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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Antifungal activity of essential oils and their synergy with fluconazole against drug-resistant strains of *Aspergillus fumigatus* and *Trichophyton rubrum*

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Abstract The aim of this study was to screen certain plant essential oils and active compounds for antifungal activity and their in vitro interaction with fluconazole against drug-resistant pathogenic fungi. The methods employed in this work included disc diffusion, broth macrodilution, time kill methods and checkerboard microtiter tests. Oil compositions were evaluated by gas chromatography-mass spectrometry (GC-MS) analysis. Transmission electron microscopy was used to assess the effect of essential oils on cellular structures of test fungi. Test fungal strains exhibited resistance to at least two drugs (fluconazole and itraconazole). Among the 21 essential oils or active compounds tested, ten showed promising antifungal activity. GC-MS analysis revealed the presence of major active compounds in the essential oils used. Cinnamaldehyde showed the most promising antifungal activity and killing potency against *Aspergillus fumigatus* MTCC2550 and *Trichophyton rubrum* IOA-9. Cinnamaldehyde showed strongest synergy with fluconazole against *A. fumigatus* and *T. rubrum* by reducing the minimum inhibitory concentration of fluconazole up to 8-fold. Zones of lysis of the cell wall and cell membrane appeared to be where cinnamaldehyde acted on fungi. This study highlights the broad spectrum antifungal activity of essential oils and active compounds and their synergy with fluconazole against drug-resistant fungi.

Keywords Antifungal drugs · Drug resistance · Essential oils · GC-MS · Synergy · Transmission electron microscopy

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Introduction

Incidence of microbial infections has increased in recent decades, especially mycoses which account for a high rate of death among patients with a weakened immune system. Opportunistic fungal infections are a serious threat to such patients and have been reported to occur at an alarming rate (Pinto et al. 2006). Infections of alveolar tissues by members of genus *Aspergillus* produce a spectrum of lung diseases known as aspergilloses. The disease involves pulmonary aspergilloma, invasive aspergillosis and allergic bronchopulmonary aspergillosis and is mainly caused by *Aspergillus fumigatus* and *A. niger* and less frequently by *A. flavus* and *A. clavatus*. Invasive aspergillosis in immunosuppressed individuals often results in death and has become a major concern among public health officials (Lutz et al. 2003; Singh and Paterson 2005; Erjavec et al. 2009; Leventakos et al. 2010; Pfaller and Diekema 2010). Also, infections of hair, skin and nails have increased considerably among pediatric and geriatric populations (Mukherjee et al. 2003; Monod 2008). Such infections are primarily caused by *Trichophyton rubrum* and other dermatophytes and are not life-threatening; however, both immunocompetent and immunosuppressed persons are affected (Vermount et al. 2008). Chronic infections of skin carry considerable morbidity and can become serious in immunocompromised patients resulting in invasive infections (Sokovic et al. 2006).

The antifungal drugs most commonly used against these diseases include amphotericin B, ketoconazole, fluconazole, terbinafine and flucytosine. Adverse side effects are associated with the use of available antifungal drugs including nephrotoxicity, hepatotoxicity and neurotoxicity (Andriole 1994). Also, therapeutic response is slow in immunocompromised patients. Fluconazole is considered to be one of the safest

antifungals used in the treatment of fungal infections but the fungistatic nature and the development of resistance in fungi have restricted the use of fluconazole (Balkis et al. 2002; Ishida et al. 2006). There are several reports on the increasing azole drug resistance in *Aspergillus* spp. and dermatophytes (Balkis et al. 2002; Howard et al. 2006; Santos and Hamdan 2007).

Resistance of human pathogens to antifungal drugs and toxicity-related problems have resulted in the need for novel anti-mycotic agents with a broad spectrum of actions and fewer dose limiting side effects. Several in vitro and in vivo studies using plant products traditionally used in ethnomedicine have shown promising antifungal activity without any side effects, especially when plant essential oils are used (Mondello et al. 2003; Sokovic et al. 2006; Park et al. 2007; Bansod and Rai 2008; Bajpai et al. 2009). Therapeutic activity from essential oils against *Candida albicans*, *Trichophyton* spp. and *Aspergillus* spp., associated with mucosal, cutaneous and respiratory tracts has been reported (Brantner and Grein 1994; Salgueiro et al. 2003; Pina-Vaz et al. 2004; Sanguinetti et al. 2007; Fontenelle et al. 2008; Pinto et al. 2009).

The increase in number of available antifungal compounds has prompted the search for better therapeutic strategies, such as the use of new antifungal agents in combinations. Combinations of two or more antifungal drugs have been exploited to achieve better therapeutic action against invasive and systemic mycoses. It is desirable to assess compounds with newer modes of action to existing antifungals and test these in combinations. The compounds under study include chitin synthase inhibitors such as nikkomycin Z, calcineurin inhibitors such as cyclosporin, and tacrolimus, in combination with fluconazole or echinocandins against *Aspergillus* spp. and *Candida* spp. (Cuenca-Estrella 2004; Baddley and Pappas 2007; Segal and Steinbach 2007). Combination therapy of available antifungal drugs with natural products is less explored, especially in the case of essential oils. The mode of action of essential oils as an antifungal agent is less understood and is expected to be different than that of available antifungal drugs (Baby and George 2008). As a result, antifungal activities of essential oils with expectations of newer target sites have been evaluated in combination with known antifungal drugs to assess possible synergy to be exploited in combination therapy. However, no systematic screening or synergy studies on plant essential oils against fluconazole resistant strains have been reported.

The reported study explores the combinational effect of essential oils with antifungal drugs against invasive and superficial fungal pathogens such as *Aspergillus* spp., *Fusarium* spp., *Mucor* spp. and *Trichophyton* spp., all of which are common disease agents in immunosuppressive individuals in India. Some of the other clinically relevant fungi such as *Lichtheimia corymbifera* and *Rhizopus oryzae* are also gaining importance but are less frequently obtained in immunosuppressed persons. In the present study, the antifungal activities of eighteen common plant essential oils and four major active compounds of these essential oils were determined against the eight strains of human pathogenic fungi. To obtain effective combinational synergy, interactive behaviors of the most potent essential oils and active compounds with fluconazole were determined against a multi-drug resistant strain of *A. fumigatus* MTCC2550 and a clinical isolate of *T. rubrum* IOA-9. Furthermore, the mode of action of oils in terms of structural changes made to the fungi was evaluated using transmission electron microscopy.

Materials and methods

Plant essential oils and drugs

Plant essential oils and their active compounds were obtained from Wyndmere Naturals, USA (*Citrus paradisi*, grapefruit; *Citrus sinensis*, orange; *Foeniculum vulgare*, sweet fennel; *Petroselinum crispum*, parsley; *Apium graveolens*, celery; *Rosmarinus officinalis*, rosemary; *Santalum album*, sandalwood; *Zea mays*, corn; and *Zingiber officinale*, ginger oils); Himalaya Drug Co. (Dehradun, India) (*Cinnamomum verum*, cinnamon; *Citrus limon*¸ lemon; and *Myristica fragrans*, nutmeg oils); Hi-Media Laboratory, Mumbai, India (oils of *Eucalyptus* sp., eucalyptus; *Mentha piperita* peppermint; and eugenol [minimum assay 98%], cinnamaldehyde [98%] and thymol [99%]), Aroma Sales Corporation (New Delhi, India) (oils of *Carum copticum*, ajowan; *Cymbopogon martini*, palmrosa; *Thymus vulgaris*, thyme; and geraniol [85%]), and Dabur India Ltd. (New Delhi, India) (*Syzygium aromaticum*, clove oil). The purity of oils and active compounds was determined by percent composition of major active compounds as revealed in gas chromatography-mass spectrometry (GC-MS) and physico-chemical analyses such as specific gravity, refractive index, optical rotation and solubility in alcohol (data not shown). Antifungal susceptibility test discs and powder drugs were obtained from Hi-Media Laboratory (Mumbai, India). Stock solutions (25 mg ml^{-1}) of fluconazole and itraconazole were prepared in 1% dimethyl sulphoxide (DMSO). Thymol was prepared as a stock solution of 30 mg ml^{-1} in 1% DMSO and the remainder of the essential oils or active compounds were diluted ten times in 1% DMSO daily before use in assays.

GC-MS of essential oils

The composition of essential oils was analyzed using GC-MS on a GCD 1800A Hewlett Packard apparatus, with an

HP-1 column (30 m×0.25 mm×0.25 μm). The temperature program was set at an initial temperature of 100–250 °C at a rate of 10 °C min^{-1} with hold time at 250 °C for 3 min, and final temperature of 250–280 °C at a rate of 30 °C min^{-1} with hold time at 280 °C for 2 min. Helium was employed as a carrier gas at the rate of 1 ml min^{-1}. Identification of major compounds was carried out on the basis of GC retention time and comparing with MS reference database of NBS75K at the Sophisticated Analytical Instrument Facility of the Indian Institute of Technology Bombay, Mumbai.

Fungal strains and media

The strains included in this study were *Aspergillus flavus* NRRL501, kindly provided from the fungal culture collection of the Agricultural Research Service, USDA, Peoria, IL, USA; *A. fumigatus* MTCC2550, *Alternaria solani* MTCC2101, *Fusarium oxysporum* MTCC284, *Mucor rouxii* MTCC386 and *T. rubrum* MTCC296 were purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. In addition, clinical isolates of *A. niger* IOA-3 and *T. rubrum* IOA-9, were obtained from Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh, India and are maintained at the departmental culture collection.

Disc diffusion assay

The disc diffusion assay with some modifications as adapted by Sokovic and van Griensven (2006) 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^{-1}) 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 $disc^{-1}$, Hi-Media), were mounted on the agar surface and the plates were incubated at 28±2 °C for 2 days. Each experiment was conducted in triplicate and average zone size was measured.

Broth macrodilution method

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 van Griensven (2006) with some modifications. We added 10 μl of spore suspension (1.5×10^5 CFU ml^{-1}) 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 °C 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. (2002). 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^{-1}). 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 °C and 120 rev min^{-1}. Viable counts were obtained from 10-fold serial dilutions of test and control solutions at 2, 4, 6, 8, 10 and up to 24 h by plating 100 μl of 10-fold dilution onto SDA plates and incubating at 30 °C 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 essential oils or active compounds was evaluated by the red blood cell (RBC) lysis assay as adapted by Luize et al. (2005) 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. Next, 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 °C 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 2,000 rev min^{-1} for 10 min and the absorbances of supernatant were read at 540 nm. Percent haemolysis was calculated as: $[\{(A-B)/(C-B)\}\times100]$, 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 essential oils or active compounds 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 2-fold dilutions, in eight numbers, of each essential 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 essential oils 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 essential oils or active compounds and fluconazole could be achieved. Also, 100 µl of inoculum from spore suspension (1.5×10^5 CFU ml^{-1}) was added to each well and plates were incubated at 30 °C for 2 days. The nature of interaction was defined quantitatively by means of fractional inhibitory concentrations (FICs) 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 (2003).

Transmission electron microscopy

Structural changes produced by cinnamaldehyde towards *T. rubrum* IOA-9 and *A. fumigatus* MTCC2550 were evaluated using transmission electron microscopy at sub-inhibitory concentrations. Briefly, 50 ml of SDB treated with 20 and 40 µg ml^{-1} of cinnamaldehyde in 500-ml Erlenmeyer flasks were inoculated with 50 µl of spore suspensions (1.5×10^5 CFU ml^{-1}) of *T. rubrum* IOA-9 and *A. fumigatus* MTCC2550 strains, respectively, and incubated at 30 °C for 72 h at 120 rev min^{-1}. Fungal material obtained was processed according to Bozolla and Russel (1999) and viewed under a Morgagni 268D transmission electron microscope at 80 kV. Ultrastructures of treated and untreated cultures were compared to assess the effect of cinnamaldehyde.

Results

Susceptibilities of fungal strains to essential oils and antifungal drugs

All the tested strains were resistant to fluconazole, itraconazole and three other antifungal drugs out of six drugs tested (Table 1). The level of resistance was higher against fluconazole compared to itraconazole with MIC and MFC values in the range of 100–200 and 200–400 µg ml^{-1}, respectively. *A. niger* IOA-3 showed the highest level of resistance to fluconazole, with MIC and MFC at 800 and 1600 µg ml^{-1}, respectively. The screening results of eighteen essential oils and four active compounds for their activity against these drug-resistant fungi are presented in Table 2. Of these essential oils, *C. copticum*, *C. verum*, *S. aromaticum*, *C. martini*, and *T. vulgaris* showed strong and broad spectrum activity against all test fungi with a zone of inhibition ranging from 11.00 to 38.66 mm. All the active compounds showed inhibitory activity greater than all the essential oils except thymol, when tested against drug-resistant fungi. Cinnamaldehyde, eugenol and geraniol showed excellent broad spectrum antifungal activity with a zone of inhibition ranging from 21.66 to 42.66 mm against all eight test strains. Cinnamaldehyde showed the highest zone of inhibition (42.66 mm) against *T. rubrum* MTCC296.

MICs and MFCs of selected essential oils and active compounds

In vitro antifungal potencies of the broad spectrum essential oils and active compounds against the drug-resistant fungi were determined in terms of MICs and MFCs (Table 3). Oils of *C. copticum*, *C. verum*, *S. aromaticum*, *C. martini* and *T. vulgaris* exhibited strong inhibitory activity against the test strains (MIC range, 72–288 µg ml^{-1}; MFC range, 144–576 µg ml^{-1}). Oil of *M. piperita* was strongly active against *A. fumigatus* MTCC2550 and moderately active against *T. rubrum* MTCC296 (MIC of 288 and 576 µg ml^{-1}, respectively). Active compounds, namely, cinnamaldehyde, eugenol and geraniol, showed activity higher than essential oils with MICs ranging from 40 to 160 µg ml^{-1} and MFCs ranging from 80 to 320 µg ml^{-1}. Cinnamaldehyde was most active against *A. solani* MTCC2101, *T. rubrum* IOA-9 and *T. rubrum* MTCC296 strains, with MICs and MFCs of 40 and 80 µg ml^{-1}, respectively.

Major active compounds of essential oils

The most active essential oils as determined by the disc diffusion and broth macrodilution methods were analyzed for their major active ingredients by GC-MS (Table 4). Cinnamaldehyde was the predominant component (79.10%) of *C. verum.* Geraniol was the prominent constituent (50.74%) of *C. martini* oil with geraniol acetate (19.21%) as the other major ingredient. Oil of *S. aromaticum* was predominately composed of eugenol (74.32%) and caryophyllenes (27.97%).

Time kill curves

The ability to kill fungal strains by most active essential oils and active compounds was compared with fluconazole

Table 1 Susceptibility of human pathogenic fungal strains to antifungal drugs

Fungal strains	Sensitivity to antifungal drugs (diameter of zone of inhibition in mm)						Fluconazole (μg ml^{-1})		Itraconazole (μg ml^{-1})	
	AMB	CLT	FLC	ITC	KTC	NYT	MIC	MFC	MIC	MFC
A. flavus NRRL501	–	14.33±1.24	–	09.00±0.81	11.00±0.81	11.00±0.81	200	400	100	200
A. fumigatus MTCC2550	–	11.00±0.816	–	–	–	12.00±0.81	200	400	100	200
A. niger (IOA-3)	13.00±0.81	10.66±0.47	–	09.00±0.47	11.00±0.81	12.33±0.47	800	1,600	100	200
A. solani MTCC2101	–	–	–	–	11.66±1.24	10.66±0.47	200	400	100	200
F. oxysporum MTCC284	–	11.66±0.47	–	–	14.00±0.81	–	ND	ND	ND	ND
M. rouxii MTCC386	12.66±0.94	–	–	–	–	13.00±0.81	ND	ND	ND	ND
T. rubrum (IOA-9)	09.00±0.81	–	–	–	14.66±1.24	14.00±0.81	200	400	200	400
T. rubrum MTCC296	16.00±0.81	–	–	–	–	11.66±0.47	200	400	200	400

All experiments were performed in triplicates and data are presented as mean ± SD

AMB amphotericin B (20 μg disc^{-1}), *CLT* clotrimazole (10 μg disc^{-1}), *FLC* fluconazole (10 μg disc^{-1}), *ITC* itraconazole (10 μg disc^{-1}), *KTC* ketoconazole (10 μg disc^{-1}), *NYT* nystatin (100 μG disc^{-1}); - indicates no zone of inhibition, *Resistant* diameter of zone of inhibition nil to ≤10 mm, *Sensitive* diameter of zone of inhibition >10 mm, *ND* not determined

against *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9. The time-dependent killing of *A. fumigatus* MTCC2550 by essential oils and active compounds revealed a difference of >1 $\log_{10}$ in viable counts compared to the control between 6 and 8 h for cinnamaldehyde (Fig. 1b). In contrast, treatment with the oils of *C. verum*, *S. aromaticum* and *C. martini* (Fig. 1a) and eugenol and geraniol (Fig. 1b) showed a difference of >1 $\log_{10}$ in viable counts compared to the control between 8 and 10 h. Fluconazole as a positive control showed no difference of >1 $\log_{10}$ up to 10 h. A similar pattern of results was also observed against *T. rubrum* IOA-9 (Fig. 2a, b).

Table 2 Antifungal activity of essential oils and active compounds

Essential oils and active compounds	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. copticum	33.33±1.69	32.66±1.24	25.66±1.24	30.00±0.81	27.66±1.69	30.66±1.24	36.00±1.41	32.33±0.15
C. verum	31.33±0.47	37.66±0.47	38.66±0.47	32.33±0.47	34.33±0.47	11.00±0.81	35.00±0.81	33.00±0.16
C. limon	09.66±0.47	16.00±0.81	–	10.66±0.47	17.66±0.47	12.33±0.47	20.33±0.47	15.66±1.24
C. paradisi	16.33±0.47	–	14.33±0.47	–	–	–	–	–
C. sinensis	12.66±0.47	–	14.66±0.47	–	–	–	15.00±0.81	–
C. martini	26.33±1.24	32.33±1.24	32.66±1.24	32.00±0.81	32.66±1.24	29.66±0.47	33.33±1.24	33.66±1.69
Eucalyptus sp.	–	12.00±1.41	19.33±1.24	15.00±0.94	15.00±0.816	09.33±1.24	20.66±0.47	16.66±2.05
M. piperita	–	37.33±0.47	–	20.00±1.41	19.66±0.47	13.66±0.47	–	23.33±1.24
M. fragrans	–	–	–	09.66±0.94	08.66±0.47	09.33±0.47	–	09.00±0.81
S. aromaticum	36.66±0.47	31.00±0.81	27.33±0.47	28.00±0.81	37.33±0.47	31.66±0.94	37.66±0.47	36.33±1.24
T. vulgaris	30.00±0.81	32.00±1.63	26.00±1.41	28.66±1.24	16.00±0.81	16.00±1.63	34.00±0.81	27.00±0.81
Z. officinale	–	–	20.33±0.47	–	12.00±0.81	–	09.66±0.942	10.66±1.24
Cinnamaldehyde	38.66±1.24	38.00±0.81	35.00±0.81	42.33±0.47	25.66±0.47	21.66±0.94	41.33±1.24	42.66±1.24
Eugenol	31.33±1.69	28.66±0.94	30.33±1.24	29.33±1.24	34.66±0.47	35.33±1.24	39.00±0.81	39.33±1.24
Geraniol	35.00±0.81	31.33±2.05	35.00±0.81	32.00±1.63	23.66±1.69	22.33±2.05	37.66±1.24	35.66±1.69
Thymol	18.66±1.69	21.66±1.24	19.00±0.81	18.66±0.47	15.66±1.24	12.66±1.69	17.33±1.24	17.66±0.94

All experiments were performed in triplicates and data are presented as mean ± SD. Each disc contains 10 μl of essential oils or active compounds – indicates no zone of inhibition. No antifungal activity was recorded in the oils of *A. graveolens* (celery) *F. vulgare* (sweet fennel), *P. crispum* (parsley), *R. officinalis* (rosemary), *S. album* (sandalwood), *Z. mays* (corn)

Table 3 Minimum inhibitory concentration and minimum fungicidal concentrations of selected essential oils and active compounds

Essential oils and active compounds	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. copticum	144	288	144	288	576	1,152	288	576	72	144	144	288
C. verum	160	320	80	160	160	320	160	320	80	160	160	320
C. limon	>2,304	>2,304	2,304	>2,304	>2,304	>2,304	>2,304	>2,304	1,152	2,304	2,304	>2,304
C. martini	288	576	144	288	144	288	288	576	144	288	144	288
Eucalyptus sp.	>2,304	>2,304	2,304	>2,304	1,152	2,304	2,304	>2,304	1,152	2,304	2,304	>2,304
M. piperita	>2,304	>2,304	288	576	>2,304	>2,304	1,152	2,304	>2,304	>2,304	576	1,152
S. aromaticum	160	320	320	640	320	640	320	640	160	320	160	320
T. vulgaris	144	288	144	288	288	576	288	576	72	144	288	576
Cinnamaldehyde	80	160	80	160	80	160	40	80	40	80	40	80
Eugenol	160	320	320	640	160	320	320	640	80	160	80	160
Geraniol	144	288	144	288	144	288	144	288	72	144	72	144
Thymol	384	768	192	384	384	768	384	768	192	384	192	384

All experiments were performed in triplicates, and MICs and MFCs are presented as mean values (μg ml^{-1})

Toxicities of essential oils and active compounds to sheep erythrocytes

Test essential oils and active compounds showed no haemolysis at their respective MFCs to test fungi. Only at a concentration two to four times higher of MFCs (2304 μg ml^{-1}) 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 5).

Synergistic interactions of essential oils or active compounds with fluconazole

The combination effects of the oils *C. verum*, *S. aromaticum* and *C. martini* and their major active ingredients such as cinnamaldehyde, eugenol and geraniol with fluconazole against *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9 are given in Table 6. All the tested essential oils and active compounds showed significant levels of synergistic interaction with fluconazole against *T. rubrum* IOA-9 (FICI values

Table 4 Constituents of essential oils as identified by GC-MS

Peak no.	*S. aromaticum*		*C. verum*		*C. martini*	
	Constituents (retention time)	Area %	Constituents (retention time)	Area%	Constituents (retention time)	Area %
1	Eugenol (6.28)	74.32	*O*-Cymene (3.94)	5.43	β-Linalool (3.66)	8.79
2	β-Caryophyllene (7.03)	4.92	Cinnamaldehyde (9.32)	79.10	Geraniol (5.450	50.74
3	Isocaryophyllene (7.09)	5.96	Cinnamicaldehyde (10.26)	5.81	Eugenol (1.86)	6.44
4	β-Caryophyllene (7.14)	7.04	Isoquinolene (13.13)	8.91	Geraniol acetate (6.73)	19.21
5	α-Caryophyllene (7.34)	1.28	α-Thujene (19.34)	0.75	β-Caryophyllene (7.23)	6.48
6	α-Caryophyllene (7.40)	4.05			α-Caryophyllene (7.53)	2.45
7	Caryophyllene oxide (8.61)	2.41			α-Nerolidol (8.21)	2.72
8					Geraniol butyrate (8.37)	0.91
9					β-Nerolidol (8.51)	4.14
10					Caryophyllene oxide (8.70)	0.59
11					β-Farnesol (9.89)	0.95
12					Butanoic acid (10.15)	0.78
13					β-Farnesol (10.92)	0.16
14					Butanoic acid (11.86)	0.22

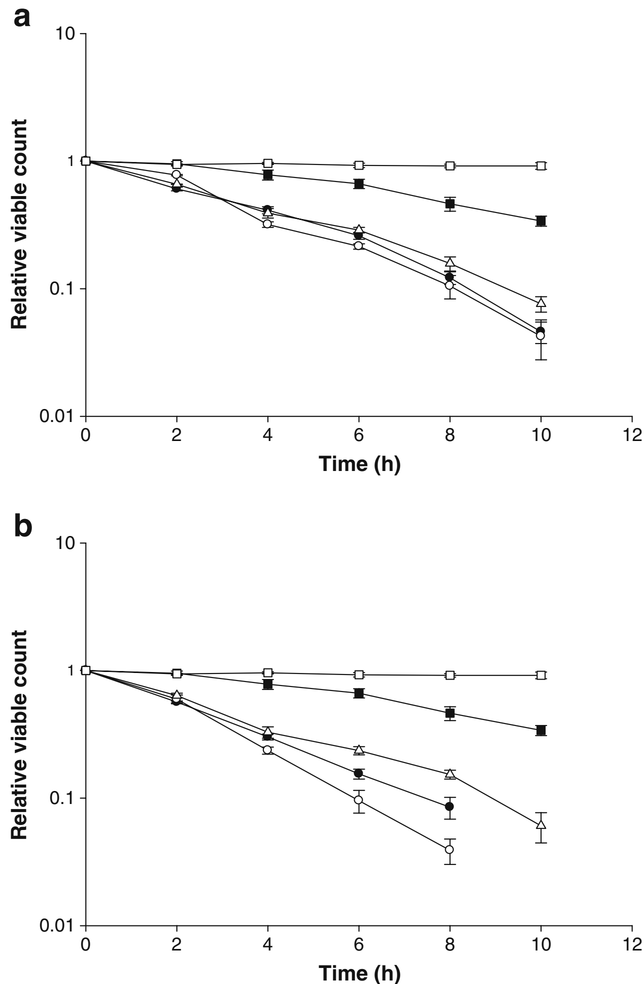

Fig. 1 **a** Time kill curves for *A. fumigatus* MTCC2550 by essential oils and fluconazole. *Open square* control, *filled square* fluconazole, *filled circle S. aromaticum* oil, *open circle C. verum* oil, *open triangle C. martini* oil. **b** Time kill curves for *A. fumigatus* MTCC2550 by active compounds and fluconazole. *Open square* control, *filled square* fluconazole, *filled circle* eugenol, *open circle* cinnamaldehyde, *open triangle* geraniol

0.312, 0.281, 0.257, 0.250 and 0.156). The oils *S. aromaticum* (0.250), eugenol (0.375) and cinnamaldehyde (0.187) exhibited synergistic interactions with fluconazole against *A. fumigatus* MTCC2550 but no interactions were observed for the oils of *C. martini* and geraniol with fluconazole. The maximum level of synergy was determined between cinnamaldehyde and fluconazole against both *T. rubrum* IOA-9 (0.156) and *A. fumigatus* MTCC2550 (0.187). Cinnamaldehyde was most effective in combination therapy, showing the strongest synergy with fluconazole and reducing the MIC of fluconazole up to 8-fold against both *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9 and a reduction in its own MIC up to 16- and 32-fold, respectively. The highest reduction in MIC (i.e. 128-fold) was recorded for oil of *S. aromaticum* in combination with fluconazole against *T. rubrum* IOA-9. No combination was found to be antagonistic against the test fungi.

Effects of cinnamaldehyde on ultrastructures of *T. rubrum* and *A. fumigatus*

The effect of sub-inhibitory concentrations of most active compounds (i.e. cinnamaldehyde) on cell constituents of *T. rubrum* IOA-9 and *A. fumigatus* MTCC2550 was observed by transmission electron microscopy. In control specimens mycelial cells showed long strands of hyphae with smooth cell walls and cell membranes with other cytoplasmic organelles intact (Fig. 3a, e). In a hyphal specimen treated with cinnamaldehyde, the ultrastructural changes were indicated as lysis of cell walls and plasma membranes

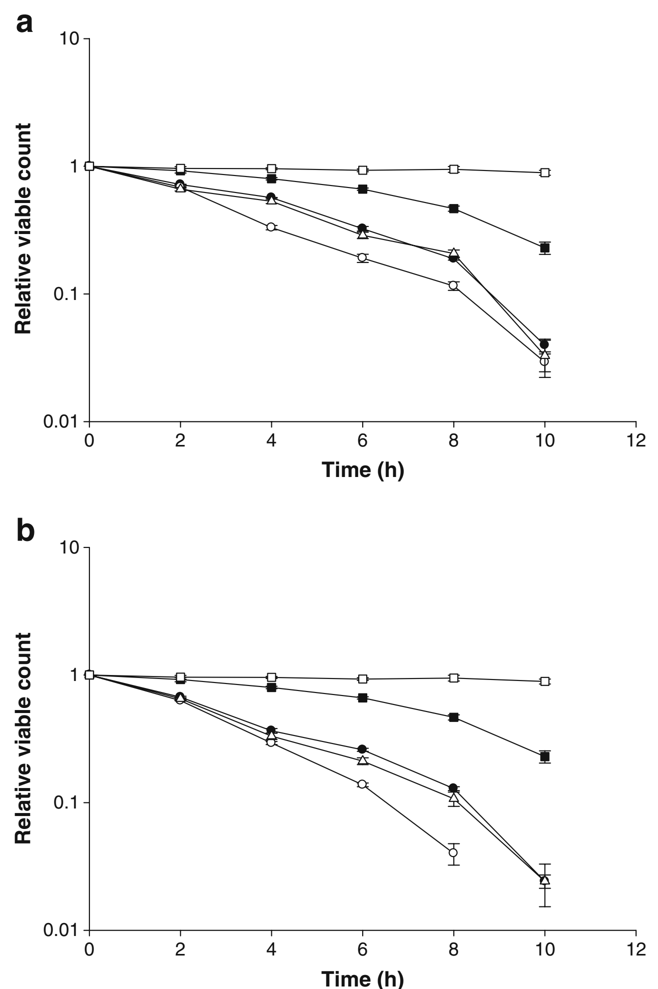

Fig. 2 **a** Time kill curves for *T. rubrum* IOA-9 by essential oils and fluconazole. *Open square* control, *filled square* fluconazole, *filled circle S. aromaticum* oil, *open circle C. verum* oil, *open triangle C. martini* oil. **b** Time kill curves for *T. rubrum* IOA-9 by active compounds and fluconazole. *Open square* control, *filled square* fluconazole, *filled circle* eugenol, *open circle* cinnamaldehyde, *open triangle* geraniol

Table 5 Haemolytic properties of essential oils and active compounds

Essential oils and active compounds	Percent haemolysis of RBC (mean ± SD)							
	Concentrations of essential oils (μg ml^{-1})							
	18	36	72	144	288	576	1,152	2,304
S. aromaticum	1.90±0.09	2.20±0.19	2.50±0.13	3.09±0.17	4.26±0.09	5.63±0.18	6.05±0.12	18.78±0.16
C. verum	1.88±0.31	2.14±0.21	2.57±0.26	3.41±0.15	4.21±0.32	4.61±0.28	6.81±0.29	22.89±0.51
C. martini	1.58±0.14	1.84±0.14	2.74±0.19	3.51±0.07	4.27±0.17	4.62±0.27	5.98±0.37	18.61±0.26
Eugenol	1.65±0.14	1.98±0.09	3.02±0.08	3.72±0.16	4.26±0.16	4.78±0.25	5.60±0.63	19.03±0.30
Cinnamaldehyde	1.84±0.19	2.87±0.07	3.39±0.12	3.96±0.05	4.40±0.17	5.06±0.20	6.22±0.19	18.11±0.40
Geraniol	1.33±0.17	2.16±0.25	2.31±0.26	2.87±0.23	3.36±0.18	3.85±0.11	4.74±0.34	14.34±0.23

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

(Fig. 3b). Expansion of endoplasmic reticula near the cell membrane and excessive vacuolization along with disintegration of cell walls were observed (Fig. 3c). Also, disintegration of mitochondria, plasma membranes, and the disorganization of cytoplasmic content due to the abnormal distribution of polysaccharides occurred (Fig. 3d, f). Disintegration of nuclear as well as cytoplasmic contents and leakage of cytosolic content were also observed (Fig. 3g, h).

Discussion

Incidence of invasive fungal infections due to *Aspergillus* spp. and *Trichophyton* spp. over the past several decades and the associated morbidity and mortality, especially in immunocompromised patients, has increased. In spite of the use of newer antifungal drugs like voriconazole and caspofungin, the rate of mortality is increasing and has led clinicians to search for more effective therapeutic

Table 6 FIC and FICI for combination of essential oils or active compounds 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
S. aromaticum with fluconazole										
S. aromaticum oil (μg ml^{-1})	320	40	0.125	0.250	Synergy	160	1.25	0.007	0.257	Synergy
Fluconazole (μg ml^{-1})	200	25	0.125			200	50	0.25		
Eugenol with fluconazole										
Eugenol (μg ml^{-1})	320	40	0.125	0.375	Synergy	80	2.5	0.312	0.281	Synergy
Fluconazole (μg ml^{-1})	200	50	0.250			200	50	0.25		
C. verum with fluconazole										
C. verum oil (μg ml^{-1})	80	20	0.250	0.50	Synergy	80	10	0.125	0.250	Synergy
Fluconazole (μg ml^{-1})	200	50	0.250			200	25	0.125		
Cinnamaldehyde with fluconazole										
Cinnamaldehyde (μg ml^{-1})	80	5.0	0.062	0.187	Synergy	40	1.25	0.031	0.156	Synergy
Fluconazole (μg ml^{-1})	200	25	0.125			200	25	0.125		
C. martini with fluconazole										
C. martini oil (μg ml^{-1})	144	9	0.062	0.562	No interaction	144	18	0.125	0.250	Synergy
Fluconazole (μg ml^{-1})	200	100	0.50			200	25	0.125		
Geraniol with fluconazole										
Geraniol (μg ml^{-1})	144	18	0.125	0.625	No interaction	72	2.25	0.062	0.312	Synergy
Fluconazole (μg ml^{-1})	200	100	0.50			200	50	0.250		

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

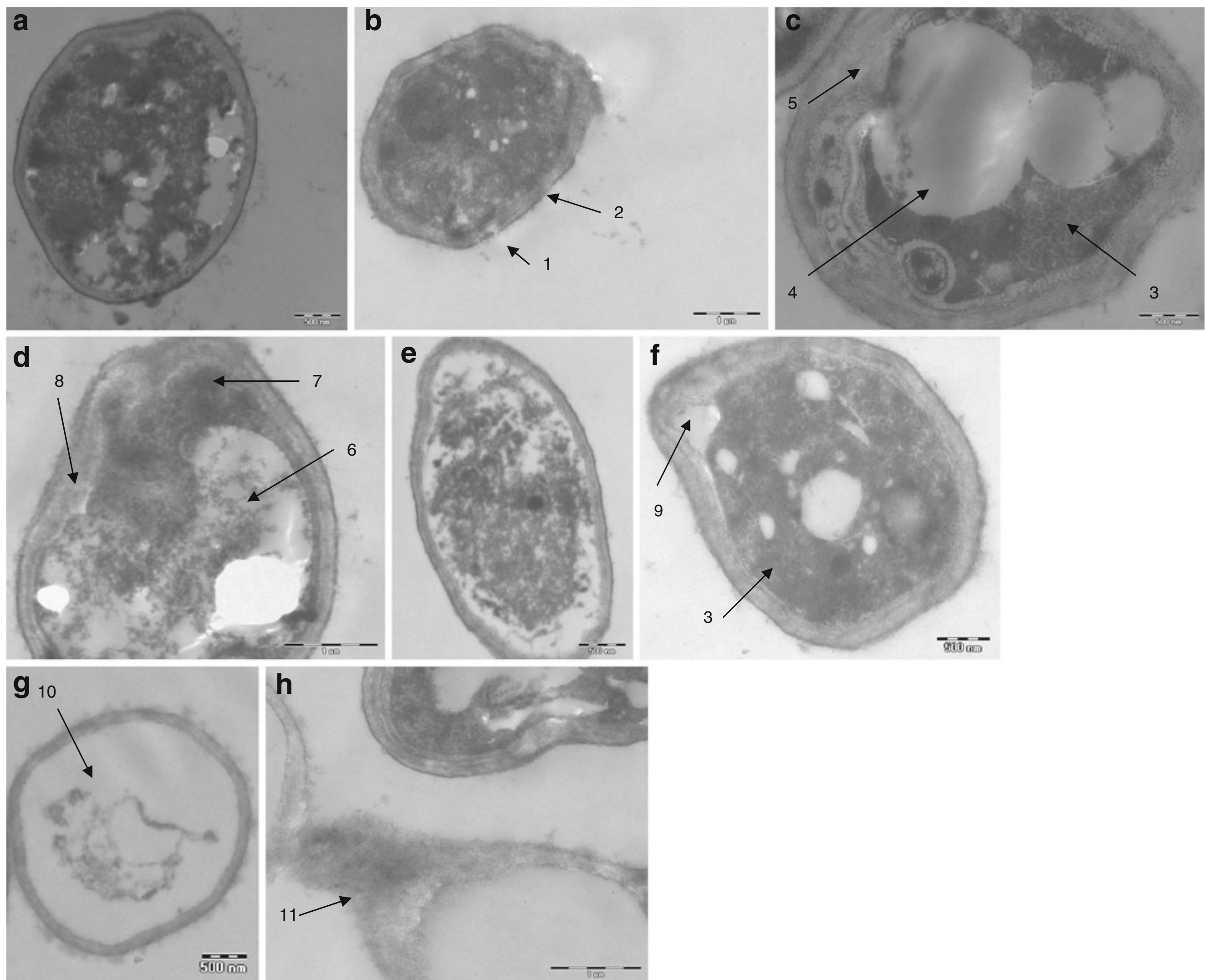

Fig. 3 Transmission electron micrographs of *T. rubrum* IOA-9 **a** control, and treated with the cinnamaldehyde at 20 μg ml^{-1} concentration **b** lysis of cell wall (*1*), lysis of cell membrane (*2*). **c** Expansion of endoplasmic reticulum near the cell membrane (*3*), excessive vacuolisation (*4*), disintegration of cell wall (*5*). **d** Abnormal distribution of polysaccharides in cytoplasm and disorganization and degradation of cytoplasmic content (*6*), disintegration of mitochondria (*7*), and disintegration of plasma membrane (*8*). *A. fumigatus* MTCC2550 **e** control, and treated with the cinnamaldehyde at 40 μg ml^{-1} concentration **f** expansion of endoplasmic reticulum (*3*), striping of plasma membrane (*9*). **g** Lack of cytoplasm, folding of nuclear membrane, disintegration of nuclear material (*10*). **h** Leakage of total cytoplasmic content (*11*)

options (Dannoui et al. 2004). Combination therapy with available antifungal drugs has been recommended and used in treatment for various mycoses (Cuenca-Estrella 2004; Baddley and Pappas 2007; Segal and Steinbach 2007; Wirk and Wingard 2008); however, combination therapy with natural products is less explored. Advantages over monotherapy include an increased potency of fungal killing, decrease in emergence of resistant strains and minimization of the dose-related toxicity of antifungal drugs (Vitale et al. 2005). In spite of their diverse activity, essential oils or active compounds in combination with antifungal drugs have been less frequently investigated. Studies have revealed synergistic interactions for antifungal activity including latex from *Euphorbia characias* with ketoconazole (Gioredani et al. 2001), oils of *Alium* and *Pelargonium graveolens* with ketoconazole against *Trichophyton* spp. (Shin and Lim 2004; Shin and Pyun 2006), *Agastache rugosa* oil with ketoconazole against *B. capitatus* (Shin and Kang 2003), and santolina oil with clotrimazole, oils of *T. vulgaris* and *Myrtus communis* with amphotericin B against *C. albicans* (Suresh et al. 1997; Giordani et al. 2004; Mahboubi and Bidgoli 2010). In this perspective, the current findings highlight the synergistic interaction of certain essential oils or active compounds with fluconazole against the drug-resistant strains of *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9.

The present study has revealed broad spectrum inhibitory activity of oils from *C. verum*, *S. aromaticum*, *C. martini*, *C. copticum* and *T. vulgaris*, and major active ingredients like cinnamaldehyde, eugenol, geraniol and thymol, against multi-drug resistant human pathogenic fungi from different genera of *Aspergillus*, *Trichophyton*, *Mucor* and *Fusarium* both in solid and liquid media. Resistance to azole drugs amongst various fungi has also been reported by several research groups (White et al. 1998; Howard et al. 2006; Snelders et al. 2008). In this regard, the above-mentioned essential oils and active compounds may act as potential antifungal agents against drug-resistant fungi. Active compounds, namely cinnamaldehyde, eugenol and geraniol, were more active than essential oils in their antifungal activities. GC-MS analysis further supports that such active constituents are present in these essential oils which collectively or synergistically interact to impart a more rapid mode of action. In our study, thymol exhibited lower antifungal activity compared to the oil of *T. vulgaris*; however, some researchers have reported higher antifungal activity for thymol (Shin and Lim 2004; Segvic et al. 2007). This difference to our results may be due to the differences in the percent composition of various compounds present in the oil of *T. vulgaris*, methods adapted for determining the MIC as well as strain-to-strain variations in the sensitivity of fungi to essential oils. This could also justify the higher antifungal activity of geraniol contrary to its lower activity as reported by some other workers (Shin and Lim 2004; Aoudou et al. 2010). The potency of cinnamaldehyde 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 and the other essential oils or active compounds. This finding indicates the fungicidal activity of essential oils and active compounds over the fungistatic nature of fluconazole. Also, these essential oils and active compounds demonstrated no toxicity to RBCs in vitro. The promising in vitro antifungal activity of these essential oils and active compounds, along with their non-toxicity to RBCs, prompted us to investigate in vitro combinations of these essential oils or active compounds with fluconazole to increase their efficacy. To our knowledge, no investigation for these particular combinations has been carried out before.

All test combinations showed synergistic interactions against the test strains except the oils of *C. martini* and geraniol against *A. fumigatus* MTCC2550. Differences occurring with the same combination may be because the geraniol and geraniol acetate shared more than 50% of the *C. martini* oil. It is possible that the behavior of *C. martini* oil is greatly affected by geraniol and that *C. martini* oil and its major ingredients share the same mechanistic differences in the interaction results. Cinnamaldehyde was most effective in the combination therapy showing strongest synergy with fluconazole against both *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9. Cinnamaldehyde also reduced the MIC of fluconazole up to 8-fold and its MIC decreased up to 16- and 32-fold. Treatment of fungal infections caused by the drug-resistant fungi may require higher treatment doses. If fluconazole is used in higher doses in monotherapy it can lead to adverse side effects such as hepatotoxicity (Groll et al. 1998). With this in consideration, our data suggests that dose-related toxicity and drug resistance against fluconazole can be overcome in combinations with essential oils or active compounds, particularly cinnamaldehyde.

The synergistic interactions of essential oils or active compounds with fluconazole may be related to the simultaneous inhibition of different target sites by test agents and fluconazole. Fluconazole acts by inhibiting the activity of enzyme 14 α-demethylase which results in the interruption of ergosterol biosynthesis. An increase in number and expansion of endoplasmic reticula serves to detoxify the drugs or pesticides inside the cell (Park et al. 2007) and indicates a stimulation response of the cell to oils as evidenced by transmission electron microscopy for *T. rubrum* IOA-9 treated with cinnamaldehyde. Further, accumulation of polysaccharide granules is responsible for the rupture of plasmalemma structure (Ghahfarokhi et al. 2004). Therefore, it appears that cell wall and cell membrane integrity, along with other membranous structures are the target sites for essential oils or active compounds. This effect may be attributed to the lipophilic properties of essential oils or active compounds and indicates their ability to penetrate the plasma membrane (Knobloch et al. 1989). It is speculated that the fungicidal action of essential oils or active compounds may reduce the antifungal burden for fluconazole. Since fluconazole is a hydrophilic azole, it is less effectively absorbed compared to lipophilic azoles like ketoconazole, clotrimazole and itraconazole by cytosolic components of fungal cells (Scheven and Schwegler 1995). Cell wall lysis by essential oils or active compounds may result in greater absorbance and a more optimal quantity available to act, which leads to improved and more rapid killing. This also supports the conversion of fluconazole from the fungistatic mode of action to a fungicidal mode. This combination may be compared with the synergistic interaction of azoles and cell wall synthesis inhibitors (i.e. echinocandins; Segal and Steinbach 2007). The exact mechanism for disruption of cell wall and membrane synthesis by oils is not understood but multiple target sites by an antifungal agent are added advantages in the combination therapy.

The broad spectrum antifungal activity of the tested essential oils and active compounds against drug-resistant filamentous fungi are encouraging in that they indicate that fungal resistance to antifungal drugs has no impact on the inhibitory activity from essential oils. In other words, the

mode of action of these essential oils or active compounds differs from that of available antifungal drugs like fluconazole. The current study suggests that fungal infections emerging due to the fluconazole-resistant fungal strains could be treated effectively by combining the appropriate essential oils or active compounds, particularly cinnamaldehyde with fluconazole. The topical application of oils could be a very effective means in treating infections of the drug-resistant strains of *Trichophyton* spp.; whereas, systemic use of oils against invasive aspergillosis requires oral administration of oils after mixing with carrier oils or suitable delivery agents (Zia et al. 2010). The oils may also be diluted appropriately to avoid any toxicity to host organs. In addition, these substances may also be inhaled in very minute quantities to treat aspergillosis. Further, in vivo experiments are needed to evaluate the therapeutic efficacy and safety of the essential oils in combination with antifungal drugs.

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