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In vitro antifungal effects of the essential oil of *Mentha piperita* L. and its comparison with synthetic menthol on *Aspergillus niger*

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The antifungal activity of *Mentha piperita* L. essential oil and its comparison with synthetic menthol on *Aspergillus niger* growth have been determined *in vitro*. The chemical compositions of essential oil of *M. piperita* provided from the aerial parts of plants grown in a village in Kerman Province in June 2012 were determined. The sample was cleaned and then dried in the shade. Essential oil was made by hydro-distillation method and analyzed by capillary gas chromatography (GC) using flame ionization (FID) and capillary gas chromatography coupled mass spectrometry (GC/MS). The main oil content from the plants of *M. piperita* was 3.26% (v/w). Twenty three (23) compounds were identified in the essential oil of *M. piperita, making* 96.25% of the total oil. The major components were menthol (38.33%), menthone (21.45%) and menthyl acetate (12.49%). For study of antifungal activity, the essential oil was tested against *A. niger* (strain PTCC = 5223) by disc diffusion method via average inhibition zone. The results showed that essential oil from *M. piperita* at 1 and 1/2 oil dilutions exhibited a strong antifungal activity than gentamycin (8 mg/ml) antibiotic on *A. niger* and exhibited a strong synthetic menthol at 10% dilution. The relative high amount of menthol and menthone in the *M. piperita* essential oil showed that they could display antifungal activity.

Key words: *Mentha piperita* L., *Aspergillus niger*, menthol, antifungal activity.

INTRODUCTION

Pathogenic fungi cause diseases in humans or vegetable organisms. Aerosolized *Aspergillus* spores are found nearly everywhere, so we are routinely and almost constantly exposed to them. Such exposure is a normal part of human condition and generally poses no adverse health effects. Nevertheless, *Aspergillus* can and does cause disease in three major ways: through the production of mycotoxins, through induction of allergenic responses and through localized or systemic infections. With the latter two categories, the immune status of the host is pivotal. Allergies and asthma are thought to be caused by an active host immune response against the presence of fungal spores or hyphae. In contrast, with invasive aspergillosis, the immune system has collapsed and little or no defence can be mounted (Machida and Gomi, 2010). *Aspergillus niger* is a haploid filamentous fungi and is a very essential microorganism in the field of biology.

In addition to producing extracellular enzymes and citric acid, *A. niger* is used for waste management and biotransformations. The fungus is most commonly found in mesophilic environments such as decaying vegetation or

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soil and plants (Schuster et al., 2002). Some strains of *A. niger* have been reported to produce ochratoxins (Abarca et al., 1994) but other sources disagree, claiming this report is based upon misidentification of the fungal species. Recent evidence suggests some true *A. niger* strains do produce ochratoxin A (Schuster et al., 2002). It also produces the isoflavone orobol. *A. niger* causes black mold of onions. Infection of onion seedlings by *A. niger* can become systemic, manifesting only when conditions are conducive. *A. niger* causes a common postharvest disease of onions, in which the black conidia can be observed between the scales of the bulb. The fungus also causes disease in peanuts and in grapes. *A. niger* is less likely to cause human disease than some other *Aspergillus* species, but, if large amounts of spores are inhaled, a serious lung disease can occur.

Peppermint (*Mentha piperita* L.) belongs to *Labiateae* family and originated from Mediterranean Regions. It is widely cultivated in the world and is a hybrid mint, a cross between water mint and spearmint (Frampton, 2009). The plant, indigenous to Europe, is now widespread in cultivation throughout all regions of the world. It is found wild occasionally with its parent species. Peppermint was first described in 1753 by Carolus Linnaeus from speci mens that had been collected in England; he treated it as a species (Linnaeus, 1753), but it is now universally agreed to be a hybrid (Harley, 1975). It is an herbaceous rhizomatous perennial plant growing 30-90 cm (12-35 in) tall, with smooth stems, and square in cross section. The rhizomes are wide-spreading, fleshy, and bare fibrous roots. The leaves are from 4-9 cm (1.6-3.5 in) long and 1.5-4 cm (0.59-1.6 in) cm broad; are dark green with reddish veins, and with an acute apex and coarsely toothed margins. The leaves and stems are usually slightly hairy. The flowers are purple, 6-8 mm (0.24-0.31 in) long, with a four-lobed corolla about 5 mm (0.20 in) diameter; they are produced in whorls (verticillasters) around the stem, forming thick, blunt spikes. Flowering is from mid to late summer. The chromosome number is variable, with 2n counts of 66, 72, 84, and 120 recorded (Huxley, 1992).

Peppermint typically occurs in moist habitats, including stream sides and drainage ditches. Being a hybrid, it is usually sterile; it produces no seeds and reproduces only vegetation, spreading by its rhizomes. If placed, it can grow anywhere, with a few exceptions. Peppermint generally grows best in moist, shaded locations, and expands by underground stolons. Young shoots are taken from old stocks and dibbled into the ground about 1.5 feet apart. They grow quickly and cover the ground with runners if it is permanently moist. For the home gardener, it is often grown in containers to restrict rapid spreading. It grows best with a good supply of water, without being water-logged, and planted in areas with part-sun to shade. The leaves and flowering tops are used; they are collected as soon as the flowers begin to open and can be dried. The wild type of the plant is less suitable for this purpose, with cultivated plants having being selected for more and better oil content. They may be allowed to lie and wilt a little before distillation, or they may be taken directly to the still. Essential oils of peppermint are used in flavors, fragrances, and pharmaceuticals. Peppermint has a long tradition of medicinal use, with archaeological evidence placing its use at least as far back as ten thousand years ago. Peppermint has a high menthol content, and is often used in tea and for flavouring ice cream, confectionery, chewing gum and toothpaste. The oil also contains menthone and menthyl esters, particularly menthyl acetate. Dried peppermint typically has 0.3-0.4% of volatile oil containing menthol (7-48%), menthone (20-46%), menthyl acetate (3-10%), menthofuran (1-17%) and 1,8-cineol (3-6%). Peppermint oil also contains small amounts of many additional compounds including limonene, pulegone, eucalyptol, caryophyllene and pinene (Leung, 1980). It is the oldest and most popular flavour of mint-flavoured confectionery. Peppermint can also be found in some shampoos, soaps and skin care products. Menthol activates cold-sensitive TRPM8 receptors in the skin and mucosal tissues, and is the primary source of the cooling sensation that follows the topical application of peppermint oil (Eccles, 1994). Peppermint oil has a high concentration of natural pesticides, mainly polygone and menthone (Krieger, 2001). Mint essential oils are generally used externally for antipruritic, astringent, rubefacient, antiseptic, and antimicrobial purposes, and for treating neuralgia, myalgia, headaches, and migraines (Hendriks, 1998).

The well-known and widely used peppermint is a cultivated natural hybrid of *M. aquatica* L. (water mint) and *M. spicata* L. (spearmint). Although a native genus of the Mediterranean Regions, 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 (Foster, 1990). Menthol is an organic compound made synthetically or obtained from peppermint or other mint oils. It is a waxy, crystalline substance, clear or white in color, which is solid at room temperature and melts slightly above. The main form of menthol occurring in nature is (−)-menthol, which is assigned the (1R,2S,5R) configuration. Menthol has local anesthetic and counterirritant qualities, and it is widely used to relieve minor throat irritation. Menthol also acts as a weak kappa opioid receptor agonist. Natural menthol exists as one pure stereoisomer, which is nearly always the (*1R,2S,5R*) form. In the natural compound, the isopropyl group is in the *trans* orientation to both the methyl and hydroxyl groups. Menthol's ability to chemically trigger the cold-sensitive TRPM8 receptors in the skin is responsible for the well-known cooling sensation it provokes when inhaled, eaten, or applied to the skin (Eccles, 1994). In this sense, it is similar to capsaicin, the chemical responsible for the spiciness of hot peppers (which stimu-

lates heat sensors, also without causing an actual change in temperature). Menthol's analgesic properties are mediated through a selective activation of κ-opioid receptors (Galeottia et al., 2002). Menthol also blocks voltage-sensitive sodium channels, reducing neural activity that may stimulate muscles (Haeseler et al., 2002). Menthol also enhances the efficacy of ibuprofen in topical applications via vasodilation, which reduces skin barrier function (Braina et al., 2006). *M. arvensis* is the primary species of mint used to make natural menthol crystals and natural menthol flakes. This species is primarily grown in the Uttar Pradesh region in India. (−)-Menthol (also called *l*-menthol or (*1R,2S,5R*)-menthol) occurs naturally in peppermint oil (along with a little menthone, the ester menthyl acetate and other compounds), obtained from *Mentha x piperita*. Japanese menthol also contains a small percentage of the 1-epimer, (+)-neomenthol. Biosynthesis of menthol was investigated in *M. x piperita*, and all enzymes involved in its biosynthesis have been identified and characterized (Croteau et al., 2005).

M. piperita has been shown to possess strong antifungal activity, even when compared to synthetic fungicides. Peppermint oil showed antifungal activity against *A. niger*, *Alternaria alternata* and *Fusarium* sp. by agar well diffusion method (Aqil *et al*, 2000). The chemical responsible for this action was menthone (Soković et al., 2009). As with many widely used natural products, the demand for menthol greatly exceeds the supply from natural sources. In organic chemistry, menthol is used as a chiral auxiliary in asymmetric synthesis. For example, sulfinate esters made from sulfinyl chlorides and menthol can be used to make enantiomerically pure sulfoxides by reaction with organolithium reagents or Grignard reagents. Menthol reacts with chiral carboxylic acids to give diastereomic menthyl esters, which are useful for chiral resolution. This study evaluated and identified the chemical compounds of *M. piperita* mainly. Also, antifungal activity of *M. piperita* has been compared with synthetic menthol and gentamicin (8 mg/ml) antibiotic standard on culture of *A. niger* (strain PTCC=5223).

MATERIALS AND METHODS

Plant material collection and extration of essential oil

The aerial parts of peppermint (*M. piperita*) were obtained from this plant grown in a village in Kerman province (Iran) at full flowering stage in June 2012. The sample was cleaned in shade condition to prevent volatility of the plant material constituents and to keep the natural color of the sample fixed. Then they were air-dried and powdered using a milling machine and kept in a cool dry place until ready for extraction of the essential oil. Afterwards, essential oil was taken from 150 g of the powdered sample in hydrodistillation method with the help of Clevenger set for three hours. The sample oils were dried with anhydrous sodium sulfate and kept in sterile sample tubes in refrigerator. The oil yield from aerial parts of peppermint plant was calculated.

Analysis of essential oil

Gas chromatography

GC analysis was performed using a model HP-439 gas chromatograph equipped with column CP Sil, whose 5CB is 25 m length; internal diameter, 0.25 mm and film thickness, 0.39 µm. Oven temperature was from 60 to 220°C at a rate of 7°C slope per minute. Injector temperature was 280°C, detector (FID) temperature was 270°C and carrier gas was helium.

Gas chromatography/mass mass spectrometry

In order to analyze and identify the combinations forming the essential oil, the chromatograph gas set attached to a mass spectrometry, Model Hewlett Packard-5973 was used. The conditions of analysis and specifications of the GC/MC set were as follows: Capillary column HP 5MS, 60 m length; internal diameter, 0.25 mm; layer thickness, 0.25 µm; thermal program of oven (3 min), 60°C to 220°C with a 6°C slope per minute; and then 220°C in 3 min; the temperature of place of injection was 280°C; carrier gas was helium; the speed of gas was 1.0 milliliter per minute; the ratio of fission, 1 to 43; the rate of injection, 0.1 µl; temperature of the reservoir of ionization, 230°C; ionization mode EI, Ionization energy, 70eV. The series of normal Alkans C8-C17 was also injected to the set under the same condition with that of essential oil injection to calculate restrictive index (RI) of components of essential oil. The Restrictive Index of components of the sample was calculated by using a computerized program. Finally, the components of essential oil were identified by comparing the mass spectrums obtained with the existing standard mass spectrums at electronic library of Wiley 2000 existing in Absolution software of GC/Ms set. Calculation of standard restrictive index was done in accordance to C8-C17 Alkans and compared with the existing standard figurers in references (Adams, 2001).

Assessment of antifungal action

The solvent showing no antifungal activity from DMSO was selected as a diluting medium for the oil. Undiluted oil was taken as dilution 1, 1/2, 1/4, 1/8 and 1/16 dilutions of the oil were made DMSO. For antifungal activity (50 µl) of each dilution was used. The antifungal activity of the essential oil was evaluated by disc diffusion method using Mueller Hinton Agar (Baron and Finegold, 1995) and determination of inhibition zones at different oil dilutions against *Aspergillus niger* (PTCC=5223). The fungal strains under experiment were obtained from the Center for Fungi and Bacteria of Iranian Scientific and Industrial Researches Organization. The antifungal property of the oil was tested by agar well diffusion method using Sabouraud Dextrose Agar (SDA). Standard reference antibiotic was used in order to control the sensitivity of the tested fungi (gentamicin 8 mg/ml). The incubation condition used was 48 to 72 h at 24°C for fungi. All the experiments were carried out in triplicate and averages were calculated for the inhibition zone diameters.

RESULTS

The study of the analysis of peppermint essential oil under investigation showed that the output of essential oil is 3.26% (v/w). The combination of essential oil, restrictive index (RI), and quantitative percentage of the compounds were identified. Thirty three (33) compounds identified in the

Table 1. Combinations identified in the essential oil of *Mentha piperita* L.

Compound name	Restrictive index (RI)	Percentage (%)
α-Pinene	937	0.65
Sabinene	975	2.23
β-Pinene	982	1.22
Myrcene	992	0.76
3-Octanol	996	0.12
α-Terpinene	1024	0.54
P-Cymene	1028	0.17
Limonene	1034	5.33
1,8-Cineole	1039	3.27
(*E*)-β-Ocimene	1045	0.59
γ-Terpinene	1068	0.45
Terpinolene	1084	0.21
Iso-menthone	1092	2.87
Linalool	1126	0.36
Menthone	1148	21.45
Menthyl acetate	1156	12.49
Menthol	1171	38.33
α-Terpineol	1203	0.57
Pulegone	1238	1.34
Piperitone	1310	0.68
β-Caryophyllene	1415	1.23
Germacrene D	1482	0.58
γ-Cadinene	1521	0.81
Total		96.25

in the essential oil of this plant were 96.25%. The combinations of menthol (38.33%), menthone (21.45%) and menthyl acetate (12.49%) with 72.27% constitute the highest percentage of essential oil (Table 1). The indexes of restrictive have been calculated by injecting the mixture of normal hydrocarbons (C8-C17) to HP-5MS column

The results of studying the antifungal impacts of *M. piperita* essential oil show that the oil of this plant has an inhibitory effect in 1, 1/2, 1/4, 1/8 and 1/16 dilutions with average diameter growth of 26, 21, 16, 12 and 8 mm respectively. The results with standard antibiotic gentamicin (8 mg/ml) with a diameter of 18 mm had inhibitory effects. Synthetic menthol in 1 % dilution had moderrate inhibitory (14 mm) effect on *Aspergillus niger* growth, but at 10 % dilution it had a strong inhibitory (20 mm) effect on fungi growth.

The results show essential oil from *Mentha piperita* at 1 and 1/2 oil dilutions exhibited strong antifungal activity than gentamycin (8 mg/ml) antibiotic on *Aspergillus niger* and exhibited strong menthol at 10% dilution. The high percentages of antifungal activities of *Mentha* oil are related with menthol, the main organic compound (Table 2).

DISCUSSION

Essential oils are natural compounds, which have extensive applications in perfumery, food and pharmaceutical industries. Essential oils of *Mentha* species possess great antibacterial and antifungal potential and could be used as natural preservatives and fungicides. In this study, the chemical composition and antifungal effects of *M. piperita* are compared with that done by other researchers.

Comparing our results with other researchers, the essential oil of *M. piperita* has been studied in Iran and in the World. In a research, the chemical compositions and antibacterial and antifungal activity of essential oils from 4 medicinal plants consist of *M. piperita*, *M. spicata*, *Anethum graveolens* and *Foeniculum vulgare*. Antibacterial and antifungal activity of these oils and their components were assayed against a variety of human pathogenic bacteria. Main components in *M. piperita* oil were menthol, limonene, 1,8-cineole, sabinene, menthyl acetate and menthone, in *M. spicata* oil carvone, menthol, limonene and menthone. *M. piperita* showed strong antibacterial and antifungal activities (Kazemi et al., 2012).

Table 2. The zone diameter of inhibition of antibiotic, mentha oil and synthetic menthol on *aspergillus niger* (mm).

Antibiotic	Dilutions of menthane oil					Synthetic menthol	
Gentamicin (8 mg/ml)	1	1/2	1/4	1/8	1/16	1%	10%
18	26	21	16	12	8	14	20

The analysis of essential oil of *M. piperita* in Iran with GS/MS revealed that main compounds of oil include menthol (19.76%), menthan-3-one (19.31%), mentho-furan + isomenthone (9.12%), 1,8-cineole + beta phellan-dren (8.8%) and mentholacetate (5.63%). Inhibitory effect of essntial oil varied among different fungi. After 48 hours, results showed no significant difference between the growth of fungi at 800 and 1600 ppm as well as between water and alcohol controls; but differences between 200 and 400 ppm were significant.

The results of this study reveal that *M. piperita* oil exhibited a significant antifungal activity (Farshbaf Moghaddam et al., 2004). In a report, the antifungal activities of essential oils and herbal extracts have been demonstrated against a range of filamentous fungi. *In vitro* antifungal activity of a combination of some essential oils extracted from herbs (*Thymus vulgaris*, *Salvia officinalis*, *Eucalyptus globulus* and *Mentha piperita*) against some filamentous fungal strains (*Metrhizium sp.*, *Ophiostoma sp.*, *Trichoderma sp.* And *Penicillium expansum*) was determined. 1,8-cineol (21.37%), thymol (13.86%), camphor (7.92%), α-thujone (7.71%), menthon (6.8%) and menthol (6.2%) were the major constituents. This combination was found to have a wide spectrum of activity against all filamentous fungi examined in this study and may be proposed for control of fungal diseases (Mousavi and Raftos, 2012). In a research, the essential oils of *Origanum vulgare* subsp. *hirtum, Mentha spicata, Lavandula angustifolia*, and *Salvia fruticosa* exhibited antifungal properties against the human pathogens *Malassezia furfur, Trichophyton rubrum*, and *Trichosporon beigelii*. Among the main components of the four oils, carvacrol and thymol exhibited the highest levels of antifungal activity (Adam et al., 1998). Antimicrobial activity of four essential oils from lemon (*Citrus lemon*), mint (*Mentha piperita)*, juniper (*Juniperus communis*) and rosemary (*Rosmarinus officinalis*) was against *Aspergillus niger*, *Fusarium oxysporum*, *Monascus purpureus* and *Penicillium hirsutum* molds.

The results of this study confirm that essential oils from aromatic plants such as lemon, mint, juniper and rosemary possess antifungal activity. The most effective against all tested strains was the mint oil (Ferdes and Ungureanu, 2012). The essential oils of *Mentha arvensis* L. and *Zingiber officinale* R. were screened against *Staphylococcus aureus* Rosenbach, *Enterococcus faecium* Schleifer and Kilpper-Bälz, *Pseudomonas aeruginosa* Migula, *Escherichia coli* Castellani and Chalmers, *Proteus mirabilis* Hauser and yeast *Candida albicans* Berkhout. The oils showed a wide spectrum of antimicrobial activity (Mickienė et al., 2011). Essential oils of peppermint were investigated for their antimicrobial properties against 21 human and plant pathogenic microorganisms. The bioactivity of the oils of 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 microor-ganisms, whereas human pathogens were only mode-rately inhibited.

Using the bioautography assay, menthol was found to be responsible for the antimicrobial activity of these oils (Iscan et al., 2002). The potential antifungal effects of *Thymus vulgaris* L., *Thymus tosevii* L., *Mentha spicata* L., and *Mentha piperita* L. (Labiatae) essential oils and their components against 17 micromycetal food poisoning, plant, animal and human pathogens are presented. In *M. piperita* oil menthol (37.4%), menthyl acetate (17.4%) and menthone (12.7%) were the main components, whereas those of *M. spicata* oil were carvone (69.5%) and menthone (21.9%). *Mentha* sp. showed strong antifungal activities. The commercial fungicide, bifonazole, used as a control, had much lower antifungal activity than the oils and components investigated.

It is concluded that essential oils of *Thymus* and *Mentha* species possess great antifungal potential and could be used as natural preservatives and fungicides (Soković et al., 2009). In a report, essential oils from peppermint (*Mentha* sp.), clove (*Syzgium aromaticum*) and eucalyptus (*Eucalyptus globus*) were evaluated for their antifungal activity against soil-borne fungi, including *Aspergillus niger, Alternaria alternata* and *Fusarium chlamydosporum* by agar well diffusion method. Maximum antifungal activity was detected in essential oil of clove oil followed by those of peppermint and eucalyptus. These observations indicate that these essential oils can be exploited as antifungal agents in the management of plant infectious diseases and post-harvest spoilage of crops (Aqil et al., 2000). In this study, the antifungal activity of essential oils of selected plant species, viz. *Piper nigrum* Linn., *Ricinus communis* Linn., *Cedrus deodara* (Roxb.) Loud., *Syzygium aromaticum* (Linn.) Merrill & Perry, *Eucalyptus globulus* Labill., *Citrus aurantium* Linn., C. *limon* (Linn.) Burm. f., *Olea europaea* Linn. and *Mentha piperita* Linn. were assayed for fungi toxicity against two genus, viz. *Aspergillus niger* and *Geotrichum candidum*. The highest and broadest activity was shown by the essential oils of *S. aromaticum*, *C.*

limon, C. aurantium and *M. piperita* as compared to standard drug, Ketoconazole. The 5 ppm concentration of essential oils of *S. aromaticum, C. limon* and *M. piperita* completely inhibited the mycelial growth of *A. niger* and *G. candidum* to the same extent as 5 ppm of Ketoconazole (Verma et al., 2011). In a study, menthol was found to be the active responsible for the antifungal effect (Edris, 2003). Essential oil samples of *Mentha x piperita* L. (peppermint) were analysed by GC/MS and assayed for their antibacterial, antifungal and antioxidant activities.

The oil samples from spring planted crops had a significantly higher menthol and lower terpinen-4-ol concentrations than those from autumn planted crops. The oil samples showed a different degree of inhibition against the twenty-five microorganisms tested. Peppermint oil exhibited a marked antifungal activity against *Aspergillus niger* (Marotti et al., 1994). *In vitro* antimicrobial activity of essential oils and the mechanisms of essential oils action on microorganisms are reported. This research gives an overview on the susceptibility of human and food-borne bacteria and fungi towards different essential oils and their constituents. Essential oils of spices and herbs (thyme, origanum, mint, cinnamon, salvia and clove) were found to possess the strongest antimicrobial properties among many tested (Kalemba and Kunicka, 2003). In a report, the effect of mint (*Mentha piperita*) essential oil (0·5, 1·0, 1·5 and 2·0%, v/w) on *Salmonella enteritidis* and *Listeria monocytogenes* in a culture medium and three model foods was studied. Mint essential oil antibacterial action depended mainly on its concentration, food pH, composition, storage temperature and the nature of the micro-organism (Tassou et al., 1995).

Conclusion

The peppermint oil recommended for large scale application is based on its strong antifungal as well as anti- *Aspergillus niger* efficacy. In this study, we find out that the antifungal effects of *Mentha piperita* essential oil under investigation compared to synthetic menthol on *Aspergillus niger* exhibited strong synthetic menthol. The high percentage of antifungal activities of *Mentha* oil is related to menthol, the main organic compound . This essential oil can be used for antifungal activity and as natural compound. The effectiveness of the oil concentration depends on the target pathogen and effects of natural compounds on fungus.

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