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Research Article

In vitro antifungal activity of oil of *Cymbopogon citratus* and citral alone and in combination with fluconazole against azole-resistant strains of *Aspergillus fumigatus* and *Trichophyton rubrum*

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ABSTRACT: **Background:** Curing of invasive and superficial mycoses by the available antifungal drugs is limiting because of the host toxicity and emergence of drug resistance in fungi. This has triggered search for anti-infective agents from natural products and alternative modes of antifungal therapy. **Objective:** The aim of this study was to evaluate *in vitro* antifungal activity of oil of *Cymbopogon citratus* and its major active compound citral alone and in combination with fluconazole against azole-resistant strains of *Aspergillus* spp and *Trichophyton* spp. **Materials and Methods:** The methodology adapted in this work included disc diffusion, broth macrodilution, time kill methods and checkerboard microtiter tests. **Results:** Test fungal strains exhibited higher levels of resistance to fluconazole and itraconazole (MIC range from 100 to 800 µg ml^{-1}). Both the oil of *C. citratus* and citral exhibited promising antifungal activity (zone of inhibition from 24.66 to 42.00 mm) and killing potency against *Aspergillus fumigatus* MTCC2550 and *Trichophyton rubrum* IOA-9. Citral showed strongest synergy with fluconazole against *A. fumigatus* and *T. rubrum* by reducing the minimum inhibitory concentration of fluconazole up to eight-fold. **Conclusion:** The study has highlighted the broad spectrum antifungal activity of oil of *C. citratus* and citral and their synergy with fluconazole against azole-resistant strains of *Aspergillus* spp and *Trichophyton* spp.

KEYWORDS: *Cymbopogon citratus*, citral, azole-resistance, synergy

INTRODUCTION

The incidence of mycoses caused by various pathogenic and opportunistic groups are on the rise in the different parts of the world as a consequence of growing number of immunocompromised patients with HIV infections, cancer chemotherapy and organ or bone marrow transplantations.[1] Due to the excessive and indiscriminate use of drugs emergence and spread of drug-resistant strains have become a common problem today. Invasive aspergillosis caused by *Aspergillus* spp is considered as a major cause of morbidity and mortality in immunocompromised hosts and mortality rates may range from 40 to 90% in high risk populations.[2,3] Other chronic fungal infections associated with the immuno-compromised patients including those of hair, skin and nails, are primarily caused by *Trichophyton rubrum*. Such infections carry considerable morbidity and can become serious in immunocompromised patients, resulting in invasive infections.[4]

With the increasing number of immunosuppressed patients at an unprecedented rate, the management of these fungal infections would be a definite challenge to mankind. Fluconazole is considered to be one of the safest antifungals used in the treatment of these fungal infections but the fungistatic nature and the development of resistance in fungi have restricted the use of fluconazole.[5] Recently, several studies have reported azole-resistance in *Aspergillus* spp and *Trichophyton* spp.[6–8]

Therefore, alternative modes of therapy and the discovery of new anti-infectives with novel modes of action from various sources including natural products are

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needed. Plant products, especially essential oils, have been traditionally used in ethnomedicine as effective antifungal agents against infectious diseases caused by yeasts and moulds, have been expected to deliver newer antifungal compounds and can be exploited as alternative therapy alone or in combination with known antifungals. In particular, oils of *Cymbopogon citratus* (lemongrass) has long been used in traditional practices by many ancient cultures. In Ayurveda, the traditional system of healing in India, lemongrass oil is used to treat hypertension, fever, stomach disorders, and to reduce pain and inflammation associated with rheumatism, cold and flu, and bacterial and fungal infections of throat, urinary and vaginal tract. [9,10]

Combinations of two or more antifungal drugs have been exploited to achieve better therapeutic action against invasive and systemic mycoses. Combination therapy has potential advantages over monotherapy in terms of reducing dose related toxicity and emergence of drug resistance.[11] The essential oils showing promising activity might synergistically interact with antifungal drugs and therefore need to be explored.

Some *in vitro* studies have reported the synergistic interaction of certain oils with fluconazole and amphotericin B against *Candida* isolates.[12,13] However, no reports on synergistic interaction of *C. citratus* with antifungal drugs against azole-resistant strains of fungi are available. Therefore, the antifungal activities of oil of *C. citratus* and its major active compound citral were determined against the eight strains of human pathogenic fungi. Further, to obtain effective combinational synergy, interactive behaviors of *C. citratus* and citral with fluconazole were determined against azole-resistant strains of *Aspergillus fumigatus* MTCC2550 and a clinical isolate of *Trichophyton rubrum* IOA-9.

MATERIALS AND METHODS

Plant essential oils and drugs

Oils of *Cymbopogon citratus* (lemongrass) and citral were purchased from Aroma Sales Corporation, New Delhi, India. The drug powder of fluconazole was purchased from Himedia Laboratories, Mumbai, India. Stock solution of fluconazole was prepared in dimethyl sulphoxide (DMSO) at a concentration of 25 mg ml^{-1} and stored at −20 ºC until used. The purity of oils and active compounds was determined by physico-chemical analyses such as specific gravity, refractive index, optical rotation and solubility in alcohol (data not shown) at Fragrance and Flavour Development Centre, Kannauj, India. Chemical composition of oil of *C. citratus* was determined by gas chromatography-mass spectrometry at Advanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi, India.[14] Essential oils were diluted ten times in 1% DMSO and used in assays.

Fungal strains

The strains included in this study were *Aspergillus flavus* NRRL501, kindly provided by fungal culture collection at Agricultural Research Service, USDA, Peoria, USA; *Aspergillus fumigatus* MTCC2550, *Alternaria solani* MTCC2101, *Fusarium oxysporum* MTCC284, *Mucor rouxii* MTCC386, *Trichophyton rubrum* MTCC296 were purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. In addition, two clinical isolates of *Aspergillus niger* (IOA-3) and *Trichophyton rubrum* (IOA-9), one each, were obtained from Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, India and are maintained at departmental culture collection. All the strains were grown in Sabouraud Dextrose Broth (SDB), solidified with 1.5% (w/v) agar, if required, at 28 ± 2 °C for 7 days.

Disc diffusion assay

The disc diffusion assay with some modifications as adapted by Sokovic and Griensven[15] was performed to determine the sensitivity of fungal strains against the antifungal drugs and essential oils or active compounds. Briefly, 100 µl of spore suspension (1.5×10^5 CFU/ml) was spread on to SDA plates and filter paper discs (8 mm, Hi-Media) impregnated with 10 µl of essential oils or active compounds, whereas for drug sensitivity, antifungal drug discs (10–20 µg/disc, Hi-Media), were mounted on the agar surface and the plates were incubated at 28 ± 2 ºC for 2 days. Each experiment was conducted in triplicate and average zone size was measured.

Broth macrodilution assay

The minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of essential oils or active compounds and drugs were determined against the test strains by the broth macrodilution method as adapted by Sokovic and Griensven[15] with some modifications. 10 µl of spore suspension (1.5×10^5 CFU/ml) was added to 1 ml Sabouraud dextrose broth containing serially diluted essential oils, active compounds or drugs along with 0.1% (v/v) Tween 80 and incubated at 28 ± 2 ºC for 2 days. MIC was defined as the lowest concentration that inhibited visible fungal growth while MFC was the concentration at which no growth was observed. Each experiment was repeated three times and mean values were calculated for MICs and MFCs.

Time kill assay

The time-dependent killing of *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9 by the most potent essential oils or active compounds and fluconazole was evaluated using the modified method of Hammer *et al.*[16] Briefly, 20 ml of PBS solution containing 1 × MFC of test agents and 0.001% (v/v) Tween 80 was inoculated with 1 ml of spore suspension (~10^6 CFU/ml). The control solution contained phosphate buffer saline with Tween 80 and fungal inoculum but no essential oils or active compounds or fluconazole. Immediately after inoculation, 100 µl was collected from solutions for viable count. Furthermore, test and control solutions were incubated at 30 ºC and 120 rpm. Viable counts were obtained from ten-fold serial dilutions of test and control solutions at 2, 4, 6, 8, 10 and up to 24 h by plating 100 µl of ten-fold dilution onto SDA plates and incubating at 30 ºC for 24 h. Each experiment was performed in triplicate and the mean colony count for each experiment was converted to values relative to the mean colony count at 0 h to normalize the data and correct the variation in starting inocula concentrations. The relative viable count was plotted against time on a log scale.

Cellular toxicity assay

The toxicity of *C. citratus* and citral was evaluated by the red blood cell (RBC) lysis assay as adapted by Luiz *et al.*,[17] with some modifications. The freshly obtained RBCs of sheep blood were washed with 1 ml of PBS (pH 7.0) and 4 ml was added to 5% (w/v) glucose solution to obtain 4% RBC suspension. 750 µl of PBS containing the desired concentration of test agent was mixed with 750 µl of RBC suspension in Eppendorf tubes and incubated at 37 ºC for 2 h. Triton X-100 (0.1% (v/v) in PBS was used as a positive control whereas 1% DMSO and PBS were used as negative controls. Tubes were centrifuged at 2000 rpm for 10 min and the absorbances of supernatant were read at 540 nm. Percent haemolysis was calculated as: [{(A – B)/(C – B)} × 100]. Where A and B are the absorbance values of supernatant from the test sample and PBS (solvent control) respectively and C is the absorbance value of supernatant from the sample after 100% lysis. Each experiment was performed in triplicate and the mean values were considered for calculation of percent haemolysis.

Interaction of *C. citratus* and citral with fluconazole

A checkerboard microtiter test was performed to evaluate the interaction of essential oils or active compounds with fluconazole against *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9. The series of two-fold dilutions, in eight numbers, of oil or active compound and fluconazole were made in SDB to obtain four times the final concentration being achieved in the microtiter well. Furthermore, 50 µl of each dilution of oil or active compounds was added to the 96 well microtiter plates in the vertical direction, while 50 µl of each dilution of fluconazole was added in the horizontal direction, so that various combinations of oil or active compounds and fluconazole could be achieved. Also, 100 µl of inoculum from spore suspension was added to each well and plates were incubated at 30 ºC for 2 days. The nature of interaction was defined quantitatively by means of fractional inhibitory concentrations (FIC) that were calculated as the MIC of the combination of essential oil or active compound with fluconazole divided by the MIC of essential oil or active compound or fluconazole alone. An FIC index (FICI) was obtained by adding both FICs. The combination result was interpreted as follows: FICI ≤ 0.5, synergistic; >0.5 – 4.0, no interaction; >4.0, antagonistic as described by Odds.[18]

RESULTS

Susceptibilities of fungal strains to essential oils and antifungal drugs

As shown in Table 1, all the tested strains were resistant to fluconazole and itraconazole. In contrast, all the test strains

Table 1: Antifungal activity of oils of *C. citratus* and citral

Test agents	Sensitivity to essential oils and active compounds (diameter of zone of inhibition in mm)							
	A. flavus NRRL 501	*A. fumigatus* MTCC 2550	*A. niger* IOA-3	*A. solani* MTCC 2101	*F. oxysporum* MTCC 284	*M. rouxii* MTCC 386	*T. rubrum* IOA-9	*T. rubrum* MTCC 296
C. citratus	28.66 ± 1.24	25.33 ± 1.24	30.66 ± 1.24	25.00 ± 0.81	27.00 ± 0.81	31.33 ± 1.24	32.66 ± 1.24	24.66 ± 2.05
Citral	32.33 ± 1.24	27.66 ± 1.24	42.00 ± 0.81	35.66 ± 1.69	37.33 ± 1.24	34.33 ± 1.69	33.00 ± 1.63	37.66 ± 1.24
Antifungal drugs								
FLC	–	–	–	–	–	–	–	–
ITC	09.00 ± 0.81	–	09.00 ± 0.47	–	–	–	–	–

All the experiments were performed in triplicates and data is presented as mean ± SD
Each disc contains 10 µl of essential oils or active compounds
FLC; fluconazole (10 µg/disc), ITC; itraconazole (10 µg/disc)
–; indicates no zone of inhibition

were susceptible to oil of *C. citratus* and citral and exhibited zones of inhibition between 24.66 to 42.00 mm. The active compound citral exhibited inhibitory activity greater than its oil *C. citratus*, when tested against drug-resistant fungi and the highest zone of inhibition (42.00 mm) was recorded against *A. niger* IOA-3. *C. citratus* exhibited a maximum zone of inhibition of 32.66 mm against *T. rubrum* IOA-9.

MICs and MFCs of essential oils and antifungal drugs

In vitro antifungal potencies of the broad spectrum oil *C. citratus* and its major active compound citral against the drug-resistant fungi were determined in terms of MICs and MFCs (Table 2). The level of resistance against flucoanzole was higher among the test strains with MIC and MFC values in the range of 200–800 μg/ml and 400–1600 μg/ml, respectively. MIC of itraconazole ranges from 100 to 200 μg/ml and MFC from 200 to 400 μg/ml against the test strains. MIC of test oil/compound ranged from 18–288 μg/ml and MFC (144–576 μg/ml) against the test strains. Citral showed activity higher than *C. Citratus*, with MIC and MFC values of 18 and 36 μg/ml respectively against *A. niger* IOA-3.

Time kill curves

The ability to kill fungal strains by oil of *C. citratus* and citral was compared with fluconazole against *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9. The time-dependent killing of *T. rubrum* IOA-9 by the test agents revealed a difference of >1 $\log_{10}$ in viable counts compared to the control between 8 and 9 h (Figure 1.a). The similar effect against *A. fumigatus* MTCC2550 was recorded for citral in 8–9 h and 10–11 h for *C. citratus* (Figure 1.b). Fluconazole as a positive control showed no difference of >1 $\log_{10}$ up to 12 h.

Toxicities of *C. Citratus* and citral to erythrocytes

Oil of *C. citratus* and citral showed no haemolysis at their respective MFCs to test fungi. Only at

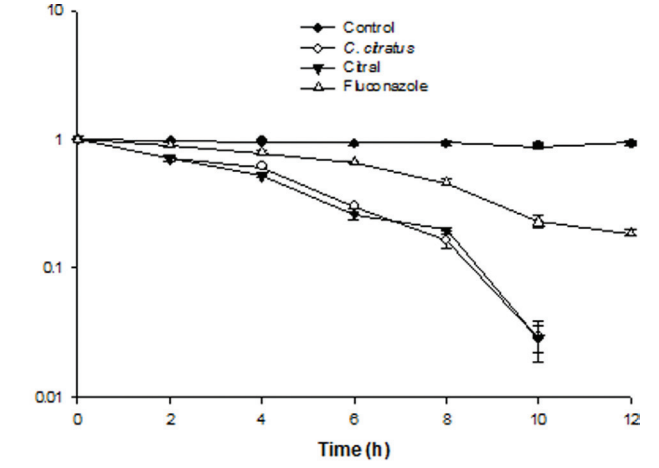

Figure 1 (a). Time kill curves for *T. rubrum* IOA-9 by *C. citratus*, citral and fluconazole.

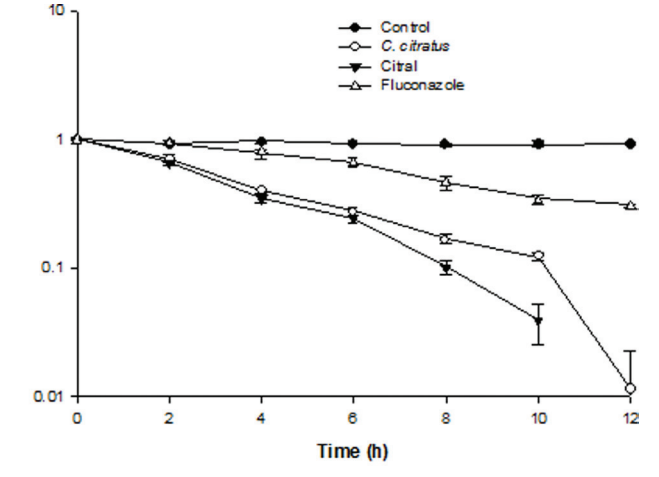

Figure 1 (b). Time kill curves for *A. fumigatus* MTCC2550 by *C. citratus*, citral and fluconazole.

a concentration two to four times higher of MFCs (2304 μg/ml) was partial haemolysis (10–25%) observed. Complete haemolysis was shown by 0.1% (v/v) Triton X-100 as a positive control and no haemolysis was exhibited by 1% DMSO and PBS as solvent controls (Table 3).

Table 2: Minimum inhibitory concentration (MIC) and minimum fungicidal concentrations (MFC) of test oils

Test agents	Test fungi											
	A. flavus NRRL 501		*A. fumigatus* MTCC 2550		*A. niger* IOA-3		*A. solani* MTCC 2101		*T. rubrum* IOA-9		*T. rubrum* MTCC 296	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. citratus	144	288	288	576	288	576	288	576	144	288	288	576
Citral	144	288	288	576	18	36	144	288	144	288	72	144
Antifungal drugs												
FLC	200	400	200	400	800	1600	200	400	200	400	200	400
ITC	100	200	100	200	100	200	100	200	200	400	200	400

All the experiments were performed in triplicates, and MICs and MFCs are presented as mean values (μg/ml)

Table 3: Haemolytic properties of *C. citratus* and citral

Test agents	Percent haemolysis of RBC (Mean ± SD)							
	Concentrations of essential oils (µg/ml)							
	18	36	72	144	288	576	1152	2304
C. citratus	1.45 ± 0.10	1.92 ± 0.10	2.54 ± 0.15	3.39 ± 0.09	4.56 ± 0.20	4.81 ± 0.12	6.68 ± 0.17	19.08 ± 0.24
Citral	1.78 ± 0.16	2.86 ± 0.15	3.80 ± 0.18	4.29 ± 0.12	4.51 ± 0.13	5.98 ± 0.21	6.96 ± 0.10	17.61 ± 1.04

All the experiments were performed in triplicate and data is presented as mean ± SD

Synergistic interactions of oil of *C. citratus* and citral with fluconazole

The combination effects of the oil of *C. citratus* and its major active ingredient citral with fluconazole against *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9 are given in Table 4. All the tested combinations showed significant levels of synergistic interaction with fluconazole against *T. rubrum* IOA-9 (FICI values 0.312) Citral exhibited highest synergy with fluconazole (FICI value 0.250) against *A. fumigatus* MTCC2550 but no interaction was observed for oil of *C. citratus*.

DISCUSSION

In spite of the use of newer antifungal drugs like voriconazole and caspofungin, the rate of mortality due to invasive fungal infections caused by *Aspergillus* spp and *Trichophyton* spp is increasing and has led clinicians to search for more effective therapeutic options.[5,19] Combination therapy with available antifungal drugs has been recommended and used in treatment for various mycoses.[19–21] However, combination therapy with natural products is less explored. Combination of drugs with different modes of action has advantages over monotherapy in terms of an increased potency of fungal killing, decrease in emergence of resistant strains and minimization of the dose-related toxicity of drugs.[11,21] In spite of their diverse activity, essential oils or active compounds in combination with antifungal drugs have been less frequently investigated. In these perspectives, the current findings highlight the synergistic interaction of oil of *C. citratus* and its major active compound citral with fluconazole against the azole-resistant strains of *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9.

The present study has revealed broad spectrum inhibitory activity of oil of *C. citratus* and citral against azole-resistant fungi from different genera of *Aspergillus*, *Trichophyton*, *Mucor* and *Fusarium*. Citral was more active than *C. citratus* in their antifungal activity. The antifungal activity of this major compound being greater than its oil indicates that inhibitory activity of oil of *C. citratus* is mainly governed by major ingredients and minor ingredients play non-significant role. The potency of citral and *C. citratus* in killing the fungi as revealed by time kill assays against *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9 was found to be higher than fluconazole. This finding indicates the fungicidal activity of *C. citratus* and citral over the fungistatic nature of fluconazole.

The promising *in vitro* antifungal activity of *C. citratus* and citral and non toxicity to RBCs encouraged us to investigate *in vitro* combinations of these agents with fluconazole to increase their efficacy. All test combinations showed synergistic interactions against the test strains except the no interaction response for *C. citratus* against *A. fumigatus* MTCC2550. However, citral, the major ingredient of oil of *C. citratus* (80.00%)[14] showed synergistic interaction alone but *C. citratus* did not. That may

Table 4: FIC and FICI for combination of *C. citratus* and citral with fluconazole against *A. fumigatus* MTCC2550 and *T. rubrum* (IOA-9)

Test combination	*A. fumigatus* MTCC2550					*T. rubrum* (IOA-9)				
	MIC_a	MIC_c	FIC	FICI	Type	MIC_a	MIC_c	FIC	FICI	Type
***C. citratus* with fluconazole**										
C. citratus oil (µg/ml)	288	18	0.062	0.562	No interaction	144	9	0.062	0.312	Synergy
Fluconazole (µg/ml)	200	100	0.50			200	50	0.250		
Citral with fluconazole										
Citral (µg/ml)	288	36	0.125	0.250	Synergy	144	9	0.062	0.312	Synergy
Fluconazole (µg/ml)	200	25	0.125			200	50	0.250		

MIC_a: MIC of one agent alone
MIC_c: MIC of agent in most effective combination

be due to presence of other minor ingredients diluting synergistic behavior of citral to a no interaction response as a whole in oil of *C. citratus*. Citral reduced the MIC of fluconazole up to eight-fold and its MIC decreased up to 8- and 16-fold against the test strains. Treatment of fungal infections caused by the drug-resistant strains of fungi may require higher treatment doses and even if fluconazole is used in higher doses in monotherapy it can lead to adverse side effects such as hepatotoxicity.[5,22] Considering this, our data suggests that dose-related toxicity and drug-resistance against fluconazole can be overcome in combinations with oil of *C. citratus* or its major active compound citral.

The synergistic interactions of *C. citratus* and citral with fluconazole may be related to the simultaneous inhibition of different target sites by test agents and fluconazole. Fluconazole targets cell membrane integrity in fungi by interrupting ergosterol biosynthesis. It has been reported in literature[14,23] that cell wall and cell membrane integrity, along with other membranous structures are the target sites for essential oils or active compounds. Fluconazole being hydrophilic azole is absorbed less by cell membrane but membrane damaging effects of essential oils or active compounds may result in greater absorbance of fluconazole. This results in improved and more rapid killing of pathogen leading to conversion of fluconazole from the fungistatic mode of action to a fungicidal mode.

CONCLUSIONS

The broad spectrum *in vitro* antifungal activity of the oil of *C. citratus* and its major active compound citral against azole-resistant filamentous fungi are encouraging in that they indicate that fungal resistance to azoles has no impact on the inhibitory activity from these oils. Our study suggests that fungal infections emerging due to the fluconazole-resistant fungal strains, especially *Aspergillus fumigatus* and *Trichophyton rubrum,* could be treated effectively by the oils of *C. citratus* or citral in combination with fluconazole. Further, therapeutic efficacy and safety of these combinations are needed to be evaluated under *in vivo*.

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