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In Vitro Antibacterial Activity and Chemical Composition of Essential Oil of *Mentha arvensis* Linn. Leaves

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Abstract: The essential oil obtained from fresh leaves of *Mentha arvensis* (Lamiaceae), a perennial aromatic herb grown in Saudi Arabia, was screened for *in vitro* antibacterial activity and chemical composition. Hydro-distilled essential oil (yield 0.71 %) was analysed for its chemical composition by gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). The antibacterial activity was tested *in vitro* on gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus pyogenes*) and gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) using agar disc diffusion assay and minimum inhibitory concentration (MIC) was also determined. The essential oil analysis displayed the presence of 21 constituents which represent 97.51 % relative peak area of the whole chromatogram. The leading components of the oil were monoterpenes (92.52 %) represented by 04 hydrocarbons (5.10 %) and 11 oxygenated derivatives (87.42 %) with menthol (56.35 %), menthone (10.90 %), methyl acetate (7.70 %) and isomenthone (5.20 %) as the major constituents. The pathogens were sensitive to the oil and showed highly effective antibacterial activity with the maximum inhibition zone against *S. aureus* (22.33 ± 1.15 mm). The minimum inhibitory concentration (MIC) of the oil was higher against gram-positive bacteria than the gram-negative bacteria. The results indicate that the fresh leaves oil of *M. arvensis* has high potential as an antibacterial agent for both pharmaceutical and pesticide industries and can also be a promising candidate for flavor and fragrance applications.

Key words: *Mentha arvensis,* essential oil, GC/GC-MS, menthol, antibacterial activity.

Introduction

The diseases caused by bacterial pathogens are a serious problem and of great concern to the medical world. The rapid development of resistant to drugs in bacteria and transmission around the world are amongst the major threats to the community and to the treatment of the diseases. Due to the immense pressure of legal authorities and consumers, the food industry has restrained the use of chemicals to minimum or have adopted more eco-friendly approach towards the preservation and increasing the shelf-life [1,2].

World Health Organization (WHO) acclaimed that large population of the world depends on the herbal medicines for the treatment of common diseases. Plants with medicinal values are extensively used in the production of medicine as they are the major source of natural organic com-

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pounds. The essential oils of the medicinal plants are the important source of antimicrobial compounds. A large number of studies have recorded the antimicrobial potential of plant compound against pathogenic microorganisms [3-6]. The essential oils having different class of compounds such as phenols, carbohydrates, ethers ketones, aldehydes and alcohols are the major source of fragrance and biological activities. Because of these properties, since ancient time, these medicinal and aromatic plants have been added to nourishment as seasoning operators as well as additives [7,8].

Mentha arvensis Linn. (Lamiaceae), commonly known as Japanese mint, Corn mint and Menthol mint is an perennial herb widely distributed in the temperate regions of Europe and western and central Asia, east to the Himalaya and eastern Siberia and North America [9-11]. *Mentha* species are mainly grown in the eastern part of Saudi Arabia and are used in herbal tea, for flavouring and as traditional medicine. *M. arvensis* is accountable for the second largest producer of essential oil in the world [12].

The chemical composition of essential oil of *Mentha* species has been explored by several scientific workers [1,13-18]. These studies have been reported in different chemical class such as oxygenated monoterpenes, monoterpene hydrocarbons, sesquiterpene hydrocarbons, sesquiterpenoids and diterpenoids. Plant species of *Mentha* has been exhibited the several biological activities [8,1,19-20].

The plants oils are composed mainly of terpenoids and also phenylpropanoids the monoterpenes are found in most abundance [21]. The *M. arvensis* essential oil having approximately 90 % monoterpenes constitutes, in which menthol represents 70-95 % of the monoterpenes [22]. It has been reported that *M. arvensis* oil contains several aroma chemicals like menthol, menthone, isomenthone, menthofuran, carvone, linalool, linalyl acetate [16-18,23-27], volatile oil like piperitenone oxide and some other compounds such as α-terpineol, p-menthone, menthol acetate [18]. These compounds have great demand in pharmaceutical, food, beverages and other related industries [28]. In conventional medicine, *M. arvensis* is used as nasal decongestant, carminative, as well as for the treatment of gastric and skin diseases [29]. The therapeutic and economic importance of essential oil extracted from *M. arvensis* has been recognized and has been continuously explored for new possible application of oil [30]. Besides, the plant *M. arvensis* has attracted much interest of researchers due to worldwide occurrence as well as several biological activities including, antibacterial antifungal, antiviral and cytotoxic activities [17,29,31-34]. In this context, there is no data available about the antibacterial and chemical composition of oil of *M. arvensis* from the location of Saudi Arabia.

The present study, reports chemical composition of the essential oil isolated by hydro-distillation from the fresh leaves of *M. arvensis* and efficacy of the oil against five bacteria (*S. aureus, B. subtilis, S. pyogenes*, *E. coli* and *P. aeruginosa*).

Materials and methods

Plant material and chemicals

The fresh leaves of *M. arvensis* were collected from the local farm (Al hafuf), Saudi Arabia in June 2015. The plant was authenticated by the Plant Taxonomist, by comparing specimen with the authentic one preserved in the Herbarium of the university (voucher specimen #11683). The chemicals, sodium sulphate (anhydrous), diethyl ether and dichloromethane were purchased from Merck (Darmstadt, Germany). Gentamycin (bacteriostatic) was acquired from Sigma Chemical Co. (St. Louis, MO, USA). Mueller Hinton Agar (MHA) media and Muller Hinton Broth (MHB) were procured from Himedia, Riyadh, Saudi Arabia.

Isolation of essential oil

The isolation of essential oil from the leaves of *M. arvensis* was carried out by the method described by Kumar *et al.* [35]. Briefly, the fresh leaves of *M. arvensis* (100 gm) were cut into small pieces and were placed in the round bottom flask containing 400 ml deionised distilled water. The sample was hydro-distilled in a Clevenger-type apparatus for 4 hours. To extract the oil from the distillate the solvent diethyl ether (Merck) was used. The ethereal layer was dried over anhydrous sodium sulphate. The removal of ether on water

bath (35°C) obtained slight yellow colored oil. The oil was transferred into a glass amber vial and stored at 4°C until analysed.

Chromatographic analysis

The 50 µL neat essential oil of *M. arvensis* was dissolved in dichloromethane (Sigma-Aldrich, USA) and a final volume was made to 1.0 ml. Identification of the oil constituents with comparisons of their GC retention indexes and their mass spectra with authentic standards [(+)-limonene and (-)-menthol)] from Sigma-Aldrich (USA), and spectroscopic (mass spectra interpretation, comparison with libraries such as Adams and NIST) evidences [36-37]. The retention indices (RI) were calculated for all volatile constituents using a homologous series of n-alkanes (C_6-C_{25}).

Gas chromatography-Flame ionization detector (GC-FID)

The quantification of constituents of essential oil was achieved through gas chromatograph (GC 5890II series, Hewlett Packard). The instrument was equipped with the flame ionization detector (FID), split/splitless injector with a split ratio of 1:50 and a data system (GC HP-ChemStation software). A fused silica capillary CP-Sil 8 CB column (Agilent Technologies, USA) with the dimension of 30 m × 0.25 mm i.d., film thickness 0.25 µm coated diphenyl-poly (methylsiloxane) phase was used. Initially, the oven temperature was raised to 70°C for 4 min and finally held at 220°C for 5 min. The temperature of the injector port was set at 210°C, while the ionization chamber was set at 230°C and FID temperature was set at 250°C. The carrier gas was helium (99.995 %, Linde, Saudi Arabia), the flow rate was set at 1.1 ml/min. In the FID, hydrogen and air were used at 30 and 300 ml/min, respectively. In this analysis makeup gas was nitrogen (30 ml/min). Percentage composition of the essential oil was calculated from the peak areas using the normalization method [35].

Gas chromatography-Mass spectrometry (GC-MS)

To identify the components of essential oil, the Perkin Elmer (Clarus 500, USA) gas chromatography coupled with (Clarus 500, USA) mass spectrometer (MS), equipped with CP-Sil 8 CB column (30 m x 0.32 mm id x 0.25 µm film thickness) was used. The oven temperature for the column was programmed identically as for the GC analysis. Mass spectra were acquired by automatic scanning in the mass range *m/z* 50-600 amu at 5.1 scan/s. For homogeneity, peaks were identified by studying the mass chromatograms of fragmentation pattern of such compound obtained by mass spectrometry analysis and also by using peak purity function of MSD software.

Antibacterial activity

Disc-diffusion assay

Antibacterial activity of the essential oil was tested against *Staphylococcus aureus* (MRSA ATCC 29213), *Bacillus subtilis* (ATCC 6633) and *Streptococcus pyogenes* (ATCC 12384) *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) obtained from the Department of Botany and Microbiology, King Saud University. These cultures were maintained on slants of Mueller Hinton Agar (MHA) media at refrigerator temperature. Antibacterial activity of essential oil was assessed by the disc diffusion assay [38-40]. The 10 µl bacterial suspension (10^6 CFU/ml) cultured in Mueller Hinton broth for 24 h was spread over the surface of MHA. Filter paper discs (6 mm in diameter) loaded with the 10 µl of essential oil and Gentamycin 40 µg (positive reference standards) separately were placed on the surface of the MHA. The plates were incubated at 37°C for 24 h, after that the zone of inhibition around the disc was measured.

Minimum inhibitory concentration (MIC)

Agar plate dilution test was used to determine the Minimum Inhibitory Concentration (MIC) of an antimicrobial agent. To determine the MIC, oils were dissolved in MHB (50 µl/ml) and serially diluted in eppendorf tubes under a laminar flow cabinet. The 10 µl of an overnight grown pure culture of the tested pathogen was transferred into each eppendorf tubes containing different concentration of oil and cultures were incubated at 37°C for 24 h. The following morning, streaking was done from all samples on MHA plates. MIC was

rated by the lowest concentration of the test solution that inhibited growth.

Results and discussion

Chemical composition of isolated essential oils

The hydro-distillation of *M. arvensis* fresh leaves produced slight yellow colored oil (yield, 0.71 %) of characteristic odor. Table 1 represents the qualitative composition of the oil analysed by GC/GC-MS. Twenty-one individual components (>0.01 %) were identified which represent 97.51 % relative peak area of the complete chromatogram. The leading components of the oil were monoterpenes (92.52 %) represented by 04 hydrocarbons (5.01 %) and 11 oxygenated derivatives (87.42 %) with menthol (56.35 %), menthone (10.90 %), methyl acetate (7.70 %) and isomenthone (5.20 %) as the major constituents. The oil was poor in oxygenated sesquiterpene compound, germacrene D-4-ol (0.90 %) and

Table 1. Chemical composition of the essential oil isolated from *M. arvensis* fresh leaves

No.	Compound	Type[a]	RI[b]	%	Identification mode
1	*cis*-3-Hexenol	OC	861	0.46	RI, MS
2	α-Pinene	MH	936	0.90	RI, MS
3	β-Pinene	MH	976	1.60	RI, MS
4	Myrcene	MH	989	0.70	RI, MS
5	3-Octanal	OC	997	1.12	RI, MS
6	Limonene	MH	1027	1.90	RI, MS, Co-inj.
7	Eucalyptol	MO	1034	0.54	RI, MS
8	Menthone	MO	1151	10.90	RI, MS
9	Neomenthol	MO	1159	3.90	RI, MS
10	Isomenthone	MO	1162	5.20	RI, MS
11	Menthol	MO	1173	56.35	RI, MS, Co-inj.
12	α-Terpineol	MO	1189	0.67	RI, MS
13	*cis*-Carveol	MO	1226	0.33	RI, MS
14	Pulegone	MO	1231	0.58	RI, MS
15	Piperitone	MO	1251	0.90	RI, MS
16	Menthyl acetate	MO	1289	7.70	RI, MS
17	Isomenthol	MO	1449	0.35	RI, MS
18	β-Farnesene	SH	1441	0.81	RI, MS
19	Germacrene D	SH	1483	1.30	RI, MS
20	Germacrene D-4-ol	SO	1572	0.90	RI, MS
21	Caryophyllene oxide	SO	1579	0.40	RI, MS
	Monoterpene hydrocarbons			5.10	
	Oxygenated monoterpenes			87.42	
	Oxygenated compounds			1.51	
	Sesquiterpene hydrocarbons			2.11	
	Oxygenated sesquiterpene			1.30	
	Total identified, %			97.51	
	Oil yield, %			0.71	

*RI, Retention Indices

MS, Mass Spectra (EI, 70 eV)

Co-inj., Comparison with retention time of standard compounds.

a: Compound type: monoterpene hydrocarbon (MH), oxygenated monoterpene (MO), oxygenated compound (OC), sesquiterpene hydrocarbon (SH), oxygenated sesquiterpene (SO).

b: Linear program retention indices determined on the CP-Sil 8 CB (30 m) column using (C_6-C_{25}) series.

caryophyllene oxide (0.40 %). Profile of different chemical class of volatile compounds of oil is shown in Figure 1.

Pino *et al.*[23] reported that the dominant components of oil of *M. arvensis* grown in Cuba were menthol (51.68 %), menthone (26.08 %) and menthyl acetate (10.55 %). Another relevant study on the plant essential oil from western Himalayan region (India) found that menthol (73.7-85.8 %), menthone (1.5-11.0 %), menthyl acetate (0.5-5.3 %), isomenthone (2.1-3.9 %), limonene (1.2-3.3 %) and neomenthol (1.9-2.5 %) were the main constituents [18.] A study from the Pantnagar (India)[24] revealed that the menthol (77.5-89.3 %) was the dominant compound of all the cultivars of *M. arvensis*, next to it were menthone (0.3-7.9 %) and isomenthone (3.7-6.1 %). Interestingly, several predominant constituents of reported earlier such as menthol, menthone, methyl acetate, isomenthone, limonene and neomenthol constituents were also identified in our sample. Menthol (56.35 %), detected as a chief constituent of oxygenated monoterpene class has been reported to display the antibacterial activity. Besides, other constituents identified in *M. arvensis* the menthone, isomenthone, menthyl acetate, neomenthol, limonene, β-pinene have also been found to possess antibacterial activity. Thus, the observed antibacterial activity of *M. arvensis* could be attributed to synergistic action of the constituents present in it. It has been noted that hydrocarbon monoterpenes shows the low activity against bacteria, whereas, oxygenated compounds like phenol-type compounds as menthol, thymol and carvacrol have more antibacterial potential. Knobloch *et al.*[41] described that oxygenated monoterpenes display high antimicrobial activity, while hydrocarbon derivatives possess low antimicrobial properties. Previous study [42], showed that greater antimicrobial potential could be ascribed to the oxygenated terpenes, especially phenolic compounds.

Antibacterial activity

The oil from *in vitro* plant of *M. arvensis* was evaluated for their antibacterial activity (Table 2). Among the bacteria strains tested, the essential oil displayed strong activity against all the tested bacterial strains except *P. aeruginosa* as evident from the zone of inhibition comparable to gentamycin. The tested strains were found to be sensitive to the oil studied and showed highly effective antibacterial activity with the maximum inhibition zone against *S. aureus* (22.33 ± 1.15 mm) and moderate inhibition displayed by *E. coli* (23.30 ± 2.89 mm) and *B. subtilis* (20.67 ± 2.89 mm). Essential oils containing terpenes are reported to exhibit microbial toxicity [43-44].

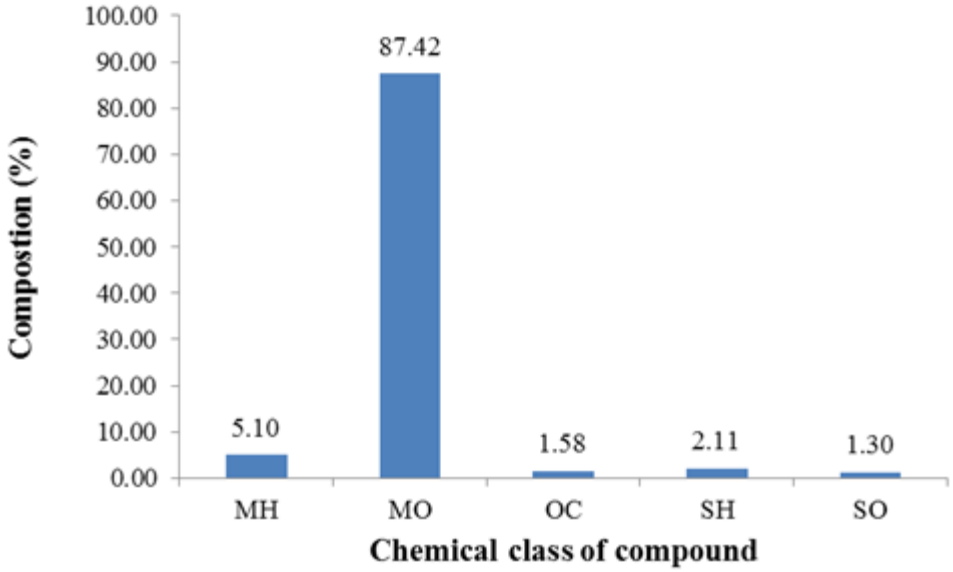

Figure 1. The chemical class composition of the essential oil of *M. arvensis*. Monoterpene hydrocarbon (MH), oxygenated monoterpene (MO), oxygenated compound (OC), sesquiterpene hydrocarbon (SH), oxygenated sesquiterpene (SO)

Table 2. *In vitro* antibacterial activity and minimum inhibitory concentration of fresh leaves of *M. arvensis* essential oil

Test Bacteria	Zone of inhibition (mm) (mean n=3) Test oil [a]	Gentamycin [b]	MIC (µl/ml)
Staphylococcus aureus	22.33 ± 1.15	25.00 ± 0.58	12.5
Bacillus subtilis	20.67 ± 2.89	25.00 ± 0.00	25.0
Escherichia coli	23.30 ± 2.89	23.33 ± 0.58	12.5
Streptococcus pyogenes	17.67 ± 2.30	15.67 ± 2.89	-
Pseudomonas aeruginosa	8.00 ± 0.00	0 ± 0	-

a: 50 µL/ml;
b: 40 µg/ml

Monoterpene hydrocarbons and oxygenated monoterpenes have been shown to display antibacterial activity through damaging the cell organelle resultant in inhibiting the important processes like ion transport and respiration [45]. Studies examining the activity of essential oils against food borne pathogens reported that the essential oils have higher antibacterial activity against gram-positive than gram-negative bacteria [46]. The maximum activity of *M. arvensis* was observed against gram-positive bacteria *S. aureus*, *B. subtilis* and *S. pyogenes* than gram-negative bacteria (*E. coli* and *P. aeruginosa*). Zang *et al*.[47] reported that the ethanol extract of *M. arvensis* has an antibacterial potential against *A. baumannii* and acts by inducing lethal cellular damage to the bacterium. The minimum inhibitory concentration (MIC) of the oil was observed to be more against gram-positive bacteria than the gram-negative bacteria tested. However, the significant value of MIC was also recorded against *B. subtilis, S. aureus* and *E. coli* strains (25.0, 12.5 and 12.5 µl/ml, respectively) [48]. Further, the studies are expected, to explain the role of oil constituents (twenty-one) which demonstrate the bactericidal impacts.

Conclusions

The hydro-distilled essential oil of *M. arvensis* was analysed by GC/GC-MS for its chemical composition. The essential oil exhibited the presence of twenty-one compounds representing 97.51 % area of the total chromatogram. The leading components of oil were monoterpenes (92.52 %) with menthol (56.35 %), as the major constituent. The antibacterial activity was tested *in vitro* condition on gram-positive and gram-negative bacteria using agar disc diffusion method. The strains were found to be sensitive to the oil and showed highly effective antibacterial activity with the strongest inhibition zone against *S. aureus* (22.33 ± 1.15 mm). The MIC of the oil in gram-positive bacteria was higher than in the gram-negative bacteria. The results indicate that fresh leaves oil of *M. arvensis* has high potential as an antibacterial agent and can be utilised in pharmaceutical and pesticide industries. Moreover, it can also be a promising candidate for flavor and fragrance applications. A further study under *in vitro* conditions is recommended to further elaborate the biological activities of *M. arvensis* essential oil for various other applications.

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