Infections associated with the biofilms of *Candida albicans* are a challenge to antifungal treatment. Combinatorial therapy involving plant molecules with antifungal drugs would be an effective complementary approach against drug-resistant *Candida* biofilms. The aim of this study was to evaluate the efficacy of three bioactive terpenoids (carvacrol, eugenol and thymol) in combination with fluconazole against planktonic cells, biofilm development and mature biofilms of *C. albicans*. Activities of the selected molecules were tested using a microplate-based methodology, while their combinations with fluconazole were performed in a checkerboard format. Biofilms were quantitated by XTT-metabolic assay and confirmed by microscopic observations. Combinations of carvacrol and eugenol with fluconazole were found synergistic against planktonic growth of *C. albicans*, while that of thymol with fluconazole did not have any interaction. Biofilm development and mature biofilms were highly resistant to fluconazole, but susceptible to three terpenoids. Sensitization of cells by sub-inhibitory concentrations of carvacrol and eugenol resulted in prevention of biofilm formation at low fluconazole concentrations, i.e. 0.032 and 0.002 mg ml\(^{-1}\), respectively. Addition of thymol could not potentiate activity of fluconazole against biofilm formation by *C. albicans*. Fractional inhibitory concentration indices (FICI) for carvacrol-fluconazole and eugenol-fluconazole combinations for biofilm formation were 0.311 and 0.25, respectively. The FICI value of 1.003 indicated a status of indifference for the combination of thymol and fluconazole against biofilm formation. Eugenol and thymol combinations with fluconazole did not have useful interaction against mature biofilms of *C. albicans*, but the presence of 0.5 mg ml\(^{-1}\) of carvacrol caused inhibition of mature biofilms at a significantly low concentration (i.e. 0.032 mg ml\(^{-1}\)) of fluconazole. The study indicated that carvacrol and eugenol combinations with fluconazole would be a potential alternative strategy for prevention and control of biofilm-associated *C. albicans* infections.

**Key words:** antifungal; biofilm; *Candida albicans*; chemosensitizer; combination therapy; drug resistance; plant molecule; terpenoid

**Introduction**

Involvement of *Candida albicans* biofilms in clinical infections is a serious problem for immunocompromised patients (Sardi et al., 2013). Being a commensal it easily form biofilms on host tissues as well as various prosthetic devices in the patient’s body (Chandra et al., 2001). It is reported to form biofilms on urinary catheters, intra-venous catheters, denture materials, central nervous system prostheses, artificial heart valves, joint prostheses, contact lenses, penile implants, and intrauterine devices as well as host tissue surfaces (Ramage et al., 2006). Compared to the planktonic (free living) cells, biofilm cells exhibit altered phenotype, due to surface-induced gene expression (Coster-ton et al., 1999). A notable feature of *C. albicans* biofilms is resistance to various antifungals, including the widely
prescribed drug fluconazole (Shinde et al., 2012). Additionally, biofilms may act as reservoirs of infectious cells to cause re-infections (LaFleur et al., 2006). Toxic side effects limit the use of high concentrations of available antifungal drugs; as such, new strategies to combat biofilm-associated C. albicans infections are a necessity (Shinde et al., 2013a).

Molecules of plant origin possess anticancer, antiparasitic, antiviral, antiallergic, and antimicrobial properties (Raut and Karuppayil, 2014). Phytochemicals are being proposed as candidates for synergy research to generate new pharmaceuticals (Wagner and Ulrich-Merzenich, 2009). Use of plant molecules against candidiasis is an attractive alternative (Inouye et al., 2012; Shreaz et al., 2011). Terpenoids of plant origin were found effective against multiple drug-resistant strains as well as biofilm growth of C. albicans (Raut et al., 2013). Few attempts have been made to study the anti-biofilm activities of terpenoids in combination with antifungal drugs (Khan and Ahmad, 2012). A combinatorial approach in antifungal therapy offers several potential advantages like increased potency, reduced dosages of individual drugs, minimized toxicities, and prevention of the emergence of drug-resistant mutant strains (Campbell et al., 2012; Shinde et al., 2013b). Carvacrol, eugenol and thymol are major constituents of essential oils derived from plants, especially of the genus Oreganum. These phenolic terpenoids are known to exhibit potent anti-Candida activities (Rao et al., 2010). But their activities in combination with fluconazole have not been investigated against C. albicans biofilms. In this study, we have analyzed the efficacy of carvacrol, eugenol and thymol in combination with fluconazole against planktonic cells, biofilm development and mature biofilms of C. albicans.

Materials and Methods

Cultures, culture conditions, media and chemicals. Candida albicans, ATCC 90028, was procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India. Activation of culture was done in 50 ml of YPD broth in a 250 ml Erlenmeyer flask. The flasks were incubated at 30°C on an orbital shaker at 120 rpm for 24 h. Cells were harvested by centrifugation for 5 min at 2,000 × g speed, washed three times and resuspended in PBS (phosphate buffered saline). RPMI-1640 medium (w/ l-glutamine w/o sodium bicarbonate), pH 7, buffered with 165 mM MOPS, was filter sterilized. Various concentrations of plant molecules were prepared in RPMI-1640 medium by double dilution. DMSO (1%) was used as a solvent. Fluconazole was used as a standard antifungal drug. Carvacrol (AR) and XTT (i.e. 2, 3-bis (2-methoxy-4-nitro-sulfophenyl)-2H- tetrazolium-5-carboxanilide) were purchased from Sigma Chem., Mumbai, India, while eugenol (AR) and thymol (AR) and all other media components were obtained from HiMedia Chem. Ltd., Mumbai, India.

MIC (minimum inhibitory concentrations) and MFC (minimum fungicidal concentrations) for planktonic growth. The effect of plant molecules on the growth of planktonic cells of C. albicans was studied by using the standard broth micro dilution methodology, as per CLSI (Clinical Laboratory Standards Institute) guidelines (CLSI, 2002). Wells without test molecules served as a control, while fluconazole was used as a standard antifungal agent. One hundred microliters of inoculum was added to 100 μl of RPMI-1640 medium containing test compounds in each well to obtain 1 × 10³ cells ml⁻¹. The plates were incubated at 35°C for 48 h. To analyze the growth, absorbance was read at 620 nm using a microplate reader (Multiskan EX, Thermo Fisher Scientific, Waltham, MA). The lowest concentration of the test molecule which cause ≥ 50% reduction in the absorbance compared to that of control was considered the MIC.

Molecules with MICs in the range 0.031 to 2 mg ml⁻¹ were selected for MFC testing. Ten microliters of cell suspension from MIC and wells containing concentrations above that were spread on YPD agar. These agar plates were incubated for 48 h at 30°C and observed for the presence of colonies. The lowest concentration which caused no appearance of colonies on the agar plates was noted as the MFC (Raut et al., 2013).

Biofilm formation and drug susceptibility. C. albicans ATCC 90028, was procured from the American Type Culture Collection (ATCC). Various concentrations of drug and plant molecules were added at the zero hour of biofilm formation (i.e. immediately after the adhesion phase) and biofilms were allowed to develop for 48 h. To analyze the effects on mature biofilms, media with a range of drug concentrations were added to the 24-h mature biofilms. The plates were further incubated for 48 h at 37°C. Density of the biofilm cells was quantitated through metabolic activity in XTT-formazan reduction assay (Raut et al., 2013).

Biofilm quantitation by XTT assay. Biofilm growth was quantitated using XTT metabolic assay (Raut et al., 2013). XTT solution was prepared by mixing 1 mg ml⁻¹ XTT salt in PBS and stored at −20°C. Prior to use, menadione solution was prepared in acetone and added to XTT to a final concentration of 4 μM. The wells containing biofilms were washed with PBS to remove non-adhered cells and incubated with 100 μl of XTT-menadione solution in the dark at 37°C for 5 h. The color formation by the water-soluble formazan product was measured at 450 nm using a microplate reader (Multiskan EX, Thermo Fisher Scientific) and indicated metabolic activity relative to biofilm growth. Wells without test compounds were considered as controls, while those without biofilms were the blanks to measure background absorbance in XTT assay. The concentration of terpenoid, which causes ≥ 50% lowering in relative metabolic activity (RMA) compared to that of the control, as measured by XTT assay, was considered the MIC for biofilm.

Checkerboard assay for combinatorial analysis of drug and plant molecules against planktonic and biofilm growth. The combinatorial efficacy of terpenoids and fluconazole was analyzed in terms of fractional inhibitory concentration indices (FICI) obtained in checkerboard assay. Dilutions of individual drug and plant molecules as well as their combinations were prepared in a checkerboard format as per standard methodology (Johnson et al., 2004; Shinde et al., 2013b). A two-dimensional array of serial concentrations of test compounds was used for preparation of dilutions of the
Terpenoids inhibit Candida biofilms and thymol were active at a 0.5 mg ml$^{-1}$ FICB = (MIC of drug B in combination / MIC of drug B alone) example, the MIC of fluconazole in combination with a to a combination of the antifungal drug and carvacrol. For MICs of fluconazole was observed when cells were exposed to the early phase of biofilms (i.e., immediately after adhesion) prevented biofilm development. Their concentration to the control (p<0.05) (Fig. 1). Carvacrol and eugenol were found to be fungicidal at 1 mg ml$^{-1}$, while 2 mg ml$^{-1}$ was the MFC of thymol. The strain used in this study, C. albicans ATCC 90028, was drug sensitive, having a fluconazole MIC of 0.001 mg ml$^{-1}$ (i.e., 1 µg ml$^{-1}$). A significant decrease in MICs of fluconazole was observed when cells were exposed to a combination of the antifungal drug and carvacrol. For example, the MIC of fluconazole in combination with a 0.031 mg ml$^{-1}$ concentration of carvacrol was found to be 0.00025 mg ml$^{-1}$ (Table 1). The FICI value for the carvacrol-fluconazole combination was calculated to be 0.374, which indicated that the combination is synergistic. Similarly, 0.031 mg ml$^{-1}$ of eugenol lowered the fluconazole MIC by four times, with a FICI value of 0.312 (Table 1). Treatment with thymol and carvacrol together was not synergistic, which was evident with a FICI value of 1.062, indicating indifference.

Terpenoid-mediated sensitization resulted in prevention of C. albicans biofilms at a low concentration of fluconazole
Fluconazole was found completely ineffective against biofilm formation. Significant biofilm growth was observed even at drug concentrations 250 times higher (i.e., 0.512 mg ml$^{-1}$) than the MIC for planktonic growth. Addition of terpenoids to the early phase of biofilms (i.e., immediately after adhesion) prevented biofilm development. Their concentration-dependent effect was indicated by a significant lowering in metabolic activity analyzed by XTT assay compared to that of the control (p<0.05) (Fig. 1). Carvacrol was found to be the most effective inhibitor of biofilm development and caused >65% inhibition at a 0.25 mg ml$^{-1}$ concentration (Table 1) and (Fig. 1a). The presence of 0.5 mg ml$^{-1}$ of eugenol resulted in a 70% decrease in relative metabolic activity compared to that of control biofilms (p<0.05) (Table 1) and (Fig. 1b). Comparatively, thymol was less efficient in preventing biofilm formation, with the MIC at 1 mg ml$^{-1}$ (Fig. 1c). Prevention of biofilms was also confirmed with microscopic observations. Only few yeast cells were seen to remain on the solid surface when treated with minimum biofilm inhibitory concentrations of the terpenoid molecules. Control wells (i.e., without test molecule) showed dense network of hyphae and yeast cells, which is characteristic of the normal C. albicans biofilms.
Table 1. MICs of three terpenoids and fluconazole alone as well as in combination, against planktonic and biofilm growth forms of *Candida albicans*.

<table>
<thead>
<tr>
<th>Terpenoid</th>
<th>MIC (mg ml⁻¹)</th>
<th>Remarks</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Alone</td>
<td>In combination</td>
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<tr>
<td><strong>Carvacrol-Fluconazole</strong></td>
<td></td>
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<tr>
<td><em>Candida albicans</em></td>
<td></td>
<td></td>
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<tr>
<td>Planktonic</td>
<td>0.001</td>
<td>0.25</td>
</tr>
<tr>
<td>Biofilm development</td>
<td>0.512</td>
<td>0.25</td>
</tr>
<tr>
<td>Mature biofilm</td>
<td>&gt;1.024</td>
<td>1</td>
</tr>
<tr>
<td><strong>Eugenol-Fluconazole</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
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<td>Mature biofilm</td>
<td>&gt;1.024</td>
<td>1</td>
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<tr>
<td><strong>Thymol-Fluconazole</strong></td>
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<tr>
<td><em>Candida albicans</em></td>
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<td>0.001</td>
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<tr>
<td>Biofilm development</td>
<td>0.512</td>
<td>1</td>
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<tr>
<td>Mature biofilm</td>
<td>&gt;1.024</td>
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</tbody>
</table>

Note: FLC, Fluconazole; CAR, Carvacrol; EUG, Eugenol; THY, Thymol; FICI, fractional inhibitory concentration indices for analyzing drug interaction.

**Carvacrol**

(a) Control

(b) MIC (0.25 mg ml⁻¹)

**Eugenol**

(c) Control

(d) MIC (0.5 mg ml⁻¹)

Fig. 2. Microphotographs confirm inhibition of *Candida albicans* biofilm formation by the terpenoids carvacrol and eugenol.

Panel a) and c) show controls without terpenoid; panel b) and d) reveal biofilm prevention in the presence of the MIC of a particular molecule. Note the reduction in density of biofilm growth at the MIC of terpenoids compared to that of dense biofilm growth in the control (Magnification × 200).
Terpenoids inhibit *Candida* biofilms

**Fluconazole and Carvacrol**

a) Fluconazole (32 µg ml⁻¹)  
b) Carvacrol (62 µg ml⁻¹)  
c) a + b

**Fluconazole and Eugenol**

d) Fluconazole (2 µg ml⁻¹)  
e) Eugenol (125 µg ml⁻¹)  
f) d + e

(Fig. 2).

A carvacrol concentration of 0.062 mg ml⁻¹ did not have inhibitory activity, but it sensitized the biofilms to bring down the fluconazole MIC to 0.032 mg ml⁻¹. Synergism between carvacrol and fluconazole against biofilm formation was evident with a FICI of 0.311 (Table 1 and Fig. 3). Similarly, combination with 0.125 mg ml⁻¹ of eugenol dramatically lowered the biofilm preventive concentration of fluconazole to 0.002 mg ml⁻¹ (i.e. 2 µg ml⁻¹), with a FICI of 0.25 (Table 1). The biofilm preventive effects of these combinations were confirmed by microscopy (Fig. 3). The FICI value of 1.003 for the combination of thymol and fluconazole indicated that there is no synergistic interaction between these two molecules against biofilm formation.

**Mature biofilms were comparatively less sensitive**

Mature biofilms were totally insensitive to very high concentrations (1.024 mg ml⁻¹) of fluconazole. Carvacrol and eugenol were effective inhibitors of mature biofilms with a MIC of 1 mg ml⁻¹. XTT-analysis showed that treatment with these compounds resulted in a significant (p<0.05) decrease in relative metabolic activity (Figs. 1a and 1b). Thymol exhibited a MIC of 2 mg ml⁻¹ against mature biofilms (Fig. 1c). Among the three terpenoids, 0.5 mg ml⁻¹ of carvacrol sensitized mature biofilms to reduce the fluconazole concentration required for inhibition by 32 times (Table 1). The FICI values >1 indicated that there is no useful interaction between eugenol and fluconazole nor between thymol and fluconazole.

**Discussion**

The efficacy of carvacrol, eugenol and thymol against virulence factors and biofilms of *C. albicans* was reported recently (Raut et al., 2013). Combinations of plant essential oils as well as their terpenoid components with commercial antifungics are known to result in synergistic antifungal activities (Amber et al., 2010; Khan and Ahmad, 2012). Interestingly, eugenol and thymol were reported to have synergistic interactions with fluconazole against the planktonic form of *C. albicans* (Ahmad et al., 2010; Guo et al., 2009), but their combinations against biofilms of *C. albicans* have not been investigated. Here, in a single comprehensive study, we have analyzed the potential of carvacrol, eugenol and thymol in combination with fluconazole against drug-resistant biofilms of *C. albicans*. The presence of low concentrations of carvacrol and eugenol (0.062 and 0.125 mg ml⁻¹) dramatically reduced the fluconazole concentrations required to prevent biofilm formation. The concentrations of carvacrol and eugenol which were active in combinations did not have any effect when used alone, suggesting their chemosensitization ability to enhance the activity of a standard antifungal drug.

Various mechanisms are supposed to be involved in the
inhibition of *C. albicans* growth by plant molecules. A study in *Saccharomyces cerevisiae* revealed that eugenol and carvacrol cause membrane disintegration, loss of ions, and interference in the TOR signaling pathway, ultimately resulting in loss of viability (Rao et al., 2010). In addition, terpenoid-mediated changes in permeability and membrane fluidity result in degradation of the cell wall, which affects adherence of *C. albicans* to solid surfaces (Macros-Arias et al., 2011). We hypothesize that membrane destabilization and intervention of specific signals by carvacrol and eugenol caused sensitization of *C. albicans* biofilms, so that the increased influx of fluconazole resulted in the inhibition of biofilm formation. Carvacrol and eugenol, two terpenoids with the phenolic functional group, were active in combination with fluconazole at considerably low concentrations. These active concentrations may not exhibit toxicities. Still, we do not attempt to suggest their direct use. Instead, the study gives insight into the molecules with potential to inhibit *Candida* biofilms and to suppress drug resistance associated with them. These structural scaffolds can be used for the synthesis of derivatives with less toxicity. The outcome of this in vitro study suggests use of terpenoid-antifungal drug combinations as a strategy for prevention of *Candida* biofilm development and also for avoidance of side effects associated with high concentrations of antifungal drugs. To confirm the practical utility of these combinations, in vivo studies are necessary.

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