wjpmr, 2018,4(7), 150-154

SJIF Impact Factor: 4.639

WORLD JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.wjpmr.com

Research Article

ISSN 2455-3301

WJPMR

EVALUATİON OF ANTİ-BİOFİLM AND ANTİFUNGAL ACTİVİTİES OF *MENTHA LONGİFOLİA* ESSENTİAL OİL AGAİNST CLİNİCAL ISOLATES OF *CANDİDA ALBİCANS*

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Article Received on 18/05/2018 **Article Revised on 08/06/2018** **Article Accepted on 29/06/2018**

ABSTRACT

Mentha longifolia is traditionally used in the treatment of various diseases such as digestive and headache disorders. In current study, the essential oil of *M. longifolia* (EOML) was investigated for their antimicrobial, anti-biofilm activity and chemical composition. The EOML chemical composition was analyzed by gas chromatography-mass spectrometry. Antimicrobial and anti-biofilm properties were determined using broth micro-dilution methods. According to the results of this study, the GC-MS profile highlights that the mains compounds were determined (61.40 %) Carvacrol, (17.38 %) o-Cymol, and (8.27 %) γ-Terpinene. The antifungal activity of the EOML was evaluated by the micro-dilution method on *Candida albicans* clinical isolates. It was determined that EOML inhibited 10 tested yeasts with Minimum Inhibitory Concentration (MIC) values in the range of 1.56-3.12 µl mL^{-1}. Minimum Biofilm Inhibitory Concentration (MBIC) value was found to be range of 3.12-12.5 (µl mL^{-1}) while the Minimum Biofilm Eradication Concentration (MBEC) value was found to be 3.12- 6.25 (µl mL^{-1}). MIC value of the *M. longifolia* essential oil was applied onto the *C. albicans* biofilm formations. It was seen that the EOML reduced the cell viability in biofilm by 20.3-71.0 %. The findings of this study show that EOML has antifungal and anti-biofilm activity against *C. albicans* isolates. For this reason, *M. longifolia* essential oil may be considered as a potential agent for the treatment of *C. albicans* infections associated with biofilm.

KEYWORDS: *Mentha longifolia, Candida albicans*, Antifungal activity, Anti-biofilm activity.

INTRODUCTION

Implante medical devices help treatment solve important problems in modern health care but the microorganisms that develop due to biofilm formation on their surfaces significantly affect the morbidity and mortality of patients. It has been reported that the sessile microorganisms in biofilms are much more resistant to antimicrobials than planktonic forms. Biofilms are potential reservoirs for pathogen microorganisms that serve as a continuous source of infection and cross-contamination. For this reason, it is important to understand the environmental conditions and mechanisms that control biofilm formation to reduce the infections associated with its formation.[1] It is known that the biofilm contributes to the antifungal resistance of Candida yeasts. In addition, resistance mechanisms developed by Candida yeasts against antifungal agents cause significant challenges in combating biofilm-associated Candida infections.[2] *Candida albicans* strains are an important opportunistic fungal pathogen causing infections associated with biofilm formation in medical implants. Because biofilms are resistant to antifungal drugs, they provide protection against host defense and make treatment difficult.[3] The problem of increased resistance to biofilm structures produced by antimicrobials and microorganisms has increased the importance of working to find new therapeutic agents. In this context, the effects of plant extracts and components on durable microorganisms and biofilm formations attract many researchers.[4]

Mentha longifolia L. (Lamiaceae) grows widely in the Mediterranean. The different extracts of *M. longifolia* are used in the cosmetology, food and pharmaceutical industries. The aerial parts of plant are used in traditional medicine, due to its antimicrobial, antispasmodic, stimulant, and carminative properties.[5]

The aim of current study was to determined anti-biofilm and antimicrobial effect of essential oil of *M. longifolia* (EOML*)* against clinical strains of *C. albicans*. It is believed that the results obtained contribute to literature and studies in order to develop alternative agents against increasing microbial resistance problem and biofilm-associated Candida infections.

MATERIALS AND METHODS

Plant material and extraction of essential oil

Mentha longifolia was gathered in July 2017 from Akhisar and Gördes districts in Aegean Region of Turkey. The plant was identified by a botanist in Süleyman Demirel University, in Isparta. The samples dried in the laboratory and it was extracted by hydrodistillation method using the Clevenger.

Microorganisms

Candida albicans strains were isolated from Cumhuriyet University Microbiology Laboratory between 2016 - 2017 and identified using the matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS) automated microbiology system.

Analysis and Identification of *M. longifolia*

EOML chemical composition was determined according to the process applied by Aksit et al.[6]. GC-MS analyzes were characterized using a Perkin-Elmer Clarus 500 Series.

Antifungal Activity

The antifungal activity of EOML was determined by the broth microdilution technique in line with the CLSI recommendation.[7] It was used to determine the minimal inhibitor concentration (MIC) of EOML against clinical isolates of *C. albicans* strains. The suspensions of yeasts were adjusted to standard of 0.5 McFarland and it was diluted at a rate of 1/100. EOML was dissolved in Mueller Hinton Broth (MHB) containing Tween 20 (0.5%). Serial two-fold dilutions were prepared with MHB at a concentration of 25-1.56 µl mL^{-1}. After incubation at 37 ° C for 24 h, a microplate reader (Thermo Scientific Microplate Photometer, Multiskan FC) was used to determine the optical density (OD) of the plates at 570 nm. The optical density of the wells are the same or decreases in MIC, which is the lowest concentration of EOML compared to the initial reading.

Inhibition of Biofilm Formation

The minimum biofilm inhibitor concentration (MBIC) is called the lowest concentration at which are protected from biofilm.[8] The effect of EOML to inhibition and eradication was measured using microdilution technique, modified from Perumal et al.[9] The overnight cultures of clinical isolates of *C. albicans* strains on TSB (Tryptic Soy Broth) containing 2% (w/v) glucose were prepared at 10^8 CFU $mL^{-1}$and then distributed 100 µL in each test well. Then 100 µL of EOML (50-1.56 µl mL^{-1}) concentration was distributed to each well. The supernatant was poured off and each well was washed three times with Phosphate-buffered saline (PBS) after incubation for 24 h, at 37 ° C. The plates were dried at room temperature during 30-40 min. The wells were stained with crystal violet 0.1% (w/v) for 15 minutes and washed with distilled water. Then the crystal violet was solubilized in ethanol and optical density of wells was read in a microplate reader at 570 nm. Cell cultures without EOML were used as positive control and only TSB was used as a negative control. It was determined as the MBIC where the absorbance of wells was less to or equal than the control absorbance. The test was performed in triplicate and taken as three reading averages.

Eradication of Biofilm Formation

The minimum biofilm eradication concentration (MBEC) is the minimum concentration that can disrupt the biofilm formation.[9] Two hundred microliters of each *C. albicans* suspension were inoculated into the wells of plate. Non-adherent medium were removed by aspiration using a micropipette after washing with PBS after 96-well microtiter plate were incubated for 48 h at 37 °C. Serial dilutions of EOML (50-1.56 µl mL^{-1}) were distributed into tested wells. Plates were incubated at 37 °C for 24 h. The wells of plate were washed with distilled water and then stained with crystal violet 0.1 % (w/v). The formation of essential oil-free biofilm was used as positive control. The concentration of biofilm formations present in the treated wells was accepted as MBEC.[10]

Cell Viability Assay

Viability of *C. albicans* within the biofilm was quantified using the tetrazolium salt XTT reduction test in a 96-well plate as previously described by Martinez and Casadevall.[11] After biofilm formation for 48 h at 37 °C, the wells were washed two times with distilled water and planktonic microorganisms were removed. EOML was diluted in TSB medium containing 0.5% Tween 20 (v/v) and 2% (w/v) glucose. EOML was applied at 48 hours of biofilm formation on 96-well plates at MIC concentrations. After incubation for 24 h at 37 °C, non adherent bacteria and medium were removed by washing with distilled water. XTT was distributed to the control and test wells. The plate was incubated in darkness for 30 min at room temperature. The metabolic activity of *C. albicans* in biofilm is determined from dehydrogenase activity, which is reduced XTT tetrazolium salt to formazan, it was resulting in a colorimetric change. The cell viability was measured by reading the wells OD at 450 nm in a microplate reader (Thermo Scientific Microplate Photometer, Multiskan FC, USA). The biofilm formation without EOML and medium with Tween 20 were used as control.

Statistical Analysis

All experiments were done in triplicate. Results were evaluated in the form of arithmetic mean ± standard deviation (x ± SD). OneWay variance analysis (ANOVA) and post-hoc Tukey analysis were used to reveal relationships between groups. Differences were considered significant for $p < 0.05$.

RESULTS AND DISCUSSION

Chemical Composition of EOML

The composition of essential oil of *M. longifolia* was shown in Table 1. Twenty-three compounds were detected in EOML. The major components of EOML

were determined (61.40 %) Carvacrol, (17.38 %) o-Cymol, and (8.27 %) γ-Terpinene. Previous studies showed that the major components of extracts of *M. longifolia* collected from different geographical regions were quite different from each other.[12-15] Hussain et al. detected in essential oil of *M. longifolia* that the major components were and piperitenone oxide (40.1%), piperitenone (16.4%), and borneol (13.3%).[15] In another study, Mkaddem et al. revealed that *M. longifolia* essential oil's major components were constituted by as pulegone (54.41%), isomenthone (12.02%), 1,8-cineole (7.41%), borneol (6.85%), piperitenone oxide (3.19%).[14] The components of the essential oil of *M. longifolia* gathered from Tunisia by Hajlaoui et al. was found to be as pulegone (47.15), 1,8 cineole (11.54%), menthone (10.7%), and α-pinene (3.57%)[13]. In another study conducted in South Africa, menthone (47.6%), pulegone (18.4%) and 1,8-cineole (16.4%) were found to be major components of *M. Longifolia.*[12] The present study results compared to previous studies, the presence of major components of *M. longifolia* essential oil were quite different from other.

Table I: Chemical composition of essential oil of *Mentha longifolia.*

	RT (min)	%	Name		RT (min)	%	Name
1	11,432	1,00	β-Thujene	**12**	19,578	0,90	endo-Borneol
2	11,709	0,72	α-Pinene	**13**	19,926	0,80	4-Terpineol
3	12,213	0,20	Camphene	**14**	20,359	0,17	α-Terpineol
4	12,887	0,62	1-Octen-3-ol	**15**	20,523	0,29	α-Terpineol
5	13,089	0,21	β-Pinene	**16**	22,518	1,04	Pulegone
6	13,29	1,02	β-Pinene	**17**	23,363	0,28	thymol
7	13,404	0,28	3-Octanol	**18**	24,321	61,40	Carvacrol
8	13,903	0,15	α-Phellandrene	**19**	26,309	0,28	3-Hydroxy-2-(2-methylcyclohex-1-enyl)propionaldehyde
9	14,302	1,32	α-Terpinene	**20**	28,157	1,65	Caryophyllene
10	14,637	17,38	o-Cymol	**21**	32,936	0,25	Spathulenol
11	15,734	8,27	γ-Terpinene	**22**	33,163	1,42	Caryophyllene oxide
				23	42,048	0,13	Naphthalene, 1,2,3,4,4a,5,6,7-octahydro-4a-methyl-

Rt(min): retention time; (%): relative percentage obtained from peak area

Antifungal Activity

Antifungal activity of EOML was assessed by microdilution method against ten clinical isolates of *C. albicans*. Minimum Inhibitory Concentration (MIC) value of essential oil was shown in Table 2. MIC values for tested strains, which were sensitive to EOML were in the range of 1.56-3.12 µl mL^{-1}. The antifungal activity may be concerned to major components of the *M. longifolia* essential oil such as Carvacrol, o-Cymol, and γ-Terpinene.

Many researchers showed in their studies that *M. longifolia* extracts were effect against pathogenic microorganisms. Saeidi et al. emphasized that *M. longifolia* extracts showed antimicrobial activity against human pathogenic bacteria with varying levels.[16] The values of MIC ranging from 0.062 to 5 mg mL^{-1}. According to Mikaili et al. the results of previous study have shown that the Mentha species have significant antimicrobial activities due to the presence in their composition.[5] Other study conducted by Yiğit et al. have shown the antimicrobial effect of *M. longifolia* extract on microorganisms.[17] In their study, antimicrobial activity was shown with 2.5 mg mL^{-1} MIC value against *C. albicans.* Mkaddem et al. reported that *M. longifolia* collected from Tunisia exhibited a high antimicrobial activity against microorganisms, such as *Saccharomyces cerevisiae* and *Candida albicans.*[14]

In our study, the essential oil obtained from *M. longifolia* collected in the Aegean Region of Turkey was found to be effective on the clinical isolates of *C. albicans*.

Anti-biofilm Activity and Reduction in Biofilm Metabolism

Biofilms are one of the major virulence factors of *C. albicans*, and show resistance levels against the majority of antifungal agents.[18] It has been reported that the sessile forms of microorganisms in the biofilm may be more resistant to antimicrobial agents than free planktonic cells. Candida species grows in a biofilm that is commonly attached to medical devices such as cardiovascular devices, articulating devices, dialysis devices, urinary catheters, and venous catheters.[19] As currently antifungals have less effective against biofilms, new drugs are urgently needed to treat candida infections associated biofilm.[20]

The inhibition and eradication activity of EOML on biofilm formation of *C. albicans* isolates was investigated in our study. According to the results, MBIC value of essential oil was found to be 3.12 (µl mL^{-1}) in two isolates, 6.25 (µl mL^{-1}) in three isolates, 12.5 (µl mL^{-1}) in five isolates while the MBEC value was found to be 3.12 (µl mL^{-1}) in two isolates, and 6.25 (µl mL^{-1}) in eight isolates. Although there are studies related to the antimicrobial activity of the other Mentha species, not much is research regarding its anti-biofilm effect of *M.*

longifolia. MIC value of EOML was applied onto the *C. albicans* biofilm formations that occurred after the 48h incubation. It was observed that the EOML reduced the cell viability in biofilm at the MIC value by 20.3-71.0 % (Table 2).

Table II: Antifungal and anti-biofilm activities of essential oil of *Mentha longifolia* against clinical isolates of *C. albicans.*

Strain	Flucanozole (µg mL-1)	MIC (µl mL-1)	MBIC (µl mL-1)	MBEC (µl mL-1)	Reduction viability in biofilm on MIC (%)
1	8	3.12	6.25	6.25	45.6±1.5
2	8	1.56	6.25	6.25	31.0±1.0
3	8	1.56	3.12	6.25	34.3±2.5
4	8	1.56	3.12	3.12	24.6±1.1
5	8	1.56	6.25	6.25	71.0±1.7
6	8	3.12	12.5	3.12	37.3±1.5
7	8	1.56	12.5	6.25	57.6±0.5
8	8	1.56	12.5	6.25	34.6±2.3
9	8	1.56	12.5	6.25	20.3±0.5
10	8	1.56	12.5	6.25	26.3±2.8

MIC, MBIC, MBEC: minimal inhibitory concentration, minimal biofilm inhibitory concentration, minimal biofilm eradication concentration, respectively

CONCLUSION

The results of current study demonstrated that *Mentha longifolia* essential oil has remarkable antifungal and antibiofilm activity against clinical isolates of *Candida albicans*. Therefore, *M. pulegium* extracts may be used as a potential agent for the treatment of biofilms associated with *C. albicans* infections.

CONFLİCT OF INTEREST

There is no conflict of interest in this study

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