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Short communication

In vitro activity of terpenes against *Candida* biofilms

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Abstract

The antibiofilm activity of 10 terpenes was tested in vitro against three *Candida* species by 24-h treatment of biofilms aged 1–5 days. Treatment of 24-h-old *Candida albicans* biofilms with carvacrol, geraniol or thymol (0.06%) resulted in >80% inhibition. Carvacrol (0.03%) inhibition was ≥75% independent of the age of the *C. albicans* biofilm. Carvacrol (0.125%) inhibition was >75% against *Candida glabrata* and *Candida parapsilosis* biofilms. Geraniol (≥0.125%) and thymol (0.06% or 0.125%) inhibition was >75% against *C. parapsilosis* biofilms whatever their age. This study demonstrates the antibiofilm activity of terpenes and points out the exceptional efficiency of carvacrol, geraniol and thymol, which could represent candidates in the treatment of candidiasis associated with medical devices.

Keywords: Yeasts; Biofilm; *Candida*; Terpenes; Candidiasis

1. Introduction

Candida albicans is a major human fungal pathogen causing both mucosal and deep tissue infections. It is the most common fungal pathogen associated with colonisation and biofilm formation on the surfaces of almost any medical devices, such as cardiac valves, dialysis access and intravenous catheters [1], but also on topical devices including lenses and dentures. Diseases produced by *C. albicans* are often associated with biofilm development [1]. *Candida* biofilms are significantly less susceptible to commonly used antifungals, and infected devices generally need to be removed [1,2]. Moreover, this resistance to antifungals increases in conjunction with maturation of the biofilm [1]. Caspofungin belongs to a new class of antifungal drugs, the echinocandins, and its significant antibiofilm potential has been demonstrated [3,4]. However, Kalya and Ahearn [5] have reported higher minimum inhibitory concentrations (MICs) and minimal fungicidal concentrations for amphotericin B, miconazole, ketoconazole and itraconazole for the cells of *C. albicans* adhering to silicone for 2 h than for planktonic cells, and the implication of the adherence step as an early stage in biofilm development has been shown for a long time.

Essential oils are known for their in vitro and/or in vivo antifungal properties [6], but their antibiofilm activity has not been studied extensively. The strong antifungal activity of some major components of essential oils, i.e. terpenes, has been described in several studies [6–8].

The aim of this study was to investigate whether terpenic derivatives can reduce the development of *C. albicans* biofilms in vitro. This study was carried out using 10 terpenic derivatives that correspond to major components of essential oils: carvacrol; 1,8-cineole; citral; eugenol; farnesol; geraniol; linalool; menthol; α-terpinene; and thymol.

2. Materials and methods

2.1. Organisms and growth conditions

In total, six isolates of *C. albicans* were studied. *Candida albicans* ATCC 66396 and ATCC 3153 strains were purchased from the American Type Culture Collection. These two strains were susceptible to fluconazole (MIC < 8 mg/L

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by Etest method). Four other strains were obtained from IHEM (Biomedical Fungi and Yeasts Collection, Brussels, Belgium) and were originally isolated from the human mouth (IHEM 9581, IHEM 9582 and IHEM 9586) or human blood (IHEM 10266). These strains were used in this study because they showed MIC values of fluconazole >256 mg/L (Etest method). One strain of *Candida glabrata* (IHEM 9556) and one strain of *Candida parapsilosis* (ATCC 22019) were also used in this study.

Yeasts were first grown for 48 h at 37 °C on Sabouraud agar slants (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). A loopful of this culture was transferred to 25 mL of Yeast Nitrogen Base medium (Difco, Detroit, MI), supplemented with 50 mM glucose (Sigma, St Louis, MO) (YNB-Glc) and incubated for 18 h at 37 °C without shaking.

Prior to use in the biofilm experiments, blastospores were harvested, washed twice in 0.1 M phosphate-buffered saline (PBS) (pH 7.2) (bioMérieux, Marcy-L'Etoile, France) and adjusted to 5×10^6 blastospores/mL.

2.2. MIC of terpenes

The 10 terpenic derivatives (carvacrol, 1,8-cineole, citral, eugenol, farnesol, geraniol, linalool, menthol, α-terpinene and thymol) were purchased from Sigma and were prepared as stock solution of 16% (v/v) in YNB-Glc and 0.1% (v/v) Tween 80 (Sigma).

The MICs of the 10 tested terpenic components were determined by broth microdilution method using YNB-Glc medium to maintain the experimental conditions related to the biofilm assay. Briefly, yeasts were first grown for 48 h on Sabouraud agar slants and *C. albicans* inocula were prepared by suspending the obtained yeasts in YNB-Glc and adjusting to a final concentration of 5×10^3 colony-forming units/mL. Serial two-fold dilutions of each terpene in YNB-Glc were prepared in 96-well microtitre trays over the range 0.01% to 8%. To enhance terpene solubility, Tween 80 was included in all assays at a final concentration of 0.05% (v/v) after inoculation [9]. The MICs were determined after incubation for 48 h at 37 °C without shaking. All tests were performed in duplicate in at least two separate experiments.

2.3. Candida sp. biofilms

Biofilm experiments were performed in untreated 96-well tissue culture plates. The wells of the culture plates were incubated for 1 h in foetal bovine serum and washed twice with YNB-Glc prior to use to produce the biofilm [3]. Briefly, 300 μL of the suspension of *Candida* sp. (5×10^6 blastospores/mL) was allowed to adhere for 1 h at 37 °C to the polystyrene wells of a 96-well culture plate. The wells were then washed twice with YNB-Glc to remove the planktonic yeasts and adherent yeasts were incubated (unless specified) for 24 h at 37 °C in 300 μL of YNB-Glc.

2.4. Treatment of C. albicans biofilms with terpene derivatives

Culture plate wells coated with 24-h-old biofilms of *Candida* sp. were incubated for an additional 24 h with each terpenic derivative at various concentrations ranging between 0.001% and 16%. Controls without terpenic treatment and controls with Tween 80 at 0.05% (v/v) were included in each experiment.

2.5. Evaluation of the influence of terpenes on biofilm development

Metabolic activity was assessed using the tetrazolium salt (XTT) assay as previously described [3]. The principle is based upon the reduction of XTT tetrazolium to tetrazolium formazan by active mitochondria from *C. albicans* yeasts in the presence of the electron-coupling agent menadione.

Briefly, the wells coated with *Candida* biofilms were washed with PBS. Then, 300 mg/L XTT (Sigma) and 0.13 mM menadione (Sigma) were added in 200 μL of PBS. Plates were incubated for 3 h at 37 °C without shaking, then gently agitated and XTT formazan was measured colorimetrically at 492 nm (micro-plate reader LP400; Sanofi Diagnostics Pasteur).

Background formazan values were determined with plates containing PBS only or containing PBS, XTT and menadione; these values did not exceed 0.005 absorbance units and therefore were not significant. All experiments were performed twice with six replicates.

2.6. Statistical analyses

An analysis of variance (ANOVA) ($P < 0.05$) and a Scheffé's test were conducted to determine differences among the test groups.

3. Results and discussion

An initial investigation was carried out to assess the efficacy of 10 terpenic derivatives to inhibit the growth of planktonic *C. albicans* yeasts. The results confirmed the potential antifungal activity of almost all the tested terpenes: MICs ranged between 0.01% and 2% (v/v), except for farnesol and 1,8-cineole that showed MICs ≥8% (v/v). Thus, we confirmed the weak antifungal activity of farnesol and 1,8-cineole, which had been previously described by several authors [6,8,9]. Our results demonstrated the strongest antifungal activity of thymol, geraniol and carvacrol, which showed MICs ≤0.03% (v/v), in agreement with observations made by other authors [10,11]. Braga and Dal Sasso [12] demonstrated by scanning electron microscopy assay that thymol affected the envelope of planktonic *C. albicans*. Terpenes alter cell permeability by penetrating between the fatty acyl chains making up the membrane lipid bilayers,