Antibiofilm activity of certain phytocompounds and their synergy with fluconazole against Candida albicans biofilms

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Objectives: The aim of this study was to evaluate four phytocompounds (cinnamaldehyde, citral, eugenol and geraniol) for their in vitro inhibitory activity against pre-formed biofilms of Candida albicans alone or in combination with fluconazole and amphotericin B. These compounds were also tested at subinhibitory concentrations for their ability to inhibit biofilm formation.

Methods: The XTT reduction assay, light microscopy and scanning electron microscopy (SEM) were employed to determine the inhibitory effect of the test compounds on biofilms. A chequerboard method was used for combination studies.

Results: Both clinical and reference strains of C. albicans (C. albicans 04 and C. albicans SC5314, respectively) displayed formation of strong biofilms. Pre-formed Candida biofilms showed ≥1024x increased resistance to antifungal drugs and 2x increased resistance to cinnamaldehyde and geraniol, but no increased tolerance of eugenol. The test compounds were more active against pre-formed biofilms than amphotericin B and fluconazole. At 0.5x MIC, eugenol and cinnamaldehyde were the most inhibitory compounds against biofilm formation. Light and electron microscopic studies revealed the deformity of three-dimensional structures of biofilms formed in the presence of sub-MICs of eugenol and cinnamaldehyde. The cell membrane appeared to be the target site of compounds in both planktonic and sessile C. albicans cells, as observed by SEM. Combination studies showed that synergy was highest between eugenol and fluconazole (fractional inhibitory concentration index=0.14) against pre-formed biofilms of C. albicans SC5314.

Conclusions: Promising antibiofilm activity was displayed by eugenol and cinnamaldehyde, which also showed synergy with fluconazole in vitro. Further evaluation in in vivo systems is required to determine whether these findings can be exploited in treating biofilm-associated candidiasis.

Keywords: eugenol, cinnamaldehyde, scanning electron microscopy, XTT reduction assay

Introduction

The majority of manifestations of candidiasis are associated in one way or another with the formation of Candida biofilms on the surfaces of inert or biological surfaces.1 Sessile (biofilm) cells display unique phenotypic traits in comparison with planktonic cells. The most notable of these is that sessile cells are notoriously resistant to antimicrobial agents and withstand host immune defences,2 and this is the main reason why biofilm-associated infections are frequently refractory to conventional antibiotic therapy. The decreased susceptibility of sessile cells to antimicrobial agents, including amphotericin B, fluconazole, itraconazole and ketoconazole, compared with that of planktonic cells has been reported extensively over the past decade.3 To overcome the problem of host toxicity and drug resistance associated with monotherapy, two-drug combination strategies have been attempted both for planktonic and biofilm cells of C. albicans, but disparate effects have been observed.4 Given these concerns, identifying antifungal agents that are effective against Candida biofilms alone or in synergy with fluconazole or amphotericin B is of great importance. Plants are known to produce phytochemicals that attenuate biofilm development through specific mechanisms.5 Therefore, in this study, we evaluated four phytocompounds of different chemical nature [phenyl aldehyde (cinnamaldehyde; 3-phenylprop-2-enal), phenyl propanoid (eugenol; 4-allyl-2-methoxyphenol) and terpenoid (citral; 3,7-dimethylocta-2,6-dienal and geraniol; 3,7-dimethylocta-2,6-dien-1-ol)] for their ability to eradicate established biofilms and to inhibit biofilm formation at sub-MICs in strains of C. albicans. The antifungal drugs...
amphotericin B and fluconazole were also tested for their antifungal activity. Further, the most effective phytocompounds exhibiting antibiofilm activity were also evaluated in combination with antifungal drugs for their synergistic activity against established biofilms.

### Materials and methods

A clinical strain (C. albicans 04) and a reference strain (C. albicans SC5314) were used. Phytocompounds, namely eugenol (minimum assay 98%), cinnamaldehyde (98%), citral (82%) and geraniol (85%), were diluted 10-fold in 1% DMSO and used in the assays. Stock solutions of amphotericin B and fluconazole were prepared in DMSO at a concentration of 250 mg/L and stored at −20°C until used. The planktonic MICs (PMICs) and sessile MICs (SMICs) of the phytocompounds and drugs were determined for the test isolates using the CLSI broth microdilution reference method (M27-A3). For testing the susceptibility of sessile cells to the compounds and drugs, the method of Ramage et al. was used with some modifications. Briefly, Candida cells were grown in Sabouraud dextrose broth (SDB) [glucose 8% (w/v)] at 37°C for 24 h. Cells were harvested and resuspended in RPMI 1640 medium with L-glutamine but without bicarbonate and buffered to pH 7.0 with MOPS to a cell density of 1.5×10^6 cfu/mL. Biofilms were formed by adding 100 µL of this standardized cell suspension to wells of microtitre plates and incubating at 37°C for 48 h. After biofilm formation, the medium was aspirated gently and non-adherent cells were removed by washing the biofilms three times with sterile PBS. Further, 0.1 mL of 2-fold serial dilutions of the test agents in RPMI 1640 medium was added to each biofilm-containing well of the microtitre plates and incubated at 37°C for an additional 48 h. The SMICs were determined by the XTT reduction assay.

For scanning electron microscopy (SEM) analysis, biofilm cells were grown in Sabouraud dextrose broth (SDB) [glucose 8% (w/v)] at 37°C for an additional 48 h. Biofilm formation was inhibited maximally by eugenol in both of the test strains. At 0.5 MIC of eugenol, biofilm formation was recorded as 18.62% and 20.65% in C. albicans SC5314. The interaction of eugenol caused shrinkage of the cell membranes of sessile cells (Figure 1b, inset 1 and inset 2). A similar effect of eugenol was also observed on planktonic yeast cells (Figure 1d and e). The test compounds showed varying levels of interaction with fluconazole or amphotericin B against biofilms of the test strains. Eugenol and cinnamaldehyde were synergistic with fluconazole and exhibited fractional inhibitory concentration index (FICI) values of 0.25 and 0.312, respectively, against C. albicans 04 biofilms. Among all the tested combinations with fluconazole, eugenol exhibited the highest synergy, with a FICI value of 0.14 against C. albicans SC5314. The interaction of

### Results

The SMICs of amphotericin B and fluconazole were increased to 512- and 1024-fold, respectively, for C. albicans SC5314. The PMICs of the phytocompounds were 90–400 and 50–360 mg/mL for C. albicans 04 and C. albicans SC5314, respectively. The SMICs of the phytocompounds were 200–400 mg/mL for C. albicans 04 and 100–360 mg/mL for C. albicans SC5314. Eugenol and citral showed similar MICs for both the planktonic and sessile cells of C. albicans strains. A >80% reduction in the viable count of sessile cells of test strains was exhibited by eugenol and geraniol in 10–12 h. Cinnamaldehyde and citral produced similar effects in 30 h and 48 h, respectively, against both the strains. Amphotericin B and fluconazole did not show this effect up to 48 h. A varying level of attenuation of biofilm formation by planktonic Candida cells was observed in the presence of the test agents (Table 1).

Biofilm formation was inhibited maximally by eugenol in both of the test strains. At 0.5× MIC of eugenol, biofilm formation was recorded as 18.62% and 20.65% in C. albicans 04 and C. albicans SC5314, respectively. Cinnamaldehyde at 0.5× MIC allowed 31.40% formation of biofilm in C. albicans 04. The other test compounds were less effective in inhibiting biofilm formation. Untreated sessile cells showed a smooth cell membrane (Figure 1a, inset), whereas treatment with eugenol caused shrinkage of the cell membranes of sessile cells (Figure 1b, inset 1 and inset 2). A similar effect of eugenol was also observed on planktonic yeast cells (Figure 1d and e). The test compounds showed varying levels of interaction with fluconazole or amphotericin B against biofilms of the test strains. Eugenol and cinnamaldehyde were synergistic with fluconazole and exhibited fractional inhibitory concentration index (FICI) values of 0.25 and 0.312, respectively, against C. albicans 04 biofilms. Among all the tested combinations with fluconazole, eugenol exhibited the highest synergy, with a FICI value of 0.14 against C. albicans SC5314. The interaction of

### Table 1. Effects of phytocompounds and drugs on biofilm formation in drug-resistant and -susceptible strains of C. albicans

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>C. albicans 04</th>
<th>C. albicans SC5314</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5× MIC</td>
<td>0.25× MIC</td>
</tr>
<tr>
<td>Eugenol</td>
<td>18.62 ± 2.91</td>
<td>28.11 ± 2.69</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>31.40 ± 2.41</td>
<td>63.53 ± 1.75</td>
</tr>
<tr>
<td>Citral</td>
<td>74.31 ± 2.27</td>
<td>84.00 ± 1.99</td>
</tr>
<tr>
<td>Geraniol</td>
<td>69.08 ± 1.48</td>
<td>84.70 ± 2.59</td>
</tr>
<tr>
<td>Antifungal drugs</td>
<td>67.59 ± 1.69</td>
<td>78.34 ± 1.93</td>
</tr>
<tr>
<td>amphotericin B</td>
<td>48.16 ± 0.97</td>
<td>67.61 ± 1.39</td>
</tr>
<tr>
<td>fluconazole</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.

Hyphaform formation values were calculated as: (mean OD_{490} of treated well)/(mean OD_{490} of untreated control well)×100.
the test compounds with amphotericin B was indifferent against both C. albicans strains.

**Discussion**

In our in vitro study, the SMIC of amphotericin B was increased 16- and 512-fold over the PMIC in C. albicans 04 and C. albicans SC5314, respectively. Similarly, the SMIC of fluconazole was increased 8- and 1024-fold, respectively. However, the SMICs of the test compounds were only 2-fold higher for cinnamaldehyde and geraniol, and 4-fold higher for citral. Remarkably, eugenol showed no increase in the SMIC compared with the PMIC for the test strains. Data obtained from time–kill assays showed the cidal activity against pre-formed biofilms was highest for geraniol and eugenol compared with the other compounds and antifungal drugs tested. We further evaluated the ability of these compounds at sub-MICs to inhibit the formation of biofilms when the compounds/drugs were added to the medium at the same time as the cells. If an agent is added at the beginning of the experiment, the agent might act before...
the biofilm is formed and inhibit its development; this could be of interest for combating recalcitrant infections involving Candida biofilms. Our data revealed a varying level of attenuation of biofilm formation by planktonic Candida cells in the presence of compounds and drugs in a dose-dependent manner. Among the tested agents, eugenol had the most inhibitory effect on biofilm formation at 0.5× and 0.25× MIC, followed by cinnamaldehyde at 0.5× MIC. In our study, SEM observations clearly indicated the interference of eugenol and cinnamaldehyde with cell membrane integrity, as evidenced by shrinkage of the cell surface in biofilm cells. A similar mode of action was also observed against planktonic cells of C. albicans. Other authors have also shown that essential oil compounds affect the cell membrane integrity of yeast cells. A decreased ergosterol content and a diminished level of ergosterol biosynthetic gene expression have been reported in mature Candida biofilms, and since sterol metabolism is the primary cellular process affected by the most widely employed antifungal drugs (amphotericin B and fluconazole), the diminished levels of ergosterol present in sessile C. albicans may reflect a physiological state more conducive to resistance in these cells. Therefore, our observations suggest that these compounds target cell membranes in both planktonic (higher sterol content) and sessile (lower sterol content) cells of C. albicans, and that their mode of action remains unaffected by the phenotypic variation in the ergosterol content exhibited by planktonic and sessile cells. However, further work is needed to explore the exact mode of action of these phytocompounds on C. albicans biofilm cells. The compounds exhibiting strong antibiofilm activity (eugenol and cinnamaldehyde) were assessed for their interaction with amphotericin B or fluconazole, to determine whether the efficacy of these drugs was increased against established biofilms of C. albicans.

In conclusion, the efficient antibiofilm activity of eugenol and cinnamaldehyde, which inhibited both pre-formed biofilms and the formation of biofilms alone, also exhibited a synergistic interaction with fluconazole against biofilms formed by the test strains. However, combination with amphotericin B was indifferent. A similar interaction result was also obtained for cinnamaldehyde. The SMIC of fluconazole was reduced substantially (32-fold), thereby indicating the effectiveness of these combinational approaches. An explanation for this is that when phytocompounds exhibiting cidal activity against biofilm cells are combined with fluconazole, the fungistatic nature of this drug is converted to fungicidal. These findings highlight the effectiveness of drug combinations against Candida biofilms.

In this study, eugenol, being a potential antibiofilm agent against pre-formed biofilms and the formation of biofilms alone, also exhibited a synergistic interaction with fluconazole against biofilms formed by the test strains. However, combination with amphotericin B was indifferent. A similar interaction result was also obtained for cinnamaldehyde. The SMIC of fluconazole was reduced substantially (32-fold), thereby indicating the effectiveness of these combinational approaches. An explanation for this is that when phytocompounds exhibiting cidal activity against biofilm cells are combined with fluconazole, the fungistatic nature of this drug is converted to fungicidal. Therefore, other structurally related compounds from the phenolic class of plant products could be screened to obtain novel antibiofilm agents against C. albicans. Further in vivo investigations are needed to uncover the therapeutic values of these phytocompounds alone or in combination with antifungal drugs against biofilm-associated candidiasis.

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Transparency declarations

None to declare.

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