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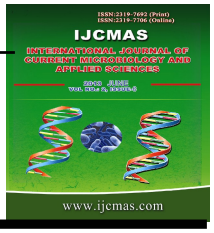

Original Research Article

Antifungal Activity of Volatiles from Lemongrass (*Cymbopogon citratus)* and Peppermint (*Mentha piperita*) Oils Against Some Respiratory Pathogenic Species of *Aspergillus*

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Keywords
Lemongrass; peppermint; antifungal activity; *Aspergillus* spp.; essential oils.

A B S T R A C T

Recently, the use of essential oils against fungal infection has gained highly importance because of acquired resistance against a large number of drugs. The aim of study was testing the biological activity of essential oil vapors against some *Aspergillus* spp., estimating chemical composition, active ingredient and mechanism of action of tested oils. Lemongrass and peppermint essential oil volatiles were preliminary evaluated via vapor phase using volatilization method. Growth inhibition, minimum inhibitory concentration (MIC), and minimum lethal concentration (MLC) of these pathogenic fungi were used to detect their susceptibility to each of the essential oils. Morphological changes of treated fungi, as well as its spores germination was observed by light and electron microscopy. Also, chemical analysis of essential oils were achieved by GC, GC-MS. Lemongrass oil was found to be highly fungicidal, as it showed the lowest MIC and MLC values and the highest growth inhibition; in a range of concentrations (15 to 20 µl/0.4l air space) it was effective in inhibiting fungal viability and spore germination. The main morphological changes caused by Lemongrass oil in *A. niger* were observed under both the light and electron microscope; these included a reduction in conidition, loss of pigmentation and disrupted conidispore structure. The results show that Lemongrass oil produces a fungitoxic effect, which supports its possible use in medicine to cure mycotic infections.

Introduction

Many of the plant species found that it can be used medically **(**Ali-Shtayeh and Abu, 1999). Essential oils are aromatic substances which are obtained from various plant parts by steam distillation; many of which exhibit antibacterial (Ozcan *et al*., 2006), antifungal (Chee and Lee, 2007) and antiviral (Khan *et al*., 2005) activity. Lemongrass (*Cymbopogon citratus)* is widely used in traditional medicine in many countries around the world. Among its attributable properties are those related to antibacterial and antifungal activities **(**Inouye *et al*., 2001, Inouye *et al*., 2006; Bansod and Rai, 2008; Revathi *et al*., 2012), as well as analgesic

and anti-inflammatory properties (Negrelle and Gomes, 2007). A large amount of literature is also available on the medicinal properties of essential oil present in peppermint (Gulluce *et al*., 2007; Rasooli, 2008). Aspergillosis results from the inhalation of spores of *Aspergillus fumigatus*. Once in the lungs the spores of this fungus germinate to form a tangled mass of fungus fibers and blood clots. Fungus spreading increase gradually leading to the destruction of lung tissue, but they do not always spread to other parts of the body (Bansod and Rai, 2008). The majority of the clinically used antibiotics, used to cure this infection, suffer from various drawbacks relating to toxicity and drug–drug interactions, lack of fungicidal efficacy, cost and finally the emergence of resistant strains caused by their frequent use. There is an urgent need towards the use of anti-fungal substances, especially with high efficiency and less toxic compared to currently used drugs **(**Rapp, 2004; Kauffman, 2006). The aim of this study was to assess the antifungal activity of the volatiles of Lemongrass and Peppermint oils against some *Aspergillus* spp. isolated from tuberculosis patients, by determining their effects on mycelia growth, spore germination, and fungal morphology.

Materials and Methods

Fungal organisms

Aspergillus niger, A. flavus and *A. fumigatus* were obtained from the Microorganisms Lab., Assiut University Hospital, Egypt. These fungi were isolated from deep sputum pulmonary tuberculosis patients of the most severe and frequently infected pulmonary tuberculosis patients; identification of the cultures was confirmed at the Assiut Mycological Unit, Assiut University, Egypt, and stock cultures were maintained on Czapek Dox agar slants, stored at 5°C.

Essential oils and authentic chemicals

Lemongrass and peppermint leaves, obtained from the College of Pharmacy Farm, Assuit University were distilled using a Clevenger-type apparatus for 2.5 hours (Guenther, 1948). The oils were then separated and dried over anhydrous sodium sulfate. Crude oils were analyzed using gas chromatography/ mass spectrometry (GC/MS) on central Laboratory, National Research Center, Gize, Egypt as suggested by Stenhagen et al, (1974). GC analysis of the essential oils was conducted using a Hewlett-Packard Model 5985 equipped with a flame ionization detector (FID). A 60 m x 0.32 mm ID fused-silica capillary column coated with DB-5 was used. The oven temperature was programmed for 50ºC to 220ºC/min. The injector and detector temperatures were 220ºC and 250ºC, respectively. Helium was used as a carrier gas at a flow rate of 1 ml/min. Values reported were an average of two analysis. The retention indices of the volatile components were calculated with hydrocarbons (C8-C20; Aldrich chemical) as references. Authentic samples of some components were injected to verify the identity of the unknown compounds beside identification using mass spectroscopy The constituents of each oil was identified by comparing their mass spectra and retention indexes with those of National Bureau of Standard (NBS) library and other published spectra (Table,1). Authentic samples of myrcene, citral, Menthone and Menthol were purchased from Aldrich Chemical Co. USA. The essential oils and their constituents were then evaluated for antifungal activity.

Assessment of the antifungal activity of oil volatiles

Preliminary screening of the antifungal activity of the oils was tested against *Aspergillus* spp. at doses of 5, 10, 15 and 20µl/0.4l air space by the inverted Petri dish technique. Doses of the oils were applied to filter paper discs (5mm diameter) which was mounted on the inside lid of a Petri dish plate. The plate diminution 140 x 23 mm, which offers 0.4l ml air space after media adding, whereas that is the available space of the closed one during this investigation. Then, a 9 mm plug taken from 7 days old culture of the test fungus was placed on the center of Czapek Dox medium in a Petri dish, and sealed with Parafilm; plates were incubated at 30ºC for 5 days. At the end of the incubation period the linear growth of the mycelium was measured and expressed as average values (mm). Controls used distilled water instead of the oil.

Assessment of oil volatiles on fungal spore germination

Doses of the essential oils were applied by gaseous contact as described above in order to determinate their potential to inhibit fungal spore germination A spore suspension (300 µl harvested from a 7 day old culture (10^6 spores/ml) was spread on glass slides which were incubated in an atmosphere of the essential oils at 30ºC for 24 hours. At the end of incubation period, each slide was fixed with lacto-phenol-cotton blue stain and observed under the light microscope for spore germination; approximately 150 spores were counted.

Assessment if minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of oil volatiles

In order to demonstrate the MIC and MLC of volatiles of lemongrass, peppermint oils (µl/0.4l air space as mention above) and their major constituents (µg/0.4l air space), a two-fold series were used. The oils were dissolved in ethyl acetate and pipetted onto a filter paper disc (5mm diameter) which was dried for 1min to remove ethyl acetate and placed into the inoculated plate, as described above. A disc impregnated with ethyl acetate alone acted as the control. Inoculated plates were incubated at 30ºC for 5 days. MICs were then recorded as the lowest concentration of the oil which inhibited fungal growth.

In order to determine the fungistatic or fungicidal (lethal) activity of the essential oils on mycelial growth, the fungal disc was transferred from the treated plates above, where no growth was observed into a new plate without oils and incubated at 30ºC for 10 days. Fungi resuming mycelial growth were considered to have shown a fungistatic response to the oil volatiles; MLCs represent the lowest essential oils concentration which allows for no fungal re-growth.

Assessment of the effect of oil volatiles on fungal morphology

Morphology alterations caused by sub-lethal dose of lemongrass oil against *A. niger* were observed in duplicate by light and electron microscope. According to The following the slide culture technique was performed (Pereira, et al, (2011). A small block of Czapek-Dox agar was transferred to a glass slides in a Petri dish and then inoculated with the test fungus. The required dose was pipetted onto a filter paper disc as described above and the plate was incubated at 30ºC for 5 days. Control assays lacking the essential oil under test were included. After incubation, the slides were observed under a stereomicroscope at 200X. In E/M studies,

electron microscope stubs were touched to the surface of *A.niger* mycelium and then examined directly using transmission electron microscopy (TEM); morphological variations were then observed in relation to the control.

Statistical analyses

Statistical analyses were performed using SPSS 17-0 software for windows (Statistical Product and Services . Solutions, Inc, Chicago, IL, USA). A p of 0.05 was set as the significant threshold for all statistical analyses.

Result and Discussion

Analysis of lemongrass and peppermint oils indicated that a citral (a mixture of neral and geranial) is the major component (70.17% and 52.96% relative to oil, respectivilly). Beside citral and myrecene (in Lemongrass oil); citral, 1,8-cineole, Isomenthone and Menthyle acetate (in Peppermint oil), both oils contained in relatively lower concentrations (Table 1).

The susceptibility of fungi to the volatiles of Lemongrass and Peppermint oils, determined by the inverted Petri dish technique, is shown in (Table, 2 and Figure, 1). Each essential oil showed varying degrees of antifungal activity. Lemongrass oil showed high significant antifungal activity against *A.niger* and *A.fumigatus* at a concentration 5µL/0.4L air space. On the other hand, *A.flavus* was only moderately inhibited by volatiles from Lemongrass oil (15µl/0.4l air space). Peppermint oil showed a weak ability to suppress the growth of *A.niger* and *A.favus* (15 and 20µl/0.4l air space, respectively). Additionally, *A.fumigatus* was continuous growth at a concentration 20µl/0.4l air space.

The results given in Table 3 show that volatiles from Lemongrass oil was strongly active against spore germination; spore germination of *A.niger* and *A. fumigatus* being significant completely inhibited by Lemongrass oil volatiles at a dose of 10 µl/0.4l air space, and spores of *A. flavus* lost their viability when exposed to concentration of 15 µl/0.4l air space. Peppermint oil volatiles exhibited weak activity against the germination of spores of *A. flavus* (72%) and *A. fumigatus* (68%) at 20 µl/0.4l air space; the same concentration lead to complete inhibition of *A. niger* spore germination.

The MIC and MLC values produced by volatiles of oils of Lemongrass and Peppermint are shown in Table 4. The lowest MIC and MLC values of lemongrass were shown significantly against *A.niger* and *A.fumigatus* (MIC 5,3 µl/0.4 l and MLC 5 µl/0.4 l), while the MIC and MLC against *A.flavus* was 13 µl/0.4 l and 17 µl/0.4 l, respectively. Peppermint oil volatiles exhibited an intermediate inhibitory effect against *A.niger* (MIC, 13 µl/0.4 l and MLC, 25 µl/0.4 l), while the highest values of MIC and MLC were observed against *A.flavus and A.fumigatus* (MIC, 18, 23 µl/0.4 l and MLC 25, 30 µl/0.4 l, respectively).

The susceptibility of *Aspergillus* spp. towards the different constituents of the oils at µg/0.4l air space is shown in Table 5. The Lemongrass constituents, citral alone and mixture of citral + myrcene together inhibited *A.flavus* and *A.fumigatus* at 7 and 4.5 µg/0.4l air space, respectively, while *A.niger* was shown to be more sensitive to citral (3.5µg) and mixture of citral and myrecene (4µg).

Table.1 the chemical composition of the essential oil from Lemongrass and Peppermint analyzed by GC-MS

Lemongrass		**Peppermint**	
Component	% Conc.	Component	% Conc.
α –pinene	0.06	α –thujene	0.04
cis-sabinene hydrate	0.11	Linalool	0.72
1-8 cineole	0.12	1,8-Cineole	8.15
Geranyl acetate	0.23	Menthyl acetate	5.28
Geraniol	0.72	Isomenthone	9.47
Terpinolene	1.52	Myrcene	0.45
β-caryophyllene	2.05	Limonene	1.8
Linalool	3.82	*trans*-sapinin hydrate	1.42
Limonene	4.12	Sabinene	0.91
3-myrcene	18.5	α-pinene	1.05
Neral*	23.92	Menthone	28.46
Geranial*	46.25	Menthol	24.5

*a mixture of Neral and geranial is called Citral

Table.2 Antifungal activity of lemongrass and peppermint essential oil against *Aspergillus* spp. growth *in vitro*.

Fungi	**Lemongrass (µl/0.4l air space)**				**Peppermint (µl/0.4l air space)**			
	5	**10**	**15**	**20**	**5**	**10**	**15**	**20**
A.niger	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	6.2^{a}	4.8^{a}	0.0^{a}	0.0^{a}
A.flavus	9^{b}	5.3^{b}	0.0^{a}	0.0^{a}	9^{b}	9^{b}	4.3^{b}	0.0^{a}
A.fumigatus	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	9^{b}	9^{b}	7.4^{c}	3.2^{b}

Means in each column having the same superscript letters are not significantly different a $p \leq 0.05$

Table.3 Inhibitory effect of Lemongrass and Peppermint oil volatiles on spore germination of *Aspergillus* spp.

Fungi	**Lemongrass (µl/0.4l air space)**			**Peppermint (µl/0.4l air space)**		
	5	**10**	**15**	**10**	**15**	**20**
A.niger	83^{b}*	100^{b}	100^{a}	11^{b}	82^{b}	100^{b}
A.flavus	31^{a}	68^{a}	100^{a}	9^{a}	48^{a}	72^{a}
A.fumigatus	96^{c}	100^{b}	100^{a}	13^{c}	42^{a}	68^{a}

Means in each column having the same superscript letters are not significantly different a $p \leq 0.05$*Results expressed in percent of spore germination inhibition in comparison with the control assay

Table.4 MIC and MLC of response to volatiles of oil of Lemongrass and Peppermint against *Aspergillus* spp.

Fungi	Lemongrass (µl/0.4l air space)				Peppermint (µl/0.4l air space)			
	MIC range	MIC	MLC range	MLC	MIC range	MIC	MLC range	MLC
A.niger	1.25-10	**5**[a]	1.25-10	**5**[a]	3.25-26	**13**[a]	6.25-50	**25**[a] **25**[a] **30**[b]
A.flavus	6.5-26	**13**[b]	8.5-34	**17**[b]	4.5-36	**18**[b]	6.25-50	
A.fumigatus	1.5- 6	**3**[a]	0.62-10	**5**[a]	5.75-46	**23**[c]	7.5-60	

Means in each column having the same superscript letters are not significantly different a p≤0.05

Table.5 MIC of major components of Lemongrass and Peppermint oil against *Aspergillus* spp.

Fungi	Lemongrass (µg/0.4l air space)					Peppermint (µg/0.4l air space)					
	MIC range	Citral	Myrcene	MIC range	Citral + Myrcene	MIC range	Menthone	MIC range	Menthol	MIC range	Menthone +Menthol
A.niger	0.87-14	**3.5**[a]	**-***	1-8	**4**[a]	1-8	**4**[a]	0.87-14	**3.5**[a]	2.5-10	**5**[a]
A.flavus	0.87-14	**7**[b]	-	2.2-9	**4.5**[b]	1-8	**4**[a]	2.25-9	**4.5**[b]	2.5-1	**5**[a]
A.fumigatus	0.87-14	**7**[b]	-	2.2-9	**4.5**[b]	1-8	**4**[a]	2.25-9	**4.5**[b]	2.5-10	**5**[a]

Means in each column having the same superscript letters are not significantly different a p≤0.05, *no activity recorded

Myrcene showed no activity against *Aspergillus* spp. The Peppermint components, menthone plus menthol exhibited the highest MIC value (5µg) against all of the fungi tested. The tested fungi also showed equal response toward menthone (4µg),while *A.niger* was more sensitive to menthol (3.5µg) compared with *A.flavus* and *A.fumigatus* (4.5µg).

When examined under light microscope after exposure to lemongrass oil volatiles (5 µl/0.4l air space) *A.niger* showed some morphological abnormalities (Figure 2ABC). Control treatments showed a regular structure and clearly visible striegmata bearing conidia. Following Lemongrass volatile oil treatment mycelia of *A. niger* appeared to present morphological changes, included decreased sporulation, less pigmentation and a reduction and distortion of conidiophores. Electron microscope examination of *A. niger* following treatment showed marked alterations in conidial spore morphology as evidenced by distorted and swollen cells (Figure 2D).

Preliminary phyto chemical screening

The antifungal activity of lemongrass and peppermint volatiles against some

Aspergillus spp., known to cause respiratory infections, were tested using the inverted Petri dish technique. A preliminary screening showed that various concentrations of all of tested volatile oils exhibited varying degrees of antifungal activity against tested pathogens. The maximum antimycotic activity was shown by Lemongrass followed by Peppermint, which proved active against both fungal mycelium and spore germination (Tables 2, 3 and Figure 1). Volatile aromatic plant components have been widely shown to exhibit greater antimicrobial activity than do nonaromatic volatile oils (Wang *et al*., 2005), and the results presented here are in general agreement with previous studies showing that Lemongrass and Peppermint volatiles exhibit antimicrobial activity (Sivropoulou, *et al.*, 1995, Cowan, 1999, Hammer *et al*., 1999, Delespaul *et al*., 2000; Inouye *et al*., 2000, Bansod and Rai, 2008). Peppermint oil has also been shown to be fungistatic, this result is consistent with findings of El Naghy *et al*., 1992, Mimica-Dukic *et al*., 2003. The major constitutes of the oils were tested in an attempt to explain which are inhibitory to the tested fungi. Generally, crude oils are more effective in inhibiting fungal growth than are the separated compounds. Lemongrass oil, citral major fraction (neural+geranial; 70.17%) exhibited the greatest antifungal activity, while myrcene showed no activity. A combination of citral and myrcene showed low MIC compared with citral alone so that a synergistic effect may have operated when the two components occurred together. Mitchell *et al*., (2010) similarly found that the activity of complete essential oils is higher than each separated compound. Citral (3,7-dimethyl-2,6-octadienal) is the major component of lemongrass oil and is present at levels of around 65–85%; it comprises a natural mixture of two isomeric acyclic monoterpene aldehydes: geranial (trans-citral, citral A) and neural (cis-citral, citral B) (Rauber *et al*., 2005). Early studies have shown that both Lemongrass and citral exhibit effective antimicrobial activity (Silva *et al.*, 2008; Saddiq and Suzan, 2010). Furthermore, the antimicrobial potential of Lemongrass has been significantly correlated with citral concentration (Onawunmi *et al.*,1984; Onawunmi,1989; Wannissorn *et al*., 1996). Edris and Ferrag (2003), Kalemba

Figure.1 Growth inhibition of *A.niger* treated with Lemongrass oil volatiles at concentrations of 5,10,15 µl/ 0.4l air space.

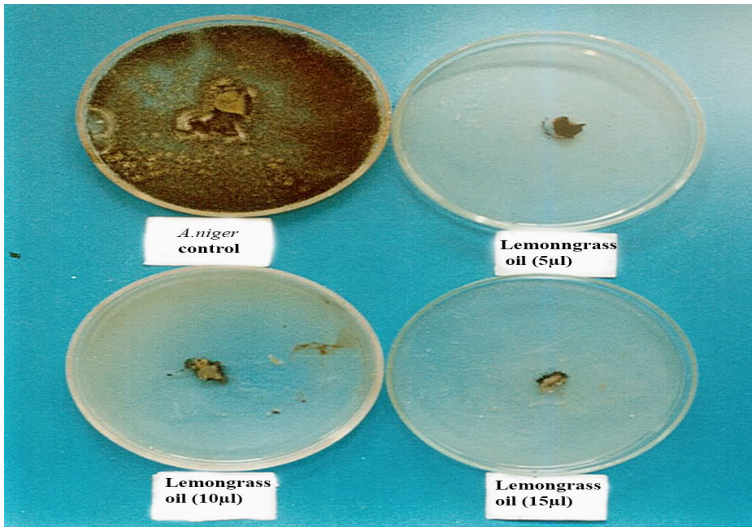
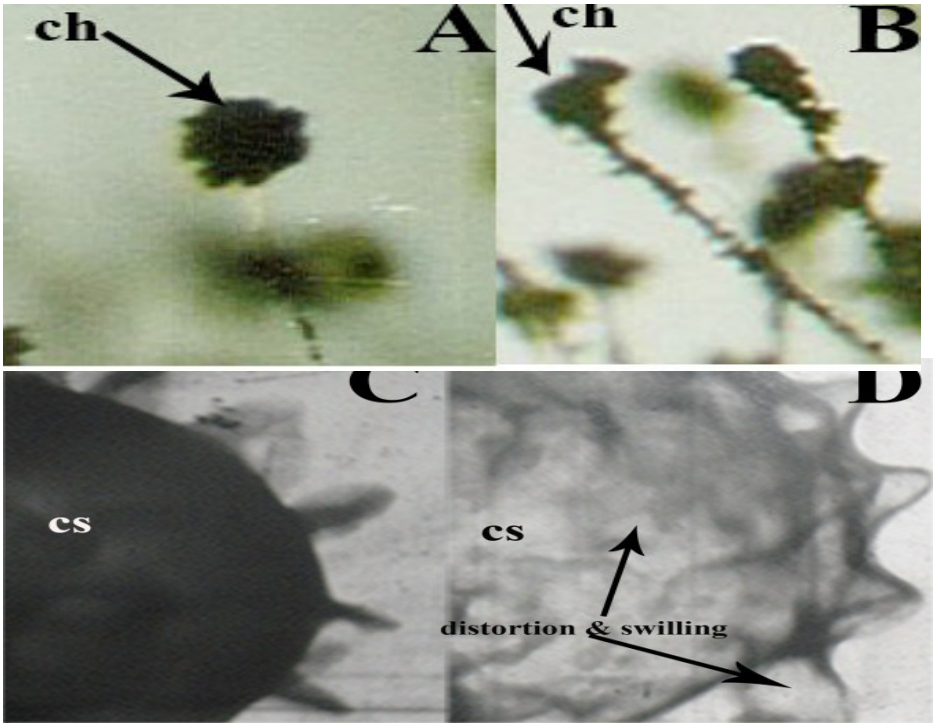


Figure.2 Morphological changes induced by Lemongrass oil vapors on *A.niger*: light microphotographs of conidial heads (ch) and conidiophores (A) without treatment (control) (B) with oil treatment. Transmission electron micrograph of conidial spore (cs) (C) without treatment (control) (D) with oil treatment. Note, distortion with swelling on surface



and Kunicka, (2003) and Hamza *et al*., (2009), have also demonstrated synergistic effects of the different compounds which are present in natural essential oils; our results are therefore in agreement with previous findings.

Previous investigations on *M. piperita* oil composition are consistent with our results in which menthol and menthone were found to be the major fungus-inhibitory compounds (Rohloff, 1999; Maffei, 1999; Gerherman *et al*., 2000; Aflatuni *et al*., 2000). Based on the findings from a variety of laboratory studies, Peppermint oil and menthol have been shown to possess action against a variety of fungi, including *Aspergillus niger, A. flavus, A. fumigatus, A. sulphureus, A. austriaca, Candida* spp., *Fusarium* spp, *Trichophyton* spp., *Penicillium chrysogenum, Mucor fragilis,* and *Rhizopus stolonifer* (Duarte *et al*., 2005; Inouye *et al*,, 2007; Agarwal *et al*., 2008; van Vuuren *et al*., 2009; Yigit *et al*., 2009). Then, antimicrobial activity of the peppermint oil samples decreased in the order of total content of 1,8-cineole, menthol and β-caryophyllene decreasing (Gochev *et al*., 2008).

Microscope studies on the susceptibility of *A.niger*, to Lemongrass show that the fungus underwent major morphological changes induced by lemongrass stress (Figure 2) which were generally consistent with the results of earlier publications and confirm the fungicidal effect of Lemongrass. Earlier studies have also demonstrated the inhibitory effect of lemongrass on hyphal growth and spore formation in *A. niger,* as well as plasma

membrane disruption and mitochondrial structure disorganization (de Billerbeck *et al*., 2001; Helal *et al*., 2006; Pawar and Thaker, 2006). Additionally, Sharma and Tripathi, 2008) showed that Lemongrass volatiles induced a loss of cytoplasm in fungal hyphae which became markedly thinner, associated with budding of hyphal tips.

The present study confirms the antifungal activity of Lemongrass and Peppermint against some respiratory *Aspergillus* spp. pathogens by the use of the measurement of radial mycelia growth, spore germination, MIC, MLC, and morphological alterations. The study suggests that the volatiles from these oils could be us effectively used in antifungal therapy against respiratory pathogens. However, before "volatile oil therapy" can be applied in clinical practice further studies are required to determine its applicability and the possibility of toxicity.

In conclusion, essential oils from Lemongrass and Peppermint could be utilized, from the vapor phase, to control the growth of pathogenic fungi. A single component of Lemongrass oil like citral can substitute the whole oil at the same dose level. Although, the myrcene have no antifungal activity, when added to citral the activity is enhanced and the dose level can be reduced. The investigation demonstrates the possibility using essential oil vapors against pathogenic fungi. The procedure provides natural, nontoxic, economically feasible and effective antifungal agents.

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