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ANTIFUNGAL ACTIVITY OF *Mentha × piperita* L. ESSENTIAL OIL

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ABSTRACT

The objective of this study was to investigate the chemical composition and in vitro antifungal activity of *Mentha × Piperita* L. (peppermint) essential oil (EO) against some plant pathogenic fungi (*Alternaria alternata*, *Penicillium expansum*, *Rhizoctonia solani*, and *Rhizopus stolonifer*). Antifungal activity of EO against the selected fungi was conducted using the agar diffusion method by adding peppermint EO concentrations (0, 250, 500, 750, 1000, and 2000 ppm). The gas chromatography-mass spectrometry (GC-MS) analysis of peppermint EO showed that the main constituent was menthol (36.4%), followed by menthone (27.7%) and menthyl acetate (11.2%). The mycelium growth of the selected fungi was significantly inhibited by peppermint EO. Light and electron microscopy studies showed that mycelium morphology was seriously changed after treatment with peppermint essential oil. The level of malondialdehyde illustrated that peppermint EO led to lipid peroxidation in the fungal pathogens. Therefore, due to its antifungal properties, peppermint EO can be used as an additive in the food industry and as an active substance in pharmaceuticals.

Key words: antifungal activity, essential oil, mycelium growth, morphology, peppermint

INTRODUCTION

Fungal rotting almostly caused by saprophytic species and plant parasites such as *Rhizopus* species, *Rhizoctonia* species, *Alternaria* species, and *Penicillium* species, is a major cause of postharvest disease, which affect the quality, short the shelf-life of fruits and vegetables, as well as during various stages of pathogenesis, some of these fungi may produce different mycotoxins that are toxic to humans and the animals that consume them [Xing et al. 2010]. Therefore, increasing the use of fungicides has become essential to control of plant diseases.

Due to disadvantage of chemical fungicides uses such as the accumulation of harmful substances in the soil, water, and organisms along with the food chains and the emergence of resistant strains of pathogens, there is a challenge to develop safer and ecofriendly alternative strategies for controlling postharvest diseases, which pose less risk to human health and the environment [Souza et al. 2009]. Plants and plant-derived metabolites have served as the starting point for the discovery and development of new alternative approaches [Gakuubi et al. 2017]. Plants are more beneficial than synthetic products, ease of use, treatment efficacy, affordable cost, and minimal side effects [Ullah et al. 2013].

Mentha × Piperita L. (peppermint) belongs to the Lamiaceae family and originated from the Mediterranean regions. It is widely cultivated in the world

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and is a hybrid mint, a cross between water mint and spearmint [Frampton 2009]. The essential oil (EO) also possesses biological activity against numerous organisms, including fungi [França et al. 2018], bacterial [Yadegarinia et al. 2006, Mehani et al. 2015], nematodes [Walker and Melin 1996], and insect pest [Ansari et al. 2000]. The objective of this study was to illustrate the antifungal properties of peppermint EO against four economically important phytopathogenic fungi and the mode of action of peppermint EO on the growth of fungal hyphae.

MATERIALS AND METHODS

Essential oil analysis

The peppermint EO was supplied by Barij Essence Pharmaceutical Company (Kashan, Iran). The compounds of the EO were then identified and quantified by gas chromatography-mass spectrometry (GC-MS). GC-MS spectrometry analysis was carried out in a GC-MS (Finnigan-Thermo Trace DSQ Mass Spectrometer) system equipped with a HP-5MS fused silica column (30 m × 0.25 mm i.d., film thickness 0.32 μm; J and W Scientific) using helium as carrier gas at a linear velocity of 1 ml/min. GC oven temperature was raised from 60 to 220°C at a rate of 3°C/min, the transfer line temperature was 250°C, the split ratio was 1 : 100, electron impact MS was 70 eV.

The compounds of peppermint EO were identified by calculation of their retention indices (RI) under temperature-programmed conditions for n-alkanes (C_8–C_{20}) and the EO on a CP-Sil 8CB column. Then retention indices of compounds were calculated using the equation:

$$I = 100 \times [n + (N - n)\,(\log t_{unknown} - \log t_n) / / (\log t_N - \log t_n)] \quad (1)$$

where *I* is Kovats retention index, *N* and *n* are the number of carbons in the larger and the smaller n-alkanes, respectively. Identification of individual compounds was made by comparison of their mass spectra and retention indices with those authentic samples, computer library (NIST), and those given in the literature [Adams 2007]. Quantification of the relative amount of the individual compounds was performed according to the area percentage method without consideration of the calibration factor. In addition, menthol was used as external standards with calibration curves included in this part.

Fungal organisms and pathogens culture. The isolates of *Rhizopus stolonifer*, *Alternaria alternata*, *Penicillium expansum* and *Rhizoctoria solani* AG4-HG II in this study were obtained from Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad in Iran, which were previously isolated from infected strawberry and tomato plants, respectively. Identification of *A. alternate* and *R. solani* AG4-HG II associated with tomato was previously investigated by Ramezani et al. [2019] and Pourmahdi and Taheri [2015], respectively. Other isolates were morphologically identified by light microscopy at the faculty. These fungi were kept on potato dextrose agar (PDA) medium and lyophilized filter papers for short-term and long-term storage, respectively.

Antifungal activity. The inhibitory effect of the peppermint EO on the mycelial growth of the selected pathogens was determined by the agar diffusion method [Liu et al. 2017]. The PDA (Zona Industriale 64026, Roseto degli Abruzzi, Italy) was autoclaved and cooled to 40–45°C. A stock solution of the peppermint EO was prepared in 5% Tween 20 (as an emulsifier). Then, the peppermint EO at different concentrations (0, 250, 500, 750, 1000, and 2000 ppm) was added in the middle of still liquid culture (PDA) followed by 5 min. of stirring in order to homogenize the medium PDA with EO. After shaking the vials, the approximately 20 mL of mixture (PDA + EO + Tween 20) is poured into 10 mm-diameter Petri dishes. The 5 mm-diameter mycelial disks taken from a 3-day-old culture of the selected pathogens were placed in the center of each Petri dish and incubated in the dark at a controlled temperature condition of 25 ±3°C. The control were prepared similarly by inoculating fresh medium with 5% Tween 20. Mycelial growth was then assayed to measure the diameter of the colonies using the cross method every day for up to 15 days. Inhibition percentage (IP) was calculated according to the equation:

$$IP = [(dc - dt) / dc] \times 100 \quad (2)$$

where dc and dt are the radial growth of the control and the treats, respectively. Blanks were run in the

same manner without the EO. Antimicrobial testing was performed in triplicate.

Assessment of the effect of peppermint oil on fungal morphology. The study of morphological changes of fungal hyphae before and after treatment with peppermint EO was performed by scanning electron microscopy (SEM) and light microscopy (LM). Morphological variations were then observed concerning the control and treatments.

In LM study, a sample of mycelium (2 mm diameter) was taken at the periphery of the colony that had grown on the PDA media with 0, 250, 500, 750, 1000, and 2000 ppm concentrations of peppermint EO after 3 days of incubation. The sample was mounted in distilled water and examined with a BX41 microscope (Olympus, Japan). Photographs were taken with the help of computer attached DP12 Olympus Digital color Camera, and SEM observations for morphological changes studies of fungal hyphae were carried out following a previously published method, with some modifications [He et al. 2011, Liu et al. 2017]. The 3 mm diameter mycelial slices obtained from 3-day culture of fungi were inoculated in Petri dishes containing 20 mm PDA with 0 and 1000 ppm concentrations of essential oil. After 4 days, mycelial slices (3 mm diameter) were taken from the margin of each culture and fixed in 2.5% glutaraldehyde in phosphate-buffered saline for 12 h at 4°C. The fixed pieces were washed thrice with sterile distilled water for more than 20 min. The pieces were then dehydrated through 2 rounds of serial dehydration in 30%, 50%, 70%, 90%, 95%, and 100% 10 min. for each alcohol dilution and 30 min. in 100% ethanol. The pieces then were coated with gold-palladium using an ion sputter-cutter (SC7620) and observed by SEM (LEO 1450 VP, Germany). This experiment was performed in triplicate.

Measurement of electrical conductivity. Assessment of cellular leakage from the fungal tissues due to peppermint EO was determined by measuring the electrical conductivity values of the incubation medium with a conductivity meter cc-511-ELMETRON [Liu et al. 2017]. The suspensions were inoculated into 100 mL potato dextrose broth (PDB) medium to a final concentration of 5×10^5 spores/mL. The inoculated PDB medium was incubated on an incubator shaker at 100 rpm for 3 days at 25 ±3°C. The mycelia were then harvested and washed with sterile distilled water. The mycelia (1 g wet weight: 10 mL sterile distilled water) were then suspended in sterile distilled water containing peppermint EO at 0, 250, 500, 750, 1000, and 2000 ppm, incubated with shaking (100 rpm) at 25 ±3°C, and subjected to conductivity measurement at 0, 1, 2, 3, 4, 5, 6, 7, 9 and 11 hours (h). As different concentrations of peppermint EO have their background conductivity, the results are expressed as changes in conductivity from the earliest measurement. This experiment was performed in triplicate.

Lipid peroxidation. Lipid peroxidation of the fungi phytopathogens plasma membranes was tested by measuring the production of malondialdehyde (MDA) using the thiobarbituric acid (TBA) method as previously described [Liu et al. 2017]. The spore suspension was inoculated into PDB medium on a rotary incubator at 100 rpm for 3 days at 25 ±3°C as described above. Peppermint EO was then added to the PDB medium to a final concentration of 0, 250, 500, 750, 1000, and 2000 ppm. The samples were mixed and incubated for 12 h under the above incubation conditions. The mycelia were then harvested for measuring the MDA content. This experiment was performed in triplicate.

Statistical analysis. The study was designed with a single factor (the effect of concentration (as a factor) on inhibition percentage, mycelium growth, and the MDA production (as dependent variables)) and multiple factors (the effects of concentration and time (hour; as factors) as well as concentration-by-hour interaction on electrical conductivity (as a dependent variable)) in a completely randomized arrangement. The normality of distribution of the trait was tested using Shapiro and Wilk's normality test except for electrical conductivity, which its mean results were compared using Duncan's multiple range test with probability level <0.05. The data were statistically analyzed with single-factor ANOVA and a general linear model in Statistical Analysis System SPSS 24.0 software. When critical differences were noted, mean results were compared using Tukey's test with probability level <0.05. Besides, graphs were prepared using Microsoft Excel 2013. It was used the commercially available EndNoteX8 software to manage references. The means and standard deviation for each concentration were calculated.

RESULTS

Chemical compositions of peppermint EO. The results of the analysis by GC-MS of the chemical composition of the EO are presented in Table 1. A total of 15 compounds were identified, corresponding to a percentage of 98.01% of all the isolated components. Among these, 2 monoterpene hydrocarbons (0.11%), 11 oxygenated monoterpenes (94.8%), 1 sesquiterpene hydrocarbon (2.1%), and 1 oxygenated sesquiterpene (1%) were included. The main 5 compounds were characterized in the EO, making up 86.7% of the oil, in compounds percentage (>5%). These contain menthol (36.4%), menthone (27.7%) as the major components, followed by menthyl acetate (11.2%), pulegone (5.9%), and cyclohexanol, 1-methyl-4--(1-methyl ethyl)- (5.5%). As the result indicate peppermint oil is rich in oxygenated monoterpenes such as menthol, menthone, and their derivatives.

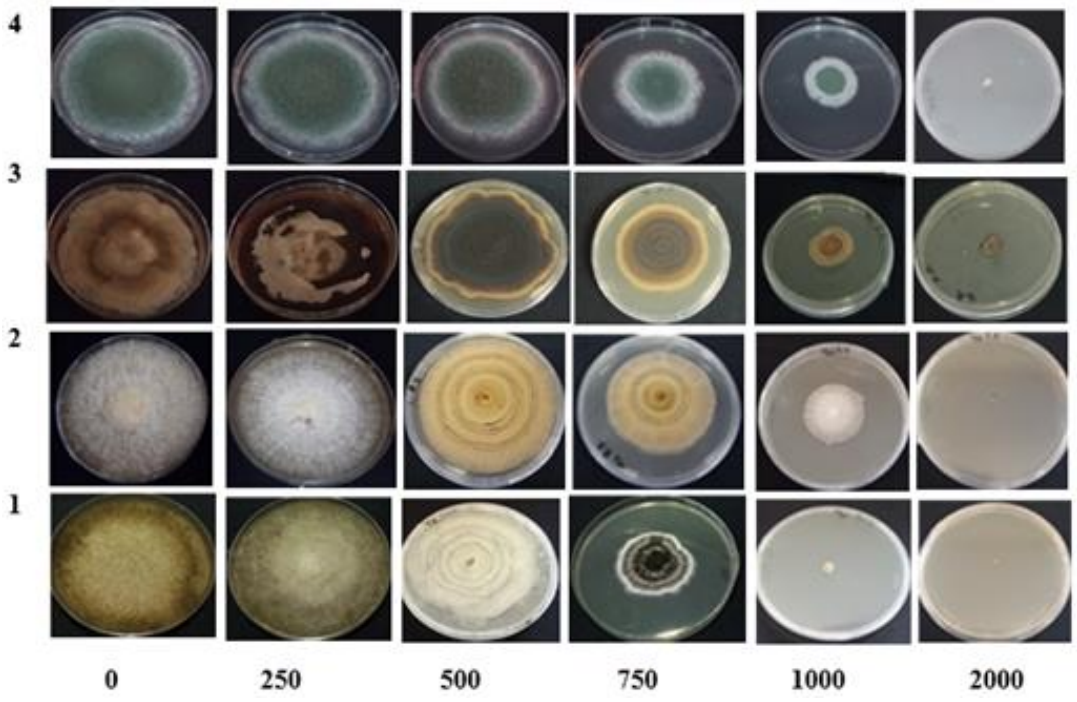
Antifungal activity. The antifungal activities of peppermint EO was assessed using the agar diffusion method by measuring the diameter of zones of mycelial growth as represented in Figure 1. Each fungus showed varying degrees of mycelial growth to the different concentrations of the EO. The rate of mycelium growth was shown to vary with different doses. Compared with the control, there were obvious decreases in the colony diameter of fungi when the EO concentration was higher than 500 ppm ($P < 0.05$). The growth of the mycelium was completely inhibited at 1000 and 2000 ppm of the peppermint EO for *Rhizopus stolonifer* and *Rhizoctonia solani*, respectively. In 2000 ppm, the mycelium growth *Alternaria alternata* and *Penicillium expansum* reduced by 74% and 91%, respectively. This result indicates that peppermint EO at concentration greater than 500 ppm can significantly inhibit the mycelium growth of *Alternaria alternata*, *Rhizopus stolonifer*, and *P. expansum*. Based on the results, *Rhizopus stolonifer* was the most sensitive microorganism and *Rhizoctonia solani* was the less sensitive one to peppermint EO.

Effect of peppermint EO on the morphology of fungal hyphae. Microscopic observations using a light microscope (LM) and scanning electron microscope (SEM) were performed to determine the structural modifications of fungi hyphae before and after treatment with peppermint EO.

Observations of fungi hyphae and conidiophores under LM with tested concentrations of peppermint EO are presented in Figure 2 (1–2–3). Examination of

Table 1. Chemical composition of peppermint EO

Components	Quantity (%)	Retention time (RT)
o-Cymene	0.01	7.84
D-Limonene	0.1	7.95
Eucalyptol	0.4	8.06
Linalool	0.1	9.8
Isopulegol	0.2	11.25
l-Menthone	27.7	11.51
Benzofuran, 4,5,6,7-tetrahydro-3,6-dimethyl-	1	11.65
Cyclohexanone, 5-methyl-2-(1-methylethyl)-, cis-	4.2	11.74
Cyclohexanol, 1-methyl-4-(1-methylethyl)-	5.5	11.82
d-Menthol	36.4	12.08
Pulegone	5.9	13.78
Carvone	2.2	13.94
Menthyl acetate	11.2	15.07
Caryophyllene	2.1	18.55
Caryophyllene oxide	1	22.64
Total	98.01	



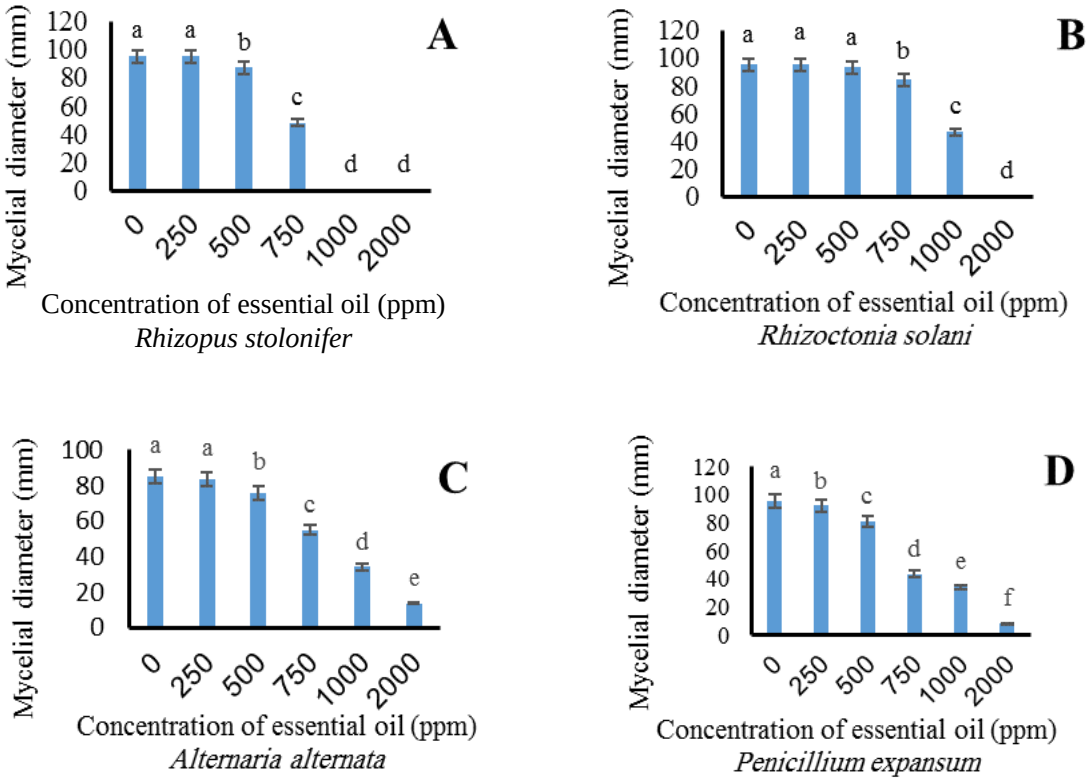


Fig. 1. The antifungal activity of peppermint EO on the mycelial growth and inhibition percentage of the celected phytopathogens. Colony diameter of fungi (above), mycelial growth (down). The colony diameter of fungi was measured daily of incubation at 25 ±3°C. Vertical bars represent standard deviations of the means. Treatments followed by different letters are statistically different by Tukey's test ($P < 0.05$)

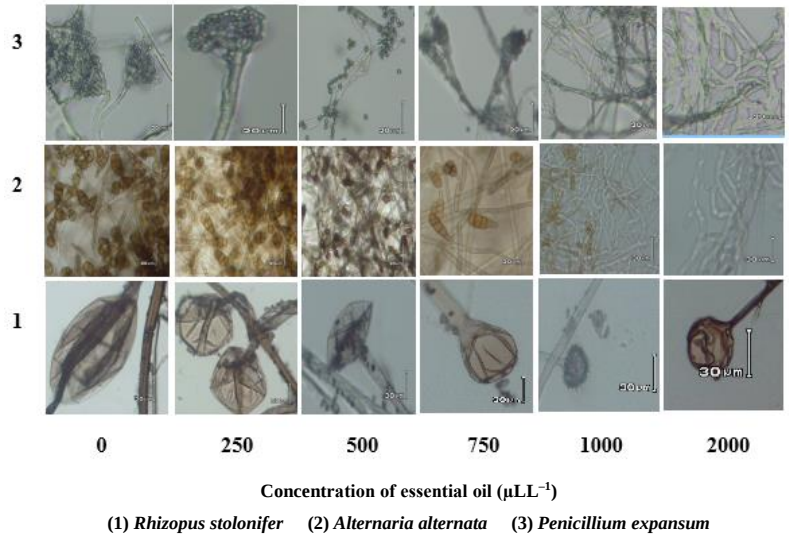


Fig. 2. Light microscopy images of *Rhizopus stolonifer*, *Alternaria alternata*, and *Penicillium expansum* without and with different concentrations of peppermint EO (250, 500, 750, 1000 and 2000 ppm). Images were obtained after the fungal growth in the Petri dish without treatment was complete

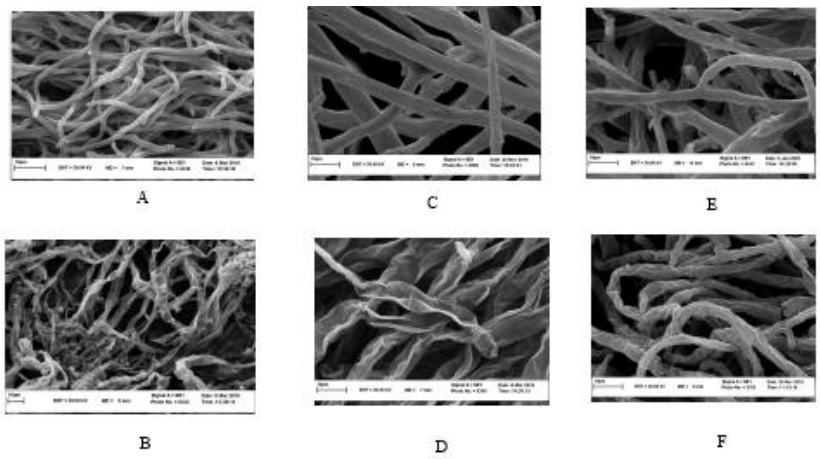


Fig. 3. Scanning electron micrographs (SEM) of fungi mycelia after 5 days of cultivation at 25 ±3°C. A. Control media, *Penicillium expansum*. B. Hyphal modifications induced by 1000 ppm peppermint EO, *Penicillium expansum*. C. Control media, *Rhizopus stolonifer*. D. Treatment with EO at 1000 ppm, *Rhizopus stolonifer*. E. Control media, *Rhizoctonia solani*. F. Treatment with EO at 1000 ppm, *Rhizoctonia solani*

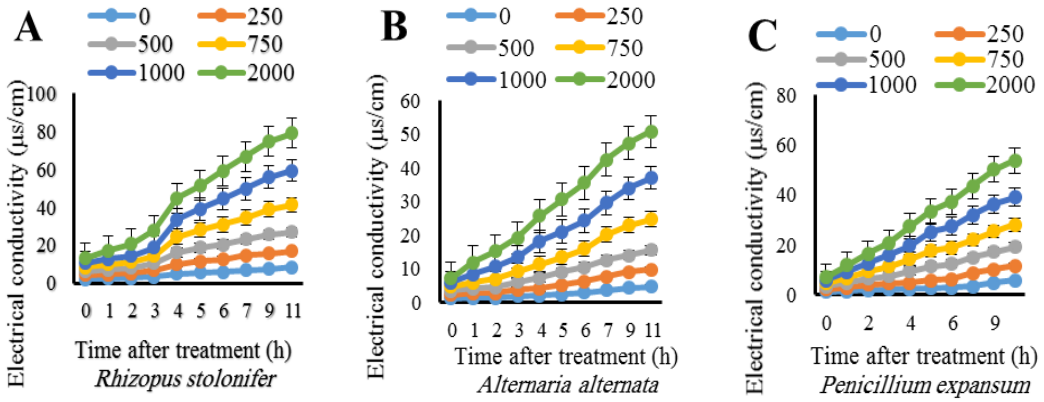


Fig. 4. Effect of different concentrations of peppermint EO on cellular leakage of phytopathogens. A. *Rhizopus stolonifer*. B. *Alternaria alternata*. C. *Penicillium expansum*. Mycelia (wet weight 2.5 g) were cultured in sterile distilled water containing different concentrations of peppermint EO or in sterile distilled water without EO (25 mL) as the control at 25 ±3°C. Samples for the electrical conductivity measurement were assayed for 11 h. Data were based on the 3 replicates and bars indicate standard deviations

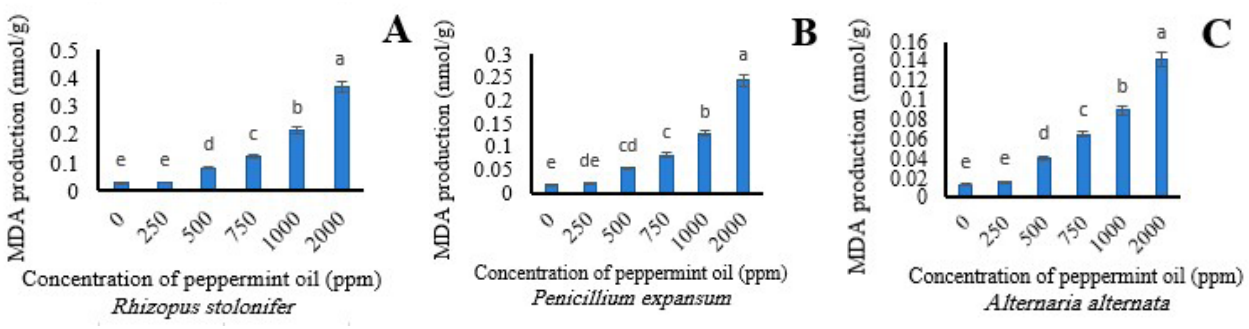


Fig. 5. Effect of different concentrations of peppermint EO on MDA production from phytopathogens. A. *Rhizopus stolonifer.* B. *Penicillium expansum*. C. *Alternaria alternata*. The levels of MDA were determined after 12 h incubation of the fungal with the treatment of peppermint oil at 25 ±3°C. Data were based on the 3 replicates and vertical bars represent standard deviations of the means. Treatments followed by different letters are statistically different by Tukey's test ($P < 0.05$)

without treatment hyphae and conidiophores of fungi by LM showed that conidiophore is colored, homogeneous, clear cytoplasm, and many ramifications for *P. expansum* and without ramifications for *R. stolonifer*. Hyphal filament structure was regular and the cells were composed of elongated hyphae. Moreover, many spores were occasionally found on the mycelia. While their hyphae and conidiophores by increasing the concentration of EO became heterogeneous with irregular cell walls, decreased sporulation, less pigmentation and hyphae size reduction, and treated hyphae were thinner than the control.

The effect of peppermint EO (0 and 1000 ppm) on the morphology of fungi hyphae (*P. expansum*, *R. stolonifer*, and *R. solani*) as well as examined by SEM are shown in Figure 3. The control fungi grown on PDA had normal morphology and regular and homogeneous hyphae with smooth cell walls (Figs 3A, 3C and 3E) while the following treatment showed morphological alterations such as irregular, flattened and empty hyphae and the hyphae lost their smoothness, and also some depressions were noted on the cell surface. Morphological changes in *R. stolonifer* hyphae was more notable and deeper depression than other tested fungi. SEM results confirmed the results of the inhibitory percentage test (Figs 3B, 3D, and 3F). Consequently, peppermint oil treatment resulted in damage to fungi hyphae.

Effect of peppermint EO on cellular leakage from the fungal tissues. The electrical conductivities of the fungal tissues were determined as shown in Figure 4. Cellular leakage increased with increasing concentrations of peppermint EO. In the control and 250 ppm, the electric conductivities of fungi were nearly constant throughout 11 h. However, the conductivity values of hyphae suspensions that were treated with 500, 750, 1000 and 2000 ppm of peppermint EO increased significantly ($P < 0.05$).

Effect of peppermint EO on lipid peroxidation. One of the important markers for determining membrane lipid peroxidation is MDA production, as shown in Figure 5. The increases of MDA observed with increasing concentrations of peppermint EO in this paper confirm the previous findings demonstrating that epsilon-poly-lysine (ε-PL) cause membrane lipid peroxidation in *Pencillium digitatum* [Liu et al. 2017], and dicarboximide fungicides on *Botrytis cinerea* [Lee et al. 1998].

DISCUSSION

Chemical compositions of essential oil. The chemical composition of peppermint EO has been the subject of numerous studies. The two main components of peppermint EO are menthol and menthone, which these are in accordance with the findings of İşcan et al. [2002] and Kostik et al. [2015] but those are different from the findings of Soković et al. [2009] and Desam et al. [2019].

Antifungal activity. In numerous studies, the inhibitory effect of EOs against phytopathogenic fungi was mainly due to the two most abundant components,

which in this research, namely menthol, and menthone. This is in accordance with the finding of İşcan et al. [2002] who tested in vitro peppermint EO for antifungal activity on *Pseudomonas syringae* pv. tomato, *P. syringae* pv. syringae, *P. syringae* pv. phaseolicola, *Xanthomonas campestris* pv. campestris, and *X. campestris* pv. phaseoli, were interestingly correlated with the menthol and menthone percentages, menthol inhibitory power was greater than menthone. Kostik et al. [2015] demonstrated that antifungal activity of peppermint EO was due to menthol and menthone, as well. The main inhibitory component of peppermint EO against the fungus *Trametes versicolor* was menthol [Matan et al. 2009]. Several authors have focused on the antimicrobial activity of menthol and/or without menthone [Al Yousef 2013, Kostik et al. 2015]. In general, biological activities of *Mentha* oils are mainly due to the presence of various chemical components, such as menthol, menthone, piperitone oxide, camphor, and linalool [Hussain et al. 2008, Bendaoud et al. 2009].

Our results are in good agreement with the findings of Kostik et al. [2015] and Hussain et al. [2008] who reported that the antifungal activity of EOs was significantly differentiated independence on kind of EO, the dose of oil, compounds of oils, and species of fungi. The percentage of inhibition due to EOs against the tested fungal organisms was dependent on the oil concentration: the more significant the decrease in the mycelial growth is, the higher the increase in the concentration of the oils. According to Charai et al. [1996], antimicrobial activity of main EO compounds is in the order: phenols (highest activity) > alcohols > aldehydes > ketones > ethers > hydrocarbons. Minimum effective concentrations of the oil against fungal pathogens due to fungus type were also different. Considering the above, the antifungal properties of the peppermint EO can be due to various compounds such as menthol (oxygenated monoterpene, alcohol), and menthone (oxygenated monoterpene, ketone) being the main components, followed by a possible synergy among their other various compounds.

Effect of peppermint EO on the morphology of fungal hyphae. These results were microscopically consistent with the results of Romagnoli et al. [2005] and Yahyazadeh et al. [2008] who demonstrated that the surface modifications in the SEM study were observed after using *Thymus patula* EO against *Botrytis cinerea* and clove oil on *Penicillium digitatum*. Jing et al. [2015] showed that *P. digitatum* treated with α-terpineol caused the loss of linearity and a warty surface of mycelia, abnormal branching, and shrunken and distorted mycelia. In several studies, SEM observations revealed that the morphology of the treated fungi caused structural alterations, such as a delay in the fungal growth, a reduction in condition, loss of pigmentation, disrupted conidiospores structure, pit formation, depression of the cell surface, degeneration of intracellular organelles, plasmolysis, and remaining sporangiophore without sporangiospores [de Oliveira Junior et al. 2012, Al Yousef 2013, Salem et al. 2016, Nishiyama et al. 2017]. Our results confirm the inhibitory effect of EOs.

Effect of peppermint EO on cellular leakage from the fungal tissues. The results are consistent with the reports of Liu et al. [2017] who indicated the electrical conductivities and cellular leakage of hyphae suspensions of *Pencillium digitatum* increase with increasing concentrations of epsilon-poly-lysine (ε-PL). Leakage of fungi hyphae was probably caused by an interaction between peppermint oil and the plasma membrane of the fungal pathogen.

CONCLUSIONS

In this paper, we demonstrated that peppermint EO exhibits inhibitory effects on the mycelial growth of the tested fungi phytopathogens such as *A. alternata*, *R. solani*, *P. expansum* and *R. stolonifer* and the measure of inhibition was widely dependent on the composition and concentration of the essential oil used. To further evaluate the detailed the SEM disclosed that peppermint EO could be applied to delay the fungal growth of the following filamentous fungi: *A. alternata*, *R. solani*, *P. expansum*, and *R. stolonifer*. SEM imaging confirmed that essential oils caused considerable morphological alterations in the tested microorganisms. Furthermore, the electrical conductivity, cellular leakage and MDA production were increased in a concentration-dependent manner in the fungal tissues treated with increasing concentrations of peppermint EO. Considering the above, the lipid peroxidation process caused cellular leakage due to peppermint EO. Natural substances from plants provide

natural, nontoxic, economically feasible, and effective antifungal agents. Due to the fact that peppermint is used as a flavor component in the food industry and aromatherapy products, and due to its antifungal properties, peppermint essential oil can be well used as a bio fungicide as well. However, due to several of the disadvantages associated with the use of essential oils include instability, evaporation, further study needs to be conducted into the development of control-release capsules and microcapsules of essential oil.

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