

Inhibitory Activity of *Myrtus communis* Oil on Some Clinically Isolated Oral Pathogens

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Key Words

Myrtus communis oil · *Streptococcus pyogenes* · *Candida albicans* · Minimum inhibitory concentration

Abstract

Objectives: To determine the antimicrobial activities of *Myrtus communis* oil (MCO) on some oral pathogens. **Material and Methods:** Thirty strains of *Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and 20 strains of *Streptococcus pyogenes* and *Candida albicans* isolated from patients with dental caries, periodontal diseases, pharyngitis and oral lesions associated with artificial dentures were used for the antimicrobial activity of MCO. The oil was prepared by hydrodistillation procedures using a Clevenger apparatus. Agar disk diffusion and broth microdilution methods were performed on various concentrations of MCO (3.9–1,000 µg/ml) using all the pathogens isolated. **Results:** All isolates were sensitive to MCO at 125–1,000 µg/ml by agar disk diffusion producing inhibition zones of 8.1–41.25 mm in diameter. All of the *S. pyogenes*, *S. mutans* and *C. albicans* strains were sensitive to 62.5 µg/ml while 70% (21/30) of *A. actinomycetemcomitans* and 66.6% (20/30) of *P. gingivalis* were resistant to these concentrations. All *S. pyogenes* and *S. mutans* strains were sensitive to

31.25 µg/ml. All *S. pyogenes* strains were sensitive to 15.6 and 7.8 µg/ml of MCO. None of the clinical isolates in this study were sensitive to 3.9 µg/ml or to a lower concentration of oil. The minimum inhibitory concentrations of MCO for *S. pyogenes*, *S. mutans*, *C. albicans*, *A. actinomycetemcomitans* and *P. gingivalis* were 29.68 ± 4.8 , 31.25 ± 0 , 46.9 ± 16 , 62.5 ± 0 and 62.5 ± 0 µg/ml, respectively. **Conclusions:** Data obtained in this study revealed a strong antimicrobial activity of MCO on the tested oral pathogens, and MCO could therefore be useful in the prevention of the related oral infections.

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Introduction

Infectious diseases are still an important cause of mortality and morbidity in developing countries. During the last 2 decades, infections caused by resistant strains of bacteria have increased dramatically [1]. Moreover, the emergence of multidrug-resistant bacteria has made it a challenge to find treatment options for such infections. New antimicrobial agents with stronger activity and less toxicity are needed to overcome these shortcomings. Many of the oils or extracts from medicinal plants have recently been thoroughly investigated as

promising agents for the treatment and prevention of various infectious diseases and human pathogens [1–5]. Among these medicinal plants, *Myrtus communis* has attracted great attention with regard to its antimicrobial activities [5–8]. *M. communis* is an evergreen shrub that belongs to the Myrtaceae family. This plant is mainly found in the Mediterranean regions, Asia, southern Europe, New Zealand, America and southern Russia [9]. In traditional medicine, myrtle leaves and flowers are used for the treatment of respiratory problems, psoriasis, herpes, urinary tract infections, diarrhea, hemorrhoids and wound infections. The pharmacological activities of *M. communis* oil (MCO) including its anti-inflammatory, antimicrobial, antioxidant and hypoglycemic properties have been widely investigated [9, 10]. To date, more than 50 active ingredients of MCO have been identified and the major components as determined by gas chromatography-mass spectrometry are α -pinene, limonene, 1,8-cineole, 4-terpineol, α -terpineol, linalool, geranyl acetate, methyl eugenol, phenolic and acetate compounds [9, 10]. According to the numerous published articles [5–8, 11] on the topic, MCO possesses strong antibacterial activities on various human pathogens including *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Listeria monocytogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Helicobacter pylori* and *Mycobacterium tuberculosis* [11]. Although the antibacterial activities of MCO on various pathogens have been reported, its effects on oral pathogens are not clearly documented. Hence, we investigated the inhibitory activities of MCO on some clinical isolates of oral pathogens that included *S. pyogenes* (β -hemolytic streptococci group A), *S. mutans*, *A. actinomycetemcomitans*, *P. gingivalis* and *C. albicans*. Samples were taken from patients with pharyngitis, dental caries, periodontal diseases, oral abscesses associated with artificial dentures and oral candidiasis and these were tested for isolation of the above-mentioned microorganisms.

Materials and Methods

Preparation of MCO

M. communis was collected from Nourabad City in the southern part of Fars Province, Iran, and was verified by the Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

A quantity of 100 g of air-dried *M. communis* leaves were chopped and immersed in 1,000 ml of sterile distilled water in a 3-liter flask. Hydrodistillation was carried out using a Clevenger glass apparatus, and the extraction process was allowed to proceed

for 3 h. Condensation took place continuously at 4°C in cold water [9]. The extracted essential oil was dried using anhydrous sodium sulfate (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and kept at 4°C in a tightly closed dark vial until used. A solution of MCO (4 mg/ml) was prepared in 10% aqueous dimethyl sulfoxide (DMSO) with Tween 80 (0.5% v/v for easy diffusion) and used as the stock solution. Twofold dilutions from the stock solution (i.e. concentrations of 3.9–1,000 μ g/ml) were prepared and used for the determination of antimicrobial activities.

Isolation of *S. mutans* from Carious Teeth

S. mutans bacteria were isolated from carious teeth as described previously [4, 12]. Briefly, the extracted carious teeth were incubated in 10 ml of Todd-Hewitt broth (THB, Merk, Germany) at 37°C and 5% CO₂ for 48 h. A mitis-salivarius-bacitracin agar (MSBA) was subcultured from the THB and incubated at 37°C and 5% CO₂ for 72 h. *S. mutans* was identified by standard bacteriological and biochemical procedures including colony morphology (greenish hemolysis), catalase, the Voges-Proskauer test, arginine dihydrolase, hippurate hydrolysis and fermentation of glucose, mannitol, raffinose melibiose and sorbitol [13]. Pure cultures of each clinical isolate of *S. mutans* were obtained in MSBA medium and kept at 4°C until used.

Isolation of Periodontopathic Bacteria

Patients with either aggressive or localized aggressive periodontitis were examined and sampled for the isolation of *A. actinomycetemcomitans* and *P. gingivalis* [3, 12]. Subgingival pocket samples were taken from the deepest part of the periodontal pocket (a probing depth of ≥ 6 mm) by insertion of a sterile paper point (Iso 35, Bocht, Offenburg, Germany). Each sample was inoculated into 4 ml of tryptic soy broth (TSB) containing 5 μ g/ml of hemin and menadione (Becton Dickinson Microbiology System) and kept under anaerobic conditions at 37°C and 5% CO₂ for 48 h. Bacteria from TSB were subcultured on tryptic soy blood agar (TSBA) plates (composed of 40 g/l tryptic soy agar, 5 mg/l hemin, 10 mg/l N-acetylmuramic acid and 50 ml/l defibrinated sheep blood) and kept under anaerobic conditions at 5% CO₂ and 37°C for 72 h. *A. actinomycetemcomitans* and *P. gingivalis* were identified according to Forbes et al. [14]. Pure cultures of each clinical isolate were prepared on TSBA plates and kept at 4°C until used.

Isolation of *S. pyogenes* (β -Hemolytic Streptococci Group A)

Throat swabs from patients with pharyngitis, mostly children <10 years of age, were obtained and cultured on sheep blood agar plates and kept at 37°C and 5% CO₂ for 48 h. The suspect colonies with β -hemolysis were subjected to the bacitracin sensitivity test for the identification of *S. pyogenes*. Pure cultures of each strain isolated from the patients were obtained on sheep blood agar plates and kept at 4°C until used.

Isolation of *C. albicans*

Samples were taken from patients with denture stomatitis, oral candidiasis and infected root canal. These were cultured on Sabouraud dextrose agar (SDA) plates and kept at 37°C for 72 h. *C. albicans* was diagnosed on the basis of colonial morphology and other conventional mycological procedures [15]. Pure cultures of *C. albicans* were prepared on the SDA plates and kept at 4°C until used. Thirty strains each of *S. mutans*, *A. actinomycetemcomitans* and *P. gingivalis* and 20 strains each of *C. albicans* and *S.*

Table 1. Antimicrobial activity on some of the oral pathogens clinically isolated by agar disk diffusion test

	<i>S. pyogenes</i> (n = 20)	<i>S. mutans</i> (n = 30)	<i>C. albicans</i> (n = 20)	<i>A. actinomycetem-</i> <i>comitans</i> (n = 30)	<i>P. gingivalis</i> (n = 30)
MCO					
3.9 µg/ml	R	R	R	R	R
7.8 µg/ml	8.3±0.5	R	R	R	R
15.6 µg/ml	9.9±0.8	R	R	R	R
31.25 µg/ml	11.8±0.9	8.8±1.2	R	R	R
62.5 µg/ml	15.9±0.9	10.0±0.9	8.1±1.2	R	R
125 µg/ml	20.8±0.4	15.8±0.6	9.9±0.8	8.8±0.3	8.1±1
250 µg/ml	28.3±0.6	21.0±0.6	17.4±0.6	15.8±0.5	11.8±0.9
500 µg/ml	34.2±0.4	28.7±0.7	24.2±0.5	20.4±0.9	17.2±0.3
1,000 µg/ml	41.25±0.8	38.8±0.9	31.4±0.7	29.4±0.6	24.3±0.7
Antibiotic disks ¹					
Vancomycin	21.5±0.8	21.2±0.9	R	R	R
Amikacin	R	R	R	9.8±0.7	9.1±0.2
Nystatin	R	R	18.02±0.8	R	R
10% DMSO	R	R	R	R	R

Figures show the inhibition zone diameter in mm (mean ± SD). R = Resistant (no inhibition zones).

¹ Vancomycin: 30 µg, amikacin: 30 µg and nystatin: 25 µg.

pyogenes were isolated from patients with various oral infections and were used for MCO antimicrobial determination by standard assays.

Antimicrobial Assays

Two basic methods of antimicrobial susceptibility testing – qualitative and quantitative – are available to diagnostic and research laboratories for the detection of bacterial sensitivity or resistance to antimicrobial agents. Agar disk diffusion, also known as the Kirby-Bauer method, is a qualitative approach that may be prone to some degree of error. However, this technique is used routinely in diagnostic bacteriology laboratories to determine the antibacterial-agent sensitivity or the resistance of bacteria isolated from patients. Microdilution and agar dilution methodologies are considered quantitative because they can measure the minimum inhibitory concentration (MIC) of an antibacterial agent. The MIC is defined as the lowest concentration of an antibiotic or antibacterial agent that inhibits the visible growth of a microorganism. Both quantitative methods are considered a gold standard for susceptibility testing because of their high level of reproducibility. In our investigation, we used the agar disk diffusion and broth microdilution methods to determine the antimicrobial activity of MCO, both qualitatively and quantitatively.

Agar Disk Diffusion

The antibacterial activities of MCO were determined by the standard disk diffusion susceptibility test on solid media. MSBA was used for *S. mutans*, sheep blood agar for *S. pyogenes*, SDA for *C. albicans* and TSBA for *A. actinomycetemcomitans* and *P. gingivalis*. *S. mutans* ATCC 25175, *A. actinomycetemcomitans* ATCC 29523 and *C. albicans* ATCC10231 were used as controls. These strains were maintained anaerobically on TSBA supplemented

with 10% defibrinated horse blood and hemin (5 µg/ml, Wako Pure Chemical Industries, Osaka, Japan).

Pure microbial cell suspensions of each clinical isolate were obtained in 5 ml THB for *S. mutans* and *S. pyogenes*, in TSB for *A. actinomycetemcomitans* and *P. gingivalis* and in Sabouraud dextrose broth for *C. albicans*. The suspension turbidity of these microorganisms was adjusted to 1.5×10^8 colony-forming units/ml (0.5 McFarland standard) and 100 µl of this suspension was seeded on appropriate solid culture media. Sterile 6-mm-diameter filter paper discs (Whatman No. 5, Macherey-Nagel, Düren, Germany) were impregnated with 50 µl of various concentrations of MCO and placed on the above culture media, followed by incubation at 37°C for 72 h. SDA containing *C. albicans* was incubated at 37°C for 72 h. The growth inhibition zones around the filter paper were measured in millimeters and the mean and standard deviation (SD) were calculated and recorded. The disks containing MCO which did not produce inhibition zones were considered as negative results. Sterile filter paper soaked in 50 µl of 10% DMSO and antibiotic disks of vancomycin (30 µg), amikacin (30 µg) and nystatin (25 µg) were also used as controls.

Broth Microdilution

The MIC of MCO against bacterial and fungal (*C. albicans*) isolates from oral infections was carried out by a broth microdilution method using 96-well cell culture plates [16]. THB was used for *S. mutans* and *S. pyogenes*, Sabouraud dextrose broth for *C. albicans* and TSB containing hemin and menadione (5 µg/ml) for *A. actinomycetemcomitans* and *P. gingivalis*. Cell suspensions of the clinical isolates were prepared in the appropriate liquid culture media and their concentrations were adjusted to 10^7 colony-forming units/ml. Twofold dilutions of MCO were prepared from the stock solution. Aliquots (200 µl) of each dilution of MCO were

Table 2. MIC ($\mu\text{g/ml}$) of antimicrobials on some of the oral pathogens clinically isolated by the broth microdilution method

Antimicrobials	<i>S. pyogenes</i> (n = 20)	<i>S. mutans</i> (n = 30)	<i>C. albicans</i> (n = 20)	<i>A. actinomycetem-</i> <i>comitans</i> (n = 30)	<i>P. gingivalis</i> (n = 30)
MCO	29.7 \pm 4.8	31.25 \pm 0	46.9 \pm 16	62.5 \pm 0	62.5 \pm 0
Vancomycin	0.66 \pm 0.24	0.95 \pm 0.47	R	R	R
Amikacin	R	R	R	24.1 \pm 1.8	29.1 \pm 1.9
Nystatin	R	R	15.0 \pm 1.7	R	R
10% DMSO	R	R	R	R	R

Values are expressed as a mean \pm SD. R = Resistant.

dispensed in 96-well cell culture plates; 100 μl of each bacterial suspension was added to each well and incubated under anaerobic conditions at 5% CO_2 and 37°C for 48 h. Microplates containing *C. albicans* were incubated under aerobic conditions at 37°C for 48 h. The absorbance was then measured at 595 nm, and the highest dilution at which no growth ($\text{OD} \leq 0.05$) was observed was defined as the MIC. All experiments were done in triplicate and the mean \pm SD recorded.

Statistical Analysis

Statistical analysis was performed by the χ^2 test and the Fisher exact test using the SPSS software package, version 11.5.

Results

The average yield of MCO on the basis of 3 successive extractions by hydrodistillation was 0.42 \pm 0.02 g/100 g of dried leaves. The essential oil was almost colorless and smelled fresh and pleasant. The results obtained by disk diffusion assay regarding the growth inhibition zones (mean \pm SD) of the tested isolates against various concentrations (3.9–1,000 $\mu\text{g/ml}$) of MCO are summarized in table 1. In this test, inhibition zones >6 mm in diameter were taken as positive results. At concentrations of 125–1,000 $\mu\text{g/ml}$, all (100%) of the microorganisms tested were found to be sensitive and produced inhibition zones ranging from 8.1 to 41.25 mm. *S. pyogenes* strains were the most sensitive isolates since they produced the widest inhibition zones against all MCO concentrations (7.8–1,000 $\mu\text{g/ml}$). All the strains of *S. pyogenes* (n = 20), *S. mutans* (n = 30) and *C. albicans* (n = 20) were sensitive to 62.5 $\mu\text{g/ml}$ of MCO, producing inhibition zones ranging from 8.1 to 15.9 mm, while 70% (n = 21) of *A. actinomycetemcomitans* and 66.6% (n = 20) of *P. gingivalis* were resistant to this concentration of oil. Moreover, at the concentration of 31.25 $\mu\text{g/ml}$, 100% of *S. pyogenes* and *S.*

mutans were sensitive within the inhibition zone 8.8–11.8 mm and only *S. pyogenes* strains were sensitive to 15.6 and 7.8 $\mu\text{g/ml}$ of oil. None of the clinical isolates in this study showed inhibition zones against 3.9 $\mu\text{g/ml}$ or lower concentrations of MCO. The agar disk diffusion test carried out on 10% DMSO showed no inhibitory activity on the oral pathogens used in this study. The results of the MIC of MCO measured by the broth microdilution method are summarized in table 2. *S. pyogenes* with an MIC of 29.68 \pm 4.8 $\mu\text{g/ml}$ followed by *S. mutans* with 31.25 \pm 0 $\mu\text{g/ml}$ were the most sensitive bacteria that we tested. The mean MIC of the oil on the *C. albicans*, *A. actinomycetemcomitans* and *P. gingivalis* clinical isolates were 46.9 \pm 16, 62.5 \pm 0 and 62.5 \pm 0 $\mu\text{g/ml}$, respectively. The MIC of MCO on the standard strains of *S. mutans* (ATCC 25175), *C. albicans* (ATCC 10231) and *A. actinomycetemcomitans* (ATCC 29523) were 31.25 \pm 0, 62.5 \pm 0 and 125 \pm 0 $\mu\text{g/ml}$, respectively.

Discussion

The MCO exhibited strong antimicrobial activity on *S. pyogenes*, *S. mutans*, *C. albicans*, *A. actinomycetemcomitans* and *P. gingivalis*. Strains of *S. pyogenes*, the major oral pathogen and causative agent of bacterial pharyngitis, were the most sensitive bacterial strains examined in this study, similar to the report by Mansouri et al. [6], who evaluated the antimicrobial activity of methanol crude extract of the leaves of *M. communis*. Essential oil extracted from the downy rose myrtle (a member of the Myrtaceae family) also revealed strong inhibitory activity on *S. pyogenes* isolated from patients with pharyngitis and exhibited an MIC ranging from 3.91 to 62.5 $\mu\text{g/ml}$ [17, 18]. Penicillin and erythromycin are the drugs of choice for *S.*

pyogenes infections; however, an increasing rate of failure of these antibiotics has been reported [19]. In such cases, MCO could be used as an alternative.

In our study, *S. mutans*, the main etiological agent of dental caries, was the second most sensitive pathogen to MCO as all its strains were sensitive to various concentrations (31.25–1,000 µg/ml), consistent with the 106.6 µg/ml concentration previously reported by Al-Anbori et al. [20] or the inhibitory efficacy of the ethanolic extract of *M. communis* leaves on *S. mutans*. Denture-related stomatitis is a very common form of oral candidiasis and is referred to as mild inflammation and erythema of mucosa beneath a denture. In our study, *C. albicans* strains isolated from cases of denture stomatitis and infected root canal were all sensitive to MCO with a mean MIC of 46.9 ± 16 µg/ml, similar to a previous report on *C. albicans* isolated from hospitalized patients with candidemia that had a sensitivity of MIC to MCO as low as 2 µg/ml [15]. On the other hand, Mahboubi et al. [21] using Turkish MCO obtained MIC values three times higher for *C. albicans*. Mert et al. [22] using N-hexane, methanol, ethanol, ethyl acetate and water extract of Turkish myrtle leaves found no inhibitory activity on *C. albicans* (ATCC 10239), though these extracts were all effective on some Gram-positive and Gram-negative bacteria. In a similar study, the ethanolic extract of Turkish myrtle leaves showed strong inhibitory effects on *C. albicans* (ATCC 10231) by agar disk diffusions showing an inhibition zone of 24 mm [23]. Moreover, *C. albicans* strains isolated from patients with vulvovaginal candidiasis were found to be sensitive to the alcoholic extract of Iranian *M. communis* leaves with an MIC of 25 mg/ml [24]. The discrepancy in MIC values reported from different institutions is attributed to the fact that the MCO chemical composition and the ingredient concentration are greatly dependent on plant growth, geographical conditions, the nature of the soil, temperature, the season of plant collection and, most importantly, the procedure of oil extraction [9, 25].

A. actinomycetemcomitans (n = 30) and *P. gingivalis* (n = 30), the two species of anaerobic Gram-negative periodontopathic bacteria isolated from dental pockets (depth ≥ 6 mm) of patients with periodontal disease, were sensitive to MCO and exhibited a mean MIC of 62.5 ± 0 µg/ml. Our results are in accordance with most similar studies, in that the MIC values of the oil for Gram-positive bacteria were significantly much less than the MIC for Gram-negative bacteria, which could be attributed to the differences in the structure of their cell walls [6, 22, 23, 26]. The antimicrobial mechanisms of the action of myrtle extract are still unclear. However, some of the major MCO components such as limonene, 1,8-cineole, α -pinene, rhodomirtone, flavonoids and the tannin of the extract are believed to play a role in the inhibitory effects of the oil [7, 18, 22]. The consumption of myrtle extract is considered safe and with the proper administration, there are no known adverse reactions or side effects other than a bitter taste and a slight burning sensation. Rare cases have reported of the intestinal administration of MCO leading to nausea, vomiting and diarrhea.

Conclusions

Data presented in this study showed strong in vitro inhibitory activity of MCO on the oral pathogens *S. pyogenes*, *S. mutans*, *C. albicans*, *A. actinomycetemcomitans* and *P. gingivalis*. MCO could be used in mouth rinses or toothpaste for the prevention of the related oral infections.

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