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ORIGINAL ARTICLE

Growth Inhibition and Morphological Alteration of *Fusarium sporotrichioides* by *Mentha piperita* Essential Oil

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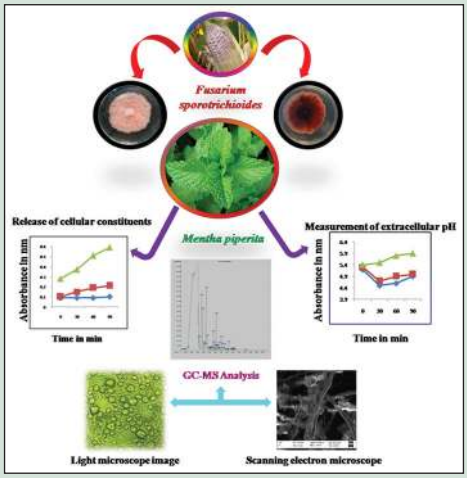
ABSTRACT

Objective: The aim of this study is to determine the phytochemical composition, antifungal activity of *Mentha piperita* essential oil (MPE) against *Fusarium sporotrichioides*. **Methods:** The phytochemical composition was conducted by gas chromatography mass spectrometry (GC MS) analysis and mycelia growth inhibition was determined by minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC), the morphological characterization was observed by scanning electron microscopy. Finally, the membrane permeability was determined by the release of extracellular constituents, pH, and total lipid content. **Result:** In GC MS analysis, 22 metabolites were identified such as menthol, l menthone, pulegone, piperitone, caryophyllene, menthol acetate, etc. The antifungal activity against targeted pathogen, with MIC and MFC 500 µg/mL and 1000 µg/mL, respectively. The MPE altered the morphology of *F. sporotrichoides* hyphae with the loss of cytoplasm content and contorted the mycelia. The increasing concentration of MPE showed increase in membrane permeability of *F. sporotrichoides* as evidenced by the release of extracellular constituents and pH with the disruption of cell membrane indicating decrease in lipid content of *F. sporotrichoides*. **Conclusion:** The observed results showed that MPE exhibited promising new antifungal agent against *Fusarium sporotrichioides*.

Key words: *Fusarium sporotrichioides*, Mentha piperita essential oil, gas chromatography-mass spectrometryGC-MS, *Mentha piperita* essential oil, scanning electron microscope

SUMMARY

- *F. sporotrichioides*, filamentous fungi contaminate to corn and corn-based products
- *F. sporotrichioides* mainly responsible for the production of T-2 toxin
- Phytochemical composition was conducted by gas chromatography-mass spectrometry analysis
- *Mentha piperita* essential oil (MPE) is commonly known as peppermint
- The *F. sporotrichioides* growth was inhibited by MPE (minimum inhibitory concentration, minimum fungicidal concentration)
- Morphological observation by scanning electron microscope.



Abbreviations Used: Cfu: Colony forming unit; DMSO: Dimethyl sulfoxide, °C: Degree celsius; *F. Sporotrichoides*: *Fusarium sporotrichioides;* EOs: Essential oils; M: Molar, g: Gram/gravity, mg: Milligram; µg: Microgram, ml: Milliliter; mm: Millimeter, min: Minutes; *M. piperita*: *Mentha piperita*, MIC: Minimum inhibitory concentration; MFC: Minimum fungicidal concentration; MAE: *Mentha arvensis* essential oil; Na_2SO_4: Sodium sulfate; pH: Potential Hydrogen; PDB: Potato Dextrose Broth; SEM: Scanning electron microscope

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INTRODUCTION

Fusarium sporotrichioides, a filamentous fungi of the section sporotrichiella, necrotrophic pathogen associated with *Fusarium* head blight of cereals.[1-3] *F. sporotrichioides*, *Fusarium acuminatum, Fusarium Sambucinum,* and *Fusarium poae* are predominant species widespread in the soil and on plants, throughout the cold and cool regions of the world. They invade cereal crops and are responsible for the production toxic metabolites such as type A trichothecenes: T-2 toxin, HT-2 toxin, T-2 tetraol, T-2 triol, scirpentriol (STO), and diacetoxyscirpenol which may contaminate animal and human food.[4-13] Trichothecene is sesquiterpenoid highly toxic; T-2 toxin and HT-2 toxin are potent inhibitor of protein synthesis and are highly cytotoxic to eukaryotes.[14] T-2 toxin, HT-2 toxin found to be contaminants in wheat, maize, barley, oats, rice, beans, and soybeans as well as in some cereal-based products and have played a role in plant diseases. These toxins are an important agricultural problem due to their detrimental effects on both human and animal health.[15-17] Trichothecene has acute symptoms in man associated with high-level intake of trichothecenes include necrotic lesions of the oral cavity, esophagus, and stomach, nausea, vomiting, abdominal pain, diarrhea, dizziness, headache, and marked leukopenia.[18,19] Within the

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outbreak of the so-called "alimentary toxic aleukia" documented.[18,20] Therefore, control of *F. sporotrichioides* growth in agricultural products is essential to reduce food-borne illness. Several low-molecular organic acids antifungal chemical have been used to control the growth of *F. sporotrichioides* during grain storage. These chemical fungicides show serious health issues in humans and animals due to their toxicity by the migration of chemical residue into food chain and they also produce resistance in fungi.[21-23] Hence, natural plant components may act as an alternative to these chemical preservatives and fungicides.

Plants essential oils have long been researched for their antibacterial, antifungal, antiviral, and antioxidant properties. Essential oils and their principle compounds, such as menthol, eugenol, cinnamaldehyde, thymol, carvacrol, terpineol, and citral, have been considered to be effective against many filamentous fungi. The several studies on essential oils cinnamon, citral, *Litsea cubeba*, *Zingiber officinale*, clove, eucalyptus, anise, peppermint, spearmint, and camphor oils show inhibitory activity against pathogenic microorganisms. cinnamon essential oil showed potent antifungal activity against mycotoxigenic fungi *Aspergillus flavus*,[24] citral, octanal, and a-terpineol volatile oils against *Geotrichum citri-aurantii*[25] and clove and Cinnamon oils were found to be effective against *A. flavus* which is responsible for aflatoxin production in maize under favorable conditions.[26-28] Growth inhibition and morphological alterations of *Fusarium verticillioides* and inhibition of fumonisin B1 by *Z. officinale*, *Rosmarinus officinalis* L., cinnamon essential oil reported by Xing *et al*., 2014,[29] Yamamoto-Ribeiro *et al*., 2013[30] da Silva Bomfim *et al*., 2015.[31]

Mentha piperita essential oil (MPE) is commonly known as peppermint. Leaves of mint plant are aromatic perennial herb cultivated in most part of the world. It is used as folk medicine and frequently used in herbal tea and for culinary purpose to add flavor and aroma. The essential oil of *M. piperita* rich in menthol, l-menthone, pulegone, piperitone, menthol acetate, piperitenone menthone, carvone, menthofuran, isomenthone, menthyl acetate, isopulegol, menthol, 3-octanol, pulegone; hence, it also used as natural antioxidants.[32-35] MPE have shown potent inhibitory activity against several microorganisms such as *Penicillium digitatum*, *A. flavus*, *Aspergillus niger*, *Candida albicans*, *Saccharomyces cerevisiae Mucor* spp., and *Fusarium oxysporum*,[33,36] further MPE reported as antifungal agent for several mycotoxigenic speceies by Silva *et al*., 2012;[37] *A. flavus* and *A. parasiticus*, Freire *et al*., in 2012[38] reported against *Aspergillus ochraceous*, *Colletotrichum gloeosporioides*, *Colletotrichum musae*, *F. oxysporum*, *Fusarium semitectum*. Currently, it has been widely used in cosmetic, food and pharmaceutical industries no studies of which we are aware have reported the effects of MPE on *F. sporotrichioides*. Therefore, the present study was undertaken to evaluate the effects of MPE against the growth of *F. sporotrichioides*. Morphological alteration was investigated by scanning electron microscopy.

MATERIALS AND METHODS

Preparation of essential oil

The plant was collected from herbal farm, Mysore, India. Leaves are washed thoroughly, dried in shade, and powdered. The essential oil was obtained from hydrodistillation using a Clevenger-type apparatus in accordance with the method recommended by the European Pharmacopoeia.[39] The extraction was performed for 180 min and 200 g of powder yield the 1000 µL of oil. The oil obtained was stored at 4°C and protected from light for subsequent use and chemical analysis.

Gas chromatography-mass spectrometry analysis of oil

The GC-MS analyses were performed in EI mode (70 eV) with an Agilent 7890 GC system, equipped with model 5975 mass selective detector (Agilent Technologies, USA). SGE BPX5 fused silica capillary columns (30 m × 0.32 mm i.d., 0.25 µm film thickness) were employed for separation; the column oven temperature was raised linearly from 80°C (hold for 2 min) to 280°C (hold for 5 min) at 20°C/min. Helium was used as carrier gas at constant flow of 1.2 mL/min. The samples were analyzed in splitless mode at injection temperature of 250°C, EI source temperature 230°C, and quadrupole analyzer at 150°C, ionization current at 235 eV.

Culture and preparation of *Fusarium sporotrichioides*

F. sporotrichioides Microbial Type Culture Collection 1894[40] was used as the test organism and was obtained from the Department of Microbiology, Defence Food Research Laboratory, Mysore. The fungus was purified and harvested at 37°C on potato dextrose broth (PDB). The spores concentration was adjusted to 5×10^5 CFU/mL using a hemocytometer.

Minimum inhibitory concentration and minimum fungicidal concentration

Minimum inhibitory concentration (MIC) determination was performed using 96-well microtiter plates. The *F. sporotrichioides* fungal inocula in 96-well microtiter plates were treated with MPE at different concentrations and incubated for 24 h; then, absorbance was measured at 575 nm. Fluconazole was used as the standard and also to compare the cell viability under inverted microscope. The minimum fungicidal concentration (MFC) was regarded as the lowest concentration that prevented growth of the fungus following 72 h incubation at 28°C in a fresh potato dextrose agar (PDA) plate, indicating more than 99.5% killing of the original inocula.[41]

Scanning electron microscopy

The 24 h old fungal culture on PDB treated with MPE at varied concentrations of control, MIC and MFC were used for scanning electron microscope (SEM) observation. The *F. sporotrichioides* cells were fixed in glutaraldehyde in 0.1 M PBS for 30 min, washed with 0.1 M PBS, and dehydrated by immersing in ice-cold ethanol for 10 min. The dehydrated samples were smeared on silver stub like a thin film and were coated by cathodic spraying (Polaron gold). The SEM observations were made using a ZEISS Instrument (EHT = 15.00 kV, signal A = VPSE G3).

Release of cellular constituents

The release of cellular constituents into the supernatant was measured following Paul *et al*.[42] method. The 2 days old mycelia from PDB were collected by centrifugation at 4000 g for 20 min, washed 3 times, and resuspended in 100 mL phosphate buffer saline (pH 7.0). The resulting suspension was treated with MPE with MIC and MFC for 0, 30, 60, and 120 min. Then, 2 mL of sample was collected and centrifuged at 12,000 g for 2 min, and 1 mL of supernatant was used to measure the cellular constituents in spectrophotometer at 260 nm.

Measurement of extracellular pH

The extracellular pH was measured by Shao *et al*.[43] method. The extracellular pH of *F. sporotrichioides* was determined by eutech pH meter. The fungal suspension 10^5 CFU/mL were added to 20 mL PDB and incubated in a moist chamber at 28°C ± 2°C for 2 days. The mixtures were centrifuged at 4000 g for 20 min, and the resulting pellet was collected, washed for 2–3 times with sterilized double-distilled water, and resuspended in 20 mL sterilized double-distilled water. After the addition of the MPE, the extracellular pH was determined.

Determination of lipid content

Total lipid content of *F. sporotrichioides* cells with the MPE at various concentrations (control, MIC, and MFC) was determined using phosphovanillin method.[44,45] The 2-day-old mycelia from 50 mL PDB were collected and centrifuged at 4000 g for 10 min. Then, the samples are lyophilized. About 0.1 g of dry mycelia were homogenized with liquid nitrogen and extracted with 4.0 mL of methanol: chloroform: water mixture (2:1:0.8, v/v/v) with vigorous shaking for 30 min. The resulting sample was centrifuged at 4000 g for 10 min. The lower phase containing lipids was mixed with 0.2 mL saline solution and centrifuged at 4000 g for 10 min. To the lipid mixture add 0.2 mL chloroform and 0.5 mL of H_2SO_4 and warm in a boiling water bath for 10 min. After that, 3 mL phosphovanillin was added and shaken vigorously, and then incubated at room temperature for 10 min. The absorbance was measured at 520 nm. Cholesterol was used as a standard.

RESULTS

Gas chromatography-mass spectrometry analysis

Phytochemical analysis was carried out by gas chromatography-mass spectrometry to determine the chemical composition of MPE. A total of 22 metabolites were identified. The chemical formulas and mass of the detected compounds chromatogram are presented in Table 1 and Figure 1.

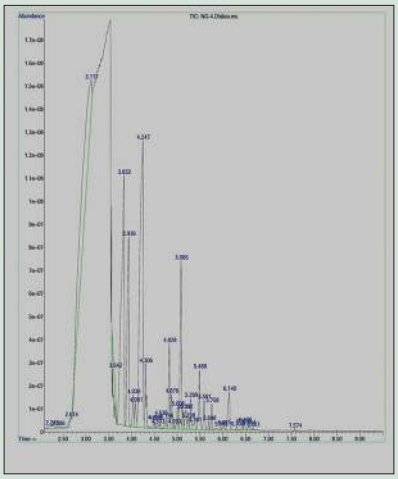

Figure 1: Chromatogram of *Mentha piperita* essential oil

Minimum inhibitory concentration

The antifungal activity of MPE was estimated using cell viability assay with fluconazole as the standards. The MIC and MFC values of MPE are shown in Table 2 and Figure 2. MPE at a concentration of 500 µg/mL inhibited mycelial growth; whereas at 1000 µg/mL (MFC), it induced fungicidal activity.

Scanning electron microscopy

The effect of the MPE on the morphology of *F. sporotrichioides* as examined through SEM is shown in Figure 3. The control fungus grown on PDA had normal tubular hyphae with regular morphology [Figure 3a and b]. After 48 h of treatment using the MIC and MFC of the MPE treated fungal mycelia showed distorted, shrunken morphology [Figure 3c and d].

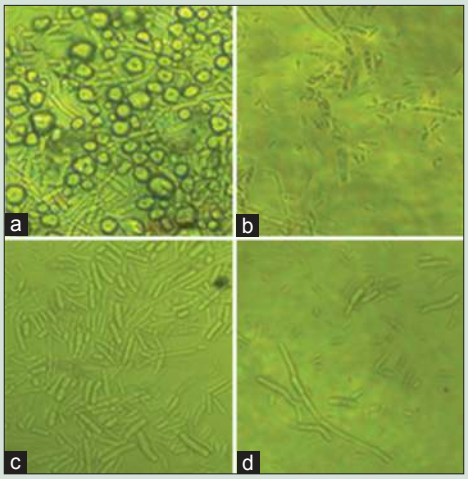

Figure 2: Inverted microscopic observation of *Fusarium sporotrichioides*: (a) Control, (b) fluconozole, (c) 500 µg/mL *Mentha piperita* essential oil, (d) 1000 µg/mL *Mentha piperita* essential oil

Release of cellular constituents

The results of the release of cell constituents when *F. sporotrichioides* was treated with MPE at three different concentrations (control, MIC, and MFC) for 0, 30, 60, and 120 min, respectively, are shown in Figure 4. A significant increase in the release of cell constituents was observed immediately after the treatment with the MPE at different concentrations compared to control.

Measurement of extracellular pH

The extracellular pH of *F. sporotrichioides* cells is shown in Figure 5. A gradual increase in extracellular pH was observed in control in 30, 60, and 90 min. The extracellular pH values in the *F. sporotrichioides* suspensions after incubation with MPE at MIC and MFC for 30 min were 4.70 and 4.55, respectively, which were significantly lower than that of the control (5.5). The extracellular pH in *F. sporotrichioides* suspensions incubation with MPE at MIC and MFC for 60 min was 4.90 and 4.60, respectively, which were significantly lower than that of the control (5.8). And finally, the extracellular pH in *F. sporotrichioides* suspensions incubation with MPE at MIC and MFC for 90 min was 5.0 and 4.50, respectively, which were significantly lower than that of the control (5.5).

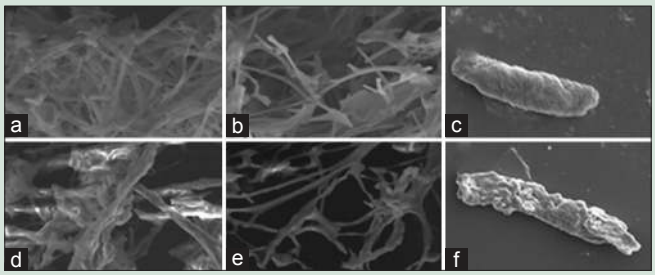

Figure 3: Scanning electron microphotography of *Fusarium sporotrichioides*: (a-c) control/untreated; (d-f) treated with *Mentha piperita* essential oil

Total lipid content

The effect of MPE on the total lipid content of *F. sporotrichioides* is shown in Table 3. The lipid content of pathogen is decreased after treating with MPE. The total lipid contents of *F. sporotrichioides* after incubation with MPE at MIC and MFC for 120 min were 120.1 ± 3.5 and 80.4 ± 4.6 mg/g dry weight, respectively, which were significantly lower than that of control (160.2 ± 3.8 mg/g dry weight).

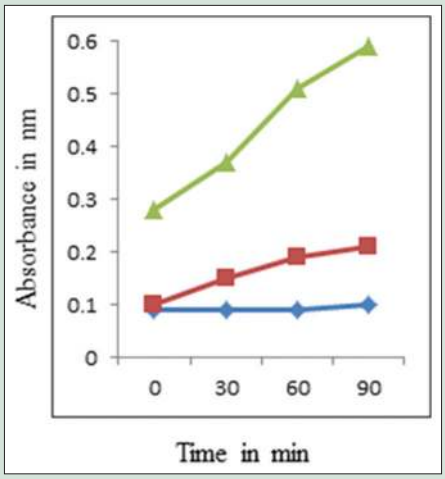

Figure 4: Effects of the *Mentha piperita* essential oil on the 260 nm absorbing material release of *Fusarium sporotrichioides* ([◊]: control; [■]: Minimum inhibitory concentration; [◄]: Minimum fungicidal concentration)

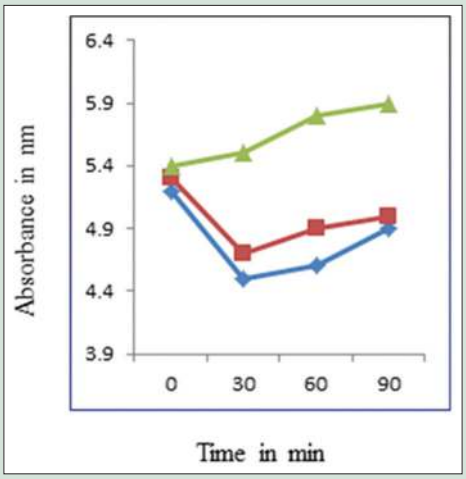

Figure 5: Effects of the *Mentha piperita* essential oil on the extracellular pH of *Fusarium sporotrichioides* ([▲]: Control; [■]: Minimum inhibitory concentration; [◊]: Minimum fungicidal concentration)

Table 1: Phytochemical constituents of *Mentha piperita* essential oil by gas chromatography-mass spectrometry analysis

Compound name	Peak area in (%)	RT (min)	Molecular formula	Molecular weight
3-hexadecene, (Z)	0.51	2.247	$C_{16}H_{32}$	224.425
Menthol, acetate	0.14	2.676	$C_{10}H1_{8O}$	154.2493
l-menthone	22.18	3.117	$C_{10}H1_{8O}$	154.2493
D-alloisoleucine	1.46	3.643	$C_6H_{13}NO_2$	131.172
Pulegone	19.33	3.832	$C_{10}H_{16}O$	152.2334
Piperitone	8.49	3.935	$C_{10}H_{16}O$	152.2334
Menthomenthene	1.15	4.038	$C_{10}H_{18}$	138.2499
9-octadecene, (E)	0.95	4.090	$C_{18}H_{36}$	252.4784
Menthol	24.96	4.250	$C_{12}H_{22}O_2$	198.3019
o-menth-8-ene	0.23	4.484	$C_{15}H_{24}$	204.3511
Piperitenone	0.17	4.519	$C_{10}H1_{4O}$	150.2176
Eugenol	0.33	4.633	$C_{10}H_{12}O_2$	164.20108
Copaene	0.38	4.759	$C_{15}H_{24}$	204.351
Cyclodecane	0.66	5.005	$C_{10}H_{20}$	140.2658
Caryophyllene	5.67	5.085	$C_{15}H_{24}$	204.36
(+)-epibicyclosesquiphellandrene	0.53	5.131	$C_{15}H_{24}$	204.3511
Methyl pivalate	0.79	5.194	$C_{11}H_{22}O_2$	186.2912
δ-cadinene	0.74	5.760	$C_{15}H_{24}$	204.3511
Caryophyllene oxide	1.98	6.138	$C_{15}H_{24}O$	220.35046
Fomepizole	0.17	6.430	$C_4H_6N_2$	82.11
Thujopsene	0.29	6.481	$C_{15}H_{24}$	204.35106

MPE: *Mentha piperita* essential oil; GC-MS: Gas chromatography-mass spectrometry; RT: Retention time

Table 2: Minimum inhibitory concentrations and minimum fungicidal concentrations of *Mentha piperita* essential oil

Treatment	MIC (µL/mL)	MFC (µL/mL)
MPE	500	1000

MIC: Minimum inhibitory concentration; MPE: *Mentha piperita* essential oil; MFCs: Minimum fungicidal concentration

Table 3: Effect of *Mentha piperita* essential oil on the total lipid contents of *Fusarium sporotrichioides*

Concentration (µL/mL)	Total lipid contents (mg/g dry weight)* MPE
Control	160.2±3.8
MIC	120.1±3.5
MFC	80.4±4.6

MIC: Minimum inhibitory concentration; MPE: *Mentha piperita* essential oil; MFC: Minimum fungicidal concentration. *Values are presented as mean ±SD

DISCUSSION

In this study, MPE was found to be effective in inhibiting the growth of *F. sporotrichioides*. In MPE rich in menthol, l-menthone, pulegone, piperitone, caryophyllene, and menthol acetate, these are the major compounds which are reported in many articles.[32,46] In addition, δ-cadinene, copaene, eugenol, methyl pivalate, menthomenthene, thujopsene, caryophyllene oxide also some of the compound identified in MPE and demonstrated antifungal activity and antibacterial activity.[47-52] Further, the anti-inflammatory, cytotoxic effects were reported by Sun *et al*. 2014.[53] The MIC values of MPE obtained in this study were 500 µg/mL and 1000 µg/mL at this particular concentration; the fungal growth was minimum as compared to untreated fungal cells. The potential mechanisms of volatile compounds as reported could damage the cell and disturb the cellular metabolism.[54] The previous report concluded that volatile compounds act as H^+ carriers and depletion of adenosine

triphosphate takes place and disturb the cellular membrane, by reacting with the active sites of cellular enzymes.[55,56] The present study clearly demonstrates that the antifungal activity of MPE supported by SEM images of MPE showing morphological changes in the fungal hyphae due to the leakage of ions, leakage of molecular substances, and lesions as well as discrepancies in cell metabolism.

Extracellular enzymes are synthesized inside the cell and then secreted outside the cell, where their function is to break down complex macromolecules into smaller units to be taken up by the cell for growth and assimilation. Extracellular pH was determined to explain the release of the cellular material and the changes of membrane permeabilization. Results showed that, after the treatment of the MPE, the release of cell constituents in the fungi suspensions visibly increased compare to control. Meanwhile, after 30 min of exposure, the MPE apparently induced the leakage of intracellular protons as evidenced by the decrease in the values of extracellular pH. These findings suggest the irreversible damage to the cytoplasmic membranes of *F. sporotrichioides*.

Cell membrane encoding lipids is a major component of the cell. It performs many important functions including increasing the stability of the membrane, reducing the permeability of water-soluble materials, and adjusting the liquidity of the membrane.[57] The decrease in membrane stability and increased in permeability of water-soluble materials suggest the decrease in lipid content.[44] The previous studies regarding volatile compounds have been reported to disrupt the lipid structures of the cells.[58] A significant decrease in lipid content of *A. niger* and *A. flavus* cells in fumigated and nonfumigated cells with 1 μL/mL of *Cymbopogon citratus* essential oil (*A. niger*) and *C. citratus* essential oil (*A. flavus*) were reported by.[44,59] In the present study, the addition of MPE significantly decreased the lipid content of *F. sporotrichioides*. These results suggest that MPE is capable of acting on the cell membrane structure and disrupt the cell membrane integrity.

CONCLUSION

The results of the present study indicate the antifungal efficacy of MPE, by changes in the morphological alteration, and growth inhibition effects on *F. sporotrichioides*. These findings suggest that MPE may be used to control *F. sporotrichioides*. Future studies with its toxic metabolites should be conducted to evaluate the potency of MPE to generate more effective natural fungicides or preventives which is less risk or safe to the environment and health.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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