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Candida albicans biofilm inhibition by synergistic action of terpenes and fluconazole

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The current treatment options for *Candida albicans* biofilm-device related infections are very scarce due to their intrinsic increased tolerance to antimycotics. The aim of this work was to study synergistic action of terpenes (eugenol, menthol and thymol) with fluconazole (FLA) on *C. albicans* biofilm inhibition. The minimum inhibitory concentration (MIC) assayed using CLSI M27-A3 broth micro-dilution method showed antifungal activity against *C. albicans* MTCC 227 at a concentration of 0.12 % (v/v) for both thymol and eugenol as compared to 0.25 % (v/v) for menthol. FLA was taken as positive control. The effect of these terpenes on metabolic activity of preformed *C. albicans* biofilm cells was evaluated using 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay in 96-well polystyrene microtiter plate. Thymol and eugenol were more effective at lower concentrations of $\geq$ 1.0 % (v/v) than menthol. Synergistic studies using checkerboard micro-dilution assay showed fractional inhibitory concentration index (Σ FIC=0.31) between thymol/FLA followed by eugenol/FLA (Σ FIC=0.37) and menthol/FLA (Σ FIC<0.5) against pre-formed *C. albicans* biofilms. Thymol with fluconazole showed highest synergy in reduction of biofilm formation than eugenol and menthol which was not observed when their activities were observed independently. Adherence assay showed 30% viability of *C. albicans* cells after 2 h of treatment with 0.05 % (v/v) thymol/FLA. Effect of thymol/FLA on *C. albicans* adhesion visualized by SEM micrographs showed disruption in number of candidal cells and alteration in structural design of *C. albicans*. Thus, the study demonstrated synergistic effect of terpenes with fluconazole on *C. albicans* biofilm, which could be future medications for biofilm infections.

Keywords: Antifungal therapy, Biofilm, *C andida albicans*, Terpenes

The uprising *Candida* biofilm infections during last decades have almost paralleled the increased use of a broad range of medically implanted devices in patients with impaired host defences. *Candida albicans* is a foremost human fungal pathogen allied with colonisation and biofilm formation on the surfaces of medical devices[1]. Due to its versatility it can behave as a commensal organism in several anatomically distinct sites which can pose a major problem from clinical point of view resulting in infections[2]. Further, in recent years there has been mounting concern about the rising pervasiveness of infections caused by yeasts that are resistant to normally used antifungal drugs. The efficacy of the majority of antifungal agents is greatly reduced if yeasts are in a biofilm as opposed to the planktonic state. Of particular concern is that biofilms display increased resistance to antifungal therapy, cause failure of implant devices, and serve as a reservoir or source for future continuing infections[3-5]. Hence there is a need to identify new methods of preventing and treating candidiasis among immunocompromised patients, both to improve treatment of established infections and to limit further development of drug resistance. Thus, new therapeutic strategies using natural products, of which essential oils are of immense importance especially in reduce oral infections[6]. These oils are complex mixtures of volatile compounds and are known for their *in vitro* and/or *in vivo* antifungal properties[7,8]. The strong antifungal activity of some major components of essential oils, i.e. terpenes has been described. Therefore, the search for new antifungal agents effective against biofilms has important clinical implications that may affect the outcome of patients suffering from these difficult-to-treat infections. Hence, in present investigation synergistic action of terpenes (eugenol, menthol and thymol) with fluconazole (FLA) has been studied on *C. albicans* with an aim to inhibit its biofilm formation.

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Materials and Methods

Fungal strain and growth conditions—C. albicans MTCC 227 used in present investigation was cultured

in yeast peptone dextrose broth (YPD) medium and incubated for 24 h at 35 °C with agitation (120 rpm). Blastospores obtained were harvested, washed twice in 0.1 M phosphate-buffered saline (PBS, *p*H 7.2) and adjusted to 1×10^7 blastospores mL^{-1} in PBS for biofilm formation[6]. All growth media chemicals used in the present investigation were purchased from Hi Media, India. 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) tetrazolium salt and Menadione were purchased from Sigma Chemical Co. (St. Louis, Mo. USA).

Minimum inhibitory concentration (MIC) of terpenes—The terpenes (eugenol, menthol and thymol) were prepared as stock solution of 16 % (v/v) in Roswell Park Memorial Institute medium (RPMI-1640) and 0.1 % (v/v) Tween-80. The MICs of the terpenes against planktonic *C. albicans* were determined by Clinical and Laboratory Standards Institute (CLSI M27-A3) broth micro-dilution method[13]. An overnight grown culture of *C. albicans* was diluted to final concentration of 2.5×10^3 blastospores mL^{-1} in RPMI 1640-MOPS medium and inoculated into commercially available, presterilised, polystyrene, flat-bottomed 96-well microtiter plate (MTP). Terpenes concentrations ranging from 0.01-8 % (v/v) were added to the 96-well MTP wells and incubated for 48 h at 35 °C. The growth in presence of terpenes was estimated using MTP reader at optical density 530 nm after incubation.

Biofilm formation of C. albicans—The effects of terpenes on biofilm cell viability were established by measurement of cell metabolic activity of *C. albicans* biofilm in 96-well MTP. A 200 µL aliquot of *C. albicans* cells suspension containing 1×10^7 blastospores mL^{-1} in RPMI 1640-MOPS medium was added in MTP wells at 35 °C for 90 min for adhesion. The medium was then aspirated from the wells and washed with sterilized PBS to remove loosely adhered cells. Further, RPMI 1640-MOPS medium (200 µL) was added to each MTP wells and incubated for 24 h at 35 °C to obtain biofilm. After that, the growth medium was carefully removed by aspiration without disrupting the integrity of biofilm, washed thrice with PBS (*p*H 7.2) to remove non-adherent cells[6].

Effect of terpenes on C. albicans biofilms—The plate with the seeded biofilm was used subsequently to determine the effect of terpenes on the metabolic activity of the biofilm. The final concentrated terpenes used in this experiment ranged from 0.01-8 % (v/v). Controls plates of seeded biofilm without terpenes were also included in each experiment. After incubation at 35 °C for 24 h, the terpenes were removed, and the treated biofilm were washed thrice with PBS followed by XTT assay.

Evaluation of the effect of terpenes on biofilm development—Metabolic activity was assessed using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) tetrazolium salt reduction, assay as described earlier[6]. Briefly, each well was incubated for 1.5 h at 35 °C with 40 µL of XTT (1 mg mL^{-1} in PBS) and 2 µL of menadione (0.4 mM in acetone) in 158 µL of PBS, *p*H 7.2. All plates were covered with aluminium foil and further incubated in the dark at 35 °C for 2 h. Aliquots (70 µL) of coloured cell-free supernatant in each well were then transferred to another blank MTP, and absorbance was determined spectrophotometrically at 492 nm. The sessile minimum inhibitory concentration ($SMIC_{90}$) was recorded as the lowest concentration resulting in a 90% reduction in absorbance when compared with control biofilm.

Assessment of drug synergy against C. albicans biofilm—The effects of terpenes and Fluconazole (FLA) on biofilm cell viability were established by measurement of cell metabolic activity of *C. albicans* biofilm in a 96-well MTP[14]. A 200 µL aliquot of a suspension of *C. albicans* cells in RPMI 1640-MOPS (1×10^7 blastospores mL^{-1}) was added in wells for biofilm formation. After incubation at 35 °C for 24 h, the growth medium was carefully removed by aspiration without disruption of the integrity of the biofilm, and the formed biofilms were carefully washed thrice with PBS (*p*H 7.2) to remove non-adherent cells. The plate with the seeded biofilms was used subsequently for a checkerboard micro-dilution assay to determine the effect of terpenes with FLA (represented as terpenes/FLA in entire text) on the metabolic activity of the biofilm. The final concentrations of terpenes ranged from 0.01-8% (v/v), and for FLA, from 0.01 to 0.5 mg mL^{-1}. After incubation at 35 °C for 24 h, the terpenes and antifungal agents were removed, and the treated biofilms were washed three times with PBS and XTT reduction assay was performed. The fractional inhibitory concentration (FIC) defined as the ratio of the MIC of an agent used in combination to the MIC of the agent used alone was calculated using the formula:

FIC = MIC (A combination)/MIC (A alone) + MIC (B combination)/MIC (B alone)

where A is MIC value of individual terpenes in % (v/v) when combined with FLA, B is MIC value of FLA in mg mL^{-1} when combined with individual terpenes.

ΣFIC < 0.5 indicates synergy; 0.5–4.0, indifference; and >4, antagonism (ΣFIC, the sum of individual FICs).

Adherence assay—The effect of terpenes/FLA on *C. albicans* adhesion was estimated by adding 200 µL of Candidal cells (1×10^7 blastospores mL^{-1} in RPMI 1640-MOPS medium) to presterilized polystyrene 96-well plate which were then treated for 0, 1, 2, or 4 h after adhesion with terpenes along with FLA (0.5 mg mL^{-1}) to adhere for 4 h at 37 °C. The media was then aspirated and wells were washed with sterilized PBS to remove loosely adhered cells. The plates were read at 492 nm by performing XTT reduction assay. The results were expressed in terms of percent cell viability compared to terpene/FLA-untreated wells, which were used as control.

Scanning electron microscopy (SEM)—Effect of thymol on *C. albicans* biofilm in comparison to control formed on PS coupons were visualized by SEM. Briefly, *C. albicans* biofilm formed on PS surface, were fixed with 2.5% (v/v) glutaraldehyde in PBS (0.1M, *p*H 7.2) for 2 h at room temperature. They were then treated with 1% (w/v) uranyl acetate for 1 h, and washed with distilled water. The samples were dehydrated with ethanol series (30, 50, 70, 90 and 100%). All samples were dried to critical point by Polaron critical point drier, coated with gold and viewed under SEM (Leo435, England)[6].

All experiments were performed in triplicate and results were expressed as mean±SD. Statistical analyses of the differences between mean values obtained for experimental groups were performed using Student's *t*-test. *P* values of 0.05 or less were considered significant.

Results

MIC of terpenes—The MIC done using CLSI M27-A3 broth micro-dilution method showed antifungal activities at a concentration of 0.12 % (v/v) for both thymol and eugenol as compared to 0.25 % (v/v) for menthol respectively, against *C. albicans* MTCC 227 (Fig. 1).

Effect of terpenes on C. albicans biofilms—The effect of terpenes (eugenol, thymol and menthol) on metabolic activity of preformed *C. albicans* biofilm cells was evaluated using XTT reduction assay in 96-well MTP. Data obtained from different concentration of terpenes mediated disruption of pre-formed *C. albicans* biofilm for 24 h at 35 °C revealed that reduction of *C. albicans* biofilm took place in a dose dependent manner. Data also showed thymol and eugenol were more effective at lower concentrations of ≥ 1.0 % (v/v) than menthol (Fig. 2).

Assessment of drug synergy against C. albicans biofilm—Synergistic studies using checkerboard micro-dilution assay showed fractional inhibitory concentration index (ΣFIC=0.31) between thymol/FLA followed by eugenol/FLA (Σ FIC=0.37) and menthol/FLA (Σ FIC<0.5) against pre-formed *C. albicans* biofilms (Table 1). Thymol with fluconazole showed highest synergy in reduction of biofilm formation than eugenol and menthol which was not observed when their activities were

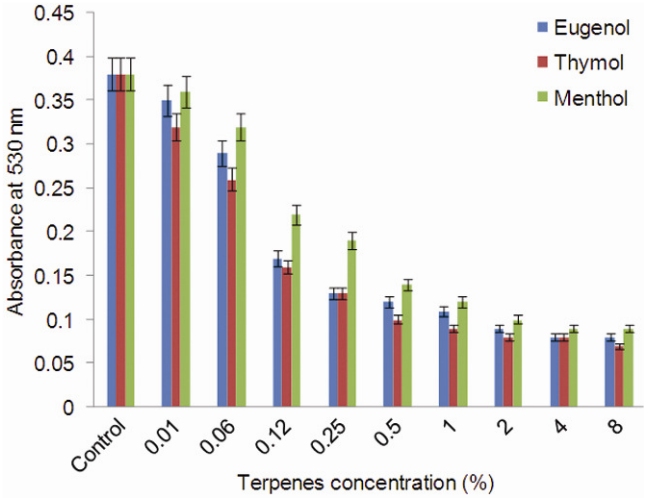

Fig. 1—MICs of different concentrations of terpenes (eugenol, thymol and menthol) on planktonic *C. albicans* after 48 h at 35 °C.

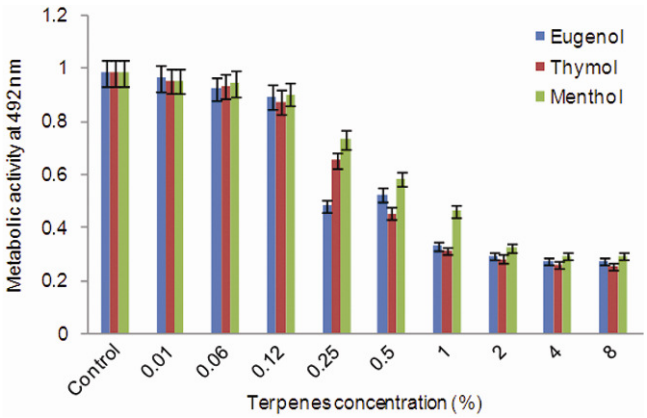

Fig. 2—Effect of different concentrations of terpenes (eugenol, thymol and menthol) on metabolic activity of *C. albicans* biofilm after 24 h at 35 °C.

observed independently. The results demonstrated that synergistic effects can also be observed in cells that have already grown in a biofilm, which are typically the most difficult to treat.

Adherence assay—In vitro studies on effect of terpenes/FLA on candidal cell adhesion after 2 h showed it to be concentration dependent (Fig. 3). Data showed 30% viability of *C. albicans* cells after 2 h of treatment with 0.05 % (v/v) thymol/FLA.

Scanning electron microscopy (SEM)—Effect of thymol/FLA at 0.05 % (v/v)/0.5 mg mL^{-1} on *C. albicans* adhesion visualized by SEM micrographs showed disruption in number of candidal cells and alteration in structural design of *C. albicans* when compared with its control (Fig. 4 a and b).

Discussion

Increased use of conventional antifungal agents, corresponding to susceptible individuals has resulted in the emergence of multidrug-resistant *Candida* strains. The present study demonstrated that synergistic activity of terpenes with fluconazole which exerted strong inhibitory effect against *C. albicans* biofilms and is in agreement with earlier data depicting the usefulness of such essential oils, complex natural mixtures extracted from several aromatic plants in having antimicrobial activities[15]. These results suggested the potential benefit of terpenes in treating immunocompromised individuals infected with *C. albicans*. Terpenes are made from combinations of several 5-carbon-base units called isoprene. The monoterpenes are formed from the coupling of two isoprene units. They are the most representative molecules constituting 90% of the essential oils and allow a great variety of structures. Among the monoterpenes, menthol, terpinen-4-ol and µ-terpineol are monocyclic alcohols; while carvacrol and thymol are phenols. The aromatic compounds such as eugenol, derived from phenylpropane, occur less frequently than the terpenes[16-18]. The antifungal properties are correlated to ability of terpenes to pass through the fungal cell wall and position between fatty acid chains of lipid bilayers, disrupting lipid packaging and altering the structure of the cell membrane[19]. While evaluating the antifungal properties of terpenes, the method for determining MIC is important; for this reason, CLSI reference method for antifungal susceptibility testing was used in the present study and fluconazole was considered as control. The metabolic activity of *C. albicans*

Table 1—Synergistic activity of terpens/FLA

Agent	$SMIC_{90}$ (%)		
	Alone	Combo	Synergistic activity (ΣFIC)
Eugenol	2.0	0.25	0.37
Thymol	1.0	0.06	0.31
Menthol	4.0	1.0	0.5

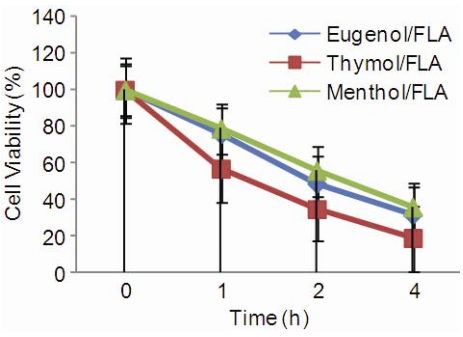

Fig. 3—Synergistic effect on combination of terpenes/FLA on candidal cells adhesion.

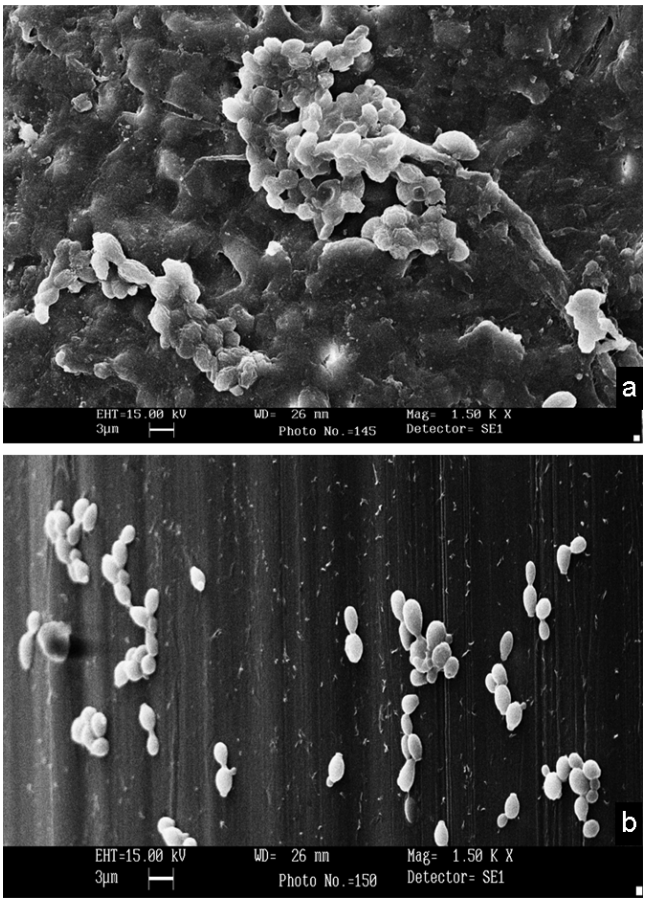

Fig. 4—SEM micrographs (a-control and b- thymol/FLA treated) of pre-formed *C. albicans* biofilm.

in biofilm was assessed using the tetrazolium (XTT) assay which based upon the reduction of XTT tetrazolium to tetrazolium formazan product by mitochondrially active *C. albicans* in presence of menadione, an electron-coupling agent[6]. Among three terpenes tested, thymol exerted strongest effect towards both the planktonic and biofilm phase of *C. albicans*. However, its effectiveness against *C. albicans* biofilm mode was achieved at threefold higher of $SMIC_{90}$ value, than the MIC for its planktonic counterparts which could be due to the resistant form of fungal growth. Braga *et al*.[20] by using scanning electron microscopy, demonstrated that thymol affected the envelope of planktonic *C. albicans*. The present study also revealed that inhibition of biofilm formation was observed when thymol and fluconazole were supplemented togather. The $SMIC_{90}$ of FLA was found to be 0.5 mg mL^{-1} when combined with thymol as compared to 2.0 mg mL^{-1} when tested alone. This can be related to enhanced antimicrobial action of FLA in presence with thymol which may be due to inhibition of ergosterol biosynthesis and alternation in permeability and membrane fluidity causing degradation of cell wall and variable effects like disruption of cytoplasm membrane, leakage of cell contents[19]. Based on the growth inhibitory effects of thymol with fluconazole in the broth micro-dilution assay, it is reasonable to assume that the effect of the combination on biofilm formation is related to an effect on *C. albicans* cell growth rather than an effect on cell adhesion. Fluconazole belongs to the azole antifungals, which inhibit biosynthesis of ergosterol, a crucial component of fungal cell membranes, leading to permeability changes. The inhibition of ergosterol biosynthesis also results in the accumulation of toxic methylated sterol intermediates and subsequently, arrests fungal cell growth[21, 22]. Even though the mode of synergic action is not clear at present, a similar mechanism may exist in the thymol with fluconazole combination against biofilm cells. Menthol has also been observed with the antifungal activity against *C. albicans*. In agreement with the present observations, other researchers have found that eugenol exhibited anti-*Candida* activity against fluconazole-resistant *Candida* isolates[23,24]. Similar results related to inhibitory action of terpenic derivatives on *C. albicans* biofilm also pointed out reduction in biofilm activity[25]. The potent antibiofilm activities of these naturally occurring active principles might convert them into promising alternatives for the topical treatment of *Candida*-associated infections as they are relatively safe, and their side effects are minor and self-limiting[26,27]. Thus, the present results indicate that terpenes, especially thymol, along with fluconazole can be a better medication for *C. albicans* biofilm related infections.

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