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Antimicrobial Screening of *Mentha piperita* Essential Oils

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Essential oils of peppermint *Mentha piperita* L. (Lamiaceae), which are used in flavors, fragrances, and pharmaceuticals, were investigated for their antimicrobial properties against 21 human and plant pathogenic microorganisms. The bioactivity of the oils menthol and menthone was compared using the combination of in vitro techniques such as microdilution, agar diffusion, and bioautography. It was shown that all of the peppermint oils screened strongly inhibited plant pathogenic microorganisms, whereas human pathogens were only moderately inhibited. Chemical compositions of the oils were analyzed by GC and GC/MS. Using the bioautography assay, menthol was found to be responsible for the antimicrobial activity of these oils.

KEYWORDS: ***Mentha piperita*; essential oils; antimicrobial activity; bioautography; TLC; GC; GC/MS**

INTRODUCTION

The well-known and widely used peppermint (*Mentha piperita* L.) (Lamiaceae) is a cultivated natural hybrid of *Mentha aquatica* L. (water mint) and *Mentha spicata* L. (spearmint). Although a native genus of the Mediterranean region, it is cultivated all over the world for its use in flavor, fragrance, medicinal, and pharmaceutical applications. Peppermint oil is one of the most widely produced and consumed essential oils (*1*−*3*).

Besides its uses in food, herbal tea preparations, and confectioneries, the medicinal uses of mint, which date back to ancient times, include carminative, antiinflammatory, antispasmodic, antiemetic, diaphoretic, analgesic, stimulant, emmenagogue, and anticatharrhal application. It is also used against nausea, bronchitis, flatulence, anorexia, ulcerative colitis, and liver complaints. Mint essential oils are generally used externally for antipruritic, astringent, rubefacient, antiseptic, and antimicrobial purposes, and for treating neuralgia, myalgia, headaches, and migraines (*2*−*8*).

With the development and wide use of synthetic and semisynthetic antibiotics, pros and cons have been experienced throughout the last 50 years which have directed research back to natural antimicrobial products as indispensable resources (*9*, *10*).

Another remaining problem for mankind is plant pathogens and their damage to agriculture. Furthermore, total yield and food quality has to be guaranteed by controlling fungi that produce mycotoxins which affect human health. Filamentous fungi can cause opportunistic systemic mycoses and other complications. Consequently, a large demand exists for fungicides, as well as antibacterial agents, in agriculture, food protection, and medicine (*9*, *11*).

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Mentha spp. have been previously investigated for their essential oil compositions by our group (*12*, *13*). Peppermint oil has been the subject of numerous other studies (*13*−*21*).

This investigation concerns the antimicrobial screening of *M. piperita* oils using different bioassay techniques in combination. The antimicrobial activities of the oils were determined and compared by using techniques such as agar diffusion, microdilution, and bioautographic agar overlay methods (*22*−*24*). The bioautography method aided in the identification of the antifungal active component. The chemical compositions of the peppermint oils were evaluated and compared using simple techniques such as thin-layer chromatography (TLC) and gas chromatography (GC). Gas chromatography/mass spectroscopy (GC/MS) analyses were also performed.

MATERIALS AND METHODS

Plant Materials. Peppermint oils *ex Mentha piperita* L. were obtained from four commercial sources: (**A**), Evcin Company, Turkey (plant material was supplied by this company from their farm in Adana, and the oil was obtained in TBAM by hydrodistillation (3.5%, w/v)); (**B**), Jet-Farms, Yakima, WA; (**C**), Mari-Linn Farms, Oregon; (**D**), Erdoğmuş Perfume Industry, imported from India. The oils of samples **B**−**D** were grown specifically for commercial peppermint oil production. Major constituents (−)-menthol (Polarome, Jersey City, NJ) (**M1**) and (−)-menthone (Fluka, Germany) (**M2**) were purchased from commercial sources.

Gas Chromatography. The GC analysis was carried out using a Shimadzu GC-9A with CR4A integrator. A Thermon 600T fused silica capillary column (50 m × 0.25 mm i.d., 0.20-μm film thickness) was used. Carrier gas was nitrogen. Oven temperature was kept at 70 °C for 10 min and programmed to 180 °C at a rate of 2 °C/min, then kept at 180 °C for 30 min. Injector and detector temperatures were 250 °C. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Gas Chromatography−**Mass Spectrometry**. The oils were analyzed by GC/MS using a Hewlett-Packard GCD system. HP-Innowax FSC

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column (60 m × 0.25 mm i.d., 0.25-μm film thickness) was used with helium as carrier gas (1 mL/min). GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min, then kept constant at 220 °C for 10 min, and then programmed to 240 °C at a rate of 1 °C/min. *n*-Alkanes were used as reference points in the calculation of relative retention indices (RRI). Split ratio was adjusted at 50:1. The injector temperature was at 250 °C. Electron impact MS were recorded at 70 eV. Mass range was from *m*/*z* 35 to 425.

Identification of individual components was achieved using the TBAM Library of Essential Oil Constituents.

Microorganisms. The following were used as test bacteria: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (NRRL B-123), *Enterobacter aerogenes* (NRRL 3567), *Salmonella typhimurium* (NRRL B4420), *Candida albicans* (Clinical Isolate, Osmangazi University, Faculty of Medicine, Eskişehir, Turkey), *Klebsiella pneumoniae, Yersinia enterocolitica*, *Listeria monocytogenes* (Ankara University, Department of Biology, Ankara, Turkey), *Bacillus cereus* (NRRL B3711), *Staphylococcus epidermidis* (ATCC 12228), *Xanthomonas campestris* pv. *phaseoli* (Ankara Zirai Araştirma Merkezi, Kemal Benlioğlu, Turkish Plant Pathogenic Bacteria: TPPB 5001), *Pseudomonas syringae* pv. *phaseolicola* (TPPB 4101), *Pseudomonas syringae* pv. *tomato* (TPPB 4212), *Pseudomonas syringae* pv. *syringae* (TPPB 4250), and *Xanthomonas campestris* pv. *campestris* (NRRL B-1459).

In the agar diffusion method, *Aspergillus flavus* (ATCC 9807), *Aspergillus paraciticus* (NRRL 2999), *Fusarium solani* (ATCC 12820), and *Sclerotium rolfsii* (Çukurova University, Faculty of Agriculture, Adana, Turkey) were used as test microorganisms to determine the antifungal activity.

All microorganisms were stored at −85 °C in 15% glycerol. The bacteria and yeast were refreshed in Mueller Hinton broth (Merck, Germany) at 35−37 °C, and afterward inoculated on Mueller Hinton agar (MHA, Mast Diagnostics, U. K.) plates for checking purity. Fungi were refreshed on Sabouraud dextrose agar (SDA)(Acumedia, MD) slants or plates and were grown at 28 °C.

Micro-Dilution Broth Method. Micro-dilution broth susceptibility assay (*22*) was used for the antimicrobial evaluation of the oils and the main compounds menthol (**M1**) and menthone (**M2**). Stock solutions of oils and pure compounds were prepared in dimethylsulfoxide (DMSO, Carlo-Erba, France). Dilution series were prepared from 5 mg mL^{-1} to 0.005 mg mL^{-1} in sterile distilled water in micro-test tubes from where they were transferred to 96-well microtiter plates. Bacterial suspensions grown overnight in double-strength Mueller Hinton broth (MHB, Merck, Germany) were standardized to approximately 10^8 CFU mL^{-1} (using McFarland no. 0.5). Each bacterial suspension (100 μL) was then added to each well. The last row containing only the serial dilutions of antimicrobial agent without microorganism was used as negative control. Sterile distilled water and medium served as a positive growth control. After incubation at 37 °C for 24 h, the first well free from turbidity was determined as the minimal inhibitory concentration (MIC). Chloramphenicol (Sigma, Germany) was used as standard antibacterial agent.

Agar Diffusion Method. An agar diffusion method (*23*) was used for screening the antifungal activity of the essential oils and the pure compounds menthol (**M1**) and menthone (**M2**). A stock solution of each oil was prepared in DMSO. Dilution series were prepared in the same way as used in the micro-dilution method described above.

Spore solutions were prepared (10^8 spore mL^{-1}) using sterile 10% Tween-80 (Aldrich, Germany) which was applied for collecting spores from plate. A Thoma counting chamber (Hawskley, U. K.) was used to determine the number of spores. An aliquot of 25 mL of SDA was poured into the plates. The spore solution (1 mL) was transferred and spread with a Drigalsky spatula on the surface of SDA plates. Using a sterile cork-borer, 9-mm-diameter wells were punched on the medium. Each well was filled with 50 μL of the oil dilutions. Plates were incubated at 28 °C for 24−48 h. Ketoconazole (Sigma, Germany) was used as standard antifungal agent.

Bioautography Method. For determining the active constituent a bioautography technique was used (*24*). The samples were subjected to TLC, and following the separation the inoculated medium was applied onto the developed TLC plate as described previously.

Table 1. Major Components of *Mentha piperita* Essential Oils[a] Identified by GC and GC/MS

	compound	**A** (%)	**B** (%)	**C** (%)	**D** (%)
1	limonene	1.9	1.3	1.0	2.1
2	1,8-cineole	5.3	4.0	3.4	4.0
3	menthone	27.9	21.2	27.2	18.4
4	menthofuran	5.5	3.8	1.3	3.2
5	isomenthone	3.5	2.9	3.8	2.9
6	linalool	2.5	4.4	4.1	4.8
7	β-caryophyllene	4.2	1.8	1.6	1.5
8	terpinen-4-ol	1.2	3.4	3.8	2.1
9	menthol	27.5	42.3	39.9	34.6
10	pulegone	6.4	1.0	1.9	14.4
11	α-terpineol/borneol	2.4	1.6	1.7	0.7

[a] **A**, *M. piperita* oil (from Turkey); **B**, *M. piperita* oil (Jet-Farms, Yakima, WA); **C**, *M. piperita* oil (Mari-Linn Farms, OR); **D**, *M. piperita* oil (Erdoğmuş Perfume Ind., imported from India).

Thin-Layer Chromatography. Precoated silica gel 60 F 254 (0.2 mm) (Reidel de Haën, Germany) plates, cut to appropriate size, on aluminum supports were used. The peppermint oils, **M1**, and **M2** were applied (1 μL) using Drummond micro-capillaries (Aldrich, Germany) onto two TLC plates and developed (90:10 v/v, *n*-hexane/ethyl acetate). In one plate, the separated compounds were visualized with UV light (365 and 254 nm) and sprayed with anisaldehyde/H_2SO_4 spray reagent followed by heating to 110 °C. The other plate was used for the bioautography assay.

Determination of the Activity. Nutrient agar (15 mL, Difco, MI) was poured onto the plate (12-cm diameter) for the formation of an agar base. One of the developed TLC plates was then placed on the agar base. *Candida albicans* was previously incubated at 37 °C for 8 h in MHB. The optical density at 600 nm (OD_{600}) of the *C. albicans* culture was measured with a UV-spectrophotometer (OD_{600} = 1 corresponds to approximately 10^7 cells/mL). Sterilized Mueller Hinton broth in addition of agar (7.5%) was used in molten form and kept at 45 °C in a water bath. Pre-grown *C. albicans* was transferred into the molten agar to obtain a final concentration of 10^5 cells/mL. Finally, the inoculated medium was poured onto TLC plates and incubated at 37 °C for 24 h. The plate was sprayed using a 1% (w/v, EtOH) tetrazolium violet (2,5-diphenyl-3-[α-naphthyl] tetrazolium chloride, Sigma, Germany) reagent and incubated at 37 °C for 1 h. Inhibition zones were visualized against the colored background.

RESULTS AND DISCUSSION

Four peppermint oils obtained from various sources were analyzed by GC and GC/MS (**Table 1**) to determine their main constituents, and evaluated for their antimicrobial properties against 21 human and plant pathogenic microorganisms using different bioassays in combination (**Table 2**).

The compositions and relative percentages of the peppermint oils (**A**−**D**) were elucidated by the aid of GC and GC/MS analyses. As a result, 12 major compounds were identified. The samples contained menthol (28−42%) and menthone (18−28%) as main constituents. Sample **D** was found to contain the lowest amounts of menthone, whereas sample **B** was found to contain the highest menthol content (**Table 1**).

The antibacterial activity of the peppermint oils, major components menthol and menthone, against the standard antimicrobial agent Chloramphenicol are shown cumulatively in **Table 2**. Microdilution broth susceptibility assay (*22*) was used for this purpose. The essential oils, menthol, and menthone (**M1** and **M2**) showed moderate inhibitory effects against human pathogenic microorganisms. *Staphylococcus aureus* was inhibited by oils **A** and **B** with a MIC value of 0.625 mg mL^{-1}. The pathogen *Listeria monocytogenes* was inhibited by oils **A**, **B**,

Table 2. Antimicrobial Activity Results[a] (MIC, mg mL^{-1})

microorganism	A	B	C	D	M1	M2	ST
Escherichia coli	2.5	1.25	1.25	1.25	1.25	5.0	0.0625[b]
Staphylococcus aureus	0.625	0.625	1.25	2.5	0.625	2.5	0.00781[b]
Pseudomonas aeruginosa	2.5	2.5	5.0	2.5	2.5	5.0	0.25[b]
Enterobacter aerogenes	1.25	1.25	1.25	2.5	1.25	5.0	0.125[b]
Proteus vulgaris	2.5	5.0	1.25	1.25	1.25	2.5	0.03125[b]
Salmonella typhimurium	1.25	1.25	1.25	2.5	0.625	5.0	0.0625[b]
Klebsiella pneumoniae	2.5	2.5	2.5	2.5	2.5	5.0	0.0039[b]
Yersinia enterocolitica	2.5	2.5	2.5	2.5	2.5	2.5	0.0039[b]
Listeria monocytogenes	0.156	0.625	2.5	0.312	0.625	1.25	0.00195[b]
Bacillus cereus	1.25	1.25	1.25	1.25	1.25	1.25	0.03125[b]
Staphylococcus epidermidis	0.625	2.5	0.625	1.25	0.625	0.625	0.00781[b]
Xanthomonas campestris pv. phaseoli	0.625	0.312	0.625	0.156	0.625	2.5	0.03125[b]
Pseudomonas syringae pv. phaseolicola	2.5	1.25	1.25	0.625	1.25	2.5	0.00391[b]
Pseudomonas syringae pv. tomato	0.07	0.07	0.625	0.312	0.07	1.25	0.03125[b]
Pseudomonas syringae pv. syringae	0.312	0.156	0.312	0.312	0.156	2.5	0.00781[b]
Xanthomonas campestris pv. campestris	0.156	0.156	0.312	0.156	0.156	1.25	0.00781[b]
Candida albicans	0.625	0.625	0.312	0.625	0.625	2.5	0.125[c]

[a] **A**, *M. piperita* oil (from Turkey); **B**, *M. piperita* oil (Jet-Farms, Yakima, WA); **C**, *M. piperita* oil (Mari-Linn Farms, OR); **D**, *M. piperita* oil (Erdoğmuş Perfume Ind., imported from India); **M1**, (–)-menthol (Polarome, Jersey City, NJ); **M2**, (–)-menthone (Fluka, Germany); **ST**, standard antimicrobial agent. [b] Chloramphenicol. [c] Ketoconazole.

and **D** with strong inhibition (MIC 0.156−0.625 mg mL^{-1}). *Staphylococcus epidermidis* was also inhibited with moderate activity having MIC values of 0.625−2.5 mg mL^{-1}, lower than that of the standard antimicrobial agent. However, all peppermint oils showed stronger inhibition (MIC 0.07−1.25 mg mL^{-1}) with the exception of sample **A,** against the following plant pathogens: *Pseudomonas syringae* pv. *tomato*, *P. syringae* pv. *syringae*, *P. syringae* pv. *phaseolicola*, *Xanthomonas campestris* pv. *campestris*, and *X. campestris* pv. *phaseoli*. These results were interestingly correlated with the menthol and menthone percentages. This finding was later confirmed by the bioautography assay.

Antifungal properties (*23*) of the *M. piperita* oils were also investigated against the standard antifungal agent Ketoconazole. All the oils and pure compounds showed very weak antifungal activity at a concentration of 5 mg mL^{-1}, which suggested strong resistance of the investigated pathogenic fungi.

The bioautography assay (*24*) was applied to all peppermint oils and pure compounds using *Candida albicans*. This experiment showed clear inhibition zones corresponding to menthol after separation on the TLC plate. Pure menthol was then subjected to the microdilution broth susceptibility assay to determine the MIC value (0.625 mg mL^{-1}) for comparison with those of the used peppermint oils (0.312−0.625 mg mL^{-1}). This showed that menthone was less active (2.5 mg mL^{-1}) than menthol against *C. albicans*, as seen in **Table 2**.

In recent years, a large of number of investigations have been performed on the antimicrobial activities of essential oils. Antimicrobial evaluations of essential oils are difficult because of their volatility, insolubility in water, and complex chemistry. Some factors are important when testing oils such as the assay technique, growth medium, the microorganism, and the oil itself (*8*, *9*, *23*, *25*−*29*).

Previous investigations on *M. piperita* oil composition are consistent with our results in which menthol and menthone were found to be the major compounds (*14*−*21*). Antimicrobial activities of Peppermint oils have also been previously investigated by different groups (*7*, *8*, *25*, *27*−*32*). The main compound menthol showed weak activity at 5.0 μL per disc against *Salmonella typhimurium* and *Rhizobium leguminosarum* using the agar plate method (*30*). Menthol was previously shown as active against *Clostridium sporogenes*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella pullorum*, *Staphylococcus aureus*, *Streptococcus faecalis*, and *Comamonas terrigena*. Menthol had inhibited those microorganisms at a concentration of 10 μL per plate using the agar plate method (*33*). Against the pathogen *Streptococcus mutans*, by using the broth technique, menthol had shown weak activity at 400 μg mL^{-1} (MIC value) (*34*). In another study, it was reported that *Fusarium sambucinium* was inhibited by menthol at a concentration of 1% (*35*).

In our study, the agar diffusion technique (*23*) was first employed to detect the activity and the associated range, whereas the micro-dilution technique (*22*) enabled the determination of the MIC values of the corresponding peppermint oils (**A**−**D**), **M1**, and **M2**. As for the bioautography technique, we believe that it can be considered as an efficient assay for the detection of antimicrobial fractions or compounds in essential oils and other plant extracts or similar mixtures, especially if high performance TLC is applied. The method allows localization of the activity even in a complex matrix and therefore permits a target-directed isolation of the active constituents (*8*, *24*, *36*).

Another conclusion resulting from these observations was that other terpenes in the essential oils may display some synergistic effects. Menthol seems to be the major constituent responsible for bioactivity within the tested major pure compounds supported by the bioautography assay.

In conclusion, it can be suggested that the use of agar diffusion, microdilution, and bioautography bioassays in combination gives information about the bioactivity itself, the MIC value, and hence these techniques in combination can be used to investigate bioactive constituents in natural products such as essential oils. As shown in this study, the agar diffusion method indicated a weak antifungal activity of *M. piperita* oil and menthol for the first time. Furthermore, antibacterial activity, especially against various plant pathogenic microorganisms, has also been reported here for the first time.

The results suggest further in vivo investigations on animal or human experimental models. We believe that it is also worthwhile to investigate different essential oils using these combined assay techniques. Another important issue is to evaluate all available menthol and menthone enantiomers in similar studies to investigate the effects of chirality on their biological activity.

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