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Chemical Composition of Hydrodistillation and Solvent Free Microwave Extraction of Essential Oils from *Mentha piperita* L. Growing in Taif, Kingdom of Saudi Arabia, and their Anticancer and Antimicrobial Activity

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ABSTRACT

The chemical composition of the essential oils was influenced by many factors including extraction methods. In this study, the effect of extraction methods; hydrodistillation, microwave assisted hydrodistillation and solvent free microwave extraction of *Mentha piperita* L. growing in Taif, KSA, on the yield and chemical composition of their essential oils were investigated. Furthermore, the oils were *in vitro* investigated as antimicrobial and anticancer agents. The results showed no great difference between the oil yields obtained by the three different methods but the methods which used microwave were rapid, saving time and energy than classical hydrodistillation. The qualitative chemical compositions of the oils were similar with little quantitative differences of some compounds between the three methods. All oils consists mainly of monoterpenes and sesquiterpenes in which carvone is the main component of *M. piperita* (carvone chemotype). All essential oils showed moderate *in vitro* anticancer activity and high antimicrobial activity. In conclusion, this considered to be the first study represented the effect of microwave extraction on the essential oil chemical composition of *M. piperita* growing in Taif, KSA. The authors recommended the usage of microwave method in the extraction of essential oils because it is energy and time saving, in addition to environment friendly.

Keywords: *Mentha piperita,* Taif, Microwave, anticancer, antimicrobial.

INTRODUCTION

The medicinal plants continue to be one of the richest bio-resources which are used in traditional and modern medicines. Variety of widely used pharmaceutical preparations contains essential oils, crude extracts, or pure compounds from numerous plants**.** Seeking for plants with medicinal properties remains of a great importance. Researchers are screening plants for a wide range of biological activities, ranging from antibiotics to antitumor[1,3].

Aromatic plants' essential oils are important class of phytochemicals which have been recognized as a great source of pharmaceutical agents and food additives. They have been used for a long time in different industries, mostly in food, perfumes, pharmaceuticals, and for centuries in traditional medicine. Essential oils are obtained from different plant parts such as seeds, buds, leaves, flowers, and fruits, composed mainly of a mixture of volatile mono- and sesquiterpenes, of low-molecular weight, and other isoprenes. Using essential oils in many fields, including phytotherapy, perfumes, cosmetics, aromatherapy, nutrition and spices encouraged many scientists to study essential oils bearing plants from the chemical and pharmacological examinations to the therapeutic aspects[4,5].

There are many factors affecting the chemical composition of the essential oils including: climate, seasonal and geographic conditions, harvest period and extraction techniques. These factors showed variation in biological activity between the same oil obtained from the same species but from different geographic regions[6,7]. Therefore, there is a need to study the chemical composition of the essential oil in its origin because the value of an essential oil has been related to its chemical composition.

The commonly techniques employed for extracting essential oils include hydrodistillation, steam distillation, solvent extraction and liquid CO_2 extraction. The composition of the extracted oil may vary from one extraction method to another. Recently, using microwave extraction for plant materials has shown remarkable research interest. Traditional extraction methods of essential oils are time consuming, and require much amounts of solvent. Following the results of the various experiments carried out it becomes obvious that extraction using microwave technology is a good alternative to conventional extraction techniques[8-10].

Labiatae family is one of the most employed medicinal plants as a worldwide source of aromatic plants and as an excellent source of extracts with strong antibacterial and antioxidant properties. Within this family, the genus *Mentha* (mint plants) provides various species of aromatic plants (about 25-30 species) commonly distributed in temperate climate regions. These species used in the food industry for flavoring, in perfumery and for pharmaceutical preparations. These species is commercially grown for its essential oil content and herbage yields. Mint plants are used in folk medicine in the treatment of many diseases as antispasmodic, choleretic, antiemetic, emmenagogue, diaphoretic, carminative, anti-inflammatory and since antiquity, it has been known to have antimicrobial proprieties[11-13].

The special geographic location and climate of the Taif governorate (1,879 m above sea level, 21°26′N 40°21′E), Kingdom of Saudi Arabia, offer an excellent environment for the cultivation of roses, fruits and ornamental plants. In this study, the effect of extraction methods; hydrodistillation (HD), microwave assisted hydrodistillation (MAHD) and solvent-free microwave extraction (SFME) on *M. piperita* L. growing in Taif, KSA on the yield and chemical composition of essential oils were investigated. In addition, anticancer and antimicrobial activities of the oils were evaluated.

MATERIAL AND METHODS

Chemicals

All solvents, chemicals and reagents were analytical and HPLC grade from Sigma-Aldrich Chemicals, USA.

Plant collection

The healthy *M. piperita* plant species under investigation grown in Taif governorate, KSA, were collected in November 2016. The collected plants were taxonomically kindly identified at Department of Biology, Faculty of science, Taif University. Voucher

specimen of the plants was deposited at Faculty of science, Taif University. The fresh leaves were cut to small pieces and become ready for essential oil extraction.

Extraction of essential oil

The essential oils for the plants under investigation were extracted using three different methods: HD, MAHD and SFME.

Hydrodistillation (HD)

Classical hydrodistillation extraction of essential oil was employed using a laboratory hot plate (Fisher Scientific, 50 Hz), five-liter flat bottom conical flask, and Clevenger system as condenser and oil collector. Five hundred grams of fresh chopped *M. piperita* leaves were immersed with 3 L of distilled water in the five-liter flat bottom glass conical flask with Clevenger system. The extraction time took about 150 minutes at 100 ˚C until no more essential oil was obtained. At the end of the experiment, the essential oil was collected, dried over sodium sulphate and filtered. The oil was stored at -20 ˚C in a brown glass vial until chemical and biological investigations.

Microwave Assisted Hydrodistillation (MAHD)

Microwave assisted hydrodistillation extraction of essential oil was carried out in a fully instrumented and controlled microwave system (Milestone NEOS-GR). Five hundred grams of fresh chopped *M. piperita* leaves were mixed with 1.5 L of distilled water in five-liter cylinder Pyrex glass. The mixture was subjected to microwave treatment at 500 W, 100 ˚C for 40 min. until no more essential oil was obtained. At the end of the experiment, the essential oil was collected, dried over sodium sulphate and filtered. The oil was stored at -20 ˚C in a brown glass vial until chemical and biological investigations.

Solvent free Microwave extraction (SFME)

Solvent-free microwave extraction was employed using the same instrument and procedure as described for MAHD. Five hundred grams of fresh chopped *M. piperita* leaves with 30 mL distilled water were introduced in five-liter cylinder Pyrex. The mixture was subjected to microwave treatment at 500 W, 105 ˚C for 40 min. until no more essential oil was obtained. At the end of the experiment, the essential oil was collected, dried over sodium sulphate and filtered. The oil was stored at -20 ˚C in a brown glass vial until chemical and biological investigations.

Chemical analysis of Essential oils

The chemical composition of essential oils under studies was obtained after analysis by gas chromatography-mass spectrometry (GC-MS) technique.

Preparation of samples and standard solutions

For each oil sample, 5 mg/mL of oil was prepared in GC grade solvent (*n*-hexane) and filtered using membrane disc filter (0.45 µm). For standards, mixture of 50 standards (1 mg/mL) was prepared in GC grade solvent (*n*-hexane) and filtered using membrane disc filter (0.45 µm).

Gas chromatography-mass spectrometry (GC-MS) conditions, samples and standards analysis

The analysis of the standards and samples were performed using gas chromatograph (GC, Model CP 3800, Varian, California, USA) coupled with a mass spectrometer (MS, Model Saturn 2200, Varian) and auto sampler (Model Combi Pal, Varian) system. The separation was done using a VF-5 fused silica capillary column (30 m x 0.25 i.d. mm, film thicknesses 0.25 µm, Varian). For MS detector, electron impact (EI) ionization system with ionization energy of 70 eV was used. Trap temperature was set at 150 ˚C and axial modulation voltage at 4.0 volts. The ions were recorded with mass range 45-400 m/z. Helium gas was used as a carrier gas at a constant flow rate of 1 mL/min. Injector and mass transfer line temperature were set at 200 and 170 °C respectively. The oven temperature was programmed for 5 min at 40 ˚C, raised gradually to 200 °C at 3 °C/min. 200-220 °C at 2 °C/min. and held for 3 min. at 220 °C, solvent delay time 3 min. the total run time 73.33 minutes. The injection volume for all samples and standards was 1 µL with a split ratio 1:20 and carried out with the auto-sampler. n-Alkane series mixture (C_8-C_{20}) was injected at the same conditions for samples and standards. Chromatograms of the standards and samples were analyzed using Varian MS Workstation software (Service Pack 1, Version 6.5).

For samples, known peaks were identified by comparing its retention time (t_R), mass spectrum and retention indices with standards. Unknown peaks were identified by matching their mass patterns with Wiley & NIST electronic library and its retention indices with review of literatures.

Biological studies

The essential oils obtained by the three methods were tested *in vitro* for their anticancer and antimicrobial activities.

Anticancer activity

The cytotoxicity of the essential oils under study were investigated *in vitro* towards three human cancer cell lines; HepG-2 (liver cancer), lung cancer (A549) and breast cancer (MCF-7); in the Cell Culture Lab, Cairo University Research Park, Faculty of Agriculture, Cairo University, Egypt. The method used was neutral red uptake assay according to Guillermo *et al.,* 2008[14]. Cells were grown under aseptic conditions with complete medium in a 25 cm^3 cell culture flask with humidified atmosphere and 5 % CO_2 at 37 ˚C. Cultured monolayer at 80 % confluence subjected to wash with PBS then trypsinized by 2 mL (0.25%) tryps in–EDTA solution, incubated for 2 min. then flask was lightly tapped to detach the cells, the reaction stopped by adding 5 mL complete culture medium. The cell suspension counted using hemocytometer, and cell viability checked by trypan blue (100% viability). The cells suspension was diluted with complete medium to have approximately 100,000 cell/mL, agitated gently and placed in a sterile reservoir. 200 µL of the cell suspension (containing ≈ 20,000 cell per well) was dispensed by multichannel pipette into the inner 60 wells of the 96 well plate, the peripherals wells were filled with PBS, then the plate incubated for 24 hours. After cell seeding and attachment, the media discarded gently and different concentrations of the compounds prepared (5, 25, 50, 75, and 100 µg/mL) by diluting with DMEM media (after dissolved 12 µL in 1 ml DMSO). 200 µL of treatment media was dispensed into 4 replicates for each concentration, other wells were filled with media only (as a negative control) and wells filled with media containing doxorubic in n-H (3 µg/mL) as a positive control. After that, the 96 well plate covered by lid, incubated at 37 ˚C for 24 hours. After the incubation period, the cultures examined under inverted microscope, recording changes in morphology of the cells due to cytotoxic effects of the test chemical and photos was taken then the medium was decanted from the wells gently without disturbing the materials. 100 µL of Neutral red medium (which was prepared and incubated at 37 ˚C for 24 h and centrifuged at 1800 rpm for 10 min. to remove any precipitated dye crystals) was added into each well and incubated again for 3 h at 37 ˚C. After incubation, the dye containing medium was decanted and each well was rinsed gently for two times with 150 µL PBS solution to remove the unabsorbed neutral red dye contained in the wells. 150 µL of destain solution was added and incubated for 10 min. with shaking. The absorbance of acidified ethanol solution containing extracted neutral red dye was measured using spectrophotometer (BioTek, ELX808). The cytotoxicity % was calculated for each concentration which reflects the inhibitory concentration of the cell proliferation.

Antibacterial activity

The agar-well diffusion method was employed for determination of antibacterial activities[15]. Four bacterial strains were used in this investigation; *Gram-negative* bacteria, *Escherichia coli* (*E. coli*) and *Aeromonas hydrophila* (*A. hydrophila*)*; Gram-positive* bacteria, *Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecium* (*E. faecium*). All bacteria were suspended in sterile water and diluted to~10^6 CFU/mL. The suspension (100 µL) was spread onto the surface of NA medium. Wells (4.6 mm in diameter) were cut from the agar with a sterile borer and 50, 100 µL of Mint oil extracts solutions dissolved in DMSO (with a concentration of 10 and 20 µg/ mL, respectively) were delivered into them. Negative controls were prepared using DMSO only. The artificial Streptomycin disc with a concentration of (10 µg) was used as positive reference standards to determine the sensitivity of each microbial species tested and to compare the relative percent of antibacterial activity. The inoculated plates were incubated at 37 ˚C for 24 hours. Antibacterial activity was evaluated by measuring the diameter of inhibition zone (DIZ) of the tested bacteria.

RESULTS AND DISCUSSIONS

Among the various plant-derived natural products, essential oils extracted from aromatic plants are extensively used as fragrances, flavor, pharmaceuticals, and food additives. Essential oils are composed of a wide range of complex mixture of bioactive chemical compounds[16]. Extraction of essential oils from plants was developed to obtain a better method for quality and quantity[17,18] .

The commonly used methods for essential oil extraction are: hydro and steam distillations, and organic solvent extraction. The previous methods have some disadvantages including time and energy consuming, degradation of some compounds through thermal or hydrolytic effects and toxic solvent residue in the oil[19,20]. Therefore, new methods have been developed for obtaining essential oils such as microwave and supercritical fluids.These methods use less energy and solvent. Using supercritical CO_2 in extraction of essential oils producing oil containing waxes and undesirable compounds. Also, this technique is highly cost and onerous[16,21]. Recently, using microwave energy in essential oil extraction has been developed due to the quick time of extraction, reduce solvent and saving energy[17,22,23]. Many areas in food processes, including our homes, are using microwave such as baking, cooking, drying, pasteurization thawing, and reheating. Several techniques depend on microwave as source of energy have been appeared and continuously developed such as; microwave hydrodiffusion and gravity (MHG)[24], solvent free microwave extraction (SFME)[17], vacuum microwave hydrodistillation (VMHD), compressed air microwave distillation (CAMD), microwave hydrodistillation (MWHD), microwave-accelerated steam distillation (MASD)[25] and microwave assisted simultaneous distillation-solvent extraction (MW-SDE)[20], microwave-assisted solvent extraction (MASE)[17,20,25,26]

In this work, the authors made a comparison study between three methods used for obtaining essential oils from *M. piperita* growing in Taif, KSA. HD is the classical hydrodistillation method using hot plate as source of heating energy while the other two methods (MAHD & SFME) microwave was used as source of heating energy but in one of them (MAHD) we have used half of the water amount that used in first method while the third method (SFME) was carried out using only 1 % of water used in the first method.

Extraction time and yield

The essential oil yields obtained by three different methods; HD, MAHD and SFME were 0.33, 0.36 and 0.31 % for *M. piperita*. It was appeared that there is no great difference between the oil yields of each species obtained by the three different methods. Our results are agreed with other studies using these techniques for different plants and other different mint species[21,24,27], while other studies showed that there was a difference in yield between SFME and HD techniques[23,28]. The obvious advantages appeared in this step of our study were time and energy saving. The extraction time for complete HD method was about 150 min. while for MAHD and SFME methods, was 40 minutes. The greater extraction time for HD method needs high energy than the lower time for the other two methods. In HD method, for reaching boiling of water, and appearing the first droplet of essential oil in the collector needs about 35 min. while by using microwave energy, the first droplet of essential oil in the collector appeared after 6 minutes. Then the microwave techniques used in our study were rapid and energy saving, in addition of reduction of the amount of water in SFME method.

Essential oils composition

The chemical compositions of the essential oils extracted from the plant under investigation by different techniques were identified using GC-MS analysis. To the best of our knowledge, this is the first comparative study on *M. piperita* essential oil extracted by three different extraction methods.

The total ion chromatograms of the *M. piperita* essential oils obtained by HD, MAHD and SFME (Fig. 1) showed good separated peaks, signal-noise is good and absence of drift of the horizontal base line. By naked eye, we can see the high similarity between the three chromatograms from the point of qualitative analysis. Table 1 showed the chemical composition, retention time (t_R), retention indices (RI) and relative percent of 48 compounds identified in the essential oil of

M. piperita extracted by three different methods. Table. 2 showed the chemical classes of the oils while Table. 3 showed the quantity (mg/mL) of some compounds in the oils. It was appeared that the qualitative chemical composition of the three oils were the same. The three oils were found containing only monoterpenes and sesquiterpenes. The monoterpene group constitutes the majority of the oils (94.86, 94.9, and 94.25 % for HD, MAHD and SFME respectively) while sesquiterpene group found with minor percent (4.83, 4.85 and 5.46 % for HD, MAHD and SFME respectively). It was obvious that the SFME method increase the extraction of sesquiterpene compounds. The detailed entire classification of monoterpene group showed that the presence of oxygenated monoterpenes which constitute the higher percent (83.30, 82.52 and 84.52 % for HD, MAHD and SFME respectively) whereas the hydrocarbon monoterpenes had lower percent (11.33, 11.96 and 84.52 % for HD, MAHD and SFME respectively). Within the oxygenated monoterpenes, monocyclic monoterpene ketones had the high percentage in the oils (72.08, 71.27 and 74.94 % for HD, MAHD and SFME respectively) whereas the monocyclic monoterpenes had higher percentage (10.17, 10.83 and 8.48 % for HD, MAHD and SFME respectively) within hydrocarbon monoterpenes. The main components of monoterpene group were carvone (70.13, 67.64 and 72.42 % and 650.16, 645.10 and 684.12 mg/ml for HD, MAHD and SFME respectively) followed by D-Limonene (9.82, 10.43 and 8.08 % and 27.56, 25.22 and 19.58 mg/ml for HD, MAHD and SFME respectively) and p-Cineol (5.03, 5.33 and 4.02 % and 10.92, 9.86 and 7.42 mg/ml for HD, MAHD and SFME respectively). It was appeared that, the SFME increased the extraction of carvone than the HD and MAHD methods. The mint species characterized by genetic variability and consequently the presence of many essential oil chemotypes for the same species[29,30]. The famous chemotypes for *M. piperita*, which each of them characterized by major compound in the oil, are menthol,

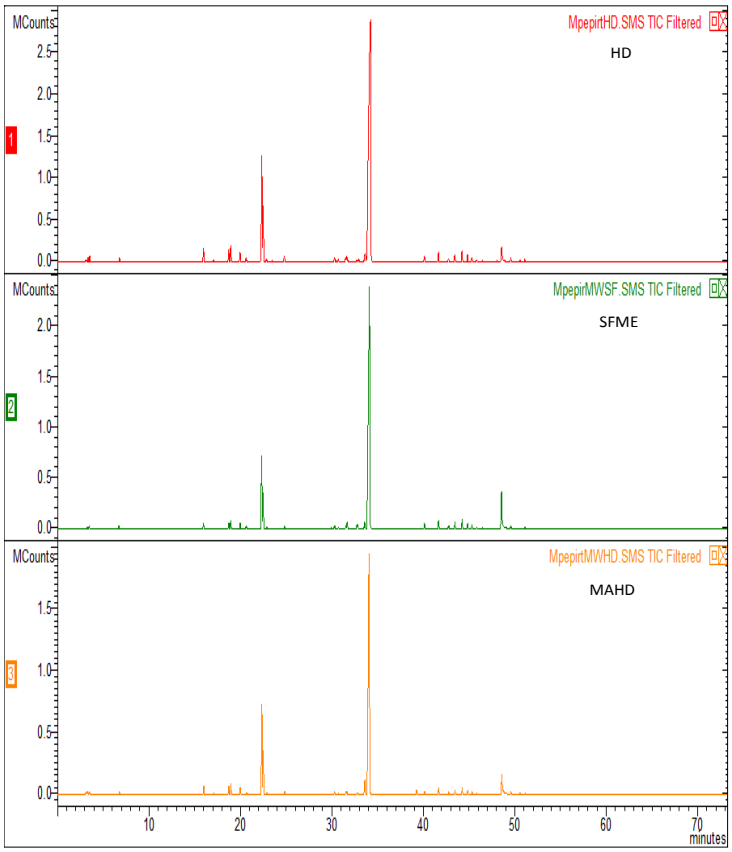

Fig. 1: GC-MS chromatograms of M. piperita essential oils extracted by HD, SFME and MAHD

Table 1: Chemical composition, retention time (t_R), retention indices (RI) and relative percent of *M. piperita* essential oils extracted by HD, MAHD and SFME

Name	t_R	RI	HD	MWHD	SFME
α-Thujene	12.71	925	0.03	0.03	0.02
α-Pinene	13.05	932	0.98	0.96	0.66
Camphene	13.91	948	0.14	0.14	0.10
Sabinene	15.19	972	0.90	0.94	0.69
β-Pinene	15.40	976	1.22	1.29	0.93
Myrcene	16.16	990	0.77	0.81	0.63
3-Octanol	16.70	1000	0.27	0.20	0.23
α-Phellandrene	16.84	1004	0.08	0.07	0.06
p-Mentha-1,3,8-triene	16.92	1006	0.11	0.09	0.08
γ-Terpinene	17.55	1016	0.00	0.06	0.07
D-Limonene	18.21	1029	9.92	10.4	8.08
1,8-Cineole	18.37	1032	5.03	5.33	4.02
cis-beta-Ocimene	18.65	1037	0.28	0.22	0.25
trans-beta-Ocimene	19.19	1048	0.11	0.10	0.09
γ-Terpinene	19.75	1058	0.09	0.11	0.12
cis-Sabinene-hydrate	20.44	1071	0.54	0.49	0.40
Terpinolene	21.15	1085	0.08	0.09	0.08
γ-Linalool	22.06	1103	0.06	0.06	0.07
Isoamyl valerianate	22.33	1108	0.02	0.03	0.03
Amyl isovalerate	22.42	1110	0.02	0.02	0.03
Limonene oxide	23.84	1139	0.08	0.08	0.08
Menthone	24.73	1158	0.01	0.04	0.01
isomenthone	25.23	1167	0.08	0.13	0.16
Borneol	25.59	1174	0.64	0.59	0.59
Isopulgeone	25.74	1177	0.16	0.16	0.14
Terpinen-4-ol	25.98	1182	0.28	0.30	0.32
α-Terpineol	26.74	1197	0.48	0.52	0.39
cis-Dihydrocarvone	26.86	1200	0.68	0.78	1.39
trans-Dihydrocarvone	27.18	1206	0.08	0.06	0.06
cis-Carveol	27.92	1223	0.48	0.33	0.88
trans-Carveol	28.62	1238	0.30	0.27	0.36
Pulegone	28.76	1240	0.94	2.47	0.78
Carvone	29.19	1251	70.26	67.9	72.6
Carvone oxide, cis	30.57	1279	0.06	0.08	0.07
α-Bourbonene	35.19	1383	0.54	0.52	0.70
β-Caryophyllene	36.72	1419	0.73	0.90	0.96
α-gurjunene	37.16	1430	0.06	0.06	0.07
α-Cubebene	37.82	1446	0.28	0.33	0.37
cis-muurola-4(14)-5-diene	38.50	1462	0.61	0.61	0.75
Germacrene D	39.28	1481	0.69	0.75	0.95

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γ-Gurjunene	39.89	1496	0.52	0.57	0.63
α-Eudesmene	40.36	1508	0.29	0.29	0.36
γ -Cadinene	40.60	1514	0.08	0.10	0.08
(-)-Calamenene	40.89	1521	0.18	0.16	0.21
α-cadinene	41.53	1538	0.07	0.07	0.08
γ-Eudesmol, 10-epi-	44.61	1617	0.52	0.22	0.20
tau.-Cadinol	45.64	1645	0.11	0.19	0.03
α-cadinol	46.15	1658	0.15	0.08	0.07
Total monoterpenes			94.86	94.9	94.25
Total sesquiterpene			4.83	4.85	5.46
Non-terpenes			0.31	0.25	0.29
Extraction time (min)			150	40	40
Yield %			0.33	0.36	0.31

Table 2: Chemical classes of *M. piperita* essential oils obtained by HD, MWHD and SFME

Chemical class	HD	MWHD	SFME
Acyclic monoterpene	1.16	1.13	0.97
Acyclic monoterpene alcohol	0.06	0.06	0.07
Monocyclic monoterpene	10.17	10.83	8.48
Monocyclic monoterpene alcohol	1.54	1.42	1.95
Monocyclic monoterpene oxide	0.08	0.08	0.08
Monocyclic monoterpene ketone	72.08	71.27	74.94
Monocyclic monoterpene ketone oxide	0.06	0.08	0.07
Bicyclic monoterpene	3.27	3.36	2.4
Bicyclic monoterpene alcohol	6.21	6.41	5.01
Oxygenated monoterpenes	83.3	82.68	84.52
Monoterpenes hydrocarbons	11.33	11.96	9.45
Total monoterpenes	94.86	94.9	94.25
Monocyclic sesquiterpene	0.69	0.75	0.95
Bicyclic sesquiterpene	1.96	2.13	2.44
Bicyclic sesquiterpene alcohol	0.78	0.49	0.3
Tricyclic sesquiterpenes	1.4	1.48	1.77
Non-oxygenated sesquiterpenes	4.05	4.36	5.16
Oxygenated sesquiterpenes	0.78	0.49	0.3
Total sesquiterpenes	4.83	4.85	5.46
Non-terpenoid	0.31	0.25	0.29

Table 3: Quantitative analysis (mg/ml) of some components of *M. piperita* essential oils obtained by HD, MWHD and SFME

Compound name	HD	MWHD	SFME
α-pinene	1.5	1.16	0.72
β--pinene	2.74	2.36	1.6
D-limonene	25.22	27.56	19.58
Eucalyptol (p-Cineol)	10.92	9.86	7.42
Cis-Sabinene-hydrate	1.44	1.14	0.78
α-Terpineol	2.14	2.36	1.74
Pulegone	4.38	11.1	3.78
carvone	650.16	645.1	686.12
β-Caryophyllene	3.24	5	5

Carvone and D-limonene chemotypes. Our results in this study proved that the essential oil of *M. piperita* growing in Taif governorate, KSA is belonging to carvone chemotype. This chemotype was previously found in some countries[31,32]. The total sesquiterpene group classified into major percent of sesquiterpene hydrocarbons (4.83, 4.85 and 5.46 % for HD, MAHD and SFME respectively) and minor oxygenated sesquiterpenes (0.78, 0.47 and 0.3 % for HD, MAHD and SFME respectively). The 14 sesquiterpene compounds identified in this group showed relative percent < 1 %. This study considered the first study on determination the chemical composition of *M. piperita* growing in Taif governorate, KSA under different extraction techniques and determined the chemotype of it.

Biological studies

Essential oils play a major role in many components used in traditional medicine, aromatherapy, and pharmaceutical preparations[3]. The essential oils in this study were investigated *in vitro* against three humane cancer cell lines (A549, HepG-2 and MCF-7) and four bacterial strains; two *Gram positive* (*E. coli* and *A. hydrophila*) and two *Gram negative* (*S. aureus* and *E. faecium*).

Anticancer activity

The essential oils from *M. piperita* leaves obtained by HD, MAHD and SFME showed variable anticancer activity toward three human tumor cell lines; A549, HepG-2 and MCF-7 at concentration 100 µg/mL (Table. 4). In general, the cytotoxic effects of the oils showed moderate activity. In many cases, there is difficulty to study the correlation between activity and agent in the form of mixture of different compounds like essential oils. This is due to; essential oil consists of many compounds that between them may play antagonistic or synergistic effect between each other. Many reports attributed the anticancer activity of aromatic plants to its content of essential oils as a major phytochemical class[33,34]. Many reports revealed that monoterpenes and sesquiterpenes showed anticarcinogenic activities[4,35,36]. The essential oils from *M. piprita* from different countries showed anticancer activity toward different carcinoma cells[37,38].

Table 4: Cytotoxicity percentage of *M. piperita* essential oils obtained by HD, MWHD and SFME (100 µg/ml) against three human tumor cell lines

Cell lines	HD	MWHD	SFME
MCF-7	34.03	56.6	50.2
HepG2	37.1	47	46.3
A549	37.8	27	21.2

Antibacterial activity

The antibacterial activities of *M. piperita* essential oils extracted by three different methods (HD, MAHD and SFME) were investigated using the agar disc diffusion method against selected pathogens as two *Gram-negative* bacteria; *E. coli* and *A. Hydrophila* and two *Gram-positive* bacteria; *S. aureus* and *E. faecium*. At first, the three essential oils with concentration 20 µg/mL were used to investigate if they have inhibition effect on the growth of the selected pathogens or not. Table. 5 showed the susceptibility pattern of the *M. piperita* essential oils (HD, MAHD and SFME) (20 µg/mL) against the bacterial isolates. The values of the Diameter of Inhibition zone (DIZ) (mm) caused by the three essential oils against four bacterial strains indicated that all essential oils treatments caused significantly antimicrobial effect comparing with that of the reference antibiotic treatment. Subsequently, the minimum inhibition concentration (MIC) of the three essential oils was calculated as 10 µg/ mL (MIC) (Data not shown) and accordingly the treatment with this concentration was used to evaluate the antimicrobial activities of the three essential oils. The (MIC) was defined as the lowest concentration that completely incubated the growth of microorganisms for 24 hours[39]. In addition, Table. 6 showed the mean diameter of the inhibition zone after the treatment with 10 µg/ mL of the three essential oils. The three essential oils showed varying degrees of antibacterial activities against these selected pathogens. For *Gram-negative* bacteria, the three essential oils exhibited equal inhibition zone (4 mm) to that caused with the reference antibiotic against the *E. coli*, while, only HD essential oil showed significant better antimicrobial activity against the *A. hydrophila* than that of the reference antibiotic. For *Gram-positive* bacteria, HD essential oil produced equal IZ length comparing with that produced by the reference antibiotic against both of *S. aureus and E. faecium.*

Both of MAHD and SFME essential oils exhibited higher antimicrobial activity against one of the two *Gram-positive* bacteria (*S. aureus and E. faecium)* and lower one against the other one, comparing with that produced by the reference antibiotic. In the present study, all three oils demonstrated promising antimicrobial activities against the most prevalent microorganisms in oral infections. *M. piperita* essential oils from many countries showed antimicrobial activity against different microbes[32,40-43] .

Table 5: Diameter of Inhibition zone (DIZ) (mm) caused by (20 µg/ml) of M. piperita essential oils (HD, MAHD and SFME) against four bacterial strains

Treatments (20 µg/ml)	*Gram-negative* bacteria		*Gram-positive* bacteria	
	E. coli	*A. hydrophila*	*S. aureus*	*E. faecium*
HD	7^{a}	14^{f}	16^{j}	8^{m}
MAHD	6^{b}	13^{e}	15^{i}	9^{n}
SFME	6^{b}	15^{g}	17^{k}	8^{m}
(Streptomycin 10 µg)	4^{a}	11^{d}	13^{h}	5^{l}

Different letters inside each column mean significant difference at $p < 0.05$.

Table 6: Evaluation of the antimicrobial activity of M. piperita essential oils (HD, MAHD and SFME) against four bacterial strains by the Diameter of Inhibition zone (DIZ) (mm) using the minimum inhibition concentration (MIC) (10 µg/ml)

Treatments (10 µg/ml)	*Gram-negative* bacteria		*Gram-positive* bacteria	
	E. coli	*A. hydrophila*	*S. aureus*	*E. faecium*
HD	4^{a}	12^{c}	13^{d}	5^{g}
MAHD	4^{a}	11^{b}	14^{e}	4^{h}
SFME	4^{a}	11^{b}	10^{f}	6^{i}
(Streptomycin 10 µg)	4^{a}	11^{b}	13^{d}	5^{g}

Different letters inside each column mean significant difference at $p < 0.05$.

CONCLUSION

There are many factors, including extraction methods, influencing the chemical composition of the essential oils which consequently affected on its biological importance. Recently, the use of microwave for natural products extraction from plant material has shown tremendous research interest and potential. This study showed the effect of extraction methods; HD; MAHD and SFME on extraction of essential from *M. piperita* growing in Taif, KSA on the yield and chemical composition of their essential oils. The results showed no great difference between the oil yield of each species obtained by the three different methods but the methods used microwave were rapid, saving time and energy than HD. It was appeared that the qualitative chemical composition of the three oils for each species was similar with little quantitative differences of some compounds between the three methods. The oil of *M. piperita* consists mainly from monoterpenes and sesquiterpenes in which carvone is the main component of *M. piperita* (carvone chemotype). All essential oils showed moderate *in vitro* anticancer activity and high antimicrobial activity. The authors recommended the usage of microwave method in the extraction of *M. piperita* essential oil because it is energy and time saving, in addition to environment friendly.

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