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Anti-Planktonic and Anti-Biofilm Formation Activities of the Essential Oil and Hydroalcoholic Extract of *Myrtus communis* L. Leaves and Fruits Against Clinical *Candida* species

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ABSTRACT

Aims Because of the increasing prevalence of fungal infections and antifungal resistance, researchers are seeking new antifungal medications and alternatives. This study aimed to assess the antifungal and anti-biofilm propertie of *Myrtus communis* L. essential oil and effect of hydroalcoholic extract on *Candida* species isolated from clinical specimens.

Materials & Methods A total of 65 *Candida* species isolated from clinical samples were evaluated in this study. The chemical composition of the essential oil was analyzed by gas chromatography-mass spectrometry (GC-MS). Besides, the antifungal and biofilm activity of *M. communis* against *Candida* isolates was compared with that of fluconazole.

Findings A total of 22 compounds, displaying 99.88% of the *Myrtus Communis* leaves OE, were identified and the major components were found to be α-pinene (51.22%), eucalyptol (16.88%), linalool (15.92%), and linalool acetate (4.03%). The main components of fruit EO were nonadecane (44.00%), heneicosane (19.60%), α-pinene (12.80%), and eucalyptol (10.08%). The minimum inhibitory concentration (MIC) of the hydroalcoholic extract of M. communis was lower against *C. parapsilosis* compared to C. albicans and C. glabrata. Biofilm formation in different *Candida* strains was inhibited at *M. communis* essential oil concentrations of 2.5-0.0156µl/mL.

Conclusion *M. communis* exhibited considerable antifungal effects against *Candida* species. Also, the inhibitory effect of *M. communis* essential oil against *C. albicans* biofilm formation was higher than that of fluconazole.

Keywords *Myrtus communis* L.; Essential oil; Hydroalcoholic extract; *Candida*; Fluconazole; Biofilm

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Introduction

In recent years, opportunistic fungal diseases, such as candidiasis, have become a significant health problem, especially in immunocompromised patients [1]. This infection may be mild and only superficial or cutaneous and may cause life-threating systemic illness. *Candida* species are reportedly the third or fourth most frequently isolated organisms in nosocomial bloodstream infections, the fourth most common cause of hospital-acquired systemic infections in the USA in nosocomial candidemia [2].The increasing prevalence of fungal infections and the use of antifungal drugs as prophylaxis can lead to drug resistance in patients [3]. One of the most commonly given antifungal drugs for candidiasis is fluconazole, a triazole antifungal agent. On the other hand, emerging non-*C. albicans* species have shown some resistance to fluconazole, ranging from 4% to 13% in *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* [4]. The use of herbal medicines has always been popular among people. Accordingly, scholars and researchers have become interested in evaluating the effects of these medicines on various health disorders, such as microbial and fungal infections [5]. The resistance of *Candida* species to conventional antifungal drugs, besides the adverse effects and high cost of these agents, has encouraged researchers to seek alternative natural compounds. Herbal medicines are commonly used in traditional medicine, as they are low-cost and readily available agents that can be used against various disorders [3, 6].

Myrtus communis L. (myrtle) is a member of the *Myrtaceae* family, which grows in different areas around the world, including the Mediterranean region, Australia, South America, Northwest India, and Iran. This plant has dark green leaves, flowers, and ovoid black fruits with medicinal properties [7]. The essential oil and extracts of different parts of myrtle, including the leaves and sometimes, berries and flowers, have been used for their tonic and balsamic qualities. They are also used in the flavor and perfume industries. Recently, major attention has been paid to myrtle for its possible antioxidant, anti-inflammatory, antibacterial, antidiabetic, and antiseptic effects [8, 9].

Biofilm formation is an important virulence factor and it has many consequences in the process of *Candida* species pathogenesis. Resistance to antifungal drugs and recurrent infection can be the result of biofilm formation [10]. To solve these problems, we need to explore potential plant-derived natural resources containing anti-bio film substances. Today, a number of essential oils have been recognized as alternative antimicrobial and anti-biofilm agent due to their virtues, such as the simplicity in extraction, selective mode of action and nontoxicity to tissue culture, rapid degradation in water and positive health impact [11]. Treatment of opportunistic fungal infections remains a challenging issue, especially in immunocompromised patients. The increasing resistance and incidence of fungal infections, besides the side effects of current classes of antifungal agents, have raised demands for developing new antifungal drugs [12]. Moreever, Plant-derived products are low-cost and have fewer side effects. The present study was designed to assess the antifungal properties and anti-biofilm activity of essential oil and hydroalcoholic extract of *M. communis* against three *Candida* species isolated from clinical samples. Also, the chemical composition of essential oil and hydroalcoholic extract of *M. communis* fruits and leaves was analyzed by gas chromatography-mass spectrometry (GC-MS).

Materials and Methods

For this purpose, 300 cases of vaginitis, 100 suspected cases of recurrent vaginitis, and 360 suspected otomycosis patients were sampled. Vaginal discharges and ear swabs samples were subcultured on Sabouraud dextrose agar and incubated at 37°C for 24 h. After that, *Candida* species were isolated from clinical specimens and identified by conventional methods (germ tube test, CHROMagar medium culture, chlamydoconidia formation test, and growth assessment at 42°C) and confirmed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using ITS1, ITS4 primers and MspI enzyme [13, 14]. The isolates were subcultured on Sabouraud dextrose agar (SDA, Merck, Germany) to confirm their purity; they were then preserved in sterile distilled water until further use. Also, *C. albicans* ATCC10253, *C. glabrata* CBS90028, and *C. parapsilosis* ATCC22019 were used for quality control.

Preparation of hydroalcoholic extract of *M. communis*

The hydroalcoholic extracts of *M. communis* were prepared separately from two parts of the plant, including the leaves and fruits. For this purpose, fresh fruits and leaves were picked, washed, and dried at room temperature. Next, 200g of the dried fruits or leaves were macerated with ethanol (Merck, Germany) and incubated at 37°C for 48 hours. The mixture was then filtered through a Whatman paper. The extract was placed under vacuum conditions for evaporation of residual alcohol, and finally, a concentrated extract remained. The prepared extracts were kept in a freezer at -70°C until use. Moreover, after plant identification, a sample (registration number: 1482) was submitted to Fars Agricultural and Natural Resources Research and Education Center in Shiraz, Iran.

Preparation of *M. communis* essential oil

First, the fresh leaves and fruits of *M. communis* L. were collected from the urban areas of Kohgiluyeh and Boyer-Ahmad Province, Iran. After adding distilled water, 200 g of leaf or fruit samples was heated for three hours in a Clevenger-type

apparatus. Finally, the collected essential oils were stored in sterile bottles and kept at 4°C under dark conditions for future experiments.

GC-MS analysis

GC-MS analysis was made on an Agilent 6890 series GC system (Agilent Technologies, Inc., USA), equipped with an Agilent mass selective detector (N-5973) and an Agilent HP 5MS capillary column with 5% methyl phenyl siloxane static phases (30m×0.25mm; film thickness, 0.25µm). The operating conditions were as follows: a column temperature of 60°C at the onset, followed by 60-246°C at a rate of 3°C/min and finally, 270°C for two minutes; injector temperature, 250°C; ionization potential, 70eV; and volume of injection, 1µL with 1.50 splits. Also, helium carrier gas was used at a flow rate of 1.5ml/min. The chemical components of *M. communis* L. essential oil were determined by comparing the mass spectra and Kovats retention indices with those available in the computer library or the literature [15].

Measurement of the minimum inhibitory concentration (MIC) against *Candida* species

Hydroalcoholic extract of M. communis

The MICs of the selected isolates to different extracts were determined according to the Clinical and Laboratory Standards Institute (CLSI) M27-A3 document. Briefly, a suspension of the fresh cultures of *C. albicans*, *C. parapsilosis*, and *C. glabrata* was prepared and adjusted by a spectrophotometer to match the turbidity standard of 0.5 McFarland. Next, *Candida* samples were diluted at 1:1000 in RMPI-1640 medium with L-glutamine, without sodium bicarbonate (Sigma-Aldrich, USA), and buffered at pH of 7.0 with 0.165 M 3-(N-morpholino) propane sulfonic acid (MOPS, Sigma-Aldrich, USA). Afterward, 100 mg of dried hydroalcoholic extract was dissolved in 1 mL of dimethyl sulfoxide (DMSO) to obtain a concentration of 100,000 µg/mL for the stock solution. A serial dilution of the extracts was prepared. The MIC was defined as the lowest concentration, causing 50% inhibition in fungal growth relative to the positive control.

Essential oil of *M. communis*

Serial dilutions of *M. communis* essential oil (50µl/mL) were prepared in the RPMI-1640 medium. Next, these serial dilutions were added to 96-well microtiter plates. Standard yeast suspensions were then added to each microtiter plate. Finally, the plates were incubated at 35°C for 48 hours. Fungal growth in each well was compared with that of the positive control.

Fluconazole

To evaluate fluconazole susceptibility *in vitro*, serial dilutions were prepared, starting from 32µg/mL. Next, 100 µL of fungal suspensions were added to each well. The plates were incubated at 35°C for 24 hours, and the MICs were determined visually. As mentioned earlier, the MIC was defined as the lowest concentration, causing 50% inhibition in fungal growth as compared to the positive control.

Anti-biofilm activity

Biofilm formation was examined according to a previous study by Salari *et al.* [16]. For this purpose, *Candida* species were cultured in SDB medium (Liofilchem) and incubated at 37°C overnight. Next, all cultures were centrifuged and washed with phosphate-buffered saline (PBS), inoculated in RPMI-1640 medium, and adjusted to 10^7cells/mL. In the adhesion phase, 100µL of the prepared suspension was transferred to 96-well microplates and incubated at 37°C for 90 minutes. Afterward, the suspensions were discarded and washed with PBS three times. Finally, 200µL of a serial dilution of fluconazole (32-0.0078µg/mL) and the essential oil (5-0.0024µl/mL) was added to each well.

MTT assay

To increase biofilm formation in *Candida* isolates, the microplates were kept at 37°C (75rpm) for 24 hours in a shaker. Then the contents were removed and washed with PBS. *Candida* biofilm metabolic activity was examined by MTT test, according to the MTT kit instructions (DNAbiotech, Iran).

The colorimetric changes (an indicator of the *Candida* biofilms metabolic activity) in the treated samples, as well as positive and negative controls, were measured. The results are reported as mean±standard deviation (SD).

Statistical analysis

Statistical analysis was performed using SPSS 16 software. Descriptive statistics and chi-squared tests were used to summarize the results.

Findings

GC-MS analysis

The GC-MS analysis presented that the main components of the essential oil of *M. communis* leaf were α-pinene (51.22%), eucalyptol (16.88%), and linalool (15.92%; Table 1).

The main constituents of *M. communis* fruit essential oil was nonadecane (44%), heneicosane (19.3%), and α-pinene (Table 2).

Antifungal susceptibility test results

Sixty-five isolates, including 39 *C. albicans*, 14 *C. glabrata,* and 12 *C. parapsilosis* isolates, were examined in this study. The lowest MIC was found in the fruit and leaf essential oils against *Candida* isolates (Table 3). Based on the results, there was no significant difference in the MICs of fruit and leaf extracts. Also, according to the CLSI breakpoints, all *Candida* isolates were sensitive to fluconazole (0.0312-2µg/mL). Also, the statistical analysis showed that the difference in the antifungal activity between all investigated cases was significant only in the leaf extract (p=0.043).

Anti-biofilm activity

The minimum biofilm inhibitory concentration (MBIC) of *M. communis* essential oil ranged from

2.5µl/mL to 0.0156µl/mL. No activity was observed against *C. glabrata* biofilms. Also, fluconazole was active against *Candida* biofilms up to a concentration of 64µg/mL. The reducing order of *Candida* biofilm susceptibility to the essential oil of *M. communis* was as follows: *C. albicans* > *C. krusei* > *C. glabrata*.

Table 1) Chemical components of *Myrtus communis* leave essential oil

Number	Compound	Retention index	Retention Index Calculated	Retention Index Standard	Composition (%)
1	α-Pinene	6.45	917.7474	932	51.22
2	β-Pinene	7.21	943.686	974	0.50
3	Myrcene	7.60	956.9966	988	0.32
4	3-Carene	7.96	969.2833	1008	0.79
5	Eucalyptol	8.58	990.4437	1031	16.88
6	β-Ocimene	9.07	1005.615	1032	0.29
7	γ-Terpinene	9.24	1010.16	1054	0.67
8	Terpinolene	10.04	1031.551	1086	0.30
9	Linalool	10.59	1046.257	1095	15.92
10	Trans-Pinocarveol	11.76	1077.54	1135	0.16
11	Terpinen-4-ol	12.72	1102.885	1174	0.25
12	α-Terpineol	13.09	1111.779	1186	2.77
13	Linalool acetate	14.30	1140.865	1254	4.03
14	(S)-(-)-Citronellic acid, methyl ester	14.62	1148.558	1261	0.43
15	(+)-4-Carene	16.82	1201.412	1002	0.79
16	Neryl acetate	17.26	1211.765	1359	0.28
17	Geranyl acetate	17.75	1223.294	1379	1.36
18	Caryophyllene	18.51	1241.176	1417	0.34
19	α-Humulene	19.38	1261.647	1452	0.87
20	Durohydroquinone	21.18	1304.077	-	0.38
21	Caryophyllene oxide	22.63	1338.849	1582	0.47
22	Humulene epoxide II	23.25	1353.717	1608	0.86

Table 2) Chemical components of *Myrtus communis* fruit essential oil

Compound	Retention index	Retention Index Calculated	Retention Index Standard	Composition (%)
α-Pinene	6.26	911.2628	932	12.80
Eucalyptol	8.63	992.1502	1030	10.08
1-Nonadecene	28.42	1480.397	1892	5.12
Nonadecane	28.84	1490.819	1900	44.00
Hexacosane	30.85	1542.487	2600	5.89
Heneicosane	32.66	1589.378	2100	19.60
Octadecane, 1-chloro-	36.24	1685.676	2079	0.83

Discussion

In the present study, GC-Mass analysis of *M. communis* showed that α-pinene and nonadecane accounted as the main component of leaf and fruit essential oil, respectively. Several studies have reported that α-pinene is the major component of the essential oil of *M. communis* L. [17-20]. On the other hand, Bouzouita *et al*. and Mhamdi *et al*. introduced 1,8-cineole as the main component of the essential oil of *M. communis* L. [21, 22]. Nevertheless, all previous studies have confirmed the antimicrobial effects of α-pinene. In the present study, α-pinene and nonadecane were the major constituents of the essential oil of *M. communis* leaves and fruits, respectively. Conversely, Qader *et al*. introduced linoleic acid methyl ester as the main component of *M. communis* fruit in Iraq [23]; it seems that geographic region influences the herbal components.

In the current study, the antifungal activity of *M. communis* essential oil was higher than that of the hydroalcoholic extract (MIC range: 0.0195-0.625 µl/mL). However, this range was inconsistent with the MICs reported by Zomorodian *et al*. (0.06-4 µl/mL) for *M. communis* L. against *Candida* species. Yadegarinia *et al*. also evaluated the antifungal activity of *M. communis* essential oil against *C. albicans* and reported a MIC of 2 µl/mL [24]. Moreover, Mahboubi and Ghazian Bidgoli reported MICs of 8 µl/mL and 16 µl/mL for *M. communis* essential oil against *C. albicans,* respectively [25].

On the other hand, Azad Hayayei *et al*. found that the total extract of *M. communis* had no inhibitory effects against *C. albicans* [26]. The observed discrepancy between previous studies may be related to the origin of *Candida* isolates or differences in the essential oil and herbal extract constituents. According to some studies, the herbicidal mechanism of the essential oil of *M. communis* may be associated with the inhibition of DNA synthesis and disruption of cytoplasmic organelles [27, 28]. The literature also suggests that the essential oil of *M. communis* can change the shape and structure of hyphae in saprophytic fungi [29].

In the present study, the MIC range of fluconazole was 0.03125-2µg/mL against *Candida* species. The results also showed that the essential oil of *M. communis* was more effective than fluconazole ($p<0.05$). In this regard, Sadeghi Nejad *et al*. evaluated the antifungal activity of hydroalcoholic extract of *M. communis* and showed the strongest activity against *C. glabrata* isolates (15-30mm) [29]. In

the current study, the hydroalcoholic extracts exhibited antifungal activity, and the lowest MIC was measured against *C. parapsilosis* (39-156µg/mL). However, Azad *et al*. found that the crude extract of *M. communis* had no anti-*Candida* effects in a concentration range of 300-600 mg/mL [26].

In the current study, the results of the antifungal susceptibility test of different *Candida* biofilms were compared between *M. communis* essential oil and fluconazole. Zarei *et al*. found that the MBICs of *Candida* strains were almost similar for fluconazole and *M. communis* [30]. However, the MBICs of fluconazole for *Candida* strains were higher than those reported by Nath Mishra *et al*. [31]. Our results indicated the increased resistance of biofilms to the essential oil of *M. communis* and fluconazole in *C. glabrata* isolates, whereas Salari *et al*. reported that 43% of biofilms formed by *C. glabrata* were inhibited by fluconazole [16]. Also, in a study by Pongracz *et al*., *Candida* biofilms were at least 100 times more resistant to fluconazole than their planktonic counterparts [32]. It seems that the difference between studies is related to the increased resistance of biofilms produced by this species.

In according to the results of the present study, the essential oil of *M. communis* can be used as a novel component in the development of new antifungal medicines. There is also hope that this herbal plant can improve resistance and recurrent cases caused by the biofilm of *Candida* species. If the antifungal effect of the *Myrtus communis* on patients with Candida fungal disease was indicated in vivo studies, this herbal plant can be proposed in the pharmaceutical industry to make antifungal medicines or as a complementary medicine for patients with Candida fungal disease.

Conclusion

M. communis has antifungal effects on *Candida* species. Also, the inhibitory effect of *M. communis* essential oil against *C. albicans* biofilm formation is greater than that of fluconazole.

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