Medical Mycology January 2012, **50,** 33–42

informa
healthcare

Anti-candidal activity of essential oils alone and in combination with amphotericin B or fluconazole against multi-drug resistant isolates of *Candida albicans*

MOHD SAJJAD AHMAD KHAN*, ABIDA MALIK† & IQBAL AHMAD*

**Department of Agricultural Microbiology, Faculty of Agricultural Sciences, and †Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh, India*

Therapy for candidiasis is becoming problematic due to the toxicities of currently available antifungal agents and the increasing prevalence of resistance among the etiologic agents. Therefore, new antifungals and alternative approaches are needed. In this study, 20 fluconazole-resistant strains of *Candida albicans* were found to have varying levels of resistance to other azoles, i.e., itraconazole (MIC of 4–128 μg/ml) and ketoconazole (2–256 μg/ml). In addition, 13 of these isolates appeared resistant to amphotericin B (32–128 μg/ml). A total of 21 plant essential oils were screened for their antifungal activity against these multi-drug resistant isolates. The oils of *Cymbopogon martini*, i.e., citral and cinnamaldehyde, exhibited strong inhibitory activity with minimum inhibitory concentrations (MIC_{50}) ranging from 90–100 μg/ml. The test oils were more effective than fluconazole and amphotericin B in inhibiting azole- and amphotericin B-resistant, as well as amphotericin B-susceptible isolates. The test oils and especially eugenol, exhibited significant synergy with fluconazole or amphotericin B against the test isolates. These findings suggest the possible effective use of certain oils alone or in combination with fluconazole or amphotericin B, against multi-drug resistant isolates of *C. albicans*.

Keywords *Candida albicans*, drug resistance, essential oils, synergy, fluconazole, amphotericin B

Introduction

Fungal infections have been increasing in recent years as a consequence of the growing number of immunocompromised patients due to HIV infection, cancer chemotherapy and organ or bone marrow transplantations [1]. In such persons, *Candida* infections are very common causing oral, vaginal and/or systemic candidiasis. Oropharyngeal candidiasis is generally frequently enountered in AIDS patients who do not have access to highly active antiretroviral therapy (HAART) [2], whereas oral candidiasis often affects cancer patients undergoing chemotherapy and/or radiotherapy [3].

Polyenes and azoles have been the antifungals of choice in the treatment of these fungal infections. However, many problems remain to be solved for most of the available antifungal drugs such as nephro- and hepato-toxicity associated with the use of amphotericin B [4]. A lipid formulation of amphotericin B is less toxic but more costly [4,5]. Azoles, particularly fluconazole, are less toxic after oral or intravenous administration and consequently are often employed [4]. However, azole therapy failures have been observed due to intrinsic resistance in *Candida* spp. such as *C. krusei* and *C. glabrata* and acquired resistance in previously susceptible strains of *C. albicans* due to their continual use in AIDS and cancer patients [6]. About 3.6–7.2 % of *C. albicans* isolates from women with vaginitis are resistant to fluconazole [7] and, oropharangeal candidiasis due to the fluconazole-resistant *Candida* has long been a problem for HIV patients [8,9]. Since the immunocompromised population is increasing in number,

Received 6 December 2010; Received in final revised form 3 March 2011; Accepted 18 April 2011

Correspondence: Iqbal Ahmad, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh-202002, India. E-mail: ahmadiqbal8@yahoo.co.in

DOI: 10.3109/13693786.2011.582890

proper medical therapy is needed to overcome problematic drug resistant strains of *C. albicans*.

Newly developed echinocandins, less toxic and having comparable activity to amphotericin B, display excellent inhibitory and cidal activity against isolates of *C. albicans* exhibiting higher levels of fluconazole resistance [10,11]. However, therapy is still expensive and often not affordable, in particular, in developing countries [12]. Therefore, considering the limitations of currently available antifungal drugs regarding toxicity, activity, cost, and emerging resistance, the search for new alternative strategies is justified.

To circumvent these problems, researchers are exploiting alternative therapeutic approaches against these infections such as the use of drug combinations. Such therapy has potential advantages over monotherapy in terms of reducing dose-related toxicity and emergence of drug resistance, the problems associated with fluconazole and amphotericin B. However, combination therapy is still immature in many countries [13].

Plant products and especially essential oils can be exploited as alternative therapies. Essential oils have long been used in ethnomedicine as effective and safe antifungal agents against *Candida* infections. Several workers have reported promising anti-candidal activity against mucosal candidiasis through the use of various essential oils [14–16]. Since natural products are less expensive and considered safer, they could be explored for their synergistic interactions with drugs of choice for treatment of *Candida* infections which might result in more cost effective and safer formulations. Some *in vitro* studies have reported the combination of oils with fluconazole and amphotericin B against candidal infections [17,18] but less work has been done with drug-resistant fungi.

It is critical to know the susceptibility of causative fungal strains to antifungal drugs and essential oils in order to optimize therapy. Therefore, in this study we determined the susceptibility of 18 clinical isolates of *C. albicans* and two reference strains to six common antifungal drugs, 17 plant essential oils, and four major active ingredients. Furthermore, to develop a safer and more potent antifungal agent, the selected essential oils were evaluated for their activity in combination with amphotericin B or fluconazole against multi-drug resistant isolates of *C. albicans*. These findings highlight the *in vitro* efficacy of certain essential oils alone and in combination with antifungal drugs.

Materials and methods

Organisms and media

In this study, 18 isolates of clinical origin and two reference strains of *C. albicans* were included. The clinical isolates (CA01-18) were recovered from patients with vaginitis, urinary tract infections, and candidemia attending the Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh. *Candida albicans* NRRLY12983 was kindly provided by the fungal culture collection at Agricultural Research Service, USDA, Peoria, USA and *C. albicans* MTCC183 was purchased from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. All isolates were characterized using morphological and physiological methods such as use of HiCrome Candida agar (Himedia Laboratories, Mumbai, India), ability to form germ tubes, and biochemical tests (such as carbon and nitrogen assimilations, urease, and nitrate reduction), and identified as *C. albicans*. The test strains were maintained on Sabouraud dextrose agar (SDA) slants at 4°C and subcultured to Sabouraud dextrose broth (SDB) prior to use.

Plant essential oils and drugs

A total of 17 essential oils and four active compounds were obtained from Wyndmere Naturals, Inc., Minnetonka, MN, USA (*Citrus paradisi*, grapefruit; *Citrus sinensis,* orange; *Foeniculum vulgare*, sweet fennel; *Petroselinum crispum,* parsley; *Apium graveolens*, celery; *Rosemarinus officinalis,* rosemary; *Santalum album*, sandalwood; *Zea mays,* corn; and *Zingiber officinale*, ginger); Himalaya Drug Co., Dehradun, India (*Cinnamomum verum*, cinnamon, *Citrus limon*¸ lemon and *Myristica fragrans*, nutmeg); Himedia Laboratories (*Eucalyptus* sp., eucalyptus; *Mentha piperita* peppermint; and pure compounds of eugenol, cinnamaldehyde); Aroma Sales Corporation, New Delhi, India (*Cymbopogon citratus*, lemongrass; *Cymbopogon martini,* palmrosa; and pure compounds of citral and geraniol); and Dabur India Ltd., New Delhi, India (*Syzygium aromaticum,* clove). The sterile paper discs, antifungal susceptibility test discs and drug powders of amphotericin B and ketoconazole were purchased from Himedia Laboratories. Fluconazole and itraconazole in powder forms were obtained from Pfizer Co. and Jansen Co, Mumbai, India, respectively. Stock solutions (25 mg/ml) of amphotericin B, ketoconazole, fluconazole and itraconazole were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C until used. All test oils obtained from Aroma Sales Corporation, New Delhi were assessed for quality using physicochemical tests such as specific gravity, refractive index, and solubility in alcohol (data not shown) at the Fragrance and Flavour Development Centre, Kannauj, India, as well as chemical composition by gas chromatography-mass spectrometry (data not shown) at the Sophisticated Analytical Instrument Facility of the Indian Institute of Technology, Mumbai, India. Essential oils were diluted ten-fold in 1% DMSO and used in the assays.

Disc diffusion assays

A disc diffusion assay, as recommended by Clinical and Laboratory Standards Institute (CLSI) M44-A2 [19], was used with some modifications to determine the susceptibility of the test isolates. Briefly, 100 µl of yeast suspension adjusted to a 0.5 McFarland standard was streaked onto SDA plates and then antifungal drug discs (10–20 µg/disc), were placed over the agar surface and incubated at 37°C for 24 h. The method of Soković and van Griensven [20] was adapted with slight modifications to determine the susceptibility of isolates against essential oils or active compounds. For this, 100 µl of yeast suspension (0.5 McFarland) was spread onto SDA plates and then paper discs (8 mm) impregnated with 10 µl of essential oils, were placed over the agar surface and incubated at 37°C for 24 h. The diameter of the zone of inhibition around the discs was recorded in mm. Each experiment was conducted in triplicate and the average zone size was calculated. Data are presented as mean ± SD.

Determination of minimum inhibitory concentration by broth macrodilution assays

The minimum inhibitory concentrations (MICs) of drugs used in the studies were determined by the CLSI broth macrodilution reference method M27-A3 [21], as modified by Colombo *et al*. [22]. Briefly, an overnight culture was adjusted to a 0.5 McFarland standard in RPMI 1640 medium with L-glutamine but without bicarbonate and buffered to pH 7.0 with MOPS. One hundred microliters of two-fold serial dilutions of test drugs (10 × concentrations) were prepared and 0.9 ml of diluted inoculum medium was added to each tube and incubated at 37°C for 48 h. Drug free control was included. For the azoles such as fluconazole, itraconazole and ketoconazole, the MIC was established as the lowest concentration that inhibited at least 80% of growth as compared with the control. For amphotericin B, the MIC was defined as the lowest concentration that inhibited visible growth. For MIC determination of the essential oils, the method of Soković and van Griensven [20] was adapted with some modifications. Essential oils were serially diluted in 1 ml SDB to achieve a range of concentrations from 50–800 µg/ml and 10 µl of yeast suspension (0.5 McFarland) was added and incubated at 37°C for 24 h. The MIC was defined as the lowest concentration that inhibited visible growth. Each experiment was repeated three times and the mean MICs were calculated.

Time kill assays

Two test strains, CA01 (resistant to both fluconazole and amphotericin B) and CA09 (resistant to fluconazole but susceptible to amphotericin B), were selected to assess time-dependent killing caused by the most potent essential oils or active compounds and antifungal drugs by the modified method of De Logu *et al*. [10]. Briefly, 2 × MIC concentrations were used. Twenty milliliters of phosphate buffered saline (PBS) solution containing the desired concentrations of the test agents were inoculated with 1 ml of yeast suspension (0.5 McFarland). The control solution contained PBS with yeast inoculum but no essential oils, active compounds or drugs. Immediately after inoculation, 100 µl was collected from the solutions for viable count. Test and control solutions were incubated at 37°C for 48 h. Viable counts were obtained from the test and control solutions at 2, 4, 6, 8, 12, 24, 36, and 48 h by plating 100 µl of 10-fold serial dilutions onto SDA plates and incubating at 37°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 for variation in starting inocula. The relative viable count was plotted against time on a log scale.

Interaction of essential oils or active compounds with antifungal drugs

A checkerboard microtiter test was performed to evaluate the interaction of essential oils or active compounds with fluconazole or amphotericin B against *C. albicans* (CA01, CA02, CA09 and *C. albicans* NRRLY12983. The method of Vitale *et al*. [23] was adapted with some modifications. Briefly, series of two-fold dilutions of each of the oils and drugs was made in SDB and DMSO, respectively, to obtain four times the final concentration to be achieved in the microtiter well. Furthermore, 50 µl of each dilution of an oil was added to the 96-well microtiter plates in the vertical direction, while 50 µl of each dilution of antifungal drugs was added in the horizontal direction, so that various combinations of essential oils or active compounds and fluconazole or amphotericin B could be achieved. In addition, 100 µl of yeast suspension (0.5 McFarland) was added to each well and plates were incubated at 37°C for 48 h. The nature of interaction was defined quantitatively by the means of fractional inhibitory concentrations (FIC) that was calculated as the MIC of the combination of essential oils or active compounds with fluconazole or amphotericin B divided by the MIC of essential oils, active compounds, fluconazole or amphotericin B 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 [24].

Results

Susceptibility of C. albicans *isolates to antifungal drugs*

Table 1 shows the susceptibility of 18 fluconazole resistant and two reference strains of *C. albicans* to six antifungal drugs using the disc diffusion method. Zones of inhibition with fluconazole, itraconazole and clotrimazole varied from nil to <16 mm, whereas, zones of inhibition to nystatin, amphotericin B and ketoconazole ranged from ≥16–31.33 mm against two, seven and nine strains, respectively. These data clearly indicated that there was a decreased susceptibility among fluconazole-resistant strains to itraconazole and clotrimazole followed by nystatin, amphotericin B, and ketoconazole.

MICs of antifungal drugs

Based on the susceptibility behavior of the test strains, the limit of drug resistance was determined using the broth macrodilution method against amphotericin B, ketoconazole, itraconazole, and fluconazole. The strains were considered resistant at the MIC value ≥1.0 μg/ml for itraconazole, and ≥ 64.0 μg/ml for fluconazole as established by CLSI breakpoints. Due to the lack of consensus on MIC breakpoints for amphotericin B and ketoconazole, the MICs for these drugs were established following the criteria suggested by other workers. The limit of resistance was considered as ≥2.0 μg/ml for amphotericin B [25] and ≥1.0 μg/ml for ketoconazole [26].

As presented in Table 2, all test strains were resistant to fluconazole (MIC in the range of 128–256 μg/ml), itraconazole (4–128 μg/ml) and ketoconazole (2–256 μg/ml). Thirteen strains were resistant to amphotericin B with MICs ranging from 32–128 μg/ml, whereas susceptible strains exhibited MICs of 0.25–1.0 μg/ml. The MIC_{50} values for amphotericin B, ketoconazole, itraconazole, and fluconazole were 64, 32, 128, and 256 μg/ml, respectively. These data further indicated the high level of azole resistance in the *C. albicans* test strains and surprisingly, co-resistance to amphotericn B.

Susceptibility of C. albicans *isolates to essential oils and active compounds*

Different susceptibility groups of *C. albicans* isolates were categorized on the basis of zones of inhibition produced by essential oils as depicted in Table 3. Out of the 21 tested essential oils and active compounds, cinnamaldehyde and oil of *C. martini* were highly active against all the test strains followed by others in the order of citral > *C. citratus* > *C. verum* = *S. aromaticum* = eugenol > geraniol, exhibiting inhibition zones > 20 mm for 14–20 of the strains. Oils of *Eucalyptu*s spp., *M. piperita*, *C. sinensis*, *C. paradisi* and *C. limon* were least active

Table 1 Susceptibility of *Candida albicans* isolates to antifungal drugs.

	Diameter of the zone of inhibition (mm)					
Strains	AMB(20)*	NYT(100)**	CLT(10)*	KTC(10)*	FLC(10)*	ITC(10)*
CA 01	19.22 ± 1.69	13.00 ± 0.81	–	14.66 ± 0.94	–	–
CA 02	23.66 ± 1.24	13.00 ± 0.81	12.33 ± 1.24	10.00 ± 0.81	–	9.33 ± 0.47
CA 03	18.33 ± 0.47	15.00 ± 0.81	11.33 ± 0.47	10.33 ± 0.47	–	–
CA 04	16.24 ± 1.24	12.00 ± 0.81	10.00 ± 0.81	11.00 ± 0.81	–	–
CA 05	11.00 ± 0 .81	11.66 ± 0.94	15.66 ± 0.47	20.00 ± 0.81	–	–
CA 06	11.66 ± 0.94	18.33 ± 0.47	13.33 ± 1.24	19.33 ± 1.24	11.33 ± 0.47	8.66 ± 0.47
CA 07	10.66 ± 0.94	11.33 ± 1.24	11.33 ± 1.24	10.00 ± 0.81	10.66 ± 0.94	8.66 ± 0.47
CA 08	19.33 ± 0.47	9.33 ± 0.47	13.66 ± 0.94	10.33 ± 0.47	–	–
CA 09	10.66 ± 0.94	10.66 ± 0.47	–	–	–	–
CA 10	9.00 ± 0.81	–	15.00 ± 0.81	19.00 ± 0.81	–	9.33 ± 0.47
CA 11	9.33 ± 0.47	10.66 ± 0.94	–	–	–	–
CA 12	10.33 ± 0.47	–	–	16.33 ± 0.47	–	–
CA 13	9.00 ± 0.81	–	10.00 ± 0.81	15.00 ± 0.81	–	–
CA 14	10.33 ± 0.47	–	–	19.66 ± 1.24	–	–
CA 15	10.33 ± 0.47	14.33 ± 1.24	12.00 ± 0.81	16.66 ± 0.47	–	–
CA 16	10.66 ± 1.24	–	13.00 ± 0.81	14.33 ± 0.47	10.66 ± 0.94	8.33 ± 0.47
CA 17	10.33 ± 0.47	–	9.33 ± 0.47	–	–	8.66 ± 0.47
CA 18	11.00 ± 0.81	13.00 ± 0.81	–	21.45 ± 2.49	–	9.33 ± 0.94
C. albicans NRRLY12983	21.33 ± 1.24	11.66 ± 1.24	13.00 ± 0.81	19.00 ± 0.81	–	12.33 ± 1.24
C. albicans MTCC183	22.00 ± 0.81	31.33 ± 1.24	10.66 ± 1.24	18.33 ± 1.24	–	–

Potency of drugs in *(μg/disc), **(u/disc), AMB, amphotericin B; NYT, nystatin; CLT, clotrimazole; KTC, ketoconazole; FLC, fluconazole; ITC, itraconazole
–, indicates no zone of inhibition.

Table 2 Susceptibility of 20 strains of *Candida albicans* in terms of MIC to antifungal drugs, essential oils and active compounds.

	MIC range (μg/ml) Total number of strains												
Test agents	0.25	0.5	1	2	4	8	16	32	64	128	256	MIC_{50}	MIC_{90}
Amphotericin B	3	2	2	–	–	–	–	3	6	4	–	64	128
Ketoconazole	–	–	–	3	–	3	2	4	2	4	2	32	256
Itraconazole	–	–	–	–	2	–	–	2	4	12	–	128	256
Fluconazole	–	–	–	–	–	–	–	–	–	3	17	256	256
	50	100	200	400									
S. aromaticum	2	3	12	3								200	400
C. verum	5	5	6	4								100	400
Eugenol	4	5	6	5								200	400
Cinnamaldehyde	7	10	3	–								100	200
	45	90	180	360									
C. citratus	6	3	6	5								180	360
C. martini	6	10	4	–								90	180
Citral	2	10	6	2								90	180
Geraniol	–	1	10	9								180	360

−, indicates no strain.

against the majority of the strains for which inhibition zones were found to be <16 mm. Oils of *A. graveolens*, *F. vulgare*, *P. crispum*, *R. officinalis*, *S. album*, *Z. mays*, *M. fragrans* and *Z. officinale* did not show any detectable activity against the test strains.

MICs of essential oils and active compounds

A total of eight essential oils and active compounds exhibiting the highest activity against the test strains by disc diffusion were assessed for their inhibitory activity in terms of MIC. As evident from Table 2, test oils were also highly active in the order of citral and *C. martini* (MIC_{50} 90 μg/ml) > *C. verum* and cinnamaldehyde (MIC_{50} 100 μg/ml) > *C. citratus* and geraniol (MIC_{50} 180 μg/ml) > eugenol and *S. aromaticum* (MIC_{50} 200 μg/ml).

Time kill assays

The potency of killing was evaluated for CA01 and CA09 strains with the most active essential oils viz. *S. aromaticum*, *C. verum*, *C. citratus*, *C. martini* and active compounds namely eugenol, cinnamaldehyde, citral, and geraniol as compared to amphotericin B and fluconazole. The time-dependent killing of CA01 at 8 h revealed a decrease of >1 $\log_{10}$ in the viable count compared to the control by citral, cinnamaldehyde and eugenol followed by *C. verum* (between 9–10 h), geraniol and *C. martini* (between 19–20 h), and *C. citratus*, *S. aromaticum* (24 h). Amphotericin B showed a similar effect by 34 h and fluconazole showed no effect within 48 h (Fig. 1a, b). Citral was most active against CA09 by reducing the viable count >1 $\log_{10}$ in 6 h followed by cinnamaldehyde and eugenol (8 h) (Fig. 2b). Activity of oils of *C. verum* and *S. aromaticum* was similar to that against CA01 (Fig. 2a). Oils of *C. martini* and *C. citratus* produced the same effect in 18 h and geraniol in 22 h, whereas, a similar effect with amphotericin B required 44 h and there was no effect with fluconazole within 48 h (Fig. 2a, b)

Synergistic interaction of essential oils or active compounds with antifungal drugs

The combined effects of the oils of *S. aromaticum*, *C. verum*, *C. citratus*, *C. martini* and their major active

Table 3 Susceptibility of *Candida albicans* isolates to different essential oils and active compounds.

Sensitivity behavior (range of zone of inhibition mm)	Test oils (number of strains)												
	CL	CN	LG	PR	ES	PT	OR	LN	GF	EL	CD	CT	GR
Least susceptible (8 – <16)	–	–	–	–	17	19	18	18	15	–	–	–	1
Moderate susceptible (16–20)	3	3	2	–	–	–	–	1	–	3	–	1	5
Highly Susceptible (>20)	17	17	18	20	–	–	–	–	–	17	20	19	14
Non susceptible (No zone of inhibition)	–	–	–	–	3	1	2	1	5	–	–	–	–

CL, *S. aromaticum*; CN, *C. verum*; LG, *C. citratus*; PR, *C. martini*; ES, *E.* sp; PT, *M. piperita*; LN, *C. limon*; OR, *C. sinensis*; GF, *C. paradise*; EL, Eugenol; CD, Cinnamaldehyde; CT, Citral; GR, Geraniol.
−, indicates no strain found susceptible.

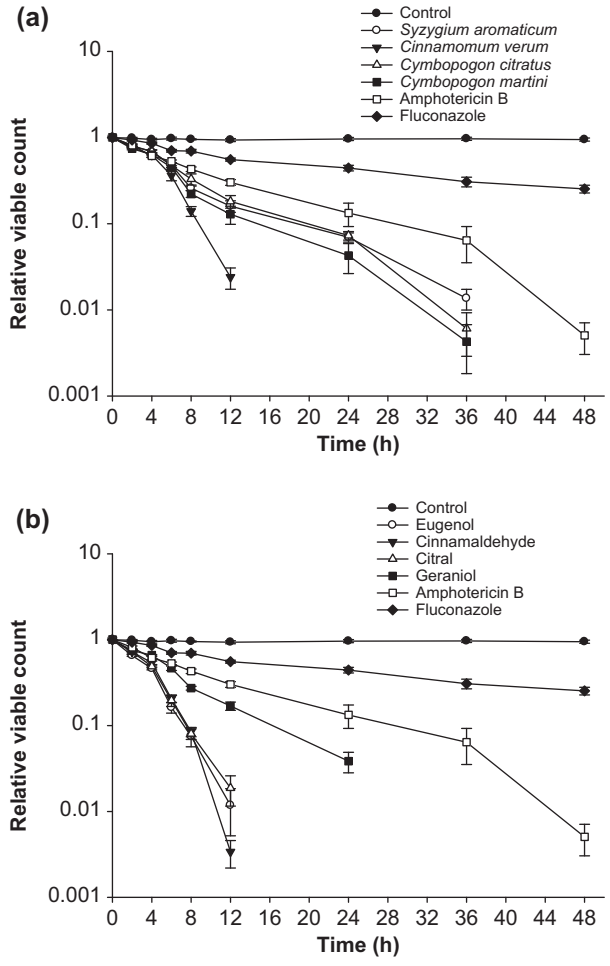

Fig. 1 (a) Time kill assays for essential oils and drugs against CA 01. (b) Time kill assays for active compounds and drugs against CA 01.

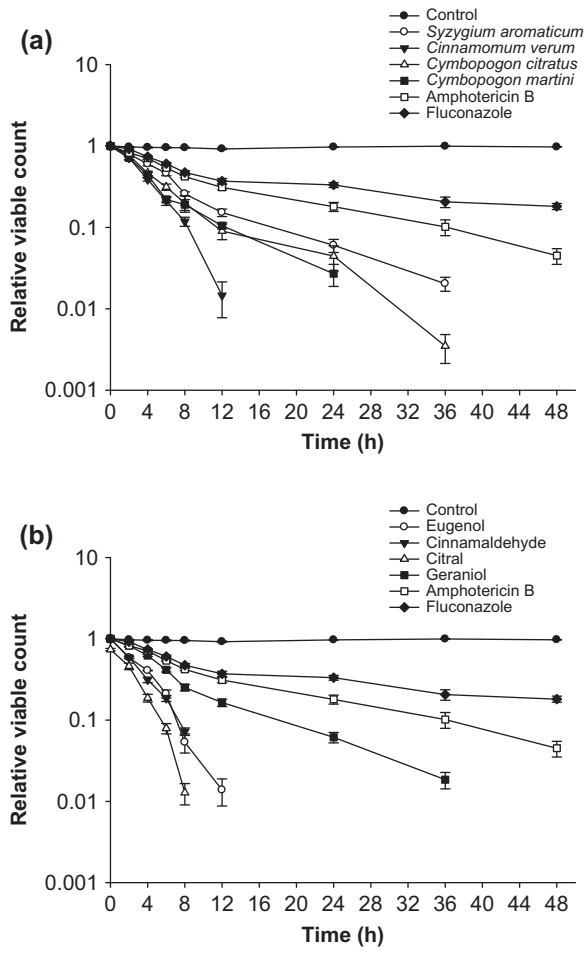

Fig. 2 (a) Time kill assays for essential oils and drugs against CA 09. (b) Time kill assays for active compounds and drugs against CA 09.

compounds like eugenol, cinnamaldehyde, citral and geraniol with fluconazole or amphotericin B against CA01, CA02, CA09 and *C. albicans* NRRLY12983 strains are shown in Tables 4 and 5. All the tested essential oils and active compounds showed varying levels of interaction with fluconazole or amphotericin B against the test strains except eugenol which was found to be synergistic with both fluconazole and amphotericin B. Oil of *S. aromaticum* showed synergistic interaction with only amphotericin B against all the test strains except CA01. Citral, cinnamaldehyde, and oils of *C. verum* and *C. citratus* exhibited both synergy and no interaction responses in combination with amphotericin B. Geraniol displayed a varying level of synergistic interaction with both fluconazole and amphotericin B against test strains except *C. albicans* NRRLY12983. Among all the tested combinations with fluconazole, eugenol (CA 02, 09) and citral (CA 01) exhibited the highest synergy by causing a reduction in the fluconazole MIC and their own by 16- to 32-fold. In combination with amphotericn B, cinnamaldehyde exhibited the highest synergy by reducing the MIC of amphotericin B by 32-fold and its own by 16-fold whereas, eugenol showed reduction of 8- and 16-fold, respectively. Interestingly, no combination was found to be antagonistic against the test strains.

Discussion

In this study, susceptibility of fluconazole-resistant test strains of *C. albicans* were found to have a cross resistance to other azoles, namely itraconazole and ketoconazole, across a wide range of concentrations (2–256 μg/ml). *Candida albicans* and non-*C. albicans Candida* spp. have both shown decreased susceptibilities in immunocompromised individuals and several workers have reported resistance to azoles [10,27–29]. Although some of the test strains were

Table 4 Interaction of essential oils and active compounds with fluconazole against *Candida albicans* strains.

	CA 01				CA 02				CA 09				*C. albicans* NRRLY12983			
Combination	A	C	FICI	T	A	C	FICI	T	A	C	FICI	T	A	C	FICI	T
CL-FLC																
CL (μg/ml)	400	100	0.750	I	200	100	0.750	I	200	100	0.750	I	400	200	1.0	I
FLC (μg/ml)	256	128			256	64			256	128			256	128		
CN-FLC																
CN (μg/ml)	400	200	0.750	I	200	100	1.0	I	100	50	1.0	I	200	25	0.375	S
FLC (μg/ml)	256	64			256	128			256	128			256	64		
LG-FLC																
LG (μg/ml)	90	11.25	0.156	S	90	11.25	0.375	S	180	45	0.375	S	90	11.25	0.375	S
FLC (μg/ml)	256	8			256	64			256	32			256	64		
PR-FLC																
PR (μg/ml)	90	11.25	0.625	I	180	90	1.0	I	45	22.5	1.0	I	180	45	0.750	I
FLC(μg/ml)	256	128			256	128			256	128			256	128		
EL-FLC																
EL (μg/ml)	400	25	0.187	S	400	12.5	0.093	S	50	3.125	0.093	S	400	100	0.375	S
FLC (μg/ml)	256	32			256	16			256	8			256	32		
CD-FLC																
CD (μg/ml)	100	100	1.5	I	50	50	1.5	I	100	100	1.5	I	100	12.5	0.375	S
FLC (μg/ml)	256	128			256	128			256	128			256	64		
CT-FLC																
CT (μg/ml)	90	5.62	0.093	S	90	5.62	0.187	S	90	5.62	0.187	S	90	11.25	0.250	S
FLC (μg/ml)	256	8			256	32			256	32			256	32		
GR-FLC																
GR (μg/ml)	360	22.5	0.312	S	180	22.5	0.187	S	360	45	0.187	S	360	180	1.0	I
FLC (μg/ml)	256	64			256	16			256	16			256	128		

CL, *S. aromaticum*; CN, *C. verum*; LG, *C. citratus*; PR, *C. martini*; EL, eugenol; CD, cinnamaldehyde; CT, citral; GR, geraniol; FLC, fluconazole
A, MIC of agent alone; C, MIC of agent in combination; T, nature of interaction; I, indifferent, S, synergy, A, antagonism.

susceptible to amphotericin B in our study, co-resistance was also clearly displayed. Other workers also reported development of resistance to amphotericin B in *C. albicans* [27,29]. Multi-drug resistance is a serious issue in the treatment of opportunistic fungal infections of immunocompromised individuals such as transplant recipients and cancer patients undergoing cytotoxic chemotherapy [30].

Therefore, considering the importance of reduced or non-susceptibility to conventional azoles and polyenes, the test isolates were screened for their susceptibility to certain essential oils and some of their major active compounds. Eight essential oils/active compounds exhibited promising anti-candidal activity. Cinnamaldehyde, citral, and oil of *C. martini* were highly active against multi-drug resistant strains of *C. albicans* with a MIC range of 45–200 μg/ml. The time kill assays reflected the efficacy of oils in killing fluconazole-resistant test strains of *C. albicans* which also exhibited resistance or susceptibility to amphotericin B. These oils appeared to be more cidal than amphotericin B.

The above test oils were also found to be nontoxic when assayed for RBC lysis [31] and may be a preferred choice in drug combinations. Hence, these oils or compounds were tested for their interactive effects with fluconazole and amphotericin B. Their synergistic effect seems to hold promise in combination therapy, preferably by combining agents with different antifungal mechanisms. Antifungal agents in combination can exhibit improved efficacy because of the increased rate of killing, a broader spectrum of action covering multiple infections by different pathogens and a reduced duration of therapy resulting in the decreased likelihood of developing resistance [32]. In our study, eugenol being a potential anti-candidal agent alone, also exhibited synergistic interaction with both fluconazole and amphotericin B. Although oil of *C. martini* was an effective anti-candidal agent alone, the combinational approach was indifferent with both fluconazole and amphotericin B. Geraniol exhibited moderate anti-candidal activity alone, and showed a significant level of synergy with both fluconazole and amphotericin B. In addition, higher MICs to amphotericin B and fluconazole in test strains were reduced substantially (16- to 32-fold) and, thereby indicated the effectiveness of these combinational approaches. As revealed in this study, the interactive responses of oils and active compounds did not appear to be affected by the susceptibility behavior of the strains to amphotericin B or fluconazole. Rather variations in the combinational effects may pertain to the nature of oils,

Table 5 Interaction of essential oils and active compounds with amphotericin B against *Candida albicans* strains.

	CA 01				CA 02				CA 09				*C. albicans* NRRLY12983			
Combination	A	C	FICI	T	A	C	FICI	T	A	C	FICI	T	A	C	FICI	T
CL-AMB																
CL (μg/ml)	400	200	1.0	I	400	25	0.375	S	200	25	0.375	S	400	25	0.312	S
AMB (μg/ml)	0.5	0.25			0.25	0.062			128	32			0.25	0.062		
CN-AMB																
CN (μg/ml)	400	25	0.312	S	200	50	0.750	I	100	50	1.0	I	200	6.25	1.03	I
AMB (μg/ml)	0.5	0.125			0.25	0.125			128	64			0.25	0.25		
LG-AMB																
LG (μg/ml)	90	22.5	0.750	I	90	11.25	0.625	I	180	22.5	0.375	S	90	22.5	1.25	I
AMB (μg/ml)	0.5	0.25			0.25	0.125			128	32			0.25	0.25		
PR-AMB																
PR (μg/ml)	90	22.5	0.750	I	180	45	0.750	I	45	11.25	0.750	I	180	45	0.750	I
AMB (μg/ml)	0.5	0.25			0.25	0.125			128	64			0.25	0.125		
EL-AMB																
EL (μg/ml)	400	25	0.187	S	400	12.5	0.156	S	50	3.25	0.312	S	400	50	0.250	S
AMB (μg/ml)	0.5	0.062			0.25	0.031			128	32			0.25	0.031		
CD-AMB																
CD (μg/ml)	100	6.25	0.093	S	50	6.25	0.250	S	100	50	1.0	I	100	12.5	0.250	S
AMB (μg/ml)	0.5	0.015			0.25	0.031			128	64			0.25	0.031		
CT-AMB																
CT (μg/ml)	90	22.5	0.750	I	90	11.25	0.187	S	90	11.25	0.250	S	90	5.625	0.312	S
AMB (μg/ml)	0.5	0.25			0.25	0.015			128	16			0.25	0.062		
GR-AMB																
GR (μg/ml)	360	45	0.375	S	180	22.5	0.375	S	360	45	0.250	S	360	90	0.750	I
AMB (μg/ml)	0.5	0.125			0.25	0.062			128	16			0.25	0.125		

CL, *S. aromaticum*; CN, *C. verum*; LG, *C. citratus*; PR, *C. martini*; EL, eugenol; CD, cinnamaldehyde; CT, citral; GR, geraniol; AMB, Amphotericin B
A, MIC of agent alone; C, MIC of agent in combination; T, nature of interaction; I, indifferent, S, synergy, A, antagonism.

their constitutional compounds and their concentrations. It may be explained by the criterion that ingredients of oils with different individual modes of action may interact alone or among themselves in oil, differently with antifungal drugs depending on the ratios and concentrations employed.

Fluconazole inhibits fungal cytochrome P450-dependent enzyme lanosterol 14-α-demethylase. Blocking ergosterol biosynthesis, as well as inhibiting P450-dependent enzymes involved in fungal respiration. Amphotericin B binds to ergosterol in the cell membrane leading to perforation and leakage of cytosol and cell death [12,32]. The synergistic combination of these drugs with oils may be explained mechanistically by the oils promoting the effects of antifungal drugs, mainly on the cell wall, plasma membrane and other membrane structures of yeast cells [33,34]. In addition, it could be possible that the cidal effect of oils lowers the levels of the drugs required to exert antifungal actions. Fluconazole, being a hydrophilic azole, does not bind or stay with the cell membrane and enters the cell to act upon it [32]. The oils damaging the cell wall and cell membrane may facilitate its entry to the cell leading to a greater effect on the ergosterol biosynthesis inhibition and adding to cell membrane destruction. This might also turn the fungistatic action of fluconazole into a fungicidal action. Regarding amphotericin B, its binding to ergosterol in the cell membrane is aided by cell wall rupture and may lead to enhanced effect of cell membrane damage. Also, dose-related toxicity of amphotericin B might be overcome in synergistic combination therapy. These combinations of fluconazole or amphotericin B with oils are comparable to their combinations with echinocandins, targeting the 1,3-β-glucan synthase enzyme and thereby disrupting cell wall synthesis [4,12]. Furthermore, efficient fungicidal activity of oils against azole- and amphotericin B-resistant or -susceptible isolates suggests that oils are effective against strategy or adaptive mechanisms of resistance exhibited by *C. albicans* isolates against these drugs.

In our findings, oils especially *C. verum*, citral, cinnamaldehyde and eugenol were quite effective against drug resistant or susceptible isolates of *C. albicans* and are potential alternatives as anti-candidal agents alone. Since these oils, especially eugenol, also exhibit strong synergistic behavior, this highlights their potential exploitation in combination therapy. Therefore, a preferred combination may be developed for oils with these drugs for treatment against *Candida* spp. irrespective of their susceptibility to individual antifungal agents. Drug resistance to azoles and

co-resistance to amphotericin B is a major problem in clinical therapy for oral and vaginal candidiasis [7,9,35,36]. These oils might be useful topically to deal with such infections. Our findings suggest that essential oils alone or in combination with antifungal drugs could provide an improved and/or safer clinical approach towards the management of fungal infections caused by drug resistant strains.

Acknowledgement

We are thankful to Dr Zehra Mohsin, Department of Gynecology, Jawaharlal Nehru Medical College and Hospital, AMU, Aligarh for providing the clinical samples for isolation of *C. albicans*. We also acknowledge the Indian Council of Medical Research, New Delhi for providing financial assistance in the form of SRF to M. S. A. Khan.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the manuscript.

References

1 Aperis G, Myriounis N, Spanakis EK, Mylonakis E. Developments in the treatment of candidiasis: more choices and new challenges. *Expert Opin Investig Drugs* 2006; **15**: 1319–1336.
2 De Repentigny L, Lewandowski D, Jolicoeur P. Immunopathogenesis of oropharyngeal candidiasis in human immunodeficiency virus infection. *Clin Microbiol Rev* 2004; **17**: 729–759.
3 Davies AN, Brailsford SR, Beighton D. Oral candidosis in patients with advanced cancer. *Oral Oncol* 2006; **42**: 698–702.
4 Groll AH, Kolve H. Antifungal agents: *in vitro* susceptibility testing, pharmacodynamics, and prospects for combination therapy. *Eur J Clin Microbiol Infect Dis* 2004; **23**: 256–270.
5 Herbrecht R, Natarajan-Ame S, Nivoix Y, Letscher-Bru V. The lipid formulations of amphotericin B. *Expert Opin Pharmacother* 2003; **4**: 1277–1287.
6 Pfaller MA, Pappas PG, Wingard JR. Invasive fungal pathogens: current epidemiological trends. *Clin Infect Dis* 2006; **43**: S3–S14.
7 Sobel JD, Zervos M, Reed BD, *et al*. Fluconazole susceptibility of vaginal isolates obtained from women with complicated *Candida* vaginitis: clinical implications. *Antimicrob Agents Chemother* 2003; **47**: 34–38.
8 Revankar SG, Kirkpatrick WR, McAtee RK. *et al*. Detection and significance of fluconazole resistance in oropharangeal candidiasis in human immunodeficiency virus infected patients. *J Infect Dis* 1996; **174**: 821–827.
9 Perea S, Lopez-Ribot JL, Wickes BL, *et al*. Molecular mechanisms of fluconazole resistance in *Candida dubliniensis* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. *Antimicrob Agents Chemother* 2002; **46**: 1695–1703.
10 De Logu A, Saddi M, Cardia MC, *et al*. *In vitro* activity of 2-cyclohexylidenhydrazo-4-phenyl-thiazole compared with those of amphotericin B and fluconazole against clinical isolates of *Candida* spp. and fluconazole-resistant *Candida albicans*. *J Antimicrob Chemother* 2005; **55**: 692–698.
11 Messer SA, Diekema DJ, Boyken L, *et al*. Activities of micafungin against 315 invasive clinical isolates of fluconazole-resistant *Candida* spp. *J Clin Microbiol* 2006; **44**: 324–326.
12 Onyewu C, Heitman J. Unique applications of novel antifungal drug combinations. *Anti-infect Agents Med Chem* 2007; **6**: 3–15.
13 Ahmad I, Khan MSA, Zahin M, *et al*. Combinational antifungal therapy and recent trends in drug discovery. In: Ahmad I, Owais M, Shahid M, Aqil F (eds). *Combating Fungal Infections: Problems and Remedy*, 1st edn. Berlin Heidelberg: Springer-Verlag, 2010: 213–240.
14 Mondello F, De Bernardis F, Girolamo A, Cassone A, Salvatore G. *In vivo* activity of terpinen-4-ol, the main bioactive component of *Melaleuca alternifolia* cheel (tea tree) oil against azole-susceptible and -resistant human pathogenic *Candida* species. *BMC Infect Dis* 2006; **6**: 158–165.
15 Noumi E, Snoussi M, Hajlaoui H, Valentin E, Bakhrouf A. Antifungal properties of *Salvadora persica* and *Juglans regia* L. extracts against oral *Candida* strains. *Eur J Clin Microbiol Infect Dis* 2010; **29**: 81–88.
16 Zhang JD, Xu Z, Cao YB, *et al*. Antifungal activities and action mechanisms of compounds from *Tribulus terrestris* L. *J Ethnopharmacol* 2006; **103**: 76–84.
17 Khodavandi A, Alizadeh F, Aala F, Sekawi Z, Chong PP. *In vitro* investigation of antifungal activity of allicin alone and in combination with azoles against *Candida* species. *Mycopathologia* 2010; **169**: 287–295.
18 Mahboubi M, Bidgoli FG. *In vitro* synergistic efficacy of combination of amphotericin B with *Myrtus communis* essential oil against clinical isolates of *Candida albicans*. *Phytomedicine* 2010; **17**: 771–774.
19 Clinical and Laboratory Standards Institute. *Reference Method for Antifungal Disc Diffusion Susceptibility Testing of Yeasts.* Proposed guideline M44-A2, Wayne, PA: Clinical and Laboratory Standards Institute USA, 2009.
20 Sokovic M, Van Griensven LJLD. Antimicrobial activity of essential oils and their components against the three major pathogens of the cultivated button mushroom, *Agaricus bisporus*. *Eur J Plant Pathol* 2006; **116**: 211–224.
21 Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*. Proposed guideline M27-A3, Wayne, PA: Clinical and Laboratory Standards Institute, 2008.
22 Colombo AL, Barchiesel F, McGough DA, Rinaldi MG. Comparison of Etest and national committee for clinical laboratory standards broth macrodilution method for azole antifungal susceptibility testing. *J Clin Microbiol* 1995; **33**: 535–540.
23 Vitale RG, Afeltra J, Dannoui E. Antifungal combinations. In: Ernst EJ, Rogers PD (eds). *Antifungal Agents: Methods and Protocols*, Totowa, NJ: Humana Press Inc, 2005: 143–152.
24 Odds FC. Synergy, antagonism, and what the chequerboard puts between them. *J Antimicrob Chemother* 2003; **52**: 1.
25 Yang YL, Li SY, Cheng HH, Lo HJ, TSARY Hospitals. The trend of susceptibilities to amphotericin B and fluconazole of *Candida* species from 1999 to 2002 in Taiwan. *BMC Infect Dis* 2005; **5**: 99–103.
26 Milan EP, Burattini MN, Kallas EG, *et al.* Azole resistance among oral *Candida* species isolates from AIDS patients under ketoconazole exposure. *Diagn Microbiol Infect Dis* 1998; **32**: 211–216.
27 Swinne D, Watelle M, Nolard N. *In vitro* activities of voriconazole, fluconazole, itraconazole and amphotericin B against non *Candida albicans* yeast isolates. *Rev Iberoam Micol* 2005; **22**: 24–28.
28 Hamza OJM, Matee MIN, Moshi MJ, *et al*. Species distribution and *in vitro* antifungal susceptibility of oral yeast isolates from Tanzanian HIV-infected patients with primary and recurrent oropharyngeal candidiasis. *BMC Microbiol* 2008; **8**: 135–143.

29 Tseng YH, Lee WT, Kuo TC. *In-vitro* susceptibility of fluconazole and amphotericin B against *Candida* isolates from women with vaginal candidiasis in Taiwan. *J Food Drug Anal* 2005; **13**: 12–16.
30 Thakur JK, Arthanari H, Yang F, *et al*. A nuclear receptor-like pathway regulating multidrug resistance in fungi. *Nature* 2008; **452**: 604–609.
31 Khan MSA, Ahmad I. Antifungal activity of essential oils and their synergy with fluconazole against drug-resistant strains of *Aspergillus fumigatus* and *Trichophyton rubrum. Appl Microbiol Biotechnol* 2011; **90**: 1083–1094.
32 Vazquez JA. Combination antifungal therapy against *Candida* species: the new frontier–are we there yet? *Med Mycol* 2003; **41**: 355–368.
33 Ishida K, De Mello JCP, Cortez DAG, *et al*. Influence of tannins from *Stryphnodendron adstringens* on growth and virulence factors of *Candida albicans*. *J Antimicrob Chemother* 2006; **58**: 942–949.
34 Pinto E, Pina-Vaz C, Salgueiro L, *et al*. Antifungal activity of the essential oil of *Thymus pulegioides* on *Candida*, *Aspergillus* and dermatophyte species. *J Med Microbiol* 2006; **55**: 1367–1373.
35 Rautemaa R, Richardson M, Pfaller M, Perheentupa J, Saxen H. Reduction of fluconazole susceptibility of *Candida albi**ca**ns* in APECED patients due to long-term use of ketoconazole and miconazole. *Scand J Infect Dis* 2008; **40**: 904–907.
36 Pinto PM, Weikert-Oliveira RC, Lyon JP, *et al*. *In vitro* antifungal susceptibility of clinical isolates of *Candida* spp. obtained from patients with different predisposing factors to candidosis. *Microbiol Res* 2008; **163**: 579–585.

This paper was first published online on Early Online on 14 July 2011.