

Cymbopogon citratus L. essential oil as a potential antifungal agent against key weed moulds of *Pleurotus* spp. spawns

J. J. Mahanta,¹ M. Chutia,¹ M. Bordoloi,¹ M. G. Pathak,² R. K. Adhikary¹ and T. C. Sarma^{1*}

¹ Division of Medicinal, Aromatic and Economic Plants, North East Institute of Science and Technology (CSIR), Jorhat-785006, Assam, India

² Division of Analytical Chemistry, North East Institute of Science and Technology (CSIR), Jorhat-785006, Assam, India

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ABSTRACT: The essential oil of *Cymbopogon citratus* L. (250–1500 p.p.m.) was tested *in vitro* for antifungal activity against seven *Pleurotus* spp. spawn-contaminating fungi, viz. *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Alternaria alternata*, *Penicillium citrinum*, *Curvularia lunata* and *Trichoderma harzianum*. Oil-enrichment resulted in significant ($p < 0.05$) reduction of growth for the pathogens examined. Fungal spore production inhibited up to 80% at 250 p.p.m. of the oil except for *C. lunata*, which was inhibited by only 30%. In the higher oil concentration (1500 p.p.m.) employed, fungal sporulation was completely retarded. Lemongrass oil reduces spore germination in *A. flavus*, *A. fumigatus*, *A. alternata*, *P. citrinum* and *T. harzianum*, with the effects dependent on oil concentration. However, lemongrass oil at 250 p.p.m. accelerated spore germination for *A. niger* and *P. citrinum*. Indeed, further increase in oil concentration shows complete inhibition. Among the pathogens, *C. lunata* was found to be most resistant strain, while *A. niger* was the most sensitive strain against lemongrass oil. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: antifungal activity; *Cymbopogon citratus*; essential oil; fungal contaminants; *Pleurotus* spp. spawn

Introduction

Mushrooms are considered to be natural and healthy food products, originating from an environmental friendly organic farming system¹ and cultivated worldwide, especially in south-east Asia, India, Europe and Africa. Mushrooms are reported to contain an appreciable amount of dietary fibre, with high protein (30–40% on a dry weight basis) and have gourmet food quality;^{2,3} they are reported to decrease the cholesterol level, lower cholesterolaemia, modulate the immune system and inhibit tumoral growth.^{4,5} Production of *Pleurotus* sp. accounted for 14.2% of the total world output (6 161 000 tonnes) of edible mushrooms.⁶

Mushroom spawn spoilage due to contaminant microflora is significant, causing 15–20% spawn loss, and poses a major problem in mushroom cultivation.^{7,8} Because of the nature of current growing conditions, i.e. the use of animal manures, plant materials, chemical fertilizers and other agricultural residues as substrates, the cultivation of the mushroom is susceptible to many competitive

organisms and weed fungi, causing substantial loss to growers due to decrease in yields.⁹

Mushroom growers commonly use synthetic pesticides and fungicides, as these are traditionally considered to be the cheapest and most effective measures to control fungal pathogens. However, because of recent reports about the deleterious effects of some synthetic pesticides on the health of consumers and the problem of fungicide resistance by pathogens, there is a demand for the development of new, safe, biodegradable alternative ‘natural’ fungicides having maximum efficacy with minimal environmental impact and danger to the consumers.^{10,11}

Essential oils (EOs) of some plants have recently proved to be successful bio-control agents that are non-hazardous, easily biodegradable and eco-friendly.^{9,12,13} There are many reports of antimicrobial, antifungal, antioxidant and radical-scavenging properties^{9,14} of spices and EOs and in some cases a direct food-related application has been tested.¹⁵ Lemongrass (*Cymbopogon citratus* L.) is a plant in the grass family that contains 1–2% EO on a dry weight basis, with wide variation of the chemical composition as a function of genetic diversity, habitat and agronomic treatment of the culture.¹⁶ The EO of lemongrass is characterized by a high content of citral (neral and geranial isomers, c. 69%), which is used as a raw material for the production of ionone, vitamin A and β -carotene.¹⁷ Lemongrass oil exhibits a broad spectrum

* Correspondence to: T. C. Sarma, Division of Medicinal Aromatic and Economic Plants, North East Institute of Science and Technology (CSIR), Jorhat-785006, Assam, India.
E-mail: drtcsarma@yahoo.com

of fungitoxicity by inhibiting several fungal species at different concentrations and its fungitoxic potency remains unaltered for 210 days of storage, after which it starts to decline. Moreover, the EO of *C. citratus* was superior to synthetic fungicides such as Agrosan GN, Dithane M-43 and copper oxychloride.^{18,19} There is therefore considerable interest in the application of lemongrass oil for the preservation of stored food crops.¹⁸

The US National Toxicology Program (NTP) reported that citral did not cause cancer in male or female rats receiving 4000 p.p.m. (~3.56 mg/ml) citral in the feed for 2 years.²⁰ The acceptable daily intake is 5 mg citral/kg body weight and citral was given Generally Recognized As Safe (GRAS) status in the USA.²⁰

The aim of this study was to determine the efficacy of *C. citratus* oil against spawn-contaminating fungi, with emphasis for the possible future use of this EO as an alternative antifungal compound.

Materials and Methods

Plant Material and Microbial Culture

Aerial parts of *C. citratus* were collected from the farm of the North East Institute of Science and Technology (NEIST), Jorhat, Assam, India, in July 2005. Maintained cultures of *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Alternaria alternata*, *Penicillium citrinum*, *Curvularia lunata* and *Trichoderma harzianum* were obtained from the Mushroom Unit of NEIST, Jorhat, isolated from oyster mushroom spawn. Cultures were maintained aseptically by subculturing onto potato dextrose agar (PDA) slants, incubated in the dark at 25 °C for 1 week and stored at 4 °C for long-term use.

Oil Isolation and GC Analysis

EO from *C. citratus* L was extracted by hydro-distillation for 3 h, using a Clevenger-type apparatus. GC analysis of the sample for the evaluation of its components was carried out on a Chemito 8510 GC instrument equipped with a data processor. A BP-5 wide-bore capillary column (30 m × 0.53 mm i.d., 1.0 µm film thickness) was used for the separation of the sample components (sample size 0.03 µl, measured using a Hamilton GC syringe of 1.0 µl cap.). Hydrogen was used as the carrier gas at a flow rate of 5 ml/min and 20 p.s.i. inlet pressure; split ratio, 1:20. The GC column oven temperature was programmed from 70 °C to 210 °C at a rate of 2.5 °C/min, with a final hold time of 5 min. Both injector (packed on column injector fitted with a wide-bore column adaptor) and detector (FID) temperatures were maintained at 230 °C. Gas chromatography–mass spectroscopy (GC–MS) analysis was carried out on a Trace DSQ MS (Thermo Electron

Corporation), using a BP-5 capillary column (30 m × 0.25 mm i.d., 0.5 µm film thickness); with helium as the carrier gas at a flow rate of 1 ml/min; split ratio 1:20. The column temperature was programmed from 65 °C to 210 °C (10 min hold) at 3 °C/min. Mass spectra were recorded in the range 50–450 amu, operating at 70 eV, and the ion source temperature was maintained at 200 °C. The constituents of the oil were identified by matching the retention times with standard reference compounds and also by matching the mass spectra fragmentation pattern with NIST Mass Spectra Library stored in the GC–MS computer library.

Test for Antifungal Activity: Agar Dilution Method

Antifungal activity on fungal colony development was obtained by a dilution method (250 p.p.m., 500 p.p.m., 1000 p.p.m. and 1500 p.p.m.) of EO (*C. citratus*) in potato dextrose agar (PDA). The oil was dissolved in 5% Tween 20 and added to the 20 ml PDA before solidification in a Petri dish. One disc (0.5 cm diameter) of mycelial plug, taken from the edge of a 4–6 day fungal culture, was placed into the Petri dish and incubated at 25 °C for 1 week. Controls consisted of 5% Tween 20 mixed with PDA and these were handled similarly, with the exception of the volatile treatment. The radial growth of the mycelium was recorded and the efficacy of the EO was evaluated by the percentage mycelial growth inhibition, using the formulae of Pandey *et al.*²¹

$$\text{Growth inhibition (\%)} = \left(\frac{\text{DC} - \text{DT}}{\text{DC}} \right) \times 100$$

where DC is the diameter of control and DT is the diameter of fungal colony with treatment.

Micro-atmosphere Test

The EO was investigated by the micro-atmosphere method to determine the antifungal activity of the vapour phase of the EO, which diffuses towards the agar in an inverted Petri dish method.²² Petri dishes were filled with PDA and one disc (0.5 cm diameter) of mycelial plug taken from the edge of a 4–6 day fungal culture was placed into the Petri dish. The Petri dishes were then inverted and 1 µl EO (250 p.p.m., 500 p.p.m., 1000 p.p.m. and 1500 p.p.m.) impregnated on sterile filter paper discs (4 mm diameter) were attached to the inverted lid (1 disc per lid). The Petri dishes were wrapped with parafilm along the rim, inverted and incubated for 7 days at 25 ± 1 °C in an incubator. The radial growth of the mycelium was recorded and results were expressed as percentage fungal colony growth.

Spore Production and Spore Germination Assay

Spores from colonies incubated for 6–10 days (until spore formation) of *A. niger*, *A. flavus*, *A. fumigatus*, *P. citrinum*, *A. alternata* and *T. harzianum*, previously exposed to lemongrass oil enrichment (250 p.p.m., 500 p.p.m., 1000 p.p.m. and 1500 p.p.m.), were collected by adding 5 ml sterile water containing 0.1% v/v Tween 80 (for better spore separation) to each Petri dish and rubbing the surface three times with a sterile L-shaped spreader. The suspension was collected and then centrifuged at room temperature at $2000 \times g$ for 5 min. The supernatant was discarded and the pellet recentrifuged until 1 ml highly concentrated spore solution remained. A haemocytometer slide was used to count spore concentration.

Fungistatic and fungicidal effects on spore viability were examined following oil treatments. Spores from 6–10 day cultures of *A. niger*, *A. flavus*, *A. fumigatus*, *P. citrinum*, *A. alternata* and *T. harzianum*, previously exposed to lemongrass oil enrichment (250 p.p.m., 500 p.p.m., 1000 p.p.m. and 1500 p.p.m.), were collected as described above. The spore suspensions were incubated at 25 °C for 24 h, and for each treatment 100 spores were examined and the extent of spore germination assessed by looking for the presence of a germ tube. The results were expressed in terms of the percentage of spore germination.

Statistical Analysis

All the experiments were repeated twice. The data were first tested for Normality and then subjected to analysis of variance (ANOVA). Significant differences between values were determined using Duncan's multiple range test ($p < 0.05$), following one-way ANOVA. Statistical analysis was performed using STAT4U v. 1.0 and graphs were produced using Graph Pad Prism v. 5.0.

Results and Discussion

The relative amount (%v/w) of EO of fresh leaves of *C. citratus* is 0.4% (SE \pm 0.006) and the chemical analysis of the EO investigated is presented in Table 1. The chemical composition of *C. citratus* oil consists of monoterpene compounds, hydrocarbons, ketones, aldehyde and esters. The GC method was used to identify and quantify these compounds. The oil was characterized by a high percentage of citral (73.3%; neral 31.3% and geranial 42.0%), followed by α -phellandrene (6.9%), geraniol (2%), neryl acetate (1.7%) and linalool (1.2%). Citral as a major component (>70%) of *C. citratus* EO was also reported by many other workers.^{14,23–25} The oil yield and its composition have minor differences from the data reported by Cimanga *et al.*²⁶ and Sacchetti

Table 1. Major composition percentage of the *C. citratus* EO

Sl. No.	Compound	RT	(%)
1	β -Pinene	2.780	0.1
2	α -Phellandrene	3.473	6.9
3	Limonene	4.233	0.1
4	(Z)- β -Ocimene	4.440	0.5
5	(E)- β -Ocimene	6.493	0.3
6	Terpinolene	5.867	0.4
7	Linalool	6.053	1.2
8	Citronellal	8.060	0.7
9	Borneol	8.667	0.7
10	Citronellol	10.427	0.5
11	Neral	10.960	31.3
12	Geraniol	11.400	2.0
13	Geranial	12.160	42.0
14	Neryl acetate	17.127	1.7
15	Caryphyllene	20.787	0.4
	Total identified		88.8

RT, Retention time.

*et al.*¹⁴ These differences may be due to genetic diversity, habitat and/or agronomic treatment.¹⁶

The EO of *C. citratus* at 250 p.p.m. significantly ($p < 0.05$) reduced the colony development of *T. harzianum* (65.65%), *A. flavus* (73.8%), *A. fumigatus* (50.6%), *A. niger* (76.5%), *A. alternata* (66.3%), *P. citrinum* (63.15%) and *C. lunata* (32.95%) (Figure 1). Moreover, the highest oil concentration employed (1500 p.p.m.) revealed complete (100%) inhibition on fungal colony development for all the pathogens in the study. EO at 1000 p.p.m. completely inhibited the growth of *A. flavus*, *A. niger*, *A. alternata* and *P. citrinum*, whereas against *T. harzianum*, *A. fumigatus* and *C. lunata* the percentage inhibition was 88–99.9%. Oil at 500 p.p.m. shows complete inhibition against *A. niger* and *A. flavus*. The plates were observed for 10 days incubation but no further fungal growth was seen in any of the treatments.

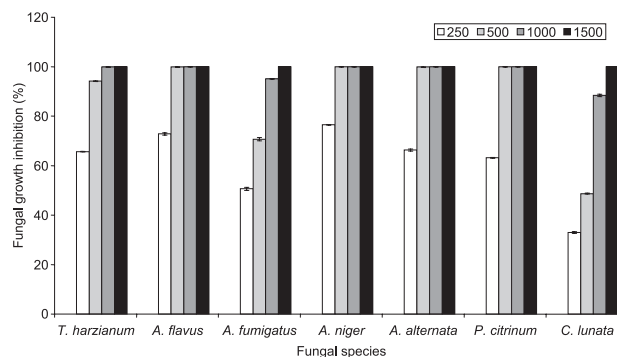


Figure 1. Impact of lemongrass (*Cymbopogon citratus* L) EO in agar dilution method on fungal growth inhibition of the seven fungal isolates causing spawn contamination raised on PDA (significant difference between the effects of *C. citratus* oil among the fungi at $p < 0.05$, ANOVA test)

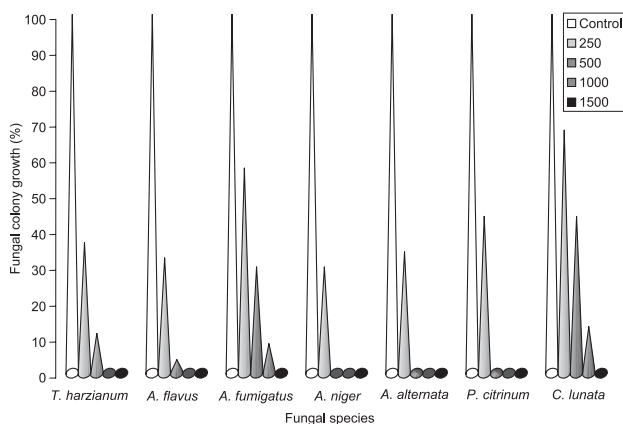


Figure 2. Antifungal activity of *C. citratus* oil in micro-atmospheric method against the seven fungal isolates (significant difference between the effects of *C. citratus* oil among the fungi at $p < 0.05$, ANOVA test)

The antifungal activity of lemongrass oil by a micro-atmospheric method (Figure 2) showed almost the same result as in the agar dilution method, showing complete inhibition at 1500 p.p.m. for all the pathogens *per se*. However, the micro-atmospheric method was more effective than the agar dilution method, complete inhibition of *T. harzianum* being observed at 1000 p.p.m. (1500 p.p.m. was required in the agar dilution method). The experimental results indicated that *C. lunata* was found to be most resistant strain among the pathogens tested, while *A. niger* was the most sensitive.

The results were qualitatively in good agreement with the antifungal activity; however, the percentage inhibition was different in *C. citratus* EO collected in other geographical areas against some pathogenic fungi, as reported by Sokovic and Griensven,⁹ Nguefack *et al.*²⁷ and Adegoke and Odesola.¹⁹ Baratta *et al.*²⁸ reported 91% inhibition of the growth of *A. niger* in liquid media when treated with 1000 p.p.m. lemongrass oil. The culture medium, the technique of testing, the biochemical sources of the plant, the age of the plant, the state of the plant material used (fresh or dried) and the isolation technique are some factors implicated in the variation of the activity.^{29,30}

The antimicrobial activity of lemongrass oil is believed to be associated with phytochemical components of the lemongrass powder, such as alkaloids, tannins and cardiac glycoside.¹⁹ EO and related substances act to make the cell membrane of the fungus permeable, causing leakage.³¹ Palhano *et al.*³² suggested that *Colletotrichum gloeosporioides* treated with citral resulted in a decrease of media pH, indicating a loss of cell membrane integrity.

Fungal sporulation in PDA was significantly ($p < 0.05$) inhibited by the EO, with spore production of 48.85% (*T. harzianum*), 26.5% (*A. flavus*), 46.9% (*A. fumigatus*), 23.25% (*A. niger*), 32.8% (*A. alternata*), 22.65% (*P.*

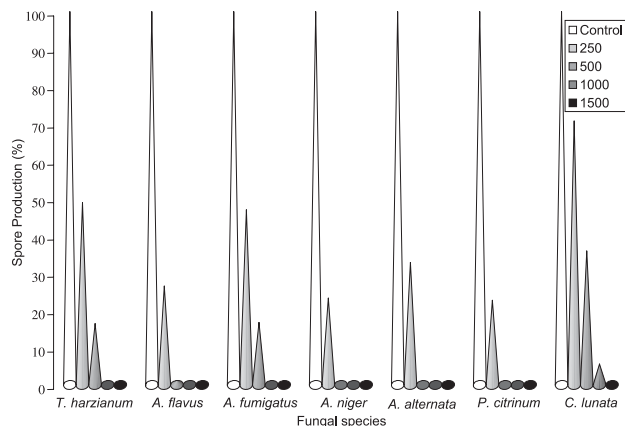


Figure 3. Effect of different concentrations of *C. citratus* oil on the spore production of the seven fungal isolates (significant difference between the effects of *C. citratus* oil among the fungi at $p < 0.05$, ANOVA test)

citrinum) and 70.7% (*C. lunata*) at 250 p.p.m. (Figure 3). Moreover, spore production was completely inhibited at 1000 p.p.m. for all the pathogens tested except *C. lunata* (5.65%). The higher oil concentration employed (1500 p.p.m.) revealed complete inhibition in spore production for all the pathogens tested. Previous studies reported that sporulation of *A. flavus* was completely inhibited by *C. citratus* (2800 p.p.m.) when used as fumigants, whereas aflatoxin production was inhibited at 100 p.p.m.²³ The variations in antimicrobial activity of EOs may be due to their different chemical compositions.

Spore germination of the pathogens in PDA was strongly inhibited in the presence of lemongrass oil (Figure 4). The inhibition of spore germination was statistically significant ($p < 0.05$) except for *A. niger* and *P. citrinum*.

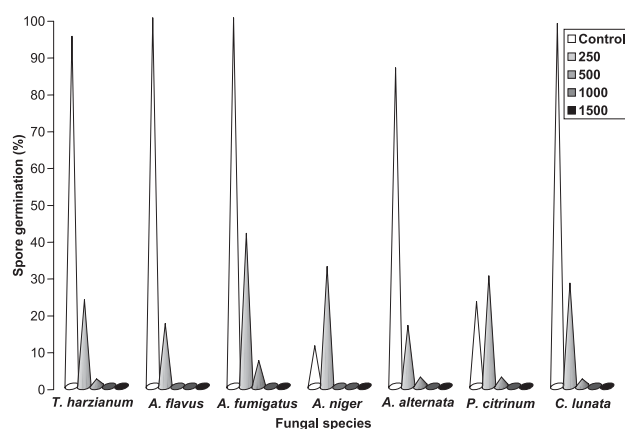


Figure 4. Effect of different concentrations of *C. citratus* oil on the spore germination of the seven fungal isolates (significant difference between the effects of *C. citratus* oil among the fungi at $p < 0.05$, ANOVA test, except for *A. niger* and *P. citrinum*, showing increase in spore germination at 250 p.p.m.)

EO at 1000 and 1500 p.p.m. showed complete inhibition of spore germination for all the pathogens tested. Lemongrass oil at 250 p.p.m. accelerated spore germination for *A. niger* and *P. citrinum*; however, a further increase in oil concentration showed complete inhibition. Palhano *et al.*³² also reported that spores of *C. gloeosporioides* treated with a low concentration of citral and lemongrass oil (0.15 mg/ml) showed a slight increase in spore germination. Filtenborg *et al.*³³ reported that the germination of *Penicillium digitatum* conidia was stimulated by certain combination of the volatiles surrounding wounded oranges, notably limonene, β -pinene, sabinene, β -myrcene, acetone, ethanol and CO₂. This might be due to the mechanism developed by some fungal pathogens of using the secondary metabolites as a signal to initiate germination, appressorium formation and infection.³⁴ The inhibitory effect of citral and lemongrass crude oil on spore germination was higher at concentrations >0.45 mg/ml.³² Mishra and Dubey¹⁸ found that the EO of *C. citratus* not only exhibited a broad antifungal spectrum but was superior to synthetic fungicides such as Agrosan FN, Dithane M43 and copper oxychloride.

Geraniol and citral isomers should probably account for the antifungal activity of lemongrass.²⁴ Kurita *et al.*³⁵ have shown that citral acts as a fungicidal agent because it is able to form a charge transfer complex with an electron donor to fungal cells, which results in fungal death. Trombetta *et al.*,³⁶ Palhano *et al.*³² and Cox *et al.*¹² reported that several monoterpenes were found to affect the structural and functional properties of the lipid fraction of the plasma membrane of fungi, causing intracellular material to leak, which proves effective against growth and conidia germination of *C. gloeosporioides* and inhibits the respiratory enzymes. Griffin *et al.*³⁷ suggested that the free OH group of phenol and alcohol might be a key to the antimicrobial activity.

It has been observed that the oil acts not only as a fungicidal agent but also inhibits sporulation of the pathogens. Treatment was effective as a solution or by contact. Even as a vapour treatment it was very effective, allowing fungal growth and sporulation to be inhibited by a small amount of oil. Suppression of spore production by oil treatment could make a major contribution to limiting the spread of the pathogen by lowering the spore load. The impact on sporulation may reflect the effects of volatiles emitted by the oil on the surface of developing mycelia, and thus the 'platform' to support spore production involved in the switch from vegetative to reproductive development.

However, there is a limitation to the potential application of EO in foods, since their effectiveness as preservatives has generally been found to decrease significantly when they are tested in real foods.³⁸ This reduction has been attributed to the high protein and fat contents of some food products, which can mask the antimicrobial effect of EO.³⁹ The availability of oxygen also affects the

antimicrobial efficacy of the oil. Indeed, Paster *et al.*⁴⁰ observed that the antimicrobial activity of oregano EO on *Staphylococcus aureus* and *S. enteritidis* was greatly enhanced when these organisms were incubated under microaerobic or anaerobic conditions. Under such condition (low oxygen tension) there would be less oxidative change in the EO.⁴⁰

The results of the present study indicate that *C. citratus* oil may be used as an alternative for synthetic chemicals that are applied to prevent and cure the most important diseases in mushroom cultivation. Further studies are required to develop strategies for practical application.

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