Phenylpropanoids of Plant Origin as Inhibitors of Biofilm Formation by Candida albicans

Jayant Shankar Raut, Ravikumar Bapurao Shinde, Nitin Mahendra Chauhan, and Sankunny Mohan Karuppayil*

DST-FIST and UGC-SAP Sponsored School of Life Sciences, SRTM University, Nanded, (MS), 431606, India

Introduction

Drug resistance associated with biofilms of Candida albicans pose a challenge to successful antifungal therapy [36]. A majority of the available antifungal drugs, including commonly prescribed azoles, are ineffective against biofilm growth of C. albicans [10]. Persistent biofilm communities survive even if the cells circulating in the bloodstream are eradicated by drug treatment. Biofilm reservoirs spread infective cells to the bloodstream to cause repeated infections [25]. Therefore, biofilm-related infections of C. albicans are a threat to patients, especially those with a compromised immune status [32]. Options of the antifungal drugs available for the successful treatment of systemic and invasive candidiasis are restricted. Side effects due to toxicity limit the use of high dosages of antifungal drugs and add to the severity of the problem. As such, new strategies need to be explored against drug-resistant biofilms [7]. The aim of this study was to evaluate the efficacy of phenylpropanoids of plant origin against planktonic cells, important virulence factors, and biofilm forms of C. albicans. Standard susceptibility testing protocol was used to evaluate the activities of 13 phenylpropanoids against planktonic growth. Their effects on adhesion and yeast-to-hyphae morphogenesis were studied in microplate-based methodologies. An in vitro biofilm model analyzed the phenylpropanoid-mediated prevention of biofilm development and mature biofilms using XTT-metabolic assay, crystal violet assay, and light microscopy. Six molecules exhibited fungistatic activity at \( \leq 0.5 \text{ mg/ml} \), of which four were fungicidal at low concentrations. Seven phenylpropanoids inhibited yeast-to-hyphae transition at low concentrations (0.031–0.5 mg/ml), whereas adhesion to the solid substrate was prevented in the range of 0.5–2 mg/ml. Treatment with \( \leq 0.5 \text{ mg/ml} \) concentrations of at least six small molecules resulted in significant \( (p < 0.05) \) inhibition of biofilm formation by C. albicans. Mature biofilms that are highly resistant to antifungal drugs were susceptible to low concentrations of 4 of the 13 molecules. This study revealed phenylpropanoids of plant origin as promising candidates to devise preventive strategies against drug-resistant biofilms of C. albicans.

Keywords: Antifungal, bioactive compound, biofilm, Candida albicans, drug resistance, phenylpropanoid
strains as well as biofilm growth of \textit{C. albicans} may be an effective strategy \cite{1, 2}.

Phenylpropanoids and terpenoids constitute the major part of bioactive molecules from medicinal plants. These molecules are secondary metabolites produced by plants, mainly in response to biotic or abiotic stress, such as infections, wounding, UV irradiation, pollutants and other environmental conditions \cite{24}. Many studies showed that phenylpropanoids play a key role as antioxidants in biological systems, exert antimicrobial activities, and also have inhibitory effects on mutagenesis and carcinogenesis \cite{19}. It was suggested that phenylpropanoids could be considered as a source for the development of antibacterial drugs \cite{18}. Efforts are being made to obtain comprehensive information on their efficacy against pathogenic biofilms \cite{13}. A few of the phenylpropanoids have been evaluated for their biofilm inhibitory potential in \textit{C. albicans}. For example, salicylic acid is reported to prevent the adherence of bacteria as well as pathogenic yeast \textit{C. albicans} to silastic catheters, in a concentration-dependent manner \cite{14}. A recent study has shown that caffeic acid and its ester derivatives exhibit biofilm inhibition at 32–64 \textmu g/ml concentrations \cite{12}. Similarly, anti-biofilm effects of eugenol and cinnamaldehyde, alone and in combination with fluconazole, have been analyzed \cite{22}. Here, in a single comprehensive study, we evaluated the antifungal properties of 13 phenylpropanoids, which are constituents of several plant essential oils. Their activities against planktonic growth, two important virulence attributes (\textit{i.e.}, adhesion and yeast-to-hyphae morphogenesis), biofilm formation, and mature biofilms of \textit{C. albicans} were analyzed. Results indicated that phenylpropanoids of plant origin exhibit noticeable efficacy against virulence factors and drug-resistant biofilms of \textit{C. albicans}.

\textbf{Materials and Methods}

\textbf{Cultures, Culture Conditions, Media, and Chemicals}

\textit{C. albicans} ATCC 90028 was procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was maintained on Yeast extract-Peptone-Dextrose (YPD) agar slants at 4°C. A single colony from the YPD agar plates was inoculated in 50 ml of YPD broth in a 250 ml Erlenmeyer flask and incubated at 30°C on an orbital shaker, at 120 rpm for 24 h. Cells from the activated culture were harvested by centrifugation for 5 min at 2,000 \times g speed, washed three times, and resuspended in PBS (10 mM phosphate buffer, 2.7 mM potassium chloride, and 137 mM sodium chloride, pH 7.4). RPMI-1640 medium (w/ L-glutamine w/o sodium bicarbonate), pH 7, buffered with 165 mM MOPS (3-[N-morpholine] propane sulphonic acid), was filter sterilized. Various concentrations of phenylpropanoids were prepared by the double dilution method. The concentration of the solvent, dimethyl sulfoxide (DMSO), was <1% in each assay system. Fluconazole (Forcan, Cipla Pvt. Ltd., Mumbai, India) and amphotericin B (Ampholyyn, Lyka Labs Ltd., India) were used as standard antifungal drugs. Farnesol, a quorum-sensing molecule in \textit{C. albicans} that inhibits yeast-to-hyphae morphogenesis and biofilm formation was also used as a control. Various phenylpropanoids, anisic acid, anisyl alcohol, anisaldehyde, salicylic acid, salicylaldehyde, vanillic acid, vanillin, cinnamic acid, cinnamaldehyde, caffeic acid, and ferulic acid (all of analytical grade) were obtained from HiMEDIA Chem. Ltd., Mumbai, India. Guaiacol, eugenol, and farnesol were procured from Sigma-Aldrich Chem. Ltd., Mumbai, India. XTT (\textit{i.e.}, 2, 3-bis-(2-methoxy-4-nitro-sulphophenyl)-2H-tetrazolium-5-carboxanilide) and menadione were from Sigma-Aldrich Chem. Ltd. All other media components were purchased from HiMEDIA Chem. Ltd., Mumbai, India.

\textbf{Minimum Inhibitory Concentration (MIC) for Planktonic Growth}

The effects of phenylpropanoids on the growth of planktonic cells of \textit{C. albicans} were studied by using the standard broth microdilution methodology, as per Clinical Laboratory Standards Institute (CLSI) guidelines \cite{9}. RPMI-1640 medium containing different concentrations (ranging from 0.015 to 4 mg/ml) of test molecules was added into 96-well plates (Costar, Corning Inc., USA). Wells without test molecule served as a control, and fluconazole (0.001 to 1.024 mg/ml) and amphotericin B (0.000015 to 0.008 mg/ml) were used as standard antifungals. One hundred microliters of inoculum was added to 100 \mu l of RPMI-1640 medium in each well to get 1 × 10^5 cells/ml. 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 Electron Corp., USA). The lowest concentration of the test molecule that caused a 50% reduction in the absorbance compared with that of control was considered as minimum inhibitory concentration for growth of \textit{C. albicans} \cite{29}.

\textbf{Minimum Fungicidal Concentration (MFC)}

Molecules for which the MIC was achieved (in the range 0.015 to 4 mg/ml) were selected for MFC testing. To determine the minimum \textit{Candida}-cidal concentrations, cells from the MIC and concentrations above were used. First, 10 \mu l of washed cell suspension from these wells was spread on YPD agar. These agar plates were incubated for 48 h at 30°C and observed for the presence of colonies. No appearance of colonies was noted as a fungicidal effect \cite{31}. The concentration of test molecule in the microplate well from which cells were taken was considered as the MFC.

\textbf{Adhesion Assay}

The effects of phenylpropanoid molecules on adherence of \textit{C. albicans} to a solid surface (\textit{i.e.}, polystyrene) were studied by using the microplate-based assay \cite{3}. Various concentrations...
(ranging from 0.015 to 4 mg/ml) of each test molecule were prepared in PBS. Fifty microliters of cell suspension were added to each well to get 1 x 10^7 cells/ml. The final volume of assay system in each well was kept at 100 µl. The plates were incubated at 37°C for 90 min at 100 rpm, in an orbital shaking incubator, to allow attachment of cells on the surface. After the incubation, wells were washed with PBS to remove non-attached cells. The density of the adherence in each well was analyzed as relative metabolic activity (RMA) using the XTT assay. More than 50% reduction in RMA compared with the control (i.e., without test molecule) was considered significant.

**Biofilm Quantitation by XTT Assay**

Biofilm cells were fixed with 99% methyl alcohol for 15 min. Supernatant was removed and wells were air-dried. One hundred microliters of 0.02% crystal violet was added to each well and allowed to stand for 15 min for staining of the biofilm. Excessive crystal violet was removed by washing the wells 2–3 times with sterile distilled water. Absorbed stain was released by addition of 150 µl of 33% of acetic acid and transferred to the fresh wells. Optical density was read at 620 nm using a microplate reader. Percentage biofilm formation was calculated by comparing the optical density of treatment with that of control biofilm. More than 50% reduction in absorbance of CV was considered as significant inhibition [6].

**Biofilm Formation**

*C. albicans* biofilms were developed on polystyrene surface of 96-well plates as per the standardized *in vitro* biofilm model [31]. A cell suspension of 1 x 10^7 cells/ml was prepared in PBS and 100 µl was inoculated in each well. In the adhesion phase, plates were incubated at 37°C for 90 min at 100 rpm to allow attachment of cells on the solid surface. Non-adhered cells were removed by washing the wells with sterile PBS. Two hundred microliters of the RPMI-1640 medium was added to each well and the plates were incubated at 37°C for 48 h to allow biofilm formation. To observe its effect on development of biofilms, RPMI-1640 medium along with various concentrations of phenylpropanoids were added to each well immediately after the adhesion phase and incubated for 48 h at 37°C. To analyze the activity against mature *C. albicans* biofilms, test molecules were added to 24 h mature biofilms and incubated for 48 h. After incubation, wells were washed to remove any non-attached planktonic cells. Wells were observed for the presence or absence of biofilm structure, using an inverted light microscope (Metzer, India). Photographs were taken by a Labomed microphotography system (Labomed, India) at ≥200 magnification. Biofilm growth was analyzed and confirmed with the XTT metabolic assay as well as the CV (crystal violet) assay.

**Statistical Analysis**

Values were the mean with standard deviations, obtained from three different observations. Values in the control and treatment groups for various molecules were compared using Student’s *t*-test. A value of *p* < 0.05 was considered statistically significant [16].

**Results and Discussion**

**Susceptibility of Planktonic Growth of *C. albicans***

Eight phenylpropanoids inhibited *C. albicans* growth in a concentration-dependent manner, in the range of 0.031–2 mg/ml (Table 1; Fig. 1). Anisyl alcohol, salicylaldehyde, and cinnamaldehyde exhibited the most potent activity at 0.031, 0.031, and 0.062 mg/ml, respectively. It was followed by an MIC of 0.5 mg/ml for anisaldehyde, guaiacol, and eugenol. Vanillin and cinnamic acid inhibited planktonic cells at 1 and 2 mg/ml, respectively. Six of the 13 plant molecules also exhibited *Candida*-cidal effects. Anisyl alcohol, salicylaldehyde, and cinnamaldehyde were most potent to kill *C. albicans* cells, exhibiting an MFC at 0.25 mg/ml. Strain ATCC 90028 was susceptible to fluconazole and amphotericin B at 1 and 0.25 µg/ml, whereas the MIC
for the known quorum-sensing molecule farnesol was obtained at 1 mg/ml. The antimicrobial activities of phenylpropanoids are mainly confined to disruption of the cell membrane [17]. Anti- Candida properties of this group of plant molecules were suggested to be due to inhibition of oxidative phosphorylation and respiratory chain functions [28]. A recent study indicated that eugenol perturbs amino acid permeases in the cytoplasmic membrane of Saccharomyces cerevisiae, resulting in inhibition of growth [11]. However, membrane disruption may be considered as the main mechanism behind the activity of phenylpropanoids against planktonic growth of C. albicans [35].

Anti-Adhesion Activity of Phenylpropanoids

Very high (i.e., 0.512 mg/ml) concentrations of fluconazole and an eight times more concentration than the planktonic MIC of amphotericin B were required to prevent C. albicans adhesion to solid substrate. Exposure to low concentrations of selected phenylpropanoids was found to prevent C. albicans adhesion to the solid surface (Table 1). Analysis by XTT metabolic assay revealed that salicylaldehyde and cinnamaldehyde were the most active inhibitors of adhesion with 0.125 and 0.25 mg/ml as effective concentrations, respectively. A 0.5 mg/ml concentration of anisyl alcohol and anisaldehyde caused significant (≥50%) reduction in adhesion of C. albicans cells (Fig. 2). Prevention of adhesion by cinnamic acid, eugenol, and guaiacol was achieved at the comparatively high concentration of 2 mg/ml. Adhesion of C. albicans cells to the host tissues or prosthetic substrates is an important step in the establishment of infection. It may lead to colonization, formation of biofilms, and induction of antifungal drug resistance [33]. Interfering in adherence of cells will prevent the development of biofilms. This study evaluated the anti-adhesion activity of 13 phenylpropanoids, which may be of significance to prevent colonization of prosthetic surfaces by C. albicans.

Yeast-to-Hyphae Morphogenesis Sensitivity to Low Concentrations of Phenylpropanoids

Seven of the selected phenylpropanoid molecules were found to prevent serum-induced yeast-to-hyphae morphogenesis at considerable low concentrations of ≤ 0.5 mg/ml (Table 1). Farnesol, a quorum-sensing molecule in C. albicans, is also a constituent of many plant essential oils. This autosignaling molecule inhibits yeast-to-hyphae dimorphism at specific concentrations accumulated in a cell-density-dependent manner [27]. Cinnamaldehyde was found to be as effective as farnesol and prevented dimorphic transition in C. albicans at 0.031 mg/ml concentration. Anisyl alcohol and salicylaldehyde were active at 0.062 mg/ml concentration. Anisaldehyde and eugenol prevented C. albicans morphogenesis at 0.125 mg/ml. Significant inhibition of yeast-to-hyphae

Table 1. MICs of selected phenylpropanoids required for significant prevention of growth, adhesion, morphogenesis, biofilm formation and mature biofilms of C. albicans.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>MIC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Planktonic growth</td>
</tr>
<tr>
<td>Anisic acid</td>
<td>4</td>
</tr>
<tr>
<td>Anisyl alcohol</td>
<td>0.031</td>
</tr>
<tr>
<td>Anisaldehyde</td>
<td>0.5</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>4</td>
</tr>
<tr>
<td>Salicylaldehyde</td>
<td>0.031</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>&gt;4</td>
</tr>
<tr>
<td>Vanillin</td>
<td>1</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>2</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>0.062</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>&gt;4</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>&gt;4</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>0.5</td>
</tr>
<tr>
<td>Eugenol</td>
<td>0.5</td>
</tr>
<tr>
<td>Farnesol</td>
<td>1</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>0.001</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.00025</td>
</tr>
</tbody>
</table>

transition was obtained with 0.5 mg/ml of guaiacol and vanillin, whereas the remaining of molecules were less effective (Fig. 3). The ability to switch from yeast to hyphae is an important virulence attribute of C. albicans. Formation of hyphae helps in foraging for nutrients and the penetration of host tissues, leading to invasive growth, which is responsible for systemic infections [34]. Filamentous forms are also an important component of the heterogeneous biofilm structure [5]. We have established morphogenesis inhibitory concentrations of 13 phenylpropanoids against C. albicans. These molecules were active at concentrations less than that of the growth inhibitory concentrations. Most of the available antibiotics kill the pathogenic microorganisms to cure the infection; however, a small population of mutants that are resistant to the antibiotics may survive and grow. This kind of natural selection is responsible for the emergence of drug-resistant populations of microorganisms. To avoid this, the prevention of specific virulence attributes instead of killing the pathogen would be a novel strategy [7]. The ability of a few phenylpropanoids to prevent the formation of C. albicans hyphae without affecting growth is of significance. Specific inhibition of morphogenesis may...
be used to restrict the pathogen (without killing it), so that the selection and emergence of a drug-resistant population of Candida can be avoided.

**Phenylpropanoids as Potential Inhibitors of Biofilm Development by C. albicans**

The efficacies of plant molecules against drug-resistant biofilm forms of C. albicans are being studied only recently [31]. Efforts are being carried to evaluate the anti-biofilm activities of phenylpropanoid molecules in C. albicans [22]. Here, the Candida biofilm preventive activities of 13 phenylpropanoids were analyzed in a single study. The prevention of biofilm formation by the standard drug amphotericin B required eight times more concentration than that of planktonic MIC. Fluconazole was completely ineffective, and significant inhibition of biofilm formation could be obtained only at a fluconazole drug concentration of 512 times that of the MIC for planktonic growth (Table 1). Addition of phenylpropanoids in the early phase (i.e., immediately after adhesion) prevented biofilm formation.

**Fig. 3.** Phenylpropanoids as inhibitors of serum-induced yeast-to-hyphal morphogenesis in C. albicans.

A microplate-based assay was used to determine the activity of the test molecules in the concentration range of 0.031–2 mg/ml.

**Fig. 4.** Anti-biofilm potential of phenylpropanoid molecules. The percentage of biofilm formation by C. albicans in the presence of the test molecules was analyzed as a function of the RMA analyzed by XTT assay.
Phenylpropanoids as Inhibitors of *Candida albicans*

This was evident from the significant reduction in metabolic activity analyzed by XTT assay. Quorum-sensing molecule farnesol prevented biofilm formation at 0.5 mg/ml concentration. Salicylaldehyde and cinnamaldehyde were potent inhibitors of biofilm development with an MIC at 0.125 mg/ml. The activities of anisyl alcohol, anisaldehyde, vanillin, and eugenol were found to be equivalent to farnesol with a biofilm inhibitory concentration of 0.5 mg/ml. Guaiacol and salicylic acid were less effective and found to restrict biofilm development at 1 and 2 mg/ml, respectively (Fig. 4). Inhibition of the biofilm network in the presence of these molecules was also evident in CV assay. The intensity of crystal violet absorbed is proportional to the cell mass (living or nonliving) as well as extracellular polymeric substances. The concentrations of phenylpropanoids that caused significant reduction of metabolic activity in the XTT assay also showed ≥50% reduction of absorbance in the CV assay (data not shown). Results of the CV assay confirmed that the reduction in XTT activity after treatment with effective molecules was not merely due to lowering of the metabolic rate, but was associated with the absence of the biofilm network. Prevention of biofilms was also confirmed with microscopic observations, which showed the absence of a characteristic biofilm structure at MICs of the phenylpropanoids. Treatment with effective concentrations of anisyl alcohol, anisaldehyde, salicylaldehyde, vanillin, cinnamaldehyde, and eugenol caused removal of *C. albicans* biofilm cells. Only a few yeast forms were seen to remain on the solid surface compared with the dense network of yeast and filamentous cells in the control (Fig. 5).

The formation of biofilms by *C. albicans* takes place through three distinct stages: the early, intermediate, and maturation phases [5]. Adhesion of yeast-form cells to a surface is followed by dimorphic transition of the yeast cells to give rise to hyphal forms. The intermediate phase of biofilm formation is characterized by cellular growth, formation of multiple layers of cells, and most importantly, elongation of filaments to form a mesh-like network of yeast, hyphae, and pseudo-hyphae. In the maturation phase, multiple layers of filamentous and yeast cells deposit an extracellular polymeric matrix (EPM) to give a three-dimensional structure embedded in the EPM [5]. The presence of multiple layers of filamentous growth is important in the formation of the three-dimensional *Candida* biofilm structure. Hyphal regulatory genes are found to be differentially regulated in biofilm growth, indicating the importance of filamentous forms in biofilm development [15]. Mutants of *C. albicans* that are unable to...
form hyphae were observed to form only basal layer and not the typical biofilm [27]. Thus, formation of hyphae from yeast cells is a key event in *C. albicans* biofilm development and maturation. It is interesting to note that the molecules grouped as potent inhibitors of biofilm development in this study are also good inhibitors of morphogenesis. Hence, screening for yeast-to-hyphae dimorphism in *C. albicans* would be a good strategy to search for inhibitors of biofilm formation. Salicylaldehyde and cinnamaldehyde prevented biofilm formation at concentrations much lower than that of farnesol, whereas four of the phenylpropanoids prevented biofilm formation at concentrations comparable to that of farnesol. These molecules should be explored further as an anti-biofilm strategy. It was speculated that plant extracts/molecules mediated their activity through repression of specific genes like ALS1, ALS3, and HWPI, which are important in biofilm formation [23]. The exact mechanisms behind the phenylpropanoid-mediated inhibition of biofilm formation are yet to be revealed.

**Activity Against Mature Biofilms of *C. albicans***

In the late stages (24 h after the adhesion), biofilms of *C. albicans* are more resistant to environmental factors as well as antifungal drugs [5]. Mature biofilms were intact at farnesol concentrations as high as 4 mg/ml and were totally insensitive to very high concentrations of fluconazole. Moreover, inhibition by amphotericin B could be achieved at a concentration 32 times that of planktonic MIC. Among the seven phenylpropanoids found active against mature biofilms, cinnamaldehyde was the most efficient and completely inhibited mature biofilms at 0.5 mg/ml (Table 1; Fig. 6). Significant reduction in the RMA of mature biofilms was exhibited by 0.5 mg/ml of salicylaldehyde. Similarly, 1 mg/ml concentrations of anisyl alcohol and eugenol as well as 2 mg/ml of anisaldehyde and guaiacol were inhibitory to mature *C. albicans* biofilms. Complete inhibition of biofilms with the phenylpropanoids resulted in eradication of the dense cellular network. Results obtained in the XTT assay were also confirmed by CV assay, where more than 50% reduction in absorbance compared with that of control indicated removal of biofilm network due to treatment (data not shown). Activity against mature biofilms seems to be due to a fungicidal effect through degradation of the cell membrane [21]. In conclusion, the present study revealed that phenylpropanoids of plant origin exhibit promising activity against the growth and virulence factors of *C. albicans*. Selected phenylpropanoids were found to be inhibitors of drug-resistant biofilms. It is a fact that the chemicals like cinnamaldehyde, salicylaldehyde, and anisaldehyde included in the study are reactive and have toxicity issues. However, we do not attempt to suggest their direct use as drug candidates. Instead, it gives insight into the probable structures that can be used as scaffolds for formation of derivatives with less toxicity. Many of the phenylpropanoids are components of spices, fruits, and vegetables, and constitute a large part of our daily diet, hence generally regarded as safe (GRAS) [8, 16]. Khan and Ahmad [22] have shown that eugenol and cinnamaldehyde would be a good strategy to search for inhibitors of biofilm formation.
have potential synergy with fluconazole against *Candida albicans* biofilms. Similarly, low concentrations of other molecules need to be evaluated for their combinatorial potential with standard antifungal drugs, as a strategy for the prevention and eradication of *C. albicans* biofilms.

**Acknowledgments**

S. M. K. acknowledges the financial support (Ref. No. SR/SO/PS-24/2006) received from the Department of Science & Technology (DST), Government of India. J. S. R. is thankful to DST, New Delhi, for providing a research fellowship.

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