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Original Research Article

Anti-biofilm and antimicrobial activity of *Mentha pulegium* L essential oil against multidrug-resistant *Acinetobacter baumannii*

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Abstract

Purpose: *To investigate the antimicrobial and anti-biofilm activities of essential oil from Mentha pulegieum L. (EOMP) on multi-drug resistant (MDR) isolates of A. baumannii, as well as its phytochemical composition, antioxidant properties and cytotoxic activity.*
Methods: *The phytochemical composition of EOMP was analyzed by gas chromatography, while its antimicrobial activities were determined by disc diffusion and broth micro-dilution methods. Minimal biofilm inhibition concentration (MBIC) and minimal biofilm eradication concentration (MBEC) tests were used for assessment of its anti-biofilm properties. Viability in the biofilm was studied using 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay, while colorimetric assay was used to assess its cytotoxicity on L929 cells*
Results: *D-isomenthone, pulegone, isopulegone, menthol and piperitenone were the major components of the plant extract. EOMP produced > 22 mm inhibition zone for the isolates, with minimum inhibitory concentration (MIC) and MBIC of 0.6 - 2.5 and 0.6 - 1.25 µL/mL, respectively, while MBEC was ≥ 10 µL/msL. EOMP damaged biofilm structures formed by A. baumannii strains at MIC by 26 – 91 %.*
Conclusion: *These results suggest that EOMP contains agents that may be useful in the development of new drugs against A. baumannii infections.*

Keywords: *Mentha pulegium, Essential oil, Anti-biofilm, Antimicrobial activity, Multi-drug resistance, A. baumannii*

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INTRODUCTION

Acinetobacter baumannii is an aerobic, non-fermentative, oxidase-negative and non-motile Gram-negative bacillus. In recent years, the rapidly increasing antimicrobial resistant pattern of this bacterium has become a serious medical problem associated with hospital-acquired infections. *A. baumannii* causes outbreaks of infection and health care–associated infections, including bacteremia, bloodstream infections, pneumonia, meningitis, urinary tract infection and wound infection [1].

Several factors may be responsible for development of virulence and resistance by *A. baumannii*. One of them is the strong biofilm activity formed by this bacterium. Biofilm

production, which is common in many bacterial species, protects pathogens from antimicrobial agents and the immune responses of their hosts. The frequency and severity *A. baumannii* infections have led to recent and heightened interest in understanding and combating the menace [2].

Sessile microorganisms in the biofilms can be much more resistant to an agent compared to the planktonic forms of the same microorganism. Therefore, it is important to understand the relationship between the biofilm and the resistance encountered in treating bacterial infections [3].

The problem of resistance against the antibiotics, which has been increasing substantially, has led need to search for new and more effective therapeutic agents. Antibiotics were discovered from the rich resources of natural products. The potential of herbal extracts and some of their constituents for overcoming antibiotic-resistant bacteria have been extensively reported [4,5].

M. pulegium L. is a flowery plant from the Lamiaceae family which is common worldwide. It is known that this plant (also known as Pennyroyal) is traditionally used for the treatment of many diseases [6]. Although there are publications related to the antimicrobial activity of the *M. pulegium* essential oil (EOMP), not much is known about its anti-biofilm activity. In this study, we aimed at determining the antimicrobial and anti-biofilm effects of the EOMP on multi-drug resistance (MDR) isolates of *A. baumannii*. In addition, the chemical composition of this oil, its antioxidant properties and cytotoxicity were determined.

EXPERIMENTAL

Plant material and extraction of EO

The aerial parts of *M. pulegium* were collected in August of 2014 in Manisa flora located in the Aegean Region of Turkey. The samples were collected from Gordes county and dried air-dried at room temperature. The plant was identified and authenticated by a qualified taxonomist in 19 Mayis University, in Samsun City, in Turkey. At the end of the drying process, the leaves and flowers were ground before the essential oil was extracted by hydro-distillation method using the Clevenger apparatus.

Analysis and identification of essential oil

The chemical composition of the essential oil was determined according to the method of Aksit *et al* [7]. The GC apparatus used was a Perkin-Elmer Clarus 500 Series GC system equipped with a flame ionization detector (FID) and BPX-5 apolar capillary column (30 m x 0.25 mm, 0.25 m i.d.) connected to a mass spectrometer.

Cultivation of L929 mouse fibroblast cells

Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO, USA) was used. L929 (ATCC cell line, NCTC clone 929), a mouse connective tissue fibroblast cell line, was cultured in DMEM containing 10 % fetal calf serum (Sigma, St. Louis, MO,USA) and 2 mM/mL L-glutamine. The medium with the cell culture had no antibiotic added. Cultivation of the cultures was performed in an incubator with 5 % CO_2 and at 37 °C until confluence was observed in the cell monolayer, which occurred after about 7 days. At all times, assays were carried out at the exponential growth phase of the cells.

Cell proliferation assay

This was carried out according to the procedure outlined by Polat *et al* [8]. In this assay, cell viability is assessed through the cleavage of tetrazolium salts, which are added to the culture medium, and for this, the water soluble tetrazolium (WST-1) labeling reagent (Roche, Mannheim, Germany) was used. Cellular enzymes cleave the tetrazolium salt to formazan, the concentration of which is directly proportional to the number of metabolically active cells in the culture.

Microtiter plates (96-well) were used to seed the cells to a concentration of 1×10^5 cells/mL in a 100 µL final volume for each well. After 24 h, the cells were treated with different concentrations of EOMP (6.25, 12.5, 25, 50, and 100 % v/v). The cells were then incubated for 24 h at 37 ^{0}C in a humidified atmosphere containing 5 % CO_2. Thereafter, 10 µL of culture medium (DMEM) in each well was added to 10 µL of the WST-1 labeling reagent. Absorbance was read at 450 nm against control (untreated cells) in a microtiter plate reader (Thermo Scientific Microplate Photometer, Multiskan FC, USA). As a background control, one well contained culture medium and WST-1 labeling reagent (10 µL of WST-1 labeling reagent and 10 µL of culture medium). The absorbance of this negative control was taken 2 h into the tetrazolium reaction. Cell viability was as in Eq 1.

Cell viability (%) = (As/Ac)100 ……(1)

where As and Ac are the absorbance of sample and negative control respectively.

DPPH (2,2-diphenyl-1-picrylhydrazyl) photometric assay

The method is based on the reduction of alcoholic solutions of the stable radical, DPPH by electron-donating antioxidants, thereby decolorizing it. The amount of DPPH that is left after a given time is inversely proportional to the radical scavenging activity of the antioxidant [9]. Five mL of 0.004 % solution of DPPH in methanol was added to 50 microliter of EOMP at different concentrations. Absorbance was read against a blank at 517 nm after 30 min of incubation at room temperature [10]. Eq 2 was used to calculate inhibition of DPPH.

Inhibition (%) = {(A0 – A1)/A0}100 ……..(2)

where A0 = control absorbance and A1 = sample absorbance. Ascorbic acid, a natural antioxidant was used as positive control. All the tests were conducted in triplicate.

Microorganisms

A. baumannii strains used in the study were isolated in the Clinical Microbiology Laboratories of Cumhuriyet University Hospital between 2013 and 2014. The bacteria were identified using the BD Phoenix (Becton Dickinson, Sparks, MD, USA) automated microbiology system and antimicrobial susceptibility tests were conducted. Resistance and susceptibility of the strains against the antimicrobial agents were assessed according to the Clinical Laboratory Standards Institute (CLSI) [11].

Evaluation of antimicrobial and anti-biofilm activity

Disc diffusion assay

Disc diffusion method was applied, with 100 µL of suspension containing 10^8 CFU/mL of bacteria on Mueller Hinton Agar (MHA). After impregnation of the disc (6 mm in diameter) with 20 µL of EOMP (10 µL/disc) at a concentration of 500 µl/mL, it was placed on the inoculated agar. The solvent used for dissolving EOMP was utilized to prepare the negative control. The inoculated plates were incubated at 37 ^{o}C for 24 h. Zone of inhibition was measured to assess the antimicrobial activity against each test organism. All the assays were repeated three times.

Microdilution assay

The broth micro-dilution method which was employed to assess the antimicrobial activities of the essential oil was done in line with the protocol of the CLSI [11]. A micro-well dilution method was taken as a basis for determining the MIC of EOMP against the bacterial strains.

Twenty four-hour broth cultures were used to prepare the inoculums of the microorganisms, and suspensions were adjusted to 0.5 McFarland standard turbidity. EOMP was dissolved in Mueller Hinton Broth (MHB) containing 0.5 % (v/v) Tween 20, and serial two-fold dilutions were prepared in 96-well plates with MHB at a concentrations varying between 0.03 and 2 % (v/v). Whereas there was MHB and cells without EOMP in the wells of the positive control, wells of negative control had only MHB. A BioTek microplate reader (Bio Tek Instruments, Inc., Winooski, VT, USA) was used to measure the absorbance of the plates at 550 nm before and after incubation for 24 h at 37 °C. The minimum inhibitory concentration (MIC) was detected as the lowest concentration of EOMP, and at this concentration, the optical density at the 24th h incubation of the inoculum remained the same or decreased when compared to the reading at the beginning.

Ten µL was taken from each well following the incubation, spot-inoculated on MHA and incubated at 37 °C for 24 h in order to determine the minimum bactericidal concentration (MBC) of EOMP. The concentration without any growth of the subculture was taken as the MBC.

Minimum biofilm inhibitory concentration (MBIC) assay

Minimum biofilm inhibitory concentration (MBIC) is described as the lowest concentration at which the antimicrobial agents prevents the biofilm formation [12]. In order to test the anti-biofilm activity of EOMP against *A. baumannii*, the microtiter plate assay was used.

The MBIC test was carried out using the method of Adukwu *et al* [13]. An aliquot (100 µL) from an overnight culture (diluted with MHB containing 1 % (w/v) glucose to 10^8 CFU/ mL) was dispensed into each test well of a 96-well plate. Then 100 µL of different concentrations of EOMP (0.03 – 2 %) were dispensed into different wells. The negative control contained only MHB whereas the positive control contained cell cultures alone without EOMP. The supernatant of the wells were decanted and each well gently rinsed three times with 300 µL of sterile distilled water and discarded after incubation at 37 °C for 24 h. The plates were dried in air for 30 min and stained with 0.1 % (w/v) crystal violet at room temperature for 30 min, washed 3 times with

distilled water and dried. Thereafter the crystal violet was solubilized in 95 % ethanol and absorbance was read in a microplate reader (Bio Tek Instruments, Inc., Winooski, VT, USA) at 550 nm. MBIC was determined as the EOMP concentration at which the absorbance is equal to or less than that of the negative control. This test was performed in triplicate and the mean (n = 3) was taken.

Minimum biofilm eradication concentration (MBEC) assay

Minimum biofilm eradication concentration (MBEC) is the lowest concentration which can damage the structure of emerging biofilm [12]. MBEC assay was carried out according to the method of Kuzma *et al* [14]. In essence, 200 µL (10^8 CFU/mL) of each strain was inoculated into each well of flat bottom 96-well microtiter plate. The wells were incubated at 37 °C for 48 h. After biofilm formation, the wells were washed four times with sterile distilled water in order to remove non-adherent cells. Serial dilutions of EOMP (0.03 – 2 %) were added to different wells (each EOMP concentration at a volume of 200 µL). Incubation of the plates was at 37 °C for 24 h and thereafter the wells were washed with distilled water and stained with crystal violet. Biofilm without EOMP served as positive control. The concentration at which established biofilms were removed from the bottom of the treated wells was taken as the MBEC.

Evaluation of biofilm eradication

Each *A. baumannii* strain (200 µL of 10^8 CFU/mL) was inoculated into each well of flat bottom 96-well microtiter plate and incubated for 48 h at 37 °C. After biofilm formation, EOMP was added to different wells at concentrations corresponding to MIC, 1/2MIC, 1/4 MIC and 1/8 MIC values. The wells were incubated for 24 h, washed twice with phosphate buffer saline and read at 550 nm. Biofilm eradication was calculated as in Eq 3.

Biofilm eradication (%) = {(Ac – As)/Ac}100 …. (2) [15]

where Ac and As are the absorbance of sample and control respectively.

Biofilm metabolism assay

Evaluation of the effect of EOMP on the metabolic activity of the biofilms formed by the bacterial strains was carried out through a modification of the tetrazolium salt (XTT) reduction test, according to the method of Chaieb *et al* [15]. EOMP at concentrations of 1.25 and 2.5 µl/mL was applied to the 48 h biofilm structures formed in 96-well plates, and after 24 h incubation, XTT reagent was added to the test and control wells. At the end of 5 h incubation period, cell viability was determined by reading the wells at 450 nm in a microplate reader.

Statistical analysis

The results are presented as mean ± standard deviation (n = 3) and assessed by one-way analysis of variance (ANOVA), while Student's t-test was applied for comparison between two groups. $P < 0.05$ were considered statistically significant.

RESULTS

GC and GC-MS data

Table 1 shows results from GC and GC-MS analysis of EOMP.

Table 1: Main components of essential oil of aerial parts of *Mentha pulegium*

Rt [a]	Compound	(%) [b]
4.407	p-Xylene	0.15
5.689	α-Pinene	0.06
6.026	Cyclohexanone, 3-methyl-	0.10
6.661	β-pinene	0.06
7.096	3-Octanol	0.32
10.489	3-Octanol, acetate	0.32
11.163	α-Terpinolen	0.10
11.299	Isopulegone	9.20
11.587	D-isomenthone	33.19
11.899	Menthol	2.98
13.729	Pulegone	51.32
14.042	Piperitone	0.83
16.348	Piperitenone	0.86

Rt[a] = retention time; (%)[b] = relative percentage obtained from peak area

Cytotoxic potential of *M. pulegium* EO

Results of cytotoxicity potential of EOMP are shown in Figure 1.The toxicity potential of the 50 % and 100 % EOMP were statistically different from that of the control (Figure 1, $p < 0.05$). However there were no significant differences in toxicity potential between the control and EOMP at concentrations of 6.25, 12.5 and 25 %.

Antioxidant capacity of EOMP

Figure 2 shows the antioxidant capacity of EOMP with respect to DPPH scavenging. The EOMP scavenged DPPH with an IC_{50} of 8.82 ± 0.67 µg/mL, compared to that of ascorbic acid with IC_{50} of 6.97 ± 1.05 µg/mL.

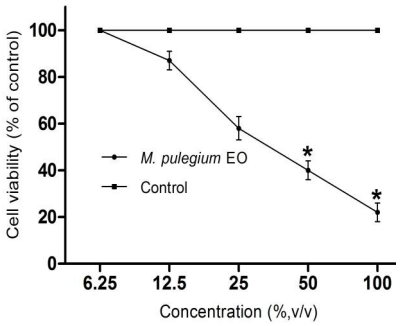

Figure 1: *In vitro* cytotoxic effect of EOMP on L929 fibroblast cells. *$p < 0.05$ compared to control

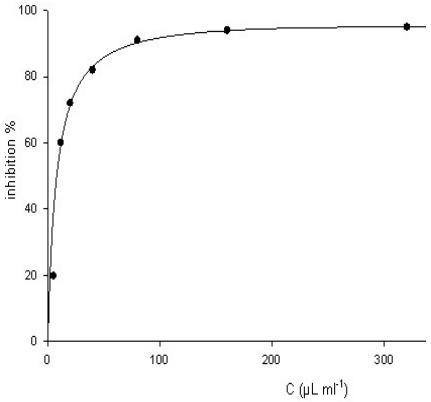

Figure 2: DPPH radical scavenging activity of EOMP. IC_{50} is the concentration of EOMP that scavenged 50 % of DPPH radical. All data are presented as the mean ± SD for three replicates

Antimicrobial and antibiofilm activities

The inhibition zones due to antimicrobial effect of EOMP on the 22 strains of *A. pulegium* tested are shown in Table 2. EOMP formed more than 22 mm inhibition zones in all the strains. The MIC values of EOMP were 2.5 µl/mL in 15 strains, 1.25 µl/mL in 2 strains, and 0.6 µl/mL in 5 strains. Values of MBC were 5 µl/mL in 18 strains and 2.5 µl/mL in 4 strains. MBIC value was 0.6 µl/mL in 2 strains, and 1.25 µl/mL in the other strains. MBEC value was ≥ 10 µl/mL in all the strains.

Reduction in biofilm formation

MIC, 1/2MIC, 1/4 MIC and 1/8 MIC values of the M. pulegium EO were applied onto the bacterial biofilm formations that emerged after the 48h incubation. It was observed that the effect of the EO on the biofilm structure decreased as the concentration reduced. It was seen that the *M. pulegium* EO damaged the biofilm structure at the MIC value by 26-91 % (Table 3).

Inhibition of metabolic activity

EOMP decreased the metabolic activity of the 48 h biofilm structures formed by MDR *A. baumannii* strains in a concentration-dependent manner (Figure 3).

Table 2: Antimicrobial and anti-biofilm activities of EOMP against MDR *Acinetobacter baumannii*

Strain	Zone of inhibition[a]	MIC (µl/ml)	MBC (µl/ml)	MBIC (µl/ml)	MBEC (µl/ml)
1	24.6±2.0	2.5	5	1.25	≥10
2	26.3±3.0	2.5	5	1.25	≥10
3	27.6±1.1	2.5	5	1.25	≥10
4	23.3±1.1	2.5	5	1.25	≥10
5	22.0±1.5	2.5	5	1.25	≥10
6	22.0±1.5	2.5	5	1.25	≥10
7	22.6±0.8	2.5	5	1.25	≥10
8	22.6±0.8	2.5	5	1.25	≥10
9	30.6±3.7	0.6	2.5	1.25	≥10
10	31.1±3.0	0.6	2.5	0.6	≥10
11	22.6±0.8	2.5	5	1.25	≥10
12	24.3±1.1	2.5	5	1.25	≥10
13	32.6±0.5	0.6	2.5	0.6	≥10
14	23.0±3.0	2.5	5	1.25	≥10
15	23.6±1.5	2.5	5	1.25	≥10
16	27±1.7	1.25	5	1.25	≥10
17	31.6±3.5	0.6	2.5	1.25	≥10
18	23.3±1.1	0.6	5	1.25	≥10
19	25.3±2.5	2.5	5	1.25	≥10
20	25.6±2.0	2.5	5	1.25	≥10
21	26.3±1.5	2.5	5	1.25	≥10
22	27.6±1.5	1.25	5	1.25	≥10

MIC = minimum inhibitory concentration, MBC = minimum bactericidal concentration, MBIC = minimum biofilm inhibitory concentration, MBEC = minimum biofilm eradication concentration; [a]values are reported as mean ± SD (mm, n = 3)

Table 3: Reduction in biofilm formation

Strain	MIC	1/2 MIC	1/4 MIC	1/8 MIC
1	85,6±1.5	56.3±3.05	25.0±1.7	8.3±1.5
2	85.0±4.3	84.3±4.7	23.3±2.5	2.3±1.1
3	86.0±7.0	89.3±1.1	16.3±3.5	5.3±2.5
4	86.6±2.0	33.3±2.5	34.0±2.0	12.6±0.5
5	80.3±0.5	75.6±3.0	25.0±3.6	13.6±2.0
6	87.6±1.5	82.6±4.5	18.3±4.0	2.3±1.5
7	89.0±1.0	84.3±5.6	27.6±3.5	4.6±3.7
8	78.0±1.7	77.0±4.0	13.6±1.1	2.6±0.5
9	26.3±2.0	5.0±1.0	NT	NT
10	41.0±2.6	2.33±0.5	NT	NT
11	91.0±2.6	90.3±2.5	28.6±0.5	24.0±2.0
12	87.0±2.0	35.0±2.6	22.6±1.5	9.6±3.5
13	36.0±2.6	8.3±1.5	NT	NT
14	84.6±3.2	78.0±8.1	25.0±5.2	25.3±2.5
15	82.0±4.0	73.0±6.2	11.3±1.5	10.3±3.2
16	56.3±6.1	26.3±7.5	24.0±3.6	NT
17	57.3±2.8	46.6±4.04	NT	NT
18	34.3±4.1	25.6±3.05	NT	NT
19	83.6±5.1	76.6±6.5	32.6±1.5	3.0±1.7
20	84.3±6.02	79.6±4.7	47.6±3.5	38.0±3.0
21	86.3 ±0.5	73.0±2.0	46.6±5.5	26.6±3.2
22	83.3±3.5	69.0±2.6	33.3±2.8	NT

Data are reported as mean ± SD (n = 3); NT =not tested

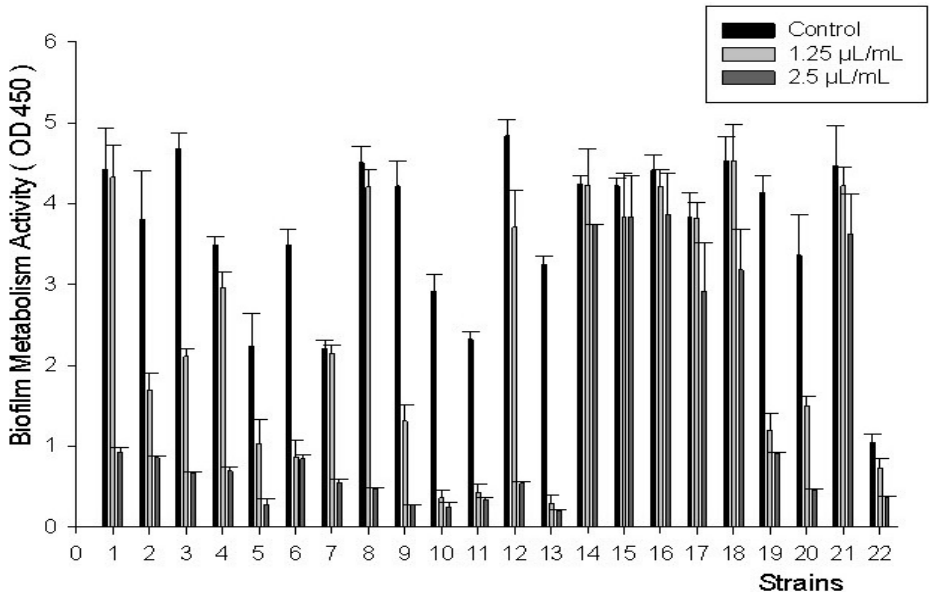

Figure 3: Changes in metabolic activity following 48 h exposure of biofilms of MDR *A. baumannii* strains to EOMP, as determined by XTT assay (control = biofilms not exposed to EOMP). All data are presented as mean ± SD for three replicates

DISCUSSION

Natural products are sources of chemical compounds that have been used as alternative medicines to conventional therapy. Currently, researchers are focusing on the pharmacological and therapeutic activities of plant products.

This study demonstrates that the essential oil of *Mentha pulegium* exhibits anti-biofilm and antimicrobial activities multi-drug resistant strains of *Acinetobacter baumannii*. In addition, the study demonstrated the antioxidant properties, cytotoxic activity and chemical composition of *M. pulegium* EO.

M. pulegium essential oil has strong antimicrobial activity on MDR *A. baumannii* isolates. It was observed that the MIC which has effect on planctonic forms are not adequately effective on the biofilm structure. In the XTT test, MIC was not completely effective on the sessile forms in the biofilm, so that viability continued. Therefore, we concluded that it will be appropriate to

investigate the anti-biofilm activity of the essential oil, and regulate the treatments accordingly. All the *A. baumannii* strains used in this study were found resistant to the tested antibiotics (amikacin, cefepime, ceftazidime, gentamycin, imipenem, meropenem). The cytotoxic potential of 50 % EOMP was significantly different from that of control. The MIC, MBIC and MBEC values of EOMP (≥0.6-2.5 µl/mL, 0.6-2.5 µl/mL and 10 µl/mL, respectively) were quite below the cytotoxic value, blocked microbial biofilm formation even at low concentrations, and eradicated emerging biofilm structures in a concentration-dependent manner even at concentrations below MIC. These results indicate that EOMP has effective antimicrobial and anti-biofilm activities against *A. baumannii*.

DPPH radical scavenging activity assay was used to evaluate the possible antioxidant properties of the *M. pulegium* essential oil. The results obtained confirm previous reports on the importance of essential oils as natural antioxidants [23-25]. A previous study reported that extracts from some *Mentha* species (*M. longifolia* (L.) Huds., *M. piperita* L., *M. pulegium* L., *M. rotundifolia* (L.) Huds., and *M. spicata* L.) have antioxidant and free radical -scavenging properties [26]. The antioxidant properties of *Mentha* extracts may be due to their phytochemical compositions. Previous studies reported that *M. pulegium* has strong antioxidant components [27]. According to result of the present study, *M. pulegium* essential oil can be considered as a strong antioxidant and a strong antimicrobial agent.

The major components of EOMP were pulegone (51.32 %), D-isomenthone (33.19 %), isopulegone (9.20 %), menthol (2.98 %) and piperitenone (0.86 %). This is in agreement with components reported from other studies on *M. pulegium* [28,29]. In 2005, Agnihotri *et al* reported pulegone (65.9-83.1 %) and menthone (8.3-8.7 %) as the major components of the oil of *M. pulegium* collected from different locations in India. Pulegone, piperitenone and isomenthone were reported as the main compounds of the oil of *M. pulegium* from Bulgaria [30,31]. It was also stated in these studies that the essential oil had antimicrobial activity against various microorganisms. The major components of the oil were similar, but may vary according to geographical regions where the plant was harvested, or due to seasonal conditions [23,28,29,32]. Some previous studies reported that pulegone, isomenthone, piperitenone exhibited high antibacterial and antifungal activities [21,33,34]. Thus the antimicrobial and anti-biofilm properties of EOMP could be attributed to its chemical components.

CONCLUSION

The findings of this study show that *M. pulegium* essential oil has strong antimicrobial and anti-biofilm activity against MDR *A. baumannii* isolates. Thus, it is a potential agent for the treatment of MDR *A. baumannii* infections. In addition, the results provide evidence that the essential oil of *M. pulegium* may be a useful source of antioxidants.

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CONFLICT OF INTEREST

No conflict of interest associated with this work.

CONTRIBUTION OF AUTHORS

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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