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Chemical Composition, Antimicrobial Activity and Potential Cytotoxic Effect of *Mentha viridis* (Spearmint) Extracts from Saudi Arabia

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ABSTRACT

Many medicinal plants have been used to treat and prevent illnesses in Saudi Arabia. The present study aimed to investigate the chemical composition of *Mentha viridis* obtained from Albaha region of Saudi Arabia and evaluate its antimicrobial and antiproliferative potential. The extract was obtained from plant fresh material and identified by gas chromatography-mass spectrometry (GC-MS). The antimicrobial and antiproliferative potential of the plant extract was analysed by performing four subsequent extracts: ethanol, petroleum ether, chloroform, and methanol. The GC-MS analysis showed carvone as a main component, as it comprised 64.82% of the plant extract. In antimicrobial activity, methanol extract showed significant activity against *Pseudomonas aeruginosa* with zone of inhibition of 15 mm. The MTT assay showed that petroleum ether and chloroform extracts have moderate cytotoxic effect against MCF-7 breast cancer cell line with IC_{50} values of 193.23 µg/mL and 131.86 µg/mL, respectively. Chloroform extract also showed mild activity against HCT-116 colorectal cancer cell line with IC_{50} value of 189.2 µg/mL. This study highlights the potential of *M. viridis* extracts as powerful bioactive phytochemicals with possible role in diseases and cancer therapy.

Keywords: Mentha viridis, antimicrobial, antiproliferative, Medicinal plant.

INTRODUCTION

Presently, the effectiveness, low cost, and fewer side effects have increased the worldwide demand of medicinal plants. Many pharmaceutical companies are engaged in large-scale pharmacologic screening of medicinal plants for developing new drugs[1]. Medicinal plants are rich resources of traditional medicines and many modern medicines, including aspirin, digoxin, quinine and morphine are obtained from willow bark foxglove, cinchona bark, opium poppy, respectively[2]. The effectiveness of medicinal plants has been proved on individual body systems, for example, they have profound antioxidant, anti-inflammatory, antimicrobial and immunostimulatory properties[1].

Mentha species is a member of the Lamiaceae (Labiatae) family and are mass distributed across all continents[3,4]. According to latest data,

Lamiaceae family is considered one of the largest families of plants that produce flowers with around 4000 species that grow worldwide[5] and is considered to have the highest number of medicinal plants[6]. The use of *Mentha* plants in treating many diseases, including common cold, fever, throat infection, bronchitis, ulcerative colitis, and digestive issues has been known for a long time[7,8]. Moreover, its use as an antimicrobial, antioxidant, anti-motion sickness, anti-inflammatory and anticarcinogenic agent has also been reported[9–11]

Mentha viridis, commonly known as spearmint, has well known industrial importance. The leaves of the *M. viridis* are used as flavouring agent in culinary purposes including iced drinks and jellies[12].

The medicinal uses of *M. virids* are also well documented. It is considered as a relaxant, antispasmodic, and soothing agent in nausea and vomiting[13]. Moreover, it is widely known as a strong stimulant and carminative[14]. M. viridis extract contains various terpenes, fatty acid esters and Vitamin E, which explains its antioxidant potential[15]. The essential oil from leaves of *M. viridis* has potent antimicrobial activity[16].

Previously, no study has been reported on *M. viridis* cultivated in Albaha region. Its medicinal properties and safe usage have made it an ideal option for studying. In addition, plants growing in different geographical and weather conditions tend to have different phytochemical composition and different biological activities. This study aims to characterize the bioactive compounds of *M. viridis* from Albaha region and investigate its extracts for antimicrobial and antiproliferative potential.

METHODS

Plant materials

The plant was obtained from local farmer market in Albaha region, Saudi Arabia in March 2019. Dr. Haider authenticated its botanical identification. An authenticated specimen was deposited at the Botany Laboratory, Department of Biology, Albaha University.

Preparation of samples for GC-MS analysis

The leaves and stem (representing fresh aerial parts of the plant), with a weight of 25 g, was transferred to 15-mL screw test tube, mixed with 5 mL methanol, capped, vortexed for 5 min, sonicated for 30 min, mixed with about 2 g anhydrous sodium sulfate, filtered through a filtration disc of the PTFE syringe, 0.22 micron thickness. The produced filtrate is then concentrated to 1 mL using room temperature Nitrogen gas in the form of a gentle stream. The extract contains both polar and nonpolar components of the plant material. A portion from the clear extract was transferred to autosampler vial. GC-MS analysis sample was prepared by injecting 1.5 µl into the vial.

The GC-MS analysis

The GC-MS analysis of bioactive compounds from plants extract was done using Clarus 500 GC-MS (Perkin Elmer, Shelton, CT, USA). TurboMass version 5.4.2.1617 was used as a software integrator & controller. An Optima® 1 GC capillary column, Crossbond® 100% dimethyl polysiloxane (30-meter × 0.25 mm ID × 0.25 µm df), Macherey-Nagel, GMBH, Duren, Germany) was used. Al Hashmi *et al.,*(2013) described a similar setting, but a few modifications were made. In this assay, Helium (purity 99.9999%) was used as a carrier gas, with 0.90 mL/min flow rate. Source (EI+): source was set to 215˚C temperature, GC inlet line was set to 265˚C temperature, with 70 eV Electron energy, and 100V trap-emission. The oven programming went as follows: 50°C temperature initially (with a 5 min hold), then raised to 260˚C (at a rate of 10˚C/min, with 5 min hold), then raised again to 280˚C (at a rate of 10˚C/min, with 2 min hold). Temperature of the injector was set to 265°C, 1.0 µL was injected, and a 50:1 ratio was used for splitting. A total MS scan from 40 to 500 m/z (500 scan/sec) was applied to acquire the sample. The eluted compounds were characterized using NIST 2008, as reported in Mosbah *et al.,*(2018).

Extraction of crude extracts

The leaves and stem (representing fresh aerial parts of the plant) were air dried at room temperature (25 ± 2°C) for about 7 days. The dried material was ground using an electric blender machine (Pulverizer HR-30B, USA). 200 g powder material was macerated in 600 mL methanol with shaking for 3 days. Then they were filtered through Whatman no1 filter paper. The residue was further extracted two times by using the same fresh solvent. All filtrates were compiled for further evaporating. The

resulting residue was air dried and further extracted with solvents of increasing polarity namely petroleum ether, chloroform, and ethanol by using similar procedure carried out for the methanol extraction. Finally, rotary evaporator (IKA RV-10, Germany) was used under reduced pressure and low temperature to evaporate solvent from each filtrate extract until dryness was achieved.[19]

Antimicrobial evaluation

The antimicrobial activity of the four plant extracts (methanol, ethanol, petroleum ether, and chloroform) was tested against standard strains of four bacteria and one fungus. King Abdulaziz University Hospital, Jeddah, KSA provided the organisms through their microbiology laboratory. These strains were: *Staphylococcus aureus* (reference: ATCC 29213) and *Bacillus subtilis* (Reference: ATCC 6633), both *Gram-positive* bacteria, as well as *Escherichia coli* (Reference: ATCC 35218) and *Pseudomonas aeruginosa* (Reference: ATCC 27853), both *Gram-negative* bacteria and finally fungus: *Candida albicans* (Reference: ATCC 76615).

Agar diffusion technique was used for the initial screening of the antibacterial and antifungal activities, as previously described (20). Briefly, Muller-Hinton agar (25 mL) containing 1 mL bacterial culture (1×10^6 CFU/mL) was used to fill Petri dishes with a capacity of 90 mm. The strains were inoculated separately. Seven holes (4 mm in diameter) were prepared in the seeded agar dishes, which were then filled with 50 µL of each extract (10 mg/mL), as well as a negative control agent (10% dimethyl sulfoxide (DMSO)). Dishes were then incubated at 37°C for 24 hours. Success of the Inhibitory activity was marked by the absence of bacterial growth in the area surrounding the holes. Triplicates were carried out against each of the tested microorganisms. The growth inhibition zones' diameters were measured using a calliper and averaged at the end of the incubation period. The mean values were tabulated.

Acquisition of cell lines and culture medium preparation

Human breast cancer MCF-7 and colorectal cancer HCT-116 cell lines were obtained from Dr. Thikryat Neamatallah, Pharmacology and Toxicology laboratory, Faculty of medicine, King Abdulaziz University, Jeddah, KSA. Dulbecco's Modified Eagles Medium (DMEM)/high glucose medium was used as a culture medium, augmented with 10% fetal bovine serum (FBS), as well as 10,000 units/mL penicillin/streptomycin (Pen/Strep) and 1% glutamine). All reagents were purchased from Thermo Fisher (Thermo Fisher Scientific UK Ltd, Leicestershire, UK) except Pen/Strep, which was obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The cell lines were cultured in 75 cm^2 flasks were used to culture the cells, and were sustained at 37°C in a 5% CO_2 humid incubator. A Class II Safety Flow Hood was used to carry out the cell culture procedure, under aseptic conditions.

MTT assay

Both types of cells were seeded at (1×10^5 cells/mL) into a plate containing 96 wells, together with 3 duplicates. The whole assay was incubated at 37°C through the night for attachment in a humid atmosphere containing 5% CO_2 as described by Mansour *et al.,*(2016), but slightly modified. In this assay, plant extracts (methanol, ethanol, petroleum ether, and chloroform) at 7 serial dilutions (1000-10 µg/mL) were introduced in 3 identical settings (triplicates) and incubated for 72 h, at a temperature of 37°C, and 5% CO_2. 0.1% DMSO was used as a vehicle to dissolve the drugs in. Untreated cells were used as control. Afterwards, 100 µL of full medium containing 10% of 3-(4,5- dimethylthiaxolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (10 mg/mL) was used as replacements for each well at each recorded time point. Cells were incubated again at a temperature of 37°C, and 5% CO_2 for 4 hours. 100 µL of DMSO was added after removing the media, and incubation at a temperature of 37°C, and 5% CO_2 was done for an additional 5 minutes. Spectra Max M3 plate reader at 570 nm was used to quantify the plates.

The following formula was used to determine the viability percentage:

Cell viability (%) = (A of treated cells/A of control cells) ×100.

Statistical analysis

Triplicates of three independent experiments were carried out and data are expressed as the mean ± SD. IC_{50} value was calculated by ED50 GraphPad Prism software (GraphPad Prism 5.0, GraphPad Software, Inc., CA, USA).

RESULTS

GC-MS Analysis

Th analysis showed that aerial parts (stem and leaves) of *M. viridis* methanolic extract had a variety of phytochemicals, which are shown in the GC-MS chromatograms (Fig. 1). Thirty-two distinct phytochemical compounds were present in *M. viridis* methanolic extract in different ratio and accounted for 98.6% of the total components. It was found that carvone was the most abundant phytochemical, as it was present in 64.82% followed by eucalyptol (10.44%), oleamide (6.34%) and phytol (5.40%).

Table 1 shows the identified components in *M. viridis* methanolic extract. Fig. 2 illustrates the chemical structure of some active components detected in *M. viridis* methanolic extract.

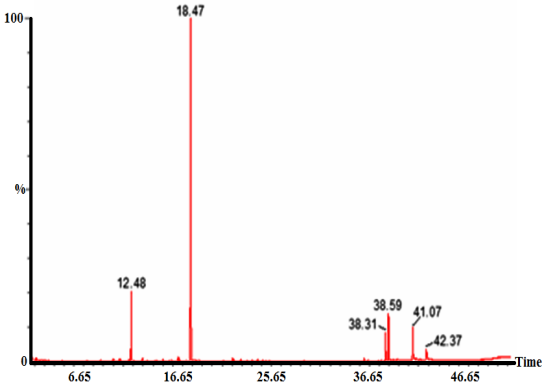

Fig. 1. Gas chromatography-mass spectrometry chromatogram of *Mentha viridis* methanolic extract. The chromatogram shows various phytocomponents peaks separated at different retention times

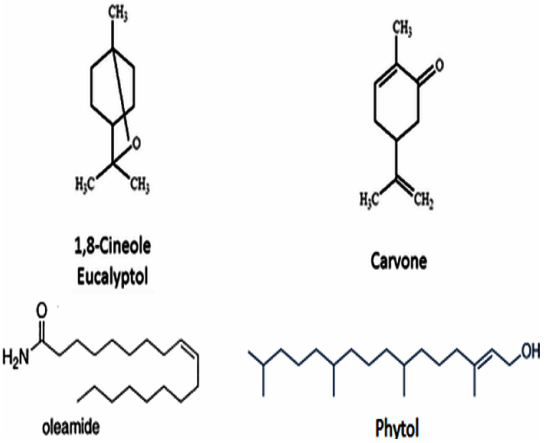

Fig. 2. Chemical structures of some bioactive constituents of *Mentha viridis* extract

Table 1: Chemical composition of *Mentha viridis* methanolic extract

Peak number	Rt, mina	Component	Area	%Area
1	7.99	2,5-Diethyltetrahydrofuran	267132	0.13
2	9.36	1R-α-Pinene	298463	0.14
3	10.62	β-Thujene	407800	0.20
4	10.72	β-Pinene	574268	0.28
5	11.27	Myrcene	498542	0.24
6	11.41	n-Octan-3-ol	751261	0.36
7	12.22	Benzeneacetaldehyde	565544	0.27
8	12.48	Eucalyptol (1,8-Cineole)	19610798	10.44
9	13.64	(E)-Sabinene hydrate	988349	0.48
10	14.03	Fenchone	72673	0.03
11	15.62	(-)-Camphor	400971	0.19
12	16.29	3-Methyl-1,2-cyclopentanedione	44459	0.02
13	16.49	Myrcenol	44459	0.02
14	16.54	Borneol	489790	0.24
15	17.15	Dihydrocarvone	368367	0.18
16	17.36	Neodihydrocarveol	152889	0.07
17	18.02	cis-Carveol	200112	0.10
18	18.47	Carvone	130497712	64.82
19	19.28	cis-Carvone oxide	117097	0.06
20	22.72	β-Bourbonene	594923	0.29
21	22.86	β-Elemene	317625	0.15
22	23.57	Caryophyllene	583306	0.28
23	24.71	(+)-Epi-bicyclosesquiphellandrene	355040	0.17
24	25.28	Germacrene D	459971	0.22
25	25.81	1,5-Heptadiene, 2,5-dimethyl-3-methylene-	203301	0.10
26	30.00	Copaene	78219	0.04
27	31.03	α-Cadinol	53101	0.03
28	36.14	Tridecanoic acid, methyl ester	520004	0.25
29	38.31	Linolenic acid, methyl ester	6695976	3.22
30	38.59	Phytol	11224343	5.40
31	41.07	Oleamide	13174507	6.34
32	42.37	Limonen-6-ol, pivalate	8055191	3.88
		Total		98.64

[a]Retention time (as minutes)
[b]Compounds listed in order of elution from a column

Antimicrobial Activities

The *In vitro* antifungal and antibacterial bioassays were carried out using the extracts from *M. viridis.* A variety of bacteria (both *Gram-positive* and *Gram-negative*) as well as fungal strains were used as test strains and agar diffusion assays were carried out to analyse the antimicrobial properties of *M. viridis*. The results were determined on the basis of formation of zones of inhibition (measured in mm) around the extract on the agar plates which were seeded with test microbial strains. The methanolic extract showed significant antibacterial activity against *P. aeruginosa* with zone of inhibition of 15 mm while ethanol, chloroform and petroleum ether extracts did not show any antimicrobial activity against the tested microorganisms. The results are given in Table 2.

Table 2: Antibacterial and antifungal activities of *Mentha viridis* extracts

Extracts	Zone diameter (mm)				
	Gram-positive bacteria	*Gram-negative* bacteria			*Fungus*
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*
Chloroform	–	–	–	–	–
Methanol	–	–	–	15 ± 0.04	–
Ethanol	–	–	–	–	–
Petroleum ether	–	–	–	–	–

[a]The diameters of the inhibition zones. Values are the means ± SD of three cultures.

MTT (IC_{50}) Assay

The antiproliferative effect of the extract of *M. viridis* on human cells' viability was analysed using MTT assay. Two cancer cell lines, human breast cancer (MCF-7) and colorectal cancer (HCT-116) cell lines were selected and tested against *M. viridis* extracts. Readings were taken using spectrophotometer Spectra Max M3 and are given in Table 3. Petroleum ether and chloroform extracts showed moderate cytotoxic effects against MCF-7 with IC_{50} values 193.23 µg/mL and 131.86 µg/mL, respectively. Chloroform extract also showed mild activity against HCT-116 with IC_{50} value of 189.2 µg/mL. Ethanol and methanol extracts showed IC_{50} higher than 200 µg/mL against the tested cell lines, which is indicative of no cytotoxicity.

Table 3: The IC_{50} of *Mentha viridis* extracts against tested human cancer cell lines

Compound	IC_{50}* (µg/mL) MCF-7	IC_{50}* (µg/mL) HCT-116
Standard drug (paclitaxel)	$0.23 \pm 2.2 \pm 10^{-6}$	$0.32 \pm 5.7 \pm 10^{-6}$
Chloroform extract	$131.86 \pm 4.2 \times 10^{-5}$	$189.2 \pm 1.13 \times 10^{-4}$
Petroleum ether extract	$193.23 \pm 5.9 \times 10^{-5}$	>200
Ethanol extract	>200	>200
Methanol extract	>200	>200

* IC_{50} is the half maximal inhibitory concentration (µg/mL). Values are the means ± SD of three cultures.

DISCUSSION

Various types of infectious bacteria and cancers are a constant threat for human health and are the leading cause of morbidity and mortality worldwide. This continuous risk calls for the need of exploring and finding new cost-effective therapies with better effectiveness. Medicinal plants have proved to be a significant source of novel therapeutic substances. Today, various plant-derived potent chemicals are being extensively used and studied for human therapeutic purposes. The present study aimed to analyse the phytochemical contents of *M. viridis* extract and evaluate its antimicrobial and anticancer properties.

GC-MS Analysis

The methanolic extract of different parts of *M. viridis* were subjected to GC-MS analysis to determine its complete phytochemical composition and to determine the ratio of each component in the respective plants. The peaks in the GC-MS chromatogram showed that about thirty distinct phytochemicals were present in the *M. viridis* extract. The most abundant component was carvone which has the highest ratio, i.e., 64.82%. Few other components were present in high quantities, including eucalyptol (1,8-cineole) (10.44%), oleamide (6.34%) and phytol (5.40%). Various studies have reported carvone as the most abundant component of *M. viridis*[22]. For example, according to the findings of Verma *et al.,* (2010), the analysis of *M. viridis* sample collected from India (specifically from the mid-hills of Himalayan region) at different stages of crop

growth showed that carvone is the most prevalent phytochemical with percentages ranging between of 59.6-72.4% in different stages of sampling. Moreover, phytochemical analysis of *M. viridis* collected from farms in Al-Kadaro region of Sudan showed carvone (64.63%) as a major component[16]. Furthermore, another study reported that carvone (50.47%) followed by 1,8-cineole (9.14%), limonene (4.87%), camphor (3.68%), and β-caryophyllene (3%) were the key component of Tunisian *M. viridis*[23].

GC-MS profiling of our study also indicated the presence of fatty acid methyl esters (tridecanoic acid, linolenic acid) terpenoids and terpenoid alcohol (eucalyptol, phytol). Most of the plant terpenoids and their derivatives are biologically active and are used extensively as traditional herbal remedies for many diseases[24]. They are also used worldwide in food, cosmetics and pharmaceutical industries[25].

Antimicrobial Activities

The significant antimicrobial activity was only shown by methanol extract whose concentration was 10 mg/ mL, forming an inhibition zone as of 15 mm when interacting with *P. aeruginosa*. However, no antimicrobial activity was noted by other extracts (10 mg/mL) obtained from *M. viridis* against the tested microorganisms. The results are similar to those by Mkaddem *et al.,* (2009) who stated that no antimicrobial activity of essential oil extract (15 µl/ mL) from Tunisian *M. viridis* was observed against *E. coli* and *S. aureus*. Referring to Silva *et al.,* (2015), the essential oil from *M. viridis* showed antimicrobial activity against *E. coli* (a *Gram–ve* bacterium) and *S.aureus* (a *Gram+ve* bacterium) forming zones of inhibition of 6 mm and 8 mm, respectively, when the minimal inhibitory concentration (MIC) was 62.5 µl/ mL. Other study showed that MIC of 12.5 mg/mL is the lowest concentration at which all the tested microorganisms are inhibited[16]. Our study found different results, which may be associated with differences in the concentration of essential oil and crude extracts of *M. viridis* used against the tested microorganisms. In addition, the disagreement was probably due to the difference in amounts and the nature of the components that presented in the essential oil and crude extracts of *M. viridis,* because Silva *et al.,*(2015) found linalool (40.70%), carvone (13.52%) and α-terpinene (8.56%) as the chief components.

MTT (IC_{50}) Assay

The antiproliferative effects of *M. viridis* extracts (10 µg/mL) on human breast cancer (MCF-7) and colorectal cancer (HCT-116) cell lines, were analysed using the MTT (IC_{50}) assay. Chloroform extract showed moderate antiproliferative activity and had IC_{50} (µg/mL) values less than 200 against both the MCF-7 and HCT-116 cell lines. Petroleum ether extract showed inhibition effect only against MCF-7 with IC_{50} values 193.23 µg/mL. However, no cytotoxic activity was observed in methanol and ethanol extracts against the tested cell lines. Sharma *et al.,*(2014), evaluated the anticancer potential of methanolic and aqueous extracts of whole plant of M. viridis against MCF-7 and HCT-116 *In vitro*, using a 100 µg/mL concentration, and sulforhodamine Blue (SRB) assay. Methanolic extract exhibited cytotoxicity against MCF-7 while aqueous extract was found active against HCT-116[27]. Our study found different results, which can be attributed to the difference in doses concentration applied against the tested cancer cell lines.

In this study, it is noticed that the non-polar solvents (petroleum ether and chloroform) used for the plant extraction exhibited more cytotoxic effects against tested cancer cells. These effects are most likely due to the presence of more bioactive terpenoids and sesquiterpenes in these solvents. It has been reported that carvone and its derivatives do not exhibit cytotoxic effects against cancer cells[28] while many of the plant terpenoids inhibit different human cancer cells and are used as anticancer drugs[25,29,30].

From the current findings we can safely conclude that extracts from *M. viridis* can be ideal candidates for novel therapeutic research. The difference observed in the antibacterial and antiproliferative properties of *M.viridis* between our results and other reported findings is certainly due to the chemical composition, methods used, strains tested, dose concentrations applied, growing conditions and regions. The emergence of novel infectious diseases and development of bacteria resistance against the available antibiotics have made it inevitable that medicinal plants, especially from novel environments, should be explored for their therapeutic potentials. The present study is among the very first studies that have investigated and explored the *M. viridis* species of Albaha Region,

KSA, and it highlights the need and importance of similar studies.

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Conflict of Interest

There are no conflicts of interest to declare.

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