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ORIGINAL RESEARCH

Evaluation of essential oil obtained from *Mentha×piperita* L. against multidrug-resistant strains

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Background: Bacterial multidrug resistance currently poses an increasingly serious threat, with important clinical consequences regarding treatment options. In 2017, the WHO released a global list of resistant bacteria, identifying multidrug-resistant (MDR) Gram-negative bacteria such as carbapenem-resistant *Enterobacteriaceae, Pseudomonas aeruginosa* or *Acinetobacter baumannii*, extended-spectrum cephalosporin-resistant *Enterobacteriaceae* as critical priorities for developing new strategies of treatment.

Purpose: The novelty presented in this study refers to the evaluation of the volatile oil obtained from the leaves of *Mentha×piperita* L., on MDR strains from hospitalized patients.

Material and methods: The essential oil was extracted by steam distillation and tested on six reference bacterial strains and also on the MDR strains collected from patients of the "Pius Brînzeu" Emergency Clinical County Hospital Timișoara. The in vitro antibacterial activity was evaluated by agar disk diffusion method and microdilution method.

Results: Testing the antibacterial activity of peppermint oil on both reference strains and isolated MDR strains from hospitalized patients demonstrated its bactericidal effect. Minimum inhibitory concentration (MIC) was lower (20 mg/mL) for *Staphylococcus aureus, Escherichia coli* and *Proteus mirabilis* and higher (40 mg/mL) for *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains. Minimum bactericidal concentration (MBC) was equal to MIC, with the exception of *Pseudomonas aeruginosa* strains, where MBC was the double of MIC.

Conclusion: The present study highlights the bactericidal activity of *Mentha×piperita* L. essential oil on all tested MDR or extensively drug-resistant Gram-positive and Gram-negative strains of *Staphylococcus aureus, Escherichia coli, Klebsiella*pneumoniae, *Proteus mirabilis, Pseudomonas aeruginosa* and *Acinetobacter baumannii*. This oil may be a therapeutic option in the near future for many infectious diseases produced by MDR bacteria.

Keywords: bacterial multidrug resistance, peppermint, essential oil, bactericidal effect

Introduction

Medicinal aromatic plants, defined as plants that contain essential oils with the property of volatilizing at room temperature, have been used since ancient times and are well known by all civilizations and cultures for their nutritional, therapeutic and cosmetic potential.[1] Moreover, through the history of mankind, mystic and religious symbols have been assigned to these plant essences.[2,3] The formation of volatile phytochemicals takes place in cells, canals, secretory bags or glandular bristles of specialized histological structures.[4] From a chemical point of view, essential oils are secondary metabolites

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formed from tens to hundreds of molecules belonging to the class of terpenoids (bio-generated by the mevalonate pathway) and phenylpropane (bio-generated by the shikimic acid pathway) derivatives.[5] The most common constituents in volatile oils are monoterpenes, sesquiterpene and phenolic compounds with oxygenated or non-oxygenated derivatives.[6] Stereochemistry is strongly reflected by the odor of the plant's secondary metabolites.[5] Essential oil (EO) can be stored in all organs of the plant (flowers, buds, leaves, fruits, seeds, bark, wood, roots), but in different quantities.[4] Commonly plants contain about 1% volatile oil, rarely more than 15%. Some of the most representative families containing essential oil plants are Lamiaceae, Apiaceae, Myrtaceae, Zingiberaceae, Lauraceae, Rutaceae, Asteraceae and Cupressaceae.[7,8] The extraction method is chosen depending on the volatile oil content, and the main procedures include: (i) distillation and/or entrainment with water vapor; (ii) extraction with volatile or non-volatile solvents; (iii) extraction with supercritical gases; (iv) mechanical methods – pressing.[9,10] The complex chemical composition is reflected by the biological activity, thus, depending on the type of constituents.[11,12] In addition to therapeutic uses, volatile oils are widely used in the cosmetics and food industry.

From the different existing mint species, *Mentha×piperita* L. (MP) is the most known and used in medical as well as industrial and culinary fields. Peppermint is an aromatic perennial herb that belongs to the *Lamiaceae* family. It has a history as a therapeutic remedy dating back to the ancient Egyptian, Roman and Greek times, being assigned with different therapeutic values.[13,14] From the botanical point of view, peppermint is a cross between *Mentha spicata* L., also known under the common name of spearmint, and *Mentha aquatica* L., also known under the common name of water mint.[15] Europe, North America and Asia are the places where this species is most commonly grown. For therapeutic purpose, dried leaves are employed with at least 1% volatile oil content. The harvest can be done twice a year in June and September, at the full maturity of the flowers. Drying is done to a maximum of 35°C to prevent volatilization.[16]

From the chemical point of view, the leaves contain essential oil (0.5–4%), flavonoids, tannins, polyphenol carboxylic acids, triterpene, tocopherols, carotenoids and minerals. The major constituents of the volatile oil obtained from this species by distillation are menthol, mentone, isomentone, methyl acetate, menthofuran, limonene, pulegone, eucalyptol and carvone.[17] The most well-known therapeutic effects for peppermint leaves conditioned in the form of tea or different types of extracts include choleretic-cholagogue, antispasmodic, stomachic, antidiabetic, antiseptic, antibacterial, antiviral, antifungal, antioxidant, antiallergenic, antitumoural, antipruritic activities.[16,18] In case of the volatile oil, the main reported biological effects are anti-infectious (bactericide, antiviral, fungicide, antimalarial, vermicidal), tonic digestive, stimulant, analgesic, local anesthetic, antispasmodic, anti-inflammatory, astringent, decongestant, vasoconstrictor, mucolytic, expectorant and carminative.[16,19–24]

The novelty presented in this study refers to the evaluation of the volatile oil obtained from the leaves of *Mentha×piperita* L., collected from the west part of Romania and obtained by distillation, on MDR strains collected from hospitalized patients.

Materials and methods

Ethical approval

This study was approved by the Ethics Committee of the "Pius Branzeu" Timisoara Emergency Clinical County Hospital (ref. no. 130/13 Sep. 2017).

Plant material

The aerial parts of MP were collected manually at the time of the plants' maximum flowering stage, in Ludeștii de Jos, Hunedoara County (Coordinates: 45°43′5″N 23°10′21″E45° 43′5″N 23°10′21″E) in July–August 2017. The plant material was dried under natural conditions and stored in double paper bags at temperatures of 3–5°C. After identification, voucher specimens (VSNH.BUASTM89/5) were deposited in the herbarium of the Department of Agricultural Technologies, Faculty of Agronomy, Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" at Timișoara.

Isolation of EOs

The EOs were extracted by steam distillation, according to the method previously described by Jianu et al.[25] The EOs were separated by decantation, dried (anhydrous sodium sulfate Sigma-Aldrich, Germany) and stored in hermetically sealed vials (−18°C) for subsequent analyses.

GC-MS characterization of *Mentha×piperita* L. essential oil (MPEO)

Analysis of MPEO was done using the gas-chromatograph equipment with the mass spectrometer (GC-MS) Shimadzu QP 2010 Plus with a capillary column that has the following characteristics: AT WAX 30 m × 0.32 mm × 1 μm. The carrier gas used was Helium with a flow rate of 1 mL/min.

The oven temperature was programmed as follows: Initial oven temperature was 40°C for 1 min, then raised to 210°C at a rate of 5°C/min, holding for 5 mins. Temperatures of the injector and ion source were 250°C and 220°C, respectively. The injection volume of 1 μL at a ratio of 1:50 was used to identify volatile compounds. MPEO constituents were identified using the NIST database[26] and the calculated linear retention indices (LRI) are presented.

Bacterial strains

The oil was tested on six reference bacterial strains: *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC BAA-196, *Klebsiella pneumoniae* ATCC 1705, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* ATCC 43300 (ThermoScientific, Lenexa, Kansas, USA), and also on the MDR strains collected from patients of the "Pius Brînzeu" Emergency Clinical County Hospital Timișoara (PBECCHT) (Tables 1 and 2).

In our study, the MDR strains came from routine clinical activity. At first, the bacteria were isolated on Columbia 5% sheep blood agar (Sanimed, Bucharest, Romania). Identification of all isolates was performed according to morphological characters of colonies and their biochemical tests obtained using the automated Vitek 2 system (bio-Mérieux, Marcy l'Etoile, France). Sensitivity to antimicrobial agents was tested according to the Clinical Laboratory and Standards Institute (CLSI) criteria, by determining the minimum inhibitory concentration (MIC) with the Vitek 2 system.[27]

For Gram-negative bacilli (GNB), phenotypic confirmation of ESBL production was done using the synergy test between extended-spectrum cephalosporins and clavulanic acid.[27,28]

Carbapenemase production was demonstrated by combined disc methods (KPC, MBL and OXA-48 Confirm kit, Rosco Diagnostica, Denmark).[27,29,30]

Table 1 Reference bacterial strains

Bacterial strains	Resistance phenotypes
Escherichia coli ATCC 25922	Wild
Escherichia coli ATCC BAA-196	ESBL
Klebsiellapneumoniae ATCC 1705	Carbapenem-resistant
Pseudomonas aeruginosa ATCC 27853	Wild
Staphylococcus aureus ATCC 25923	Wild
Staphylococcus aureus ATCC 43300	MRSA

Abbreviations: ESBL, extended-spectrum beta-lactamases; MRSA, methicillin-resistant *S. aureus*.

For methicillin-resistant *Staphylococcus aureus* (MRSA) strains, cefoxitin screening was performed. The constitutive macrolide-lincosamide-streptogramin B (MLSBc) phenotype was determined based on its resistance to erythromycin and clindamycin. inducible macrolide-lincosamide-streptogramin B (MLSBi) resistance phenotype was either detected on Vitek AST-P592 cards or by the appearance of a D-region around the clindamycin disk in the vicinity of erythromycin, which was associated with erythromycin resistance (on disk diffusion test).

Inclusion into the MDR group was made according to the definition proposed by Magiorakos (2012). Thus, MDR was defined as acquired resistance to at least one agent in three or more antimicrobial categories, while XDR were considered as bacterial strains that remained susceptible to at most two antimicrobial categories.[31]

After phenotyping, the MDR strains were stored on a microbank (Pro-Lab Diagnostics, Toronto, Canada) at −80°C. Reconstitution was accomplished by discharging cryobiles on Columbia +5% sheep blood agar medium (Sanimed, Bucharest, Romania) and by incubating for 24 hrs at 37°C. After reconstitution, the susceptibility of strains to peppermint oil was tested.

In vitro antibacterial activity

The antimicrobial activity of this oil was evaluated by agar disk diffusion method and microdilution method as previously described.[32,33]

Disk diffusion method

The bacterial suspensions were adjusted with physiological saline to a concentration of 0.5 Mac Farland (10^8bacteria/mL) and 100 μL of these suspensions were placed on the surface of Mueller–Hinton agar (Sanimed, Bucharest, Romania). Ten microliters of oil were added to a blank paper disk (BioMaxima, Lublin, Poland), and then deposited on the surface of the Mueller–Hinton plates inoculated with the microbial suspensions and consequently incubated at 37°C for 24 hrs. The reading of the inhibition zones was done in millimeters. All tests were performed in triplicate for each bacterial strain. Gentamycin 10 μg (BioRad, Marnes la Coquette, France) was used as a positive control, and for negative control, we used a blank paper disk that was not impregnated.

Table 2 MDR bacterial clinical isolates

Bacterial species	ID	Resistance phenotypes	Source
Acinetobacter baumannii	AB1	Carba-R + Fq + Ag + SXT	Bronchoalveolar lavage
Acinetobacter baumannii	AB2	Carba-R + Fq + Ag + SXT	Bronchoalveolar lavage
Escherichia coli	EC1	PASE + SXT + Fq	Urine
Escherichia coli	EC2	ESBL + SXT + Fq	Wound secretion
Escherichia coli	EC3	ESBL + Fq	Urine
Escherichia coli	EC4	ESBL + SXT + Fq	Urine
Klebsiella pneumoniae	KP1	CASE + SXT + Fq	Urine
Klebsiella pneumoniae	KP2	ESBL + Fq	Bronchoalveolar lavage
Klebsiella pneumoniae	KP3	Carba-R + Fq + Ag + SXT	Bronchoalveolar lavage
Proteus mirabilis	PM1	PASE + Ag + SXT	Wound secretion
Pseudomonas aeruginosa	PA1	CASE + Ag + Fq	Wound secretion
Pseudomonas aeruginosa	PA2	PASE + Ag + Fq	Otic discharge
Staphylococcus aureus	SA1	MRSA + M	Nasal swab
Staphylococcus aureus	SA2	MRSA + MLSBc	Nasal swab
Staphylococcus aureus	SA3	MRSA + MLSBi	Wound secretion

Abbreviations: ID, identification; PASE, penicillinase hypersecretion; CASE, cephalosporinase hyperproduction;, ESBL, extended-spectrum beta-lactamases, Carba-R, carbapenem resistance; Ag, aminoglycosides resistance; Fq, fluoroquinolones resistance; SXT, folate pathway inhibitors resistance; MRSA, methicillin-resistant *S. aureus*; M, macrolides resistance; MLSB, macrolide-lincosamide-streptogramin B resistance phenotypes (c-constitutive, i-inducible).

Dilution method-determination of MIC and minimum bactericidal concentration (MBC)

The broth dilution assay was done as recommended by the (CLSI).[26] In seven test tubes, serial two-fold dilutions of the oil (80, 40, 20, 10, 5, 2.5, 1.25 mg/mL) in Mueller–Hinton broth (Sanimed, Bucharest, Romania) were done and was added with the bacterial inoculum (5×10^5 bacteria/mL). After incubating the test tubes at 37°C for 24 hrs, the MIC (the lowest concentration without visible growth) was determined. The MBC was determined by sub-cultivation of 1 µL on Columbia agar +5% sheep blood and was considered as the lowest concentration which killed 99.9% of the initial inoculum.

Statistical analysis

Correlation between parameters (Pearson coefficients) was obtained by using the program "Microsoft Excel 2010".

Results

The chemical composition of MPEO

Figure 1 and Table 3 present the GC-MS (gas-chromatograph with mass spectrometer) profiles of MPEO and the percentages of volatile components in order of elution. In MPEO, 17 compounds (over 0.08%) were identified comprising 99.896% of the total MPEO composition. Monoterpene hydrocarbons (MH) represent 34.229%, monoterpene oxygenates (MO) 60.826%, sesquiterpene hydrocarbonates (SH) 4.635% and sesquiterpene oxygenate (SO) 0.206% of the total compounds (Table 3).

The GC-MS profile evidenced the major compounds of MPEO: p-Mentha-6,8-diene-2-one (57.920%) and p-Mentha-1,8-diene (29.576%). Also, other characteristic phytocompounds of MPEO could be detected as described in Table 3.

The values of the diameters' inhibition zones, MIC and MBC for peppermint oil against 21 bacterial strains are listed in Table 4.

Testing the antibacterial activity of peppermint oil on both reference strains and isolated MDR strains from hospitalized patients demonstrated its bactericidal effect. Out of the 21 strains tested, 16 were represented by GNB (*Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa*), whereas the remaining five were represented by *Staphylococcus aureus*. The MIC of the oil tested was lower (20 mg/mL) for *Staphylococcus aureus, Escherichia coli* and *Proteus mirabilis* and higher (40 mg/mL) for *Klebsiella pneumoniae, Pseudomonas aeruginosa*

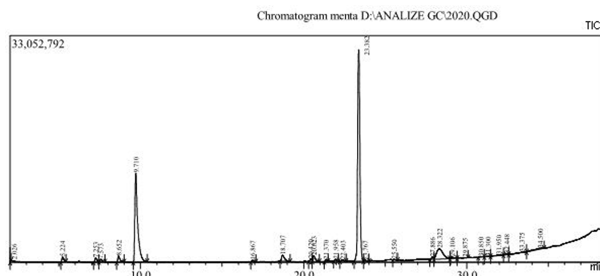

Figure 1 Chromatogram of MPEO.

Table 3 The chemical composition of MPEO (% of total)

Nr.	Compounds	Type	Retention time	LRI	%
1.	α-Pinene	MH	5.224	1006	1.282
2.	β-Pinene	MH	7.253	1089	1.013
3.	Thujene	MH	7.573	1102	0.699
4.	β-Myrcene	MH	8.652	1142	1.659
5.	p-Mentha-1,8-diene	MH	9.710	1180	29.576
6.	Menthone	MO	16.867	1436	0.461
7.	β-Bourbonene	SH	18.707	1505	2.895
8.	Trans-p-Mentha-6,8-diene-2-one	MO	20.454	1574	0.454
9.	Caryophyllene	SH	20.623	1580	1.166
10.	Pulegone	MO	21.370	1610	0.387
11.	β-Farnesene	SH	21.958	1634	0.494
12.	p-Mentha-6,8-diene-2-one	MO	23.380	1693	57.920
13.	Gamma Elemene	SH	23.765	1709	0.080
14.	Carvone oxide, cis-	MO	25.545	1786	0.119
15.	Jasmone (Z)-	MO	27.882	1891	0.288
16.	p-Mentha-6,8-diene-2-ol	MO	28.321	1915	1.197
17.	Caryophyllene oxide	SO	29.085	1961	0.206
Total of compounds					99.896%
Monoterpene hydrocarbonates (MH)					34.229%
Monoterpene oxygenate (MO)					60.826%
Sesquiterpene hydrocarbonates (SH)					4.635%
Sesquiterpene oxygenate (SO)					0.206%

Table 4 Antibacterial activity of the essential oil studied

Bacteria	Phenotypic mechanism of resistance	Disk diffusion method (inhibition zonesin mm)	MIC (mg/mL)	MBC (mg/mL)
Acinetobacter baumannii AB1	XDR: Carba-R + Fq + Ag + SXT +	25	40	40
Acinetobacter baumannii AB2	XDR: Carba-R + Fq + Ag + SXT	26	40	40
Escherichia coli ATCC 25922	Wild	39	10	10
Escherichia coli ATCC BAA-196	MDR: ESBL	32	20	20
Escherichia coli EC1	MDR: PASE + SXT + Fq	35	20	20
Escherichia coli EC2	MDR: ESBL + SXT + Fq	32	20	20
Escherichia coli EC3	MDR: ESBL + Fq	32	20	20
Escherichia coli EC4	MDR: ESBL + SXT + Fq	35	20	20
Klebsiella pneumoniae ATCC 1705	MDR: Carba-R	30	40	40
Klebsiella pneumoniae KP1	MDR: CASE + SXT + Fq	31	20	20
Klebsiella pneumoniae KP2	MDR: ESBL + Fq	30	40	40
Klebsiella pneumoniae KP3	XDR: Carba-R + Fq + Ag + SXT	30	40	40
Proteus mirabilis PM1	MDR: PASE + Ag + SXT	35	20	20
Pseudomonas aeruginosa ATCC 27853	Wild	31	20	40
Pseudomonas aeruginosa PA1	MDR: CASE + Ag + Fq	28	40	80
Pseudomonas aeruginosa PA2	MDR: PASE + Ag + Fq	27	40	80
Staphylococcus aureus ATCC 25923	Wild	42	5	5
Staphylococcus aureus ATCC 43300	MDR: MRSA	32	20	20
Staphylococcus aureus SA1	MDR: MRSA + M	33	20	20
Staphylococcus aureus SA2	MDR: MRSA + MLSBc	31	20	20
Staphylococcus aureus SA3	MDR: MRSA + MLSBi	32	20	20

Abbreviations: ID, identification; PASE, penicillinase hypersecretion; CASE, cephalosporinase hyperproduction; ESBL, extended-spectrum beta-lactamases; Carba-R, carbapenem resistance; Ag, aminoglycosides resistance; Fq, fluoroquinolones resistance; SXT, folate pathway inhibitors resistance; MRSA, methicillin-resistant *S. aureus*; M-macrolides resistance; MLSB, macrolide-lincosamide-streptogramin B resistance phenotypes (c-constitutive, i-inducible).

and *Acinetobacter baumannii* strains (Table 4). MBC was equal to MIC, with the exception of *Pseudomonas aeruginosa* strains, where MBC was the double of MIC.

In order to evaluate the influence of functional molecules of oil and the possible antibacterial activity of those compounds, Pearson correlation between inhibition zones (mm) of principal bacterial strains (*Acinetobacter baumannii, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*) and the chemical constituent derivates from menthone (p-Mentha-1,8-diene, p-Mentha-6,8-diene-2-one, menthone and trans-p-Mentha-6,8-diene-2-one) were established.

The analysis of correlation (Table 5) highlights a very strong (r=1) positive correlation between the strains of *Acinetobacter baumannii* and the total compounds which includes menthone in molecule that suggests the effect of these compounds on bacterial cell growth. Also, a strong positive correlation (r=0.867) was obtained between *Klebsiella pneumoniae* and menthone type compounds. In these cases, we can presume a mutual connection between these chemical compounds and their antibacterial effect. Low positive correlations (r=0.248 and r=0.179, respectively) were identified between pairs: *Pseudomonas*/menthone type compounds and *Staphylococcus*/menthone type compounds, while a low negative correlation (r=−0.046) was identified between *E. coli* and menthone type compounds.

Discussion

Regarding the chemical composition of MPEO, in our study, monoterpene hydrocarbons (MH) represent 34.229%, monoterpene oxygenates (MO) 60.826%, sesquiterpene hydrocarbonates (SH) 4.635% and sesquiterpene oxygenate (SO) 0.206% of the total compounds (Table 3). The major detected compounds of MPEO were the following: p-Mentha-6,8-diene-2-one (57.920%) and p-Mentha-1,8-diene (29.576%) (Table 3). Similar composition was reported by Ashrafi et al who recorded a menthol content in MPEO of 45.05% and menthol 17.53%.[34] The study published by Yu et al reported that the major constituents are within the phytochemical classes of oxygenated monoterpenes (73.9–94.8%), monoterpenes type hydrocarbons (1.0–21.9%) and sesquiterpenes (0.5–16.6%).[35] Reddy et al found menthol (36.02%) as the main chemical compound in MPEO, followed by menthone (24.56%).[36] Similar composition was reported in other studies.[37,38] In Italian MPEO, menthol (32.4%) and menthone (26.6%) were determined,[39] while linalool and linalyl acetate were detected as major compounds of Tunisian MPEO.[40]

Table 5 Pearson coefficients (r) between bacteria strains and chemical composition expressed as menthone and menthone derivates

Bacteria	Menthone and menthone derivates
Klebsiella pneumoniae	0.867
Pseudomonas aeruginosa	0.248
Staphylococcus aureus	0.179
Escherichia coli	−0,049
Acinetobacter baumannii	1

With respect to bacterial multidrug resistance, this currently poses an increasingly serious threat with important clinical consequences regarding the treatment options. Over recent years, infections caused by MDR bacteria have become endemic in many health care units and hospital-acquired outbreaks involving such microorganisms are being reported worldwide.[41–43]

Because of the impact of rising antimicrobial resistance, since 2001, the World Health Organization (WHO) concluded that high priority should be given to measures that aimed to slow the emergence of MDR, these measures being particularly important given that the development of antimicrobial agents has been reduced over the last years.[44] In 2017, the WHO released a global list of resistant bacteria, identifying MDR Gram-negative bacteria such as carbapenem-resistant *Enterobacteriaceae, Pseudomonas aeruginosa* or *Acinetobacter baumannii*, extended-spectrum cephalosporin-resistant *Enterobacteriaceae* as critical priorities for developing new strategies of treatment.

For these reasons, we initiated this multidisciplinary study in order to find a new therapeutic alternative for MDR bacteria-induced infections.

In choosing the bacterial strains obtained from the hospitalized patients, we have tried to show different phenotypes of resistance, either within the same class or in different classes of antibiotics, in order to report the efficacy of the MP oil on several MDR strains. Thus, most of the *Enterobacteriaceae* and *Pseudomonas* clinical isolates were MDR, secreting various beta-lactamases, while the strains of *Acinetobacter baumannii* were XDR.

Different mechanisms could determine the antimicrobial resistance; these include the enzymatic degradation of antimicrobial agents, such as beta-lactamases in the case of beta-lactam resistance or modifying enzymes in

aminoglycosides resistance. The alteration of antimicrobial targets in case of MRSA or fluoroquinolones resistance. Moreover, changes in bacterial membrane permeability can lead to resistance to many antimicrobial agents.[45]

The antibacterial activity of essential oils is incompletely known, but some mechanisms have been proposed in literature over time, such as the alteration of the membrane permeability of pathogens by disrupting transport systems and energy production.

The antimicrobial effect of the essential oil of MP has been studied in various types of microorganisms, both Gram-positive and Gram-negative bacteria.

Mimica-Dukic et al obtained a low MIC (8 µL/mL) on *E. coli* strains, while Hammer et al reported an MIC of 25 µL/mL for *E. coli* and 12 µL/mL for *S. aureus*.[46,47] A few groups, including that of Aridogan et al reported antibacterial activity only for *S. aureus* and not for *E. coli*.[48]

These differences could be due to dissimilarity in the oils' chemical composition collected from different parts of the world.

The antimicrobial action of essential oils has been explained mainly by the presence of terpenes, alcohols, aldehydes and esters. From the terpenes, phenolic compounds, in particular, thymol and carvacrol, appear to be able to increase plasma membrane permeability to cellular metabolites.

Soković et al used volatile oil obtained from *Mentha×piperita* L., collected from Pančevo, Serbia.[20] This geographic region is similar to the area from which the essential mint oil used in our study was obtained. All the mint oils tested showed bacteriostatic activity in concentration of 1 µg/disc. The inhibition zones that were obtained for MP oils were 16.0–25.0 mm and 13.0–25.0 mm. The antibacterial effect was tested for the following human pathogenic bacteria: *Micrococcus flavus, Enterobacter cloacae, Escherichia coli* O157:H7, *Proteus mirabilis, Pseudomonas aeruginosa, Salmonella enteritidis, Salmonella typhimurium, Staphylococcus aureus* and *Streptococcus epidermidis*. Oils from MP exhibited much higher antibacterial activity with the same MIC (1.0–3.0 µg/mL) and MBC (1.5–5.0 µg/mL).[20]

However, in the literature, there are fewer studies on the antimicrobial activity of essential oils of MP on MDR bacterial strains. Shalayel et al have evaluated the antibacterial activities of ethanol, ethyl acetate, methanol and chloroform peppermint extracts on ten MDR pathogenic bacterial clinical isolates, reporting the bactericidal effect of those extracts, especially ethyl acetate extracts, against MDR *S. pyogenes, E. faecalis, MRSA, MRSE* and carbapenem-resistant *E. coli* and *K. pneumoniae*.[49]

A potent antibacterial activity of tea tree oil against MDR microorganisms, which presented a high level of synergism with oxacillin against MRSA, was also observed.[50]

Moreover, dithiodiketopiperazine derivates (isolated from cultures of *Trichoderma harzianum* and *Epicoccum nigrum* from *Zingiber officinale* and *Salix* sp.) manifested antibacterial, antifungal and cytotoxic activity against several MDR microorganisms.[51]

For the moment, little is known about the functional molecules responsible for the antibacterial activity of MPEO and their mechanism of action, menthol and menthone being the major constituents responsible for the antibacterial activity and the most described in the literature.[52–54] Moreover, association of menthol, menthone, limonene, neomenthol, carvone and 1,8-cineole with other minor constituents could induce a synergistic antibacterial activity, although the part of the plant used (root, leaves, etc.), composition of the used extract, concentration of the active substance and the type of microorganism are crucially important factors for the potency of the antimicrobial action.[55–57]

In this study, MP oil had a bactericidal effect on all bacterial tested strains, regardless of the resistant phenotypes these strains exhibited against the currently applied anti-infective agents. Different beta-lactam resistant phenotypes of the tested bacteria were associated with minor differences in the diameters of the inhibition zones, but with significant variations in MIC and MBC, thus confirming the importance of determining the MIC/MBC for the determination of antibacterial activity of substances. Resistance to aminoglycosides, fluoroquinolones, sulfamides, macrolides or lincosamides did not influence the antibacterial activity of MP oil. As in other studies, the bacterial species significantly influenced the test results, with the most sensitive strains to MP oil represented by *S. aureus, E. coli* and *Proteus mirabilis*, while *Pseudomonas aeruginosa* strains exhibited the highest MIC and MBC values.[58]

Regarding the correlation between chemical and microbiological parameters, a strong correlation was recorded between menthone and menthone compounds (p-Mentha-1,8-diene, p-Mentha-6,8-diene-2-one, trans-p-Mentha-6, 8-diene-2-one) and the bacterial strains of *Klebsiella pneumoniae* and *Acinetobacter baumannii*, that suggest a

potential effect of menthone and its derivatives in inhibition of bacterial cell growth. Regarding the development of *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* cells in correlation with the chemical composition, low values of Pearson coefficients were observed. These findings lead to the idea that a high level of menthone and menthone derivates is responsible for a pronounced inhibition of *Klebsiella pneumoniae* and *Acinetobacter baumannii* strains, without affecting the development of *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* strains. The microbiological effect of *Mentha piperita* essential oil can be explained by the synergism exercised by the minority components of the oil and the compounds derived from menthone. Our studies agree with previous data obtained by our group that reported the influence of synergism of chemical compounds on the microbiological activity of essential oils belonging to *Lamiaceae* family.[59]

Due to the bactericidal effect of MP oil demonstrated on MDR bacteria, it could become a new antimicrobial agent. Volatile MPEO could be used locally, by inhalation in the respiratory tract, or in treatment of various skin infections. Further studies are necessary to investigate the potential toxicity of MP oil and standardize the inhibitory effect of MP oil against MDR pathogens.

Conclusion

The present study highlights the bactericidal activity of MPEO on all tested Gram-positive and Gram-negative strains of MDR or XDR: *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa* and *Acinetobacter baumannii*. This oil may be a therapeutic option for more and more frequent infections caused by MDR bacteria in the future. In this purpose, studies that investigate also the possible adverse effects of this essential oil should be conducted.

Abbreviations

Ag-, aminoglycosides resistance; c, constitutive; Carba-R, carbapenem resistance; CASE, cephalosporinase hyperproduction; CLSI, Clinical Laboratory and Standards Institute; ESBL, extensive spectrum beta-lactamase; EO, sential oil; Fq-, fluoroquinolones resistance; GC-MS, gas-chromatograph with mass spectrometer; i, inducible; ID, identification; LRI, linear retention indices; M-, macrolides resistance; MBC, minimum bactericidal concentration; MDR, multidrug-resistant; MLSB, macrolide-lincosamide-streptogramin B; MIC, minimum inhibitory concentration; MH, monoterpene hydrocarbonates; MO, monoterpene oxygenates; MP, *Mentha×piperita* L.; MPEO, *Mentha×piperita* L. essential oil; MRSA, methicillin-resistant *S. aureus*; PASE, penicillinase hypersecretion; SH, sesquiterpene hydrocarbonates; SO, sesquiterpene oxygenate; SXT, folate pathway inhibitor resistance; WHO, World Health Organization; XDR, extensively drug resistant.

Disclosure

The authors report no conflicts of interest in this work.

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