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Susceptibility of Microorganisms to *Myrtus Communis* L. Essential Oil and its Chemical Composition

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ABSTRACT

The antimicrobial effects of essential oil isolated from *Myrtus communis* L. against *Escherichia coli, Staphylococcus aureus, Streptococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumoniaee, Bacillus subtilis, Bacillus licheniformis, Candida albicans* and *Saccharomyces cerevisiae* were studied. The disc diffusion method was used to evaluate the zone of microbial growth inhibition at various concentrations of the oil. The minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the oil were determined. The oil was found to have an antimicrobial effect. Kinetics of microbial destruction exhibited that the oil could kill microorganisms within 45 minutes of exposure. GC and GC/MS analysis of the oil revealed 32 components. The major components were α-Pinene (29.4%), Limonene (21.2%), 1,8-Cineole (18%), Linalool (10.6%), Linalyl acetate (4.6%) and α-Terpineole (3.1%). The high monoterpenes hydrocarbons such as α-Pinene and Limonene seem to contribute to the strong antimicrobial activity of *Myrtus communis* L. Further research on antimicrobial effects of essential oils with a view to substituting the common chemical compounds is promising.

Keywords: Antimicrobial, Essential oil, *Myrtus communis L.*

INTRODUCTION

Recently, there has been a profound interest in the antimicrobial properties of extracts from aromatic plants, particularly essential oils (Collins *et al*., 1993; Milhau *et al*., 1997). Essential oils are rich sources of biologically active compounds (Bishop and Thornton, 1997). Many oils and extracts from different plants have been investigated for their antimicrobial properties against a series of bacteria and yeasts (Lattaoui and Tantoui-Elaraki, 1994; Bagci and Digrak, 1996; Hammer, 1998; Khan *et al*., 2001; Erdemoglu and Sener 2001). Essential oils have been found to be antibacterial (Kivanc and Akgul, 1986), antifungal (Pandey *et al*., 1996) and therapeutic in cancer treatment (Crowell, 1999). Some oils have been shown to have an effect on behavior (Gallup, 1993), while others have applications in food preservation (Faid *et al.*, 1995). Some oils have pharmacological properties (Craig, 1999; Crowell, 1999), and some are used in aromatherapy (Buttner, 1996). Thus the use of natural antimicrobial compounds seems to be important not only in the preservation of food but also in the control of human and plant diseases of microbial origin. Bacterial and fungal infections pose a greater threat to humans, animals and plants, hence the need to find natural, inexpensive and effective antimicrobial agents. The quantitative composition and the relative proportions of the

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oil components are widely influenced by the genotype, the ontogenic development and by the environmental and growing conditions (Rhyu, 1979, Piccaglia *et al*., 1991). In the light of the above facts it seems necessary to evaluate the chemical composition and antimicrobial activities of the oil of Iranian *Myrtus communis L.*

MATERILS AND METHODS

Cultures and Media

Escherichia coli (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 13183), *Bacillus subtilis* (ATCC 9372) and *Bacillus licheniformis* (ATCC 9789) were grown on nutrient agar, *Streptococcus faecalis* (ATCC 10541) was grown on brain heart infusion agar, and *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (NCYC 1414) were cultured on yeast malt agar plates. Microbial suspensions were then made from the agar plates using sterile relevant broths at a concentration of approximately 10^8 CFU/mL. Subsequent dilutions were made from the above suspensions. Both spectrophotometer absorbance readings at 580 nm and plate counts using broth dilution blank, and agar plates were used to confirm the viable cell concentration.

Oil Isolation

Myrtus communis L. was collected from the National Botanical Garden of Iran in November 2000. The fresh aerial parts were hydrodistilled for 90 minutes in full glass apparatus. The oils were isolated using a Clevenger-type apparatus. The extraction was carried out for two hours after a four-hour maceration in 500 mL of water. The sample yielded 0.45% w/w oil. The oil was stored in dark glass bottles in a freezer until it was used.

Oil Analysis

The essential oil was analyzed by a GC (9-A-Shimadzu) and GC/MS (Varian-3400) column (DB-1, 60 mm × 0.25 mm fused silica capillary column, film thickness 0.25 μm) using a temperature program of 40°-220°C at a rate of 4°C/min, an injector temperature of 260°C and using the carrier gas helium. The constituents were identified by comparison of their mass spectra with those in the computer library and with authentic compounds. The identifications were confirmed by comparison of their retention indices with those of authentic compounds or with literature data.

Oil Dilution

Various solvents such as ethanol, methanol, acetone, butanol and diethyl ether were tested for their antimicrobial activities using the disc diffusion method. Methanol was selected, as a diluting medium for the oils as it did not show any antimicrobial activity. This solvent also served as a control. ½, ¼, and 1/8 dilutions of oils were made with methanol. Undiluted oil was taken as dilution 1.

Antimicrobial Analysis

The fresh oil was tested for its antimicrobial activities. The disc diffusion method was used for antimicrobial screening as follows. Sterile Mueller-Hinton agar medium was used for the antimicrobial assay of *E. coli*, *S. aureus*, *P. aeruginosa*, *Klebsiella pneumoniae*, *B. subtilis* and *B. licheniformis.* Brain heart infusion agar was used for the *S. faecalis* and yeast malt agar for the *C. albicans* and *S. cerevisiae* antimicrobial assays. The media in plates were allowed to solidify and then the microbial suspension was streaked over the surface of the medium using a sterile cotton swab. The disc size used was 6mm (Whatman No. 1) paper. Under aseptic conditions, the discs were placed on

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the agar plates and then 10 µL from each of the oil dilutions were put on the discs. The plates were then incubated for 24 - 48 hours at 37 ^{O}C in order to get reliable microbial growth. Microbial inhibition zones were measured using vernier calipers. The Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were assessed according to the modified procedure of Kivanc and Akgul (1986). MIC was determined by a broth dilution method in test tubes as follows. 5mL of 10^7 cells/mL microbial suspensions were incubated in a series of tubes containing 50µL of decreasing concentrations of the oil. The highest dilution (lowest concentration), showing no visible growth, was regarded as MIC. Cells from the tubes showing no growth were subcultured on nutrient agar plates to determine whether the inhibition was reversible or permanent. MBC was determined as the highest dilution (lowest concentration) at which no growth occurred on the plates.

Kinetics of Microbial Destruction

50 µL of the each oil dilution, as determined by MBC, was added to 5 mL of each microbial suspensions containing 10^7 cells per mL and were then incubated at relevant microbial growth temperatures for between five and 60 minutes at the increments of five minutes each in an incubator shaker. The spore forming bacilli were exposed to the oil as vegetative cells. Samples were taken after the time intervals and were cultured on growth media for 24-48 hours. Microbial colonies were counted after the incubation period and the total number of viable cells per mL was calculated. The calculation was converted to a percentage of remaining live cells using routine mathematical formulae.

RESULTS AND DISCUSSION

Average values of the three trials constitute the results. Variable zones of microbial growth inhibited by various dilutions of *Myrtus communis L.* essential oil were noted

Table 1. Microbial inhibition zones (mm) and Minimal inhibitory (MIC) and bactericidal concentrations (MBC) of essential oil from *Myrtus communis L.* at various dilutions.

Micro-organisms and	Oil dilutions and corresponding microbial inhibition zones (mm)			
MIC/MBS	1	1/2	1/4	1/8
E. coli	15	13	11	8
MIC/MBC	+/+	+/+	+/-	-/-
S. aureus	-	-	-	-
MIC/MBC	-/-	-/-	-/-	-/-
S. faecalis	-	-	-	-
MIC/MBC	-/-	-/-	-/-	-/-
P. aeruginosa	-	-	-	-
MIC/MBC	-/-	-/-	-/-	-/-
K. pneumoniae	11	8	-	-
MIC/MBC	+/+	-/-	-/-	-/-
B. subtilis	17	14	10	8
MIC/MBC	+/+	+/+	+/+	+/-
B. licheniformis	24	18	13	9
MIC/MBC	+/+	+/+	+/+	+/-
C. albicans	24	20	15	11
MIC/MBC	+/+	+/+	+/+	+/-
S. cerevisiae	29	20	13	9
MIC/MBC	+/+	+/+	+/+	+/-

+ = Effective - = Not effective

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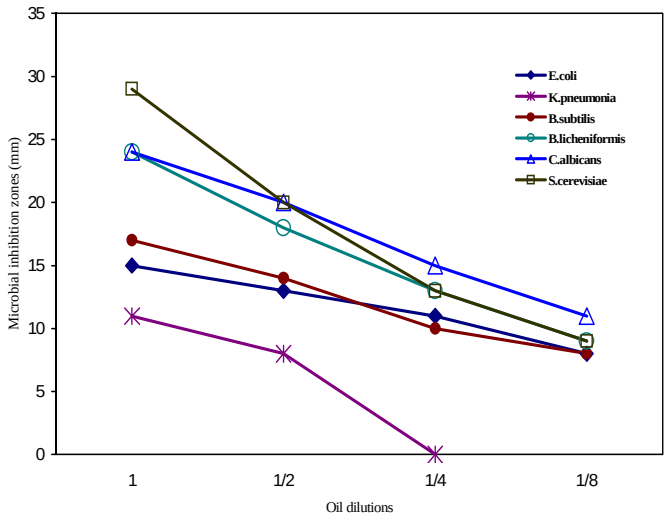

Figure 1. Effect of oil dilutions on microbial growth.

(Table 1, Graph 1). Although the oil had good antimicrobial activity, the fungi showed greater susceptibility than the bacteria on disk diffusion plates. Of nine microbial strains tested, six showed variable susceptibility (Table 1) and were killed at different dilutions of the essential oil (Figure 2). The fresh oil was tested for its bacteriostatic or bactericidal effects employing MIC and MBC techniques (Table 1). Undiluted oil was lethal to all microorganisms tested except for *S. aureus*, *S. faecalis* and *P. aeruginosa*. Dilution ½ showed deadly effect against *E. coli*, *B. subtilis*, *B. licheniformis*, *C. albicans*, and *S. cerevisiae*. ¼ dilution was bacteriostatic against *E. coli*. Lethal effects were noted at ¼ dilution and static effects were observed at 1/8 dilutions against *B. subtilis*, *B. licheniformis*, *C. albicans* and *S. cerevisiae* (Table I). The inhibitory effect of oil dilutions on microbial growth varies from one microorganism to another (Figure 1) which shows the susceptibility of bacteria and yeasts to essential oils (Kivanc and Akgul, 1986). It is evident from Figure 1 that the inhibitory effect of oil

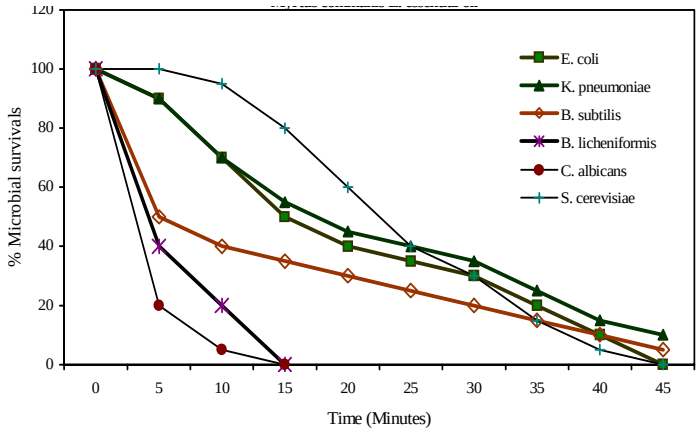

Figure 2. Kinetics of microbial destruction at Minimal Bactericidal Concentrations of *Myrtus* communis L. essential oil.

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Table 2. Chemical composition of the essential oil of *Myrtus communis* L.

No	Compound	RI	%
1	Isobutyl isobutyrate	892	0.7
2	α-Thujene	922	0.25
3	α-Pinene	931	29.4
4	Sabinene	971	0.6
5	Myrcene	981	0.3
6	δ-3-Carene	998	0.2
7	p-Cymene	1013	0.4
8	Limonene	1025	21.2
9	1,8-Cineole	1028	18.0
10	(E)- -Ocimene	1038	0.1
11	γ-terpinene	1051	0.6
12	Terpinolene	1082	0.3
13	Linalool	1089	10.6
14	α -Campholenal	1122	Trace
15	Trans-Pinocarveole	1130	Trace
16	δ-Terpineole	1154	Trace
17	Terpinene-4-ol	1169	0.5
18	α-Terpineole	1180	3.1
19	Trans-Carveole	1213	0.4
20	Cis- Carveole	1217	Trace
21	Geraniol	1242	1.1
22	Linalyl acetate	1248	4.6
23	Methyl geranate	1310	0.2
24	α-terpinyl acetate	1342	1.3
25	Neryl acetate	1351	Trace
26	Methyl eugenol	1369	1.6
27	β-Caryophyllene	1430	0.2
28	α-humulene	1463	0.2
29	Spathulenol	1562	Trace
30	Caryophylleneb epoxide	1586	0.1
31	Humulene epoxide II	1608	Trace
32	Acetocyclohexane dione (2)	1704	0.5

RI- Retention indices Trace = Less than 0.1%

dilutions was greater on *C. albicans* and *S. serevisiae.* The encapsulated *K. pneumoniae* was resistant to the oil dilutions. *E. coli* and spore forming bacilli i.e. *B. licheniformis* and *B. subtilis* were moderately affected by all the dilutions of the oil (Figure 1). The kinetics of microbial destruction showed that *K. pneumoniae, E. coli, S. serevisiae,* and *B. subtilis* (Figure 1) were the last microorganisms being killed (Figure 2). *Candida albicans* and *Bacillus licheniformis* were the most susceptible organisms, being killed within 15 minutes of exposure to the essential oil (Figure 2). The results clearly indicate that the susceptibility of microorganisms to the lethal effect of essential oils do not necessarily depend on the size of growth inhibition zones. Although the essential oil exhibited significant antimicrobial activity, the level of inhibition could be lower in diffusion plate tests (Pandey *et. al*., 1996).

The differences observed in the susceptibility of various microorganisms to essential oil suggests antimicrobial properties of various chemical components of the oils. This becomes more evident with the different reactions of various microbial strains to the oil of the same plant species. This supports the suggestion of Lawrence (1993) and Shu and Lawrence (1997) concerning the dependence of oil composition on the plant species, the chemotypes and the climatic

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conditions. It also implies the possibility of different medicinal uses of the same plant species grown in different regions (Mohagheghzadeh *et al*., 2000). Chemical analysis of the oil components resulted in the identification of 32 components (Table 2). The major components were α-Pinene (29.4%), Limonene (21.2%), 1,8-Cineole (18%), Linalool (10.6%), Linalyl acetate (4.6%) and α-Terpineole (3.1%). This study favors the report that the essential oils with high monoterpenes hydrocarbons are very active against microrganisms (Balchin *et al*., 1998). The results indicate the potential antimicrobial properties of essential oils and hence a hope to see the emergence of antimicrobial compounds from natural sources in the near future.

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حساسیت میکروارگانیسمها به اسانس مورد و ترکیبات شیمیایی آن

ا. رسولی، م. ل. موسوی، م. ب. رضائی و ک. جایمند

چکیده

گیاه مورد با روش تقطیر با بخارآب اسانس‌گیری و تاثیر ضدمیکروبی اسانس آن بر روی *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Bacillus licheniformis*, *Streptococcus faecalis*, *Candida albicans* و *Saccharomyces cerevisiae* مطالعه شد. تاثیر ضد میکروبی اسانس با روش دیسک پلیت در رقتهای مختلف مطالعه گردید. اسانس موثر در رقتهای مختلف در برابر سقه‌های میکروبی قرارگرفتند تا MBC و MIC آن را تعیین نماییم. از نظر زمانی اسانس در مدت حداکثر ٤٥ دقیقه بهترین تاثیر خود را نشان داد. ترکیبات اسانس با دستگاه گاز کروماتوگراف/طیف سنج جرمی (GC/MS) شناسایی شد و ۳۲ ترکیب شناسایی شد که ترکیبات اصلی آن را α-*Pinene* (29.4%), *Limonene* (21.2%), *1,8-Cineole* (18%), *Linalool* (10.6%), *Linalyl acetate* (4.6%) and α-Terpineole (3.1%) تشکیل می‌دهند. تاثیر ضد میکروبی مواد مونوترپنی مانند آلفا پینن و لیمونن محتمل به نظر می رسد. مطالعه بیشتر خواص ضد میکروبی اسانسها با هدف یافتن جایگزین بهتر برای مواد شیمیایی متداول امیدوار کننده است.

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