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Research Article
Chemo-geographical Variations in the Composition of Volatiles and the Biological Attributes of *Mentha longifolia* (L.) Essential Oils from Saudi Arabia

[1]Farooq Anwar, [2,3]Khalid M. Alkharfy, [4]Najeeb-ur-Rehman, [1]Elsadig Hassan Khamis Adam and [5]Anwar-ul-Hassan Gilani

[1]Department of Pharmaceutical Chemistry, College of Pharmacy, Prince Sattam bin Abdulaziz University, 11942 Al-Kharj, Saudi Arabia
[2]College of Pharmacy, Prince Sattam bin Abdulaziz University, 11942 Al-Kharj, Saudi Arabia
[3]Department of Clinical Pharmacy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia
[4]Department of Pharmacology, College of Pharmacy, Prince Sattam bin Abdulaziz University, 11942 Al-Kharj, Saudi Arabia
[5]Pakistan Council for Science and Technology, G-5/2, Islamabad, Pakistan

Abstract

Objective: The objective of study was to assess the variation of volatiles and biological activities (antioxidant, antimicrobial, cytotoxic and thrombolytic) of essential oils from aerial parts of *Mentha longifolia* harvested from five regions of Saudi Arabia such as Al-Kharj, Al-Qassim, Dammam, Abha and Al-Madinah. **Methodology:** Within the regions, the hydro distilled essential oil yield varied from 0.66-1.52% (dry weight basis) with least contribution from Abha and the highest from Al-Qassim populations. The isolated oils were then analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) which confirmed the identification of a total of 18-33 volatile components in different *M. longifolia* ecotypes, representing 96.7-98.9% of the total oil composition. The oils were analytically characterized by occurrence of carvone (35.30-71.51%) as a principal compound, followed by limonene (5.73-28.45%) and 1, 6 dihydrocarveol (0-12.33%). **Results:** Results showed that trans-dihydrocarvone, 1,8-cineole, β-caryophyllene, β-bourbonene, germacrene D and bicyclosesquiphellandrene were also detected in considerable amounts. The oils mainly, showed notable ($p<0.05$) quantitative variations and were dominated by oxygenated monoterpenes (53.76-79.65%) followed by monoterpene hydrocarbons (8.63-32.13%), sesquiterpene hydrocarbons (2.79-18.41%) and then oxygenated sesquiterpenes (0.77-2.18%). The oils (10.0 μg mL^{-1} concentration) exhibited notable antioxidant potential as assessed by the determination of DPPH• scavenging capacity (54.9-89.7%; IC_{50} 4.4-8.5 μg mL^{-1}) along with total phenols (0.7-4.7% mg GAE/100 g). Moreover, the oils, relative to major compound, carvone and reference compounds, showed moderate antifungal activity against *A. niger*, *A. flavus* and *F. solani* but were weekly active against bacteria such as *E. coli* and *S. aureus*. The oils exhibited moderate to good thrombolytic activity with clot lysis of 11.6-68.4% and low cytotoxicity (1.7-5.1%) *in vitro*. Except, antioxidant assays, the biological attributes of the oils tested were found to be fairly correlated with the contents of oxygenated monoterpenes and/or monoterpene hydrocarbons. **Conclusion:** Overall, the current findings revealed noteworthy variation in the (bioactives) composition and biological characteristics of the tested essential oils that can be mainly linked to the morphological and biochemical diversity of *M. longifolia* plants depending on the agro-ecological conditions of the areas/regions selected. The presently examined *M. longifolia* chemotypes from Saudi Arabia can be explored as a prospective source of carvone-rich essential oil with promising antioxidant, antimicrobial and thrombolytic activities. These results may also provide a scientific basis and support for the ethno medicinal uses of this species in traditional healing.

Key words: Wild mint oil, hydrodistillation, GC-MS, carvone-rich chemotypes, total phenolics, DPPH• scavenging, cytotoxic, thrombolytic

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Corresponding Author: Farooq Anwar, Department of Pharmaceutical Chemistry, College of Pharmacy, Prince Sattam bin Abdulaziz University, 11942 Al-Kharj, Saudi Arabia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Saudi Arabia is known as one of the major centers of plant biodiversity in Arabian Peninsula[1,2] due to diverse natural environment and topographic conditions such as rocky and sandy deserts, valleys, mountains, salt-pans and meadows. Due to high ecological diversity, a wide variety of indigenous medicinal plants and herbs with variable morphological characteristics are distributed in different regions of the country[3,4]. According to an ethnobotanical survey on the diversity in Saudi Arabian flora it has been documented that Lamiaceae family is one of the main sources of medicinal and aromatic herbs naturalized under diverse agro-ecological conditions in several parts of the Kingdom[4].

Most of the Lamiaceae herbs, apart from culinary and perfumery uses have also been traditionally employed as folk remedy for the treatment of cough, diarrhea, constipation and headaches[5,6]. Now there is more focus on the investigation of biological activities, especially the antioxidant/antimicrobial effects of essential oils derived from Lamiaceae herbs[7-9].

According to studies, chemicals and biological activities of Lamiaceae plant based essentials oils not only varied within the species and its varieties but also among different agro-climatic and geographical regions[7,8,10-12]. Such variations in the composition of Lamiaceae plant essential oils can be mainly linked to genetic as well as environmental factor that also define the genetic expressions and affect the chemical composition of the oils[13].

One of the important members of Lamiaceae family, *Mentha longifolia* (L.) Huds, which is distributed in many parts of the world such as Europe, Mediterranean regions, Australia, Asia and Africa also grows widely in Saudi Arabia[13,14]. Traditionally, *M. longifolia,* also known as wild mint, has been utilized as food flavoring and medicinal agent in the native medicine systems of several civilizations to treat cough, colds, asthma, headaches and digestive disorders due to its carminative, antihypertensive, antispasmodic and stimulant properties[15-17]. Recently, there is increasing scientific interest in the evaluation of potential pharmacological such as antimicrobial[8,15,18,19] antioxidant[15,20], anti-inflammatory[16] and biopesticidal[21] activities of *M. longifolia*-derived extracts and/or essential oils.

According to studies from different countries, the oil yield and composition of oil bioactives in *M. longifolia* varies depending upon the agro-climatic and geographical factors[16,20,22-24]. Typically, the content (yield) of essential oil from this species varies between 0.9-1.8%[22]. The essential oils of *M. longifolia* from different countries are reported to contain a complex profile of volatile constituents. The GC-MS analysis of *M. longifolia* oils depicted menthol, carvone, menthone, piperitone oxide, pulegone, piperitenone, d-limonene and 1,8-cineole as dominating components, nevertheless, a considerable chemo-geographical variation in the qualitative and quantitative composition of this species oil across different countries has been observed[25,18,20]. It has also been revealed that such chemo-geographical variation in the composition of bioactives of *M. longifolia* oil might have an imperative role in defining the biological and pharmaceutical characteristics of the oil from different ecotypes[18].

Locally known as Al-Madinah mint, Habak or Hassawi, *M. longifolia* is widely cultivated in different regions of Saudi Arabia for its traditional food and medicinal uses. Few latest studies investigated the biological and therapeutic activities such as antimicrobial, anti-viral, anti-cancer and antioxidant characteristics of ethanol and methanol extracts of *M. longifolia* from Saudi Arabia[26-28]. In an another recent study[13] the composition of *M. longifolia* essential oil isolated from the plants of Northern areas of Kingdom of Saudi Arabia was examined; however, no methodical and detailed studies as such have been made earlier to explore and quantify the differences in the yield and chemicals (bioactives) composition as well as biological activities (attributes) of volatile oil from *M. longifolia* populations harvested from different agro-climatic regions of Saudi Arabia. The present study was therefore, an attempt to assess the variations in the yield, bioactive chemicals and biological properties of volatile essential oils isolated from *M. longifolia* plants harvested in five regions of Saudi Arabia. Moreover, efforts have been made to correlate the biological attributes with the chemical composition of the oils investigated.

MATERIALS AND METHODS

Plant material: The aerial parts of *M. longifolia* (L.) Hudson were collected and/or procured from different agro-climatic regions including Al-Kharj, Al-Qassim, Dammam, Abha and Al-Madinah during August/September 2015 through the local agricultural farms and market sources. Three diverse samples of plant material were taken from each region and pooled. The data for some important environmental (temperature, humidity and rain fall) and geographical (elevation, longitude, latitude) factors in the selected regions are shown in Table 1. The collected plant materials were further identified and authenticated by Dr. Usman Makke (College of Pharmacy, Prince Sattam bin Abdulaziz University (PSAU), Al-Kharj). After washing with tap water to remove any debris or dirt, the materials were shade-dried in a well ventilated Pharmaceutical Chemistry Laboratory at College of Pharmacy, PSAU.

Table 1: Agro-climatic factors in the regions selected for *Mentha longifolia* plant sampling

Environmental/geological	Al-Kharj	Al-Qassim	Dammam	Al-Madinah	Abha
August, 2015					
Minimum temperature (°C)	27	29	31	33	15
Maximum temperature (°C)	44	47	45	43	27
Humidity (%)	13	18	54	12	52
Rainfall (mm)	8	8	72	6	20
September, 2015					
Minimum temperature (°C)	25	25	30	32	18
Maximum temperature (°C)	42	46	43	44	32
Humidity (%)	17	19	52	14	42
Rainfall (mm)	13	13	73	53	77
Elevation (m)	439	621	9	608	2270
Longitude (°E)	47	43	50	39	42
Latitude (°N)	24	26	26	24	18

Source: http://weatherdatacenter.com/Humidity/Medina/August-2015, http://www.al-taif.climatemps.com/humidity.php, https://en.wikipedia.org/wiki/Medina, http://www.accuweather.com/en/sa/dammam/297095/august-weather/297095, https://weather-and-climate.com/average-monthly-Rainfall-Temperature-Sunshine,al-kharj-riyadh-province-sa,Saudi-Arabia, http://weatherdatacenter.com/Humidity/Medina/August-2016

Chemicals, reagents and other materials: Folin-Ciocalteu reagent (2 N), 2,2,-diphenyl-1-picrylhydrazyl (90.0%), butylated hydroxyanisole (BHA,99.0%), carvone, homologous series of n-alkanes (C9-C24), Triton-x100 and Phosphate Buffer Saline (PBS) (pH 7.4) were obtained from Sigma Chemical Company. (St. Louis, MO, USA). All other chemicals and solvents (analytical grades) were obtained from Sigma Aldrich or Scharlau Chemie (Barcelona, Spain) and/or Merck (Darmstadt, Germany). The culture media (nutrient agar for bacteria and potato dextrose agar for fungi) and standard antibiotic discs used were purchased from Oxoid Ltd., (Hampshire, UK). Streptokinase (1.5×10^6 IU) was purchased from Pfizer Laboratories, Ltd., Pakistan. The pure microbial strains including two bacterial and three pathogenic fungal strains such as *Staphylococcus aureus* (Gram positive bacteria), *Escherichia coli* (Gram negative bacteria), *Aspergillus niger, Aspergillus flavus* and *Fusarium solani* were employed.

Isolation of *Mentha longifolia* essential oil via hydrodistillation: The dried *M. longifolia* plant material was crushed in a coffee grinder (80-90 mesh) and quantitatively fed into a Clevenger-Type apparatus (Gulf Scientific Glass Industry, Manama, Bahrain). The essential oil was isolated by hydro-distillation on a heating mantle for 4 h using de-ionized water. The recovered oil distillates were freed of traces of moisture, if any, by drying over anhydrous sodium sulfate (Na_2SO_4, 99%). Finally, the recovered oil was filtered and kept at -4°C in Teflon capped glass vials until used for further experiments[7].

Gas chromatography and Gas Chromatography-Mass Spectrometry (GC-MS) analyses: A GC-2010 Plus gas chromatograph fitted with QP 2010/Ultra Mass Spectrophotometer (Shimadzu, Tokyo, Japan) and Shimadzu AOC. 20i Auto injector were used for GC-MS analysis. The essential oil chemical constituents were separated on fused silica Rtx-5 MS (RESTEK, Bellefonte, PA, USA) capillary column (30 m×0.25 mm, 0.25 µm film coating of 5% phenyl methylpolysiloxane). The auto injector was operated at 290°C. A 1.0 µL test essential oil (diluted in methanol 1:4 v/v) sample was injected in the splitless mode. The column temperature was initially set at 80°C and then raised to 220°C with linear increment of 4°C min^{-1}, held for 4 min and then further progressed by the rate of 5°C min^{-1} to 290°C and finally held for 2 min. The carrier gas (Helium) was used at a flow rate of 0.75 mL min^{-1}. For mass spectra detection, an Electron Ionization (EI) mode (ionization energy, 70 eV) was used whereas, the mass scanning (m/z) range varied over 40-500. The MS transfer line temperature was adjusted at 320°C. The quantitative data (percentage composition) of the oils was obtained electronically from the GC-FID (Clarus 600 gas chromatograph, Perkin-Elmers, Norwalk, CT, USA) area percentages (normalization method) without using any correction factors. For GC-FID, the column chromatographic conditions being employed were the same as used for GC-MS analysis. The composition of oil constituents identified was computed as a relative percentage of the total peak areas[7].

Compounds authentication/identification: The essential oil compounds (volatiles) were identified based on the comparison of their retention indices relative to standard series of n-alkanes (C9-C24). The compounds were additionally identified/authenticated by relating their MS data with those of the NIST 05 Mass Spectral Library and published Mass Spectral (MS) data and wherever appropriate, by co-injecting reference standards[29,7].

Total Phenolics Content (TPC) and DPPH• radical scavenging activity: Antioxidant Activity (AA) of the tested oils was followed by the determinations of Total Phenol (TP) and DPPH• radical scavenging activity. Amount of TP in the tested *M. longifolia* oils was determined colorimetrically following Folin Ciocalteu Reagent (FCR) method with minor changes[11]. Briefly, the oil (10 mg/1 mL of methanol), placed in a test tube was mixed with 0.5 mL of FCR and deionized water (7.5 mL). The mixture was retained at room temperature (ambient condition) for ca. 10 min followed by adding 1.5 mL of solution of sodium carbonate (20% w/v). This mixture was incubated (in a water bath) at 40°C for 20 min and after that cooled using ice bath. The absorbance (A) of the colored complex reaction mixture formed was taken at 755 nm using a UV/VIS spectrophotometer (V-630, JASCO International Co. Ltd., Hachioji, Tokyo, Japan). The contents of TP were determined using Gallic Acid (GA) standard calibration curve constructed by running a series of standard solution prepared within the concentration range of 0.25-6.50 mg L^{-1} ($R^2 = 0.9982$). The results were presented as GAE mg/100 g of essential oil.

The antioxidant potential of *M. longifolia* oils, along with the major component (carvone) was assessed by means of measuring their redox potential and capacity to scavenge 2,2'-diphenyl-1-picrylhydrazyl stable free radicals (DPPH•). This test, with slight modification, was performed according to the method as described in previous study[7]. Different concentrations of methanolic (0.5-10 μg mL^{-1} methanol) solutions of the test *M. longifolia* oils were mixed with 1 mL of freshly prepared 90 μM DPPH solution. The volume was made up to 4 mL by adding 95% MeOH. After shaking, the reaction mixture was incubated in the dark for 30 min at ambient temperature. The absorbance of the incubated mixture and the blank (control) was taken/recorded at 515 nm by means of a UV/VIS spectrophotometer (V-630, JASCO International Co. Ltd., Hachioji, Tokyo, Japan). Butylated Hydroxyl Anisole (BHA), a food grade synthetic antioxidant, was employed as a positive control in parallel measurement. Percent (%) DPPH◦ scavenging power and related IC_{50} values of the oils were computed by the equation stated elsewhere[7,8].

Antimicrobial activity: In order to evaluate antimicrobial potential, the tested *M. longifolia* oils and the main component, carvone were tested against the selected strains of microorganisms (bacteria and fungi) following disc diffusion assay[30,8]. The bacterial and fungal strains were cultured (overnight, ca.15 h) at 37 and 30°C in nutrient agar (NA, Oxoid) and Potato Dextrose Agar (PDA, Oxoid), respectively. In this assay, a 100 μL (suspension) of each of the tested microbial strain (containing 10^4 Colony Forming Units (CFU) mL^{-1} of fungi spores and 10^8 CFU mL^{-1} of bacteria cells) were spread on to PDA and Nutrient Agar (NA), respectively. The filter paper discs (with diameter 6 mm), separately soaked with *M. longifolia* oil (15 μL mL^{-1}) and/or major component (carvone) were placed on the agar plate, which had formerly been inoculated with the microorganisms under test. A negative control has the discs without samples, while drugs such as Ampicillin and Fluconazole (each 30 μg per disc) were employed as positive controls (reference compounds) for comparison of the antibacterial and antifungal activities, respectively. The plates (petri dishes) were retained at 4°C for 2 h and then incubated at 30°C for a period of 48 h for fungal strains while at 37°C for 24 h for bacteria. The antimicrobial (antibacterial and antifungal) activities were measured by computing the diameter of the growth inhibition zones in millimeters together with 6 mm paper disc diameter relative to the controls employed. Triplicate measurements were made and averaged data reported.

Thrombolytic activity: The thrombolytic activity of the tested *M. longifolia* oils was evaluated using streptokinase (SK) as a reference standard following the method of Prasad *et al.*[31] with slight changes. Sterilized deionized water was added to standard streptokinase vial and mixed thoroughly. A 100 μL (3×10^4 IU) from this suspension was used for *in vitro* thrombolysis experiment. Venous blood samples (1 mL), taken from healthy human volunteers (n = 7), without noting a history of oral contraceptive (OCP) or anticoagulant therapy, were shifted/transferred to micro centrifuge tubes (pre-weighed) and incubated at 37°C for 45 min for clot formation. Briefly, each test essential oil (100 μL) was added in sterile micro centrifuge tube containing the pre-weighed blood clots. In parallel experiment, 100 μL streptokinase (3×10^4 IU) was employed as a positive control, whereas the negative (non-thrombolytic) control contained 100 μL sterilized deionized water. The tubes were incubated at 37°C for 90 min followed by examination for clot lysis and weight of released clot. The clot lysis was calculated and expressed in percentage using the following Eq. 1:

$$\text{Percent clot lysis} = \frac{\text{Weight of released clot}}{\text{Clot weight}} \times 100 \quad (1)$$

Cytotoxicity/Haemolytic activity: The cytotoxicity, in terms of the haemolytic activity of *M. longifolia* essential oils was determined according to the protocol of Malagoli[32] with slight modification. Approximately 3 mL of freshly obtained and heparinized human blood was centrifuged (1000×g) for 5 min. The supernatant (plasma) was poured off (decanted). The Red Blood Cells (RBCs) were washed (three times) with 5 mL of chilled (4°C) sterile isotonic Phosphate Buffer Saline (PBS) (pH 7.4) and then suspended in chilled PBS (20 mL). A hemocytometer was used to count RBCs which were maintained at 7.10×10^8 cell mL^{-1} for each assay. A 20 µL volume of *M. longifolia* essential oil was placed in Eppendorf tube along with blood cell suspension (180 µL). The mixture was incubated at 37°C for 35 min, tubes were kept in ice bath for 5 min followed by centrifugation (5 min at 1,150×g). After this, the supernatant (ca.100 µL) was decanted (taken off) and diluted with chilled PBS (0.9 mL μL^{-1}) and the eppendorfs were cooled in ice bath. A 200 µL mixture from each Eppendorf was placed in 96 well plates for measurements. For each assay, Phosphate Buffer Saline (PBS) and 0.1% Triton X-100 were employed as negative and positive control, respectively. The absorbance of the final reaction mixture was taken at wavelength of 576 nm with a BioTek, µ CuantTM instrument (BioTek, Winooski, VT, USA). Replicate measurements were made and the percentage of RBCs lysis for each oil sample was computed and reported as average.

Statistical analysis: The data were expressed as Mean±Standard Deviation for triplicate experiments. One way analysis of variance (ONE Way ANOVA) was used to evaluate the statistical differences of the means among the different agro-climatic regions. A probability ($p<0.05$) was used to denote statistically significant differences. For cytotoxic and thrombolytic activities, the statistical variations, relative to the controls were analyzed by applying Dunnett test.

RESULTS AND DISCUSSION

Essential oil yield and chemical composition: As can be seen in the last row of Table 2, the yield of hydro-distilled essential oil from aerial parts of *M. longifolia* harvested from different regions of Saudi Arabia varied from 0.66-1.52% dry weight basis indicating a partially significant ($p<0.05$) differences of the means among the regions selected. The minimum oil yield was recorded from samples of Abha, while the maximum from Al-Qassim followed by Al-Kharj (1.40%). Such variations in the oil concentration can be linked to morphological diversity of plants based on the divergent agro-climatic and typographical conditions of the regions (Table 1). Abha is relatively a colder and semi-arid climatic zone situated in the fertile mountainous area of South-Western Saudi Arabia at an elevation of ca. 2270 m a.s.l. Al-Qassim is popular for its typical sandy desert climate and is recognized for its cold, rainy winters and hot summers with harsh agro-climatic conditions; it is central part of Saudi Arabia and the land's height is about 600-750 m a.s.l. Al-Kharj is situated 77 km South of Riyadh and also has hot dry summers and cool somewhat rainy winter weather. Al-Kharj is well known throughout Saudi Arabia for its dairy industry and agriculture production. Madinah is characterized by a hot desert/arid climate and little rain fall whereas the soil surrounding this city is mostly basalt and hills, especially in the South, are volcanic ash. Dammam is situated in the Eastern part of the Kingdom of Saudi Arabia and has mild to warm winter while extremely hot summer temperatures and is rich in oil reservoirs. The city of Dammam is on the coastal belt of Persian Gulf and is characterized by relatively a higher level of humidity.

According to research reports, the essential oil yield of different plants including the Lamiaceae herbs not only varies due to genetic factors, nevertheless, environmental and geographical variables that influence genetic expression and plant tissue development stages, also affect the yield and chemical compositions of oils[33,13]. Hajlaoui *et al.*[34] reported the essential oil content of *M. longifolia*, based upon geographical and climatic parameters may vary between 0.9-1.8%. The essential oil yield from *M. longifolia* obtained from eight different locations in South Africa ranged between 0.36-1.46%[25]. Another study by Sharopov *et al.*[23] reported the oil yield of different *M. longifolia* populations to be 0.5-0.9% from Tajikistan. Salman *et al.*[13] analyzed *M. longifolia* plants from North areas of Saudi Arabia and reported the oil yield in the range 0.50-0.9%. Meanwhile, Al-Okbi *et al.*[20] investigated *M. longifolia* plants from Egypt had an oil yield as high as 0.99%. However, *M. longifolia* wild populations from different provinces of Iran had relatively a higher oil yield in the range of 1.39-4.05%[16]. In another report, the samples of *M. longifolia,* harvested at three maturity stages such as pre-flowering, post-flowering and full flowering stages from Sudan exhibited an oil yield of 1.40, 1.20 and 1.80%, respectively. The samples of wild *M. longifolia*, analyzed from Pakistan by Hussain *et al.*[8] and Iqbal *et al.*[35], exhibited oil yield in the range of 0.7-1.08%. The essential oil yield from *M. longifolia* plants harvested from Western Himalaya regions of India was noted to be 0.88%[36]. As far as comparison is concerned, the present oil yield (0.66-1.52%) of *M. longifolia* from different agro-climatic areas of Saudi Arabia, with few exceptions is within the range of those reported in the literature from different countries.

Table 2: Chemo-geographical variations in the composition and yield of *Mentha longifolia* essential oil from different regions of Saudi Arabia

			Composition (%)				
Essential oil constituent	RT*	RI#	Al-Kharj	Al-Qassim	Dammam	Abha	Al-Madinah
α-pinene	5.03	948	1.05	0.54	0.85	0.76	0.65
Sabinene	6.08	897	0.76	0.49	0.67	3.32	1.25
β-pinene	6.25	943	1.33	0.84	1.11	1.15	0.94
Limonene	8.26	1018	12.03	6.13	28.45	5.73	7.77
β-myrcene	6.52	958	0.79	0.34	1.05	0.67	0.65
3-octanol	6.63	979	0.53	-	-	0.77	0.63
1,8-cineol	8.37	1059	4.50	4.22	-	3.38	3.24
Trans-β-ocimene	8.50	976	-	-	-	0.36	0.26
γ-terpinene	9.64	998	-	0.29	-	0.48	0.39
Linalool	11.92	1082	-	-	0.40	0.82	-
Isoborneol	15.47	1138	0.63	-	0.50	-	0.69
Terpinen-4-ol	15.99	1137	0.68	0.61	-	1.12	0.98
α-terpineol	16.67	1143	0.37	0.75	-	-	0.36
Trans-dihydrocarvone	16.94	1179	0.48	3.08	4.48	-	
1,6 dihydrocarveol	17.08	1196	-	-	-	12.33	6.84
Cis-carveol	18.75	1206	1.48	1.90	1.77	1.5	5.31
p-menth-2-en-9-ol	18.93	1201	-	-	-	-	0.55
Carvone	19.88	1190	71.51	63.76	45.99	35.30	46.77
Bornyl acetate	20.91	1277	-	-	-	0.30	0.30
Carvacrol	21.56	1262	-	-	-	0.67	-
Trans-dihydrocarvyl acetate	22.50	1335	-	-	-	4.25	0.6
Verbenone	23.03	1119	-	-	0.62	-	-
2-cyclohexen-1-ol	23.78	1346	-	0.30	-	4.66	1.15
β-bourbonene	24.63	1339	0.77	1.94	2.25	2.88	2.14
β-elemene	24.85	1398	0.43	1.92	0.27	1.85	1.63
β-caryophyllene	25.86	1494	0.79	2.76	1.44	3.57	2.75
β-copaene	26.18	1216	-	0.31	0.34	0.47	0.35
Cis-muurola-3,5-diene	26.76	1440	-	0.81	0.48	0.78	1.08
α-humulene	27.04	1579	-	-	-	0.35	0.22
Bicyclosesquiphellandrene	27.33	1435	0.46	1.87	0.95	2.24	2.43
Germacrene D	27.97	1515	0.34	2.05	2.79	1.74	2.35
γ-elemene	28.47	1431	-	1.33	0.45	1.15	1.67
Cis-calamenene	29.27	1537	-	1.07	0.78	1.32	0.91
Caryophyllene oxide	31.20	1507	-	-	-	0.46	0.21
Cubedol	32.11	1484	-	0.63	0.35	1.05	0.75
α-cadinol	33.24	1580	-	0.60	0.42	0.67	0.65
Trans-phytol	44.59	2045	-	-	0.26	0.85	0.37
Total constituents identified			98.93	98.54	96.67	96.95	96.84
Grouped components composition (%)							
Monoterpene hydrocarbons			15.96^{c}	8.63^{a}	32.13^{d}	12.47^{b}	11.91^{b}
Oxygenated monoterpenes			79.65^{d}	74.62^{c}	53.76^{a}	64.33^{b}	66.79^{b}
Sesquiterpene hydrocarbons			2.79^{a}	14.06^{c}	9.75^{b}	16.35^{d}	15.53^{c}
Oxygenated sesquiterpenes			-	1.23^{b}	0.77^{a}	2.18^{d}	1.61c
Acyclic diterpenoids			-	-	0.26^{a}	0.85^{c}	0.37b
Oil yield (g/100 g)			1.40 ± 0.09^{c}	1.52 ± 0.08^{c}	0.90 ± 0.09^{b}	0.66 ± 0.10^{a}	0.70 ± 0.10^{a}

Means for grouped components followed by different superscript letters in the same row represent significant difference ($p < 0.05$). Identification of compounds is based on matching of RI and MS data following by Adams[29] and NIST Library, *Chemical compounds are listed in the order of their elution from Rtx-5 MS column, #Retention indices relative to C9-C24 n-alkanes on Rtx-5 MS column, RT: Retention time, RI: Retention index

Mass spectrum of major compound (carvone) detected in the tested *M. longifolia* essential oils is shown in Fig. 1. The chemicals of the tested *M. longifolia* essential oils from different regions of Saudi Arabia was probed by GC-MS (Fig. 2a-e) and revealed quantitative variations for most of the identified components among the regions (Table 2). Overall, 18, 24, 23, 32 and 33 components were identified using GC-MS (Typical GC-MS chromatograms are shown in Fig. 2) in *M. longifolia* oils isolated from samples of Al-Kharj, Al-Qassim, Dammam, Abha and Al-Madinah, representing 98.93, 98.54, 96.67, 96.95 and 96.84% of the total oil composition, respectively. The tested *M. longifolia* essential oils were dominated by carvone followed by limonene and 1,6 dihydrocarveol with their contribution varying over a wider range, 35.30-71.51, 5.73-28.45 and 0-12.33%, respectively. The essential oil from Al-Kharj contained the highest level

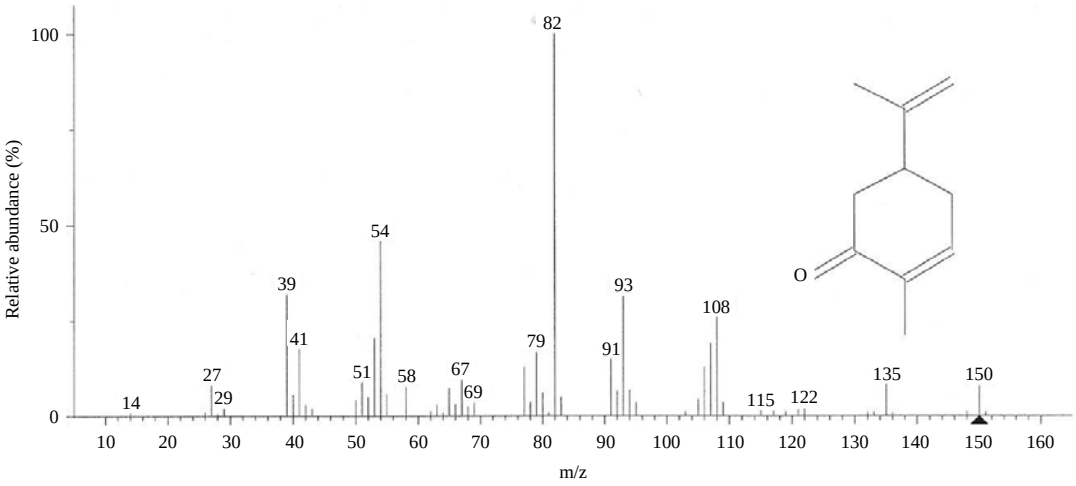

Fig. 1: Mass spectrum of major compound (carvone) detected in the tested *M. longifolia* essential oils

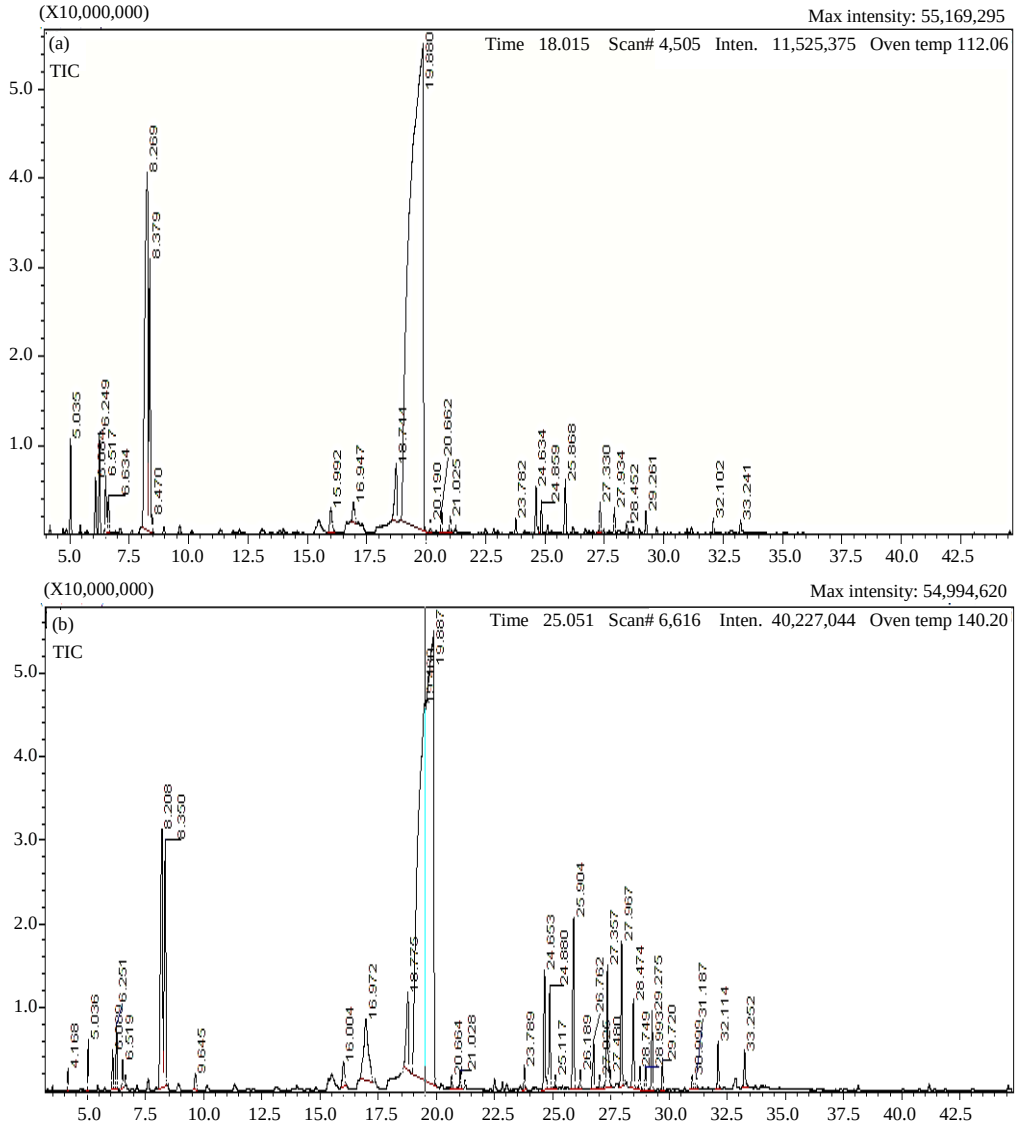

Fig. 2(a-e): Continue

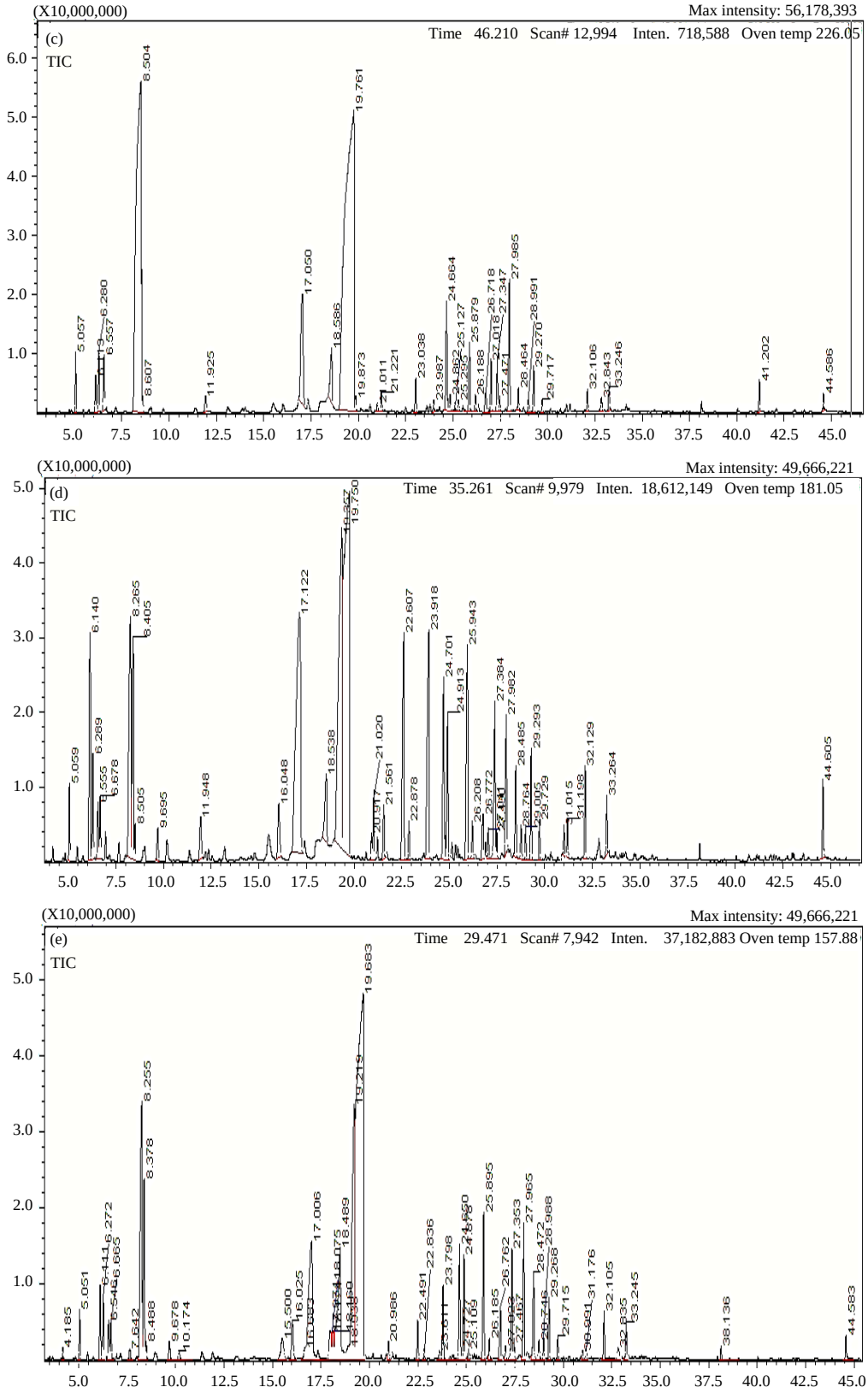

Fig. 2(a-e): GC-MS chromatogram of *M. longifolia* essential oil from different agro-climatic regions (a) Al-Kharj, (b) Al-Qassim, (c) Dammam, (d) Abha and (e) Al-Madinah

of principal component, carvone, while Abha based sample had the least. On the other hand, the essential oil from Dammam samples exhibited the maximum amount of limonene (second major compound) followed by Al-Kharj, Madinah, Al-Qassim and Abha samples. In addition, trans-dihydrocarvone, 1,8-cineol, β-caryophyllene, β-bourbonene, germacrene D and bicyclosesquiphellandrene were also identified in considerable amount. The presently studied *M. longifolia* chemotypes from Saudi Arabia can be locally explored as a potential source of carvone-rich essential oil. Carvone is known one of the most important compounds in the essential oils from several species of mint; particularly *Mentha spicata* (spearmint) oil is richest source (50-80%) of carvone. Carvone has potential uses as antimicrobial and antioxidant agents and insect repellent[37].

A considerable variation in the contents of most of the identified components in the present analysis of essential oils from the selected *M. longifolia* ecotypes can be linked to morphological diversity of plants depending upon the variable agro-climatic factors of the habitats. The morphological diversity in the investigated species is strongly linked to the extent of agro-ecological variations. Moreover, it can also be assumed that the selected *M. longifolia* populations may belong to different varieties (genetic factoring) and thus have variable essential oil yield and composition. In fact agro-climatic and geographical conditions such as temperature, rainfall, humidity, habitat elevation, plants maturity stage, sample drying and extraction mode are some of the known factors which have significant effect on the yield and chemical composition of plant essential oils[33]. Considerable differences in the quantitative composition of essential oils from different chemotypes of *M. longifolia* from several regions of the world such as Tajikistan[23], Iran[38,16,24], South Africa[25,39], France[40,41], Greece[42,43], Crete[44] and Serbia[15,45] have previously been appraised.

Table 2 shows that on grouped components aggregate basis, the tested *M. longifolia* oils from different regions were mainly characterized by the presence of oxygenated monoterpenes (53.76-79.65%) followed by monoterpene hydrocarbons (8.63-32.13%), sesquiterpene hydrocarbons (2.79-18.41%) and oxygenated sesquiterpenes (0.77-2.18%). A small fraction of acyclic diterpenoids (0.26-0.85%) was also detected in Dammam, Abha and Al-Madinah based oil samples among others. The contents of Oxygenated Monoterpenes (OM), Monoterpene Hydrocarbons (MH) and Sesquiterpene Hydrocarbons (SH) varied considerably among the regions selected. For example, OM were mainly comprised of carvone, dihydrocarveol and 1,8-cineol while MH chiefly contained limonene and β-pinene. On the other hand, SH were dominated by β-caryophyllene, germacrene D and bicyclosesquiphellandrene, whereas cubedol and α-cadinol were mainly noted in OS.

The highest contents of OM were recorded in Al-Kharj, whereas the least in Al-Qassim based *M. longifolia* essential oils. The oils from Dammam and Abha contained the highest level of MH and SH, respectively. Meanwhile, OS were higher in Abha samples; however, this fraction was not detected in Al-Kharj samples.

The content of OM (53.76-79.65%) of the major class of components (grouped component), in the present analysis of *M. longifolia* oils is in line with the findings of earlier studies on this species from different countries. In comparison to present contents of OM (53.76-79.65%), *M. longifolia* oils analyzed from Pakistan[8,35], Sudan[22], Tunisia[46], Egypt[20], India[36,47,48], Iran[38,49], Bosnia and Herzegovina[50] and South Africa[25] contained 67.24-75.93, 81.5, 89.18, 86.24, 74.0-85.3, 74.2-93.5, 87.1 and 56.5-89.6% of MO (total oil basis), respectively. Likewise, the present findings, in addition to comprising oxygenated monoterpenes as a major fraction, *M. longifolia* oil from different regions were consisted of variable amounts of other important classes of compounds such as monoterpene hydrocarbons, sesquiterpene hydrocarbons and oxygenated sesquiterpenes[22,25,8,46].

The composition of *M. longifolia* oils from different regions of the world has been studied and noted that this species has relatively a higher extent of chemicals[23]. Previously, it has been reported that agro-climatic and agro-ecological factors that define morphological characteristics exhibit strong impact the qualitative and quantitative composition of *M. longifolia* essential oil[16,23]. *Mentha longifolia* oils are recognized to contain various terpenoids with piperitone, piperitone oxide, pulegone, piperitenone, carvone, limonene, menthone, 1,8-cineole and β-caryophyllene as dominating constituents; however, based on the extent of morphological diversity, a greater degree of chemo-geographical variations have been noted in the composition of essential oil from *Mentha* species[23,20]. In this context, a number of *M. longifolia* chemotypes have been identified from different regions with their essential oils dominated by principal compound such as carvone (34-78.9%) from Sudan[22], Croatia[51], Crete[44], Iran[38], India[48] and Greece[43], piperitenone oxide (25-66%) from Pakistan[8,35], India[47], Italy[52], Kazakhstan[53], South Africa[25], Iran[16] and Morocco[54], piperitone oxide (58-65%) from Turkey[55], Greece[42], Bosnia and Herzegovina[50] and Egypt[20], pulegone (11-62.5-70.54%) from Jordan[56], Tunisia[46] and Saudi Arabia[13], trans-dihydrocarvone (23.64%) from Serbia[15]. Moreover, two chemotypes of *M. longifolia* from Tunisia[57] and Iraq[19], producing menthol

Table 3: Essential oil main chemical constituents and grouped components in some important chemotypes of *M. longifilia* from different countries

		Grouped components/class composition (%)				
Countries	Main oil constituent	OM	MH	OS	SH	References
Pakistan	Piperitenone oxide (40.1-64.6%), piperitenone (1.97-16.4%), borneol (4.36-13.3%)	75.31-75.96	7.03-7.24	0.91-1.51	14.25-14.94	Hussain *et al.*[8]
Pakistan	Piperitenone oxide (28.3%), piperitenone (24.9%), germacrene D (8.16%)	67.24	7.31	5.05	17.19	Iqbal *et al.*[35]
South Africa	Piperitenone oxide (0-65.7%), cis-piperitone oxide (0-35.7%), menthofuran (0.9-61.6%)	56.5-89.6	0.3-15.5	0.6-3.5	4.4-16.7	Viljoen *et al.*[25]
Egypt	Piperitone oxide (59.05%), piperitenone oxide (40%),1,8-cineol (16.07%)	86.24	2.85	4.35	4.15	Al-Okbi *et al.*[20]
Tunisa	Pulegone (54.41%), isomenthone (12.02%), 1,8-cineol (5.4%)	89.18	2.19	0.5	6.16	Mkaddem *et al.*[46]
Sudan	Carvone (67.3%), limonene (13.5%), 1,8-cineol (5.4%)	81.5	14.7	1.0	0.8	Younis and Basher[22]
Serbia	Trans-dihydrocarvone (23.64%), piperitone (17.33%), cis-dihydrocarvone (15.68%)	82.51	4.79	2.41	2.48	Dzamic *et al.*[15]
Iran	Carvone (61.8%), piperitone oxide (3.3%), limonene (19.4%)	74.2	23.2	Not reported	1.6	Monfared *et al.*[38]
Iran	Piperitenon (35.8%),piperitenon oxide (16.6%), piperitone (6.3%), 1,8-cineole (28.2%)	93.5	4.8	Not reported	0.6	Moradalizadeh *et al.*[49]
India	Trans-piperitone epoxide (74%), piperitenone oxide (21.2%), germacrene D (9.8%)	74	2.6	0.4	18	Verma *et al.*[36]
India	Piperitenone oxide (54.23%), cis-piperitone oxide (7.04%), trans-piperitone oxide (24.06%)	85.3	8	1.5	4.77	Singh *et al.*[47]
India	Carvone (61.12-78.70%), dihydrocarveol (0.4-9.4%), cis-carvyl acetate (0.16-6.43%)	77.45-84.01	0.32-2.07	1.43-10.52	4.39-11.19	Mathela *et al.*[48]
Bosnia and Herzegovina	Piperitone oxide (63.58%), 1,8-cineol (12.03%)	87.1	3.06	5.57	6.79	Niksic *et al.*[50]
Saudi Arabia	Carvone (35.30-71.51%), limonene (5.73-28.45%), 1,6-dihydrocarveol (traces-12.33%)	53.76-79.65	8.63-32.13	0.77-2.18	2.79-18.41	Present study

OM: Oxygented monoterpenes, MH: Monoterpene hydrocarbons, OS: Oxygenated sesquiterpenes, SH: Sesquiterpene hydrocarbons

rich oil, while two others from South Africa[58,39] with high content of menthone (31-51%) have also been identified. According to Gulluce *et al.*[59] and Verma *et al.*[36] essential oils from *M. longifolia* from Turkey and India were rich in cis-piperitone epoxide (18%) and trans-piperitone epoxide (48.7%), respectively. It has been revealed that due to distinct climate several chemotypes, based upon the quantitative variations of constituents within a taxon is a common characteristic in most of the *Mentha* hybrids and species[33]. Table 3 provides a comparison of the contents of major constituents and classes of components identified in some of the important *M. longifolia* chemotype essential oils from different regions of the world, relative to the present data.

DPPH• radicals scavenging power and total phenolics: The antioxidant activity of the essential oils isolated from different chemotypes of *M. longifolia* was monitored by assessing their capacity to scavenge stable DPPH• radicals along with contents of total phenolics (Table 4). The DPPH• radicals scavenging power of the tested oils, determined over the concentration ranged from 0.50-10.0 μg mL^{-1} was progressed virtually in a linear fashion with respect to increase in concentration ($R^2 = 0.9139$-0.9697). Typically, under the test conditions, the oils (at concentration 10.0 μg mL^{-1}) scavenged DPPH• radicals to an extent of 54.9-89.7% (IC_{50} 4.4-8.5 μg mL^{-1}) with least contribution from Al-Kharj, while the highest from Al-Madinah samples (Table 4). The major component of *M. longifolia* oils, carvone and a synthetic antioxidant compound, butylated hydroxyanisole (BHA), tested in parallel experiments for comparison purposes, exhibited 73.6 and 91.5% scavenging activity, respectively.

The contents of total phenolic (0.7-4.70 mg GAE/100 g) varied significantly ($p<0.05$) within the oils analyzed (Table 4). The oil from Al-Kharj chemotype showed the least content of total phenols, whereas the oil from Al-Qassim had the highest followed by Dammam, Abha and Al-Madinah ecotypes oils. Partially, the oils with higher total phenols offered better DPPH• radical scavenging power; however, a strongly positive correlation could not be developed between the amount of phenols and scavenging capacity of the oils tested. Such a random pattern of correlation between total phenols and DPPH• radical scavenging reflects that the tested *M. longifolia* oils have acted as free radical scavengers not only due to phenolics, nevertheless, other volatile constituents such as oxygenated monoterpenes and monoterpene hydrocarbons possibly have also contributed to the free radical scavenging capacity of the oils.

Mentha longifolia plant extracts and essential oils are known to be a potential source of antioxidant components; however, mostly the methanol extracts from these species have been evaluated as strong antioxidant agents than the respective oils[60,18,20]. In compassion to essential oils, a good antioxidant activity of methanolic extracts of *M. longifolia* has mainly been attributed to phenolics[59,60]. Selected phytochemical, especially the plant phenolic which are widely distributed in foods such as fruits, vegetables, cereals and aromatic herbs are now gaining high level of recognition for their antioxidant properties and multiple medicinal effects[61,62,8,7]. The phenolics in aromatic plants due to containing hydroxyl groups exhibit redox properties and thus act as hydrogen donors and natural reducing agents. However, the antioxidant properties of *Mentha longifolia* essential oils are not only due to the occurrence of phenolics, nonetheless, oxygenated monoterpenoids such as carvone, 1,8 cineol, carveol and monoterpene hydrocarbons (limonene, δ-terpinene, γ-terpinene and para cymene) and monoterpene ketones (menthone and isomenthone) also significantly contribute to free radical scavenging activity and thus antioxidant potential of the oils[60,8,50,20].

The essential oils and extracts from *M. longifolia* have previously been evaluated from different regions for their antioxidant potential using different assays such as estimation of total phenols, DPPH free radical (DPPH•) scavenging, measurement of percent inhibition of linoleic acid oxidation and reducing power assays[18,63,64,8]. The DPPH• scavenging assay, due to its simplicity and quick response has most often employed to evaluate the antioxidant potential of *Mentha* plant extracts and essential oils; however, a wide variation in the scavenging potential have been recorded across the regions[59,64,63]. Due to morphological diversity and chemo-geographical differences in the composition together with choice of antioxidant assay and dilution factors, it is rather difficult to analytically compare the antioxidant properties of tested *M. longifolia* oils among different regions. Previously, Gulluce *et al*.[59] investigated the methanol extracts of *M. longifolia* had much better DPPH• scavenging potential than its essential oil. Mimica-Dukic *et al*.[65] reported that DPPH• potential of *M. longifolia* oils can be linked with monoterpene ketones such as menthone and isomenthone. According to Dzamic *et al*.[15], Serbian *M. longifolia* oil showed significant antioxidant activity and scavenged DPPH• to a level as high as 50% (IC_{50} = 0.66 mL mL^{-1} of solution). *Mentha longifolia* oils investigated from Bosnia and Herzegovina reduced the purple-colored DPPH• into its neutral form (DPPH-H) in a concentration-dependent (IC_{50} =10.5 μg mL^{-1}) fashion[50]. In another study, the antioxidant activity of *M. longifolia* oil and different solvent extracts, in terms of DPPH◦ scavenging was investigated from Pakistan. The results revealed that methanol extract had higher antioxidant activity; however, the essential oil (IC_{50} 21.8 5 μg mL^{-1}) exhibited a comparable scavenging activity with that of dichloromethane (DCM) extract (IC_{50} 21.2 μg mL^{-1})[35]. In another investigation[46], however, the essential oil from Tunisian *M. longifolia* chemotypes exhibited quite lower DPPH• activity (IC_{50}>8 mg mL^{-1}) that was however, comparable with Turkish counterpart oil (IC_{50}>10.7 mg mL^{-1}).

In a previous study, antioxidant potential of methanol extracts of *M. longifolia*[27] has also been evaluated from Saudi Arabia; however, rarely data is available on the antioxidant attributes of essential oils isolated from locally naturalized plants of this species with which to compare the results of the present analysis. The data of the present study supported the potential uses of *M. longifolia* as natural antioxidant ingredient for food science applications. Currently, there are greater focus on the utilization of plant based natural antioxidants due to their potential health benefits and safety[61,7,8,15,18]. In this direction, extracts and essential oils form many plants and aromatic herbs have been identified as potential source of functional food and nutraceuticals with promising antioxidant activities[15,18].

Antimicrobial activity: Infectious diseases are mainly caused due to infections from pathogenic and biological agents. Antimicrobials prevent or slow down the incidence and transmission of infectious diseases. Due to growing antimicrobial resistance (AMR), now there is increasing interest in the search of safer and more effective antimicrobial agents. Many traditional medicinal plants extracts/constituents and essential oils are now gaining recognition for the treatment of infectious diseases due to their antioxidant and antimicrobial properties[5,7,8]. In the present study, it was evaluated the essential oils from different chemotypes of *M. longifolia* from Saudi Arabia for antimicrobial potential against two bacterial strains including *Staphylococcus aureus* and *Escherichia coli* and three strains of pathogenic fungi such as *Aspergillus niger*, *Aspergillus flavus* and *Fusarium solani*. The tested *M. longifolia* essential oils (at concentration of 15 μL mL^{-1}) displayed variable antibacterial and antifungal potential in relation to oil chemotype and microbial strains tested (Table 4). The oils, compared with major component carvone (17.5 mm) and standard drug ampicillin (42.0 mm), fairly inhibited the growth of Gram negative pathogen (*E. coli*) with inhibition zone diameter ranged from 17.0-25.0 mm. However, antibacterial activity of these oils against Gram positive *S. aureus* was quite weak with small inhibition zone diameters (7.0-17.0 mm) indicting that this Gram positive

Table 4: DPPH• scavenging capacity, Total Phenolic Contents (TPC) and antimicrobial activity of *Mentha longifolia* essential oils from different agro-climatic regions of Saudi Arabia

	Essential oil							
Parameters	Al-Kharj	Al-Qassim	Dammam	Abha	Al-Madina	Carvone	Ampicillin	Fluconazole
DPPH• scavenging (%)	54.9±0.7^{a}	78.7±0.9^{b}	86.7±0.6^{c}	78.7±1.0^{b}	89.7±0.8^{c}	73.6±0.7^{b}	-	-
TPC (mg GAE/100 g)	0.7±0.1^{a}	4.7±0.14^{e}	3.6±0.20^{d}	2.8±0.15^{c}	1.7±0.2^{b}	-	-	-
Antimicrobial activity DIZ (mm)$^{\Lambda}$								
Escherichia coli	22.5±0.8^{b}	20.5±1.0^{b}	25.0±0.8^{c}	17.0±0.5^{a}	21.0±0.7^{b}	17.5±0.5^{a}	42.0±1.0^{d}	-
Staphylococcus aureus	7.0±0.2^{a}	12.0±0.4^{c}	13.0±0.8^{c}	11.0±0.7^{b}	17.0±0.5^{d}	10.0±0.7^{b}	30.5±1.2^{d}	-
Aspergillus niger	33.4±1.0^{d}	30.0±0.7^{d}	26.8±1.4^{c}	22.0±0.9^{b}	23.5±0.9^{b}	18.0±0.5^{a}	-	41.0±0.9^{e}
Aspergillus flavus	25.5±0.8^{c}	24.9±0.6^{c}	28.7±0.9^{d}	21.6±0.5^{b}	22.4±0.8^{b}	19.3±0.4^{a}	-	35.2±1.1^{e}
Fusarium solani	23.0±0.8^{c}	21.4±0.7^{b}	19.2±0.6^{b}	19.5±0.5^{b}	20.0±0.6^{b}	16.2±0.5^{a}	-	29.0±0.7^{d}

The values given are Mean±SD for three determinations. Means with different superscript letters within the same row represent significant difference at $p<0.05$, DIZ (mm)$^{\Lambda}$: Diameter of inhibition zone including disc diameter of 6 mm

bacterium was less sensitive to the oils. *Mentha* oil from Al-Kharj population, although richer in carvone and with good activity exhibited against *E. coli*; however, did not show any noticeable activity against *S. aureus*.

On the other hand, the tested oils showed relatively a better antifungal activity against three pathogenic molds including *A. niger*, *A. flavus* and *F. solani* with inhibition zone diameters ranged from 22.0-33.4, 21.6-28.7 and 19.2-23.0 mm, relative to the major component, carvone, which produced inhibition zone diameters of 18.0, 19.3 and 16.2 mm, respectively. In comparison, the standard drug fluconazole strongly inhibited the growth of fungi (inhibition zone diameters of 41, 35.2 and 29.0 mm, respectively). With few exceptions, the tested essential oils, richer in oxygenated monoterpenes with carvone as major constituent, exhibited relatively a better antibacterial and especially antifungal activities supporting the potential utilization of this active compound (carvone) as antimicrobial agent in food and pharmaceutical industry. However, as can be analyzed from some random activities data trends, not only the carvone but some other compounds such as monoterpene hydrocarbon (especially limonene-the second major compound in the oil), along with 1,8-cineol, trans-dihydrocarvone, cis-carveol, 1,6 dihydrocarveol, pinene and germacrene D might also have influenced the antimicrobial potency of the tested oils. For example, Dammam based *M. longifolia* essential oil, regardless of relatively lower contents of oxygenated monoterpenes and carvone, exhibited a potent activity against *E. coli*, *A. niger* and *A. flavus* among others which may be attributed to higher contents of monoterpene hydrocarbons (limonene) in this typical chemotype. Such random antimicrobial activity trends may also be linked to the extent of sensitivity of the microorganisms to the oils tested depending on the species strain specificity. Overall, the whole oils exhibited stronger antimicrobial activities than the major component, carvone, tested in parallel experiment that supports a positive contribution due to synergistic effects of different oil components. *Mentha longifolia* is widely used for carminative and food flavoring purpose as well as to control skin disease; the present results supported the scientific rational for the traditional medicinal uses of this herb for the treatment of skin disorders and wound healing.

Mentha plants have shown considerable antibacterial, antiviral and antifungal activities which have mainly been attributed to oxygenated monoterpenoids along with monoterpene hydrocarbons and sesquiterpene hydrocarbons[46,18]. In fact, *M. longifolia* essential oils from

Table 5: Cytotoxic and thrombolytic activities of *Mentha longifolia* essential oils from different agro-climatic regions of Saudi Arabia

	Essential oil							
Parameters (%)	Al-Kharj	Al-Qassim	Dammam	Abha	Al-Madinah	Streptokinase	Triton X-100	Negative control
Clot lysis	68.4±1.5***d	52.4±1.4***c	21.6±0.5**b	11.6±0.8*a	51.8±1.0***c	87.7±1.2***	-	2.20±0.1
Cytotoxicity	3.2±0.5**c	1.7±0.3*a	2.4±0.4*b	2.4±0.3*b	5.1±0.5**d	98.7±0.7***	0.86±0.1	

Values given are Mean±SD for three determinations. Means with different superscript letters within the same row represent significant difference ($p<0.05$). *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs negative control (deionized water/PBS)

different regions have shown widely variable antimicrobial activities against microorganisms such as *Salmonella typhimurium*, *S. aureus*, *E. coli*, *Aspergillus flavus*, *Fusarium oxysporum*, *Aspergillus niger*, *Microsporum canis* and *Mucor ramamnianus*[15,18]. Typically, Viljoen *et al.*[25] investigated antimicrobial potential of *M. longifolia* oils from different regions of South Africa and noted a considerable antimicrobial variability (a moderate to good activity) against *S. aureus* and *E. coli* depending upon chemo-geographical variations in the composition of oil. Dzamic *et al.*[15] investigated *M. longifolia* oils (at concentration 10 μL mL^{-1}) from Serbia as a good antimicrobial agent against *Aspergillus* and *Fusarium* species. In another study, Mkaddem *et al.*[46], *M. longifolia* essential oil chemotypes from Tunisia were found to show good antimicrobial activities with inhibition zone of 32-43 and 20-29 mm against two strains of fungi (*M. ramamnianus* and *A. ochraceus*) and four strains of bacteria (*S. aureus*, *L. monocytogenes*, *E. coli* and *K. pneumonia*), respectively. According to another study concluded by Niksic *et al.*[50], *M. longifolia* oils (1-10%) exhibited significant antibacterial activity against multi-resistant Gram negative bacteria such as *E. coli, Salmonella enterica* and *Pseudomonas aeruginosa* by producing 13-19, 10-20 and 19-25 mm zone of inhibition, respectively.

Thrombolytic and cytotoxic activities: Atherothrombosis, which is characterized by formation of a blood clot within an artery (blood vessel) as a result of atherosclerosis is a wide spread and challenging problem in medical sciences. Several thrombolytic drugs are in use to dissolve the clots formed and/or suppress the clot formation in the blood vessels; however, their uses, especially on long term basis have restrictions and can lead to serious and in certain cases fatal consequences[66]. Supplementation and consumption of numerous natural foods, with antithrombotic (antiplatelet/anticoagulant) effects is linked to reduce the incidence of coronary heart diseases stroke[67,31]. In the present study, an *in vitro* thrombolytic activity model was followed to appraise the thrombolytic activity (percent clot lysis) of the tested essential oils from different chemotypes of *M. longifolia* using streptokinase as a positive control. The tested oils, compared to streptokinase and negative control, exhibited a significant but variable thrombolytic activity in terms of percent clot lysis of 11.6 and 68.4% (Table 5). The reference compound (streptokinase) exhibited 87.7% clot lysis, whereas, sterilized deionized water, used as a negative control, showed a negligible effect (2.2%).

The clot lysis potential of the essential oils varied quite significantly ($p<0.05$) with in the regions depending upon chemo-geographical variability. The highest percent of clot lysis was shown by essential oil from Al-Kharj *M. longifolia* chemotype followed by Al-Qassim (52.4%) and Al-Madinah chemotype (51.8%), whereas, the oil from *Mentha* population of Abha and Dammam contributed only 11.6 and 21.6%, respectively. Typically, carvone richer *M. longifolia* oil chemotype from Al-Kharj and Ak-Qassim regions had higher extent of clot lysis among others. However, a strong correlation could not be developed between the thrombolytic potential and carvone content of all chemotype oils reflecting that not only oxygenated monoterpenes, rather other classes of terpenoids such as monoterpene hydrocarbons and sesquiterpenes hydrocarbons and oxygenated sesquiterpenes might have also played a significant role in clot lysis. Rarely essential oils form Lamiaceae plants have been evaluated for thrombolytic activity, however, a previous investigation from Bangladesh revealed the methanol, ethanol, chloroform and acetone extracts from *Mentha* species such as *Mentha spicata*, *Mentha arvensis* and *Mentha viridis* exhibited 30.29-32.56, 30.02-32.04, 29.05-31.87 and 27.55-30.29%, clot lysis, respectively[68]. In this study, the investigated local chemotypes of *M. longifolia* from Saudi Arabia can also be explored for isolation of natural thrombolytic agents.

Cytotoxic activity: Assessment of toxicity of foods and medicinal plants is of key importance in order to explore their pharmacological and nutraceutical applications. As such treating the cells with a cytotoxic agent can lead to different health disorders such as loss of cell membrane integrity and cell mortality as result of cell lysis[66]. The mechanical stability of the membrane of Red Blood Cells (RBCs) is an acceptable indicator towards evaluating *in vitro* cytotoxic effects of pharmaceutical compounds and phyto-extracts[69,70].

In the present study, the cytotoxic effects of *M. longifolia* essential oils were studied by examining their haemolytic activity against human Red Blood Cells (RBCs) relative to triton X-100 as positive control, while Phosphate Buffer Saline (PBS) acted as a negative control (Table 5). The positive control (Triton X-100) exhibited almost 100% lysis (98.27%), whereas, PBS showed only 0.86% lysis of RBCs. The present results demonstrated the tested essential oils produced form different *M. longifolia* chemotypes caused haemolysis of only 1.7-5.1% conforming that the oils have low toxicity. *Mentha* plants have been utilized over a long time as traditional food and medicinal commodity. In some previous study it has been reported that extracts from some species of *Mentha* including *M. spicata*, *M. arvensis* and *M. viridis* are quite safe for food applications due to low cytotoxic activity[71,68].

CONCLUSION

In the present study, based on chemo-geographical variations, five carvone-rich chemotypes of *M. longifolia* essential oil, with variable oil yield were identified for the first time from different agro-climatic regions of Saudi Arabia. The GC-MS profiling elucidated these *M. longifolia* chemotype oils to be dominated by oxygenated monoterpenes, followed by monoterpene hydrocarbons and then sesquiterpene hydrocarbons. The tested essential oils, relative to the major component, carvone and reference compounds have appreciable DPPH scavenging potential and exhibited moderate to good antimicrobial activity against two strains of bacteria and three pathogenic fungi. The oils also possessed significant blood clots lysis activity *in vitro* as well as have very low cytotoxic effects against human blood erythrocytes (RBCs). Overall, depending upon morphological and biochemical diversity of different *M. longifolia* chemotypes, the tested essential oils exhibited notable variations in the yield, chemical composition and biological attributes. On the basis of antioxidant value and potential bioactivities, which were validated in the present study, these chemotypes of *M. longifolia* form Saudi Arabia can be explored as a valuable source of carvone-rich essential oil with nutraceutical and pharmaceutical prospects. In addition, these finding support the possibility of isolating and characterizing novel antimicrobial and thrombolytic active agents from these *M. longifolia* chemotypes. However, *in vivo* biological activities of these chemotype oils are further recommended so as to establish whether or not, active components from this species could be explored for specific pharmaceutical/nutraceutical applications. Further studies may also be conducted to elucidate the mechanism behind the biological effects of *M. longifolia* oils.

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