nosocomial infections have started to exhibit antiobiotic resistance [4,12,16,29]. Surveillance data reported by the National Nosocomial Infection Surveillance shows that the distribution of pathogens causing nosocomial infections varies with time and

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location [28]. The top antiobiotic resistant pathogens causing noso-comial infections include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* strains, third-generation cephalosporin-resistant *Escherichia coli*, imipenem and quinolone-resistant *Pseudomonas aeruginosa,* and azole-resistant *Candida* species [15,28].

In the face of increasing antibiotic resistance, natural products and phytochemicals are being examined as potential sources of novel antimicrobial agents. A retrospective look at the issue shows that plants have been, traditionally, used to prevent and cure infectious diseases. They synthesize aromatic chemicals and secondary metabolites that serve as part of their defense mechanisms against microbes. *Myrtus communis* L. (Myrtaceae) is an evergreen shrub that grows in Iran and many other countries. In folk medicine, it has been used as an antiseptic, disinfectant and hypoglycemic agent. Aqueous and methanolic extracts from *M. communis* have proved effective in dealing with cardiovascular disorders and exhibited antimicrobial activities [3,14]. Moreover, different parts of the mentioned plant have been used in both the cosmetic as well as food industry as flavoring for meat and sauces. The leaves are 3-5 cm long with a pleasantly fragrant essential oil that has known antifungal [25], antibacterial [27,30], and antioxidant [32] activities. This study characterizes the chemical components of the essential oil from *M. communis* leaves collected from southern Iran and screens its antimicrobial effects.

Materials and methods

Essential oil preparation

M. communis leaves were collected before flowering stage from southern regions of Iran, around Jahrom in the Fars province in May 2008. The voucher specimen was identified by Dr. M. Moein based on the morphological description and known samples which collected previously and deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Shiraz University of Medical Sciences, Iran (No. PM.2).

Briefly speaking, powder (300 g) from dried *M. communis* leaves was distilled in water for 4 h using a Clevenger-type apparatus (yield 0.69 %). The yellow oil was dried over anhydrous sodium sulphate, filtered, and stored at 4°C until analysis.

Essential oil analysis by Gas chromatography Mass Spectrometry (GC-MS)

The essential oil obtained from the distillation was analyzed by GC-MS (Helwett-Packared-6890, Co., USA) using a capillary column (HP-5MS, phenyl methyl silicon, 25 m × 0.25 mm, 1 µL injection). Helium was used as the carrier gas (1.2 ml/min). The GC oven program was as follows: initial temperature 50°C for 5 min, programmed rate 3°C/min up to 200°C. The injector temperature was 250°C. For MS detection, an electron ionization system with ionization energy of 70 eV was used. Injector and MS transfer line temperatures were set at 250°C. *n*-Alkanes were used to calculate Kovat´s Indices (KI) for the detected compounds.

Tentative identification of the compounds was based on the comparison of their relative retention time and mass spectra with those from Wiley 275 and Adams data libraries for GC-MS [1].

Antimicrobial susceptibility testing

Fungal and microbial strains

Seven American Type Culture Collection (ATCC) strains of fungi including *Candida albicans* (ATCC 10261), *C. tropicalis* (ATCC 750), *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 90030), *C. parapsilosis* (ATCC 4344), *Aspergillus flavus* (ATCC 64025), and *A. fumigatus* (ATCC 14110) were used in the current study. In addition, the antimicrobial activities of the essential oil against 39 strains of yeasts, identified by PCR-RFLP [23,24] were examined. Additionally included in the study is determining *in vitro* activity of essential oil against standard species of *Staphylococcus aureus* (ATCC 25923 and ATCC 700698), *Entrococcus faecalis* (ATCC11700), *Streptococcus pyogenes* (ATCC8668), *S. pneumonia* (ATCC33400), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella flexneri* (NCTC 8516), *Salmonella enterica* subsp. *enterica* **(**ATCC 14028), and clinical isolates of *S. aureus*, *E. faecalis*, *E. faecium*, *E. coli*, and *P. aeruginosa*.

Determination of the antimicrobial activities

Using microdilution method according to the CLSI guidelines [7,8,9], the antimicrobial activities of the essential oil against above mentioned microorganisms were determined. Briefly speaking, in order to determine the antifungal activities of the essential oil [7,8], serial dilutions of the isolated essential oil (32.0 to 0.06 µL/mL) were prepared in 96-well microtitre plates using RPMI-1640 media (Sigma, St. Louis, USA) buffered with MOPS (Sigma, St. Louis, USA). Similarly, as to determine the antibacterial activities [9], serial dilutions of the isolated essential oil (0.25 to 128 µL/mL) were prepared in Muller-Hinton media (Merck, Darmstadt, Germany) and Tryptic soy broth (Merck, Darmstadt, Germany). Test yeast or bacteria strains were suspended in media and the cell densities were adjusted to 0.5 McFarland standards at 530 nm wavelength using a spectrophotometeric method (this yields stock suspension of 1-5 × 10^6 cells/ml for yeasts and 1-1.5 × 10^8 cells/ml for bacteria). The working inoculums (0.1 ml) were added to the micotiter plates and the plates were incubated in a humid atmosphere of 32°C for 24-48 h and 37°C for 24 h for the yeasts and bacteria, respectively. Uninoculated medium (200 µL) was included as a sterility control (blank). The growth controls (medium with inoculums but without essential oil) were also included. The growth in each well was compared with that of the control growth well. MICs of the tested micro-organisms were visually read based on broth microdilution method as recommended by CLSI [7,8,9]. Each experiment was performed in triplicate.

In addition, media from wells with fungi showing no visible growth was further cultured on sabouraud dextrose agar (Merck, Darmstadt, Germany) and from wells with bacteria showing no visible growth on tryptic soy agar (Merck, Darmstadt, Germany) to determine the minimum fungicidal concentration (MFC) and minimum bactericidal concentration (MBC). MBCs and MFCs were determined as the lowest concentration yielding no more than 4 colonies, which corresponds to a mortality of 98 % of the microbes in the initial inoculums.

Oxacillin disks were used (Mast Diagnostics, Merseyside, UK) for detection of methicillin resistant *S. aureus*, vancomycin (Mast Diagnostics, Merseyside, UK) for vancomycin resistant *Enterococci* spp, ciprofloxacin and imipenem for quinolone and imipeneme resistant *P. aeruginosa* and ceftriaxone for third generation resistant *E. coli* [10].

Results

Extraction of the essential oil from the *M. communis* leaves produced a yellow essential oil at 0.69 % yield. Approximately 24 compounds, representing 97.1 % area of the oil, were identified. GC-MS analysis revealed that the major constituents of the oil were α-pinene (39.2 %), 1,8-cineole (22.0 %), and linalool (18.4 %) (Table 1). The oil was enriched with monoterpene hydrocarbons (47.58 %) and oxygen-containing monoterpenes (47.85 %). Sesquiterpenes and oxygen-containing sesquiterpenes were detected at 0.80 % area and 0.32 %, respectively.

The antibacterial activities of *M. communis* essential oil are shown in Table 2. The essential oil inhibited the growth of all Gram positive cocci (MIC_{90}) at a concentration range of 0.5-32 µL/mL. Furthermore, the essential oil exhibited bactericidal activity (MBC) for all Gram positive cocci at concentrations ranging from 1 to 64 µL/mL, except for one strain of *Enterococci* with an MBC of 128 µL/mL.

The growth of all Gram negative bacteria was inhibited by *M. communis* essential oil at the concentration range of 8-32 µL/mL (MIC), except for *Salmonella entrica* (MIC 64 µL/mL). For Gram negative bacteria, the MICs and MBCs were the same for each given bacteria. No significant differences in susceptibility to the essential oil were found between antibiotic-resistant and antibiotic-sensitive bacteria.

The antimicrobial activities of *M. communis* essential oil against fungi are shown in Table 3. For the clinical and standard yeasts tested, MICs for the essential oil were in the range of 0.03-4 µL/mL, with isolates of *C. glabrata* demonstrating the lowest MIC_{50} and MIC_{90} values (0.03 and 0.06 µL/mL, respectively). All of the isolates showed MFC values of ≤ 8 µL/mL. The essential oil inhibited the growth of *A. fumigatus* and *A. flavus*, with MIC_{50} values of 4 and 16 µL/mL, respectively.

Table 1. Chemical composition of essential oil distilled from *M. communis* L. leaves

Compound	KI [a]	% (Peak area) [b]
α-Pinene	940	39.2
δ-3-Caren	952	6.1
β-Phellandrene	980	0.1
Myrcene	993	0.1
Sabinene	1012	0.1
Limonene	1029	0.3
1,8-Cineole	1039	22.0
β-(z)Ocimene	1053	0.1
trans-Linalool oxide (furanoid)	1078	1.2
Terpinolene	1090	0.1
Linalool	1109	18.4
cis-Sabinene hydrate	1184	0.1
α-Terpineol	1204	2.2
Linalyl acetate	1264	3.6
Geraniol	1268	0.3
Thymol	1315	0.4
α-Terpinyl acetate	1354	0.6
Neryl acetate	1369	0.2
Geranyl acetate	1389	0.9
trans-Caryophyllene	1418	0.2
α-Humulene	1454	0.5
Flavesone	1544	0.1
Caryophyllene oxide	1581	0.1
Humulene epoxide II	1608	0.2

[a] Kovacs Indices to C9-C25 n-alkanes on the HP5MS column.

[b] The amount of each compound was calculated based on the total essential oil chromatogram area peak.

Table 2. Antibacterial activity (MIC and MBC) of essential oil distilled from *M. communis* L.

Bacteria (Number of Strains)	MIC 90 GM[a] µL/mL (range)	MBC GM[a] µL/mL (range)
Gram positive		
Methicillin resistant *S. aureus* (MRSA) (6)	4 (4)	5.65 (4 - 8)
Methicillin sensitive *S. aureus* (MSSA) (6)	5.65 (4 - 8)	8 (8)
Vancomycin resistant *E. feacalis* (2)	11.31 (4 - 32)	45.2 (32 - 64)
Vancomycin sensitive *E. feacalis* (5)	14.25 (4 - 32)	64 (32 - 128)
Vancomycin resistant *E.faecium* (4)	27 (16 - 32)	107.63 (64 - 128)
S. mutans ATCC 35668	0.5	8
S. pneumonia ATCC 33400	0.5	4
S. pyogenes ATCC 8668	1	1

table 2. continued.

Bacteria (Number of Strains)	MIC 90 GM[a] µL/mL (range)	MBC GM[a] µL/mL (range)
Gram negative		
Third generation cephalosporin resistant *E. coli* (6)	27.85 (16 - 32)	27.85 (16 - 32)
Third generation cephalosporin sensitive *E. coli* (5)	25.4 (8 - 32)	25.4 (8 - 32)
Multidrug resistant *P. aeruginosa* (6)	14.25 (8 - 16)	14.25 (8 - 16)
Sensitive strain *P. aeruginosa* (5)	14.25 (8 - 16)	14.25 (8 - 16)
S. flexneri NCTC 8516	16	16
S. enterica ATCC 14028	64	64

[a] GM: Geometric Mean

Table 3. Antifungal activity (MIC and MFC) of essential oil distilled from *M. communis* L.

	Essential Oil *M. communis* (µL/mL)	Fluconazole	Itraconazole
Fungi (Number of Strains)	MIC50 GM(Range)	R ≥ 64 µg/mLL	R ≥ 1 µg/mL
	MIC90 GM(Range)	SDD: 16-32 µg/mL	SDD: 0.25-0.5 µg/mL
	MFC GM(Range)	S ≤ 8 µg/m	S ≤ 0.12 µg/mL
C. albicans (19)	0.72 (0.25 - 2)	6	12
	1.44 (0.5 - 4)	1	1
	3.85 (0.5 - 8)	12	6
C. tropicalis (8)	0.53 (0.06 - 4)	3	3
	1.07 (0.12 - 8)	2	3
	3.08 (1 - 8)	3	2
C. dubliniensis (5)	0.32 (0.06 - 1)	0	0
	0.65 (0.12 - 2)	0	0
	1.74 (0.5 - 8)	5	5
C. glabrata (4)	0.34 (0.03 - 1)	0	0
	0.69 (0.06 - 2)	0	1
	1.39 (0.12 - 4)	4	3
C. parapsolosis (5)	0.75 (0.06 - 2)	0	0
	1.50 (2 - 4)	0	2
	2.63 (4 - 8)	5	3
C. guilliermondii (1)	1	0	0
	2	0	1
	4	1	0
C. krusei (1)	0.25	0	0
	1	0	0
	2	1	1
Aspergillus spp. (2)	8 (4 - 16)	0	0
	-	0	0
	-	2	2

GM: Geometric Mean; R: number of resistant strains
SDD: number of susceptible-dose dependent strains; S: number of susceptible strains

Discussion

In the course of time, a number of studies have been conducted investigating the chemical composition of the essential oil from *M. communis* leaves [6,18]. Like that of Rasooli *et al*., [27] and Messaoud *et al*., [22], α-pinene was determined as the major constituent of the essential oil in the recent study, while others have reported 1,8-cineole [2,5,11,26,32] or myrtenyl acetate [13,17] as the major constituent of the essential oil.

In the present study, *M. communis* essential oil exhibited antimicrobial activities against a wide number of microorganisms particularly those antibiotic resistant strains responsible for nosocomial infections. Consistent with previous studies, the essential oil was active against both methicillin resistant and sensitive *S. aureus* at concentrations ranging from 4-8 µL/mL, [21,30,32].

In nosocomial infections, *E. faecalis* were isolated more often than *E. faecium*. However, *E. faecium* strains exhibited more resistance to antibiotics than *E. faecalis*. Rasooli *et al.*, found no inhibitory effect of the essential oil against *S. aureus* and *E. faecalis* by the agar diffusion method [27]. However, in this study, the essential oil inhibited the growth of both *E. faecalis* and *E. faecium* at a concentration ranging from 4-32 µL/mL. Moreover, the MICs and MBCs of vancomycin resistant *E. faecium* were about three times as much as those of vancomycin resistant *E. faecalis*.

The MICs of *M. communis* essential oil for Gram positive bacteria were lower than those for Gram negative bacteria. These differences may be due to the multi-layered cell membrane structure and presence of lipopolysaccharide in the cell membrane of Gram negative bacteria. Moreover, the essential oil had both inhibitory and bactericidal activities against all the examined Gram negative bacteria with equal MICs and MBCs. One of the Gram negative bacteria examined, *P. aeruginosa*, is an opportunistic bacteria with low susceptibility to many antibiotics [28]. Rasooli *et al.*, found no inhibitory effect of the essential oil against *P. aeruginosa* [27]. However, in the present study, the essential oil exhibited bacteri-cidal activity against *P. aeruginosa* at a concen-tration ranging from 8-16 µL/mL. This result is comparable with that of the study conducted by Owlia *et al.*, who reported an MIC of 64 µg/mL against the same standard *P. aeruginosa* strain [26].

Taking a contrastive look at the issue shows that although some previous studies have reported that the essential oil of *M. communis* had no inhibitory effects against the growth of *E. coli* [14,30], others have found that the growth of *E. coli* was inhibited by the essential oil [18,27]. The present study, supporting the latter view, shows that the essential oil exhibited bactericidal effects against both third generation cephalosporins resistant (TGCsR) and third generation cephalosporins sensitive (TGCsS) *E. coli* at a concentration ranging from 8-32 µL/mL. The results of our study are, to some extent, in line with that of the study by Rasooli *et al*., who reported bacteriostatic (MIC 128 µL/mL) and bacteriocidal (MBC 256 µL/mL) activities of the essential oil against *E. coli* [27]. The lower MICs in the present study, as compared to Rasooli *et al*., may be due to the difference in the oil constituents or the methods used to assay antimicrobial activity [27]. Unlike the present study, Rasooli *et al*., used the agar diffusion test, which can be affected by many factors such as interactions between the test agent and agar, and/or reduced permeability of the antimicrobial agent in agar. Therefore, it is not recommended to use this method for determination of MIC of essential oils [20].

The proportion of nosocomial infections, especially bloodstream infections, caused by *Candida* species other than *C. albicans* is increasing [16]. The essential oil inhibited the growth of all tested yeasts at a concentration ranging from 0.06 - 4 µL/mL. These results are consistent with those of Yadegarnia *et al*., who reported the same MIC (2 µL/mL) of *M. communis* essential oil for *C. albicans* [32]. In this study, *M. communis* essential oil was fungicidal for *C. albicans* at 0.5-8 µL/mL. Yadegarnia *et al*., reported a consistent value of 4 µL/mL [32], while Rasooli *et al*., reported a fungicidal activity at 128 µL/mL [27].

Mohammadi *et al*., reported that the essential oil of *M. communis* had inhibitory effects on the growth of *Aspergillus* species with MICs ranging from 32-256 µL/mL. Lower MICs were determined in the present study and may reflect a

difference in essential oil constituents or in the antimicrobial determination methods [25].

α-Pinene has been previously reported to have antimicrobial effects on Gram positive bacteria with MICs values between 5 and 20 μL/mL [19]. Their MICs are almost the same as those determined in this study except for the MICs of *Streptococci* strains that were 5-10 times greater than those determined in the present study. In this study, the second major constituent identified in the *M. communis* oil was 1,8-cineole exhibiting antimicrobial activities with MICs ranging from 2-23 mg/mL like that of Van Vuuren and Viljoen [31]. The fact that the whole essential oils have lower MICs than the isolated constituents suggest that there may be synergistic effects when the whole essential oil is used. Many of the constituents of the essential oil appear to have structures and modes of action that are distinct from the antibiotics tested here, suggesting that cross-resistance with these antibiotics may be minimal.

Conclusions

Herbal remedies used in traditional folk medicine may have a contribution in overcoming the growing problem of microbial resistance to antimicrobial agents. Taking the implication into account, *M. communis* essential oil may be used to develop novel antimicrobial agents on the basis of the bactericidal and fungicidal activities of the oil shown in this study as well as other works. Further studies are, doubtlessly, needed to determine the efficacy of the active components of this essential oil.

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