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Effect of a casbane diterpene isolated from *Croton nepetaefolius* on the prevention and control of biofilms formed by bacteria and *Candida* species

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ABSTRACT

This study aimed to evaluate the effect of the compound 1,4-dihydroxy-2E,6E,12E-trien-5-one-casbane (CD1CN), a casbane diterpene isolated from *Croton nepetaefolius* stalks, on the biofilm formation or preformed biofilms of bacteria and yeasts. Minimum inhibitory concentration results showed that CD1CN inhibited the growth of single cultures of *Staphylococcus aureus* and *Candida albicans* at 125 and 500 μg/mL, respectively, as well as dual cultures of *S. aureus* with *C. albicans* or *Candida glabrata* at 500 and 250 μg/mL, respectively. In general, CD1CN reduced biofilm biomass when applied to preformed biofilms or when applied during the biofilm formation of single and dual cultures in concentrations ranging from 31.25 to 250 μg/mL, depending on the culture. CD1CN was more effective in reducing the cfu of *S. aureus* in single and dual biofilms (62.5–250 μg/mL) than that of *Pseudomonas aeruginosa*, although this reduction was also significant. For yeasts, CD1CN was generally more effective in reducing *C. glabrata* cfu in single or dual cultures when compared to *C. albicans*. SEM images of the dual-species biofilms confirmed these results. In conclusion, CD1CN could be an effective alternative to conventional antimicrobial agents against infectious biofilms, in particular those attributed to mixed cultures.

1. Introduction

It is well known that a significant proportion of human nosocomial infections are associated with biofilms (Høiby, 2011; Murphy and Clegg, 2013). Biofilms exhibit elevated resistance to host defenses and administered antimicrobial agents (Høiby et al., 2010). These communities are frequently composed of multiple species, including bacteria and yeasts, with several studies describing the association of a range of clinical specimens (Peters et al., 2010; Elias and Banin, 2012).

Candida albicans is the fourth leading cause of nosocomial bloodstream infections in patients in intensive care units and in neutropenic patients (Kett et al., 2011; Bonnin, 2012). *C. albicans* is often found with bacterial species in polymicrobial biofilms where extensive interspecies interactions are likely to occur (Douglas, 2002). For instance, *Candida* and *Pseudomonas* have been coisolated from the cystic fibrosis lung or serious burn wounds (Gupta et al., 2005; Valenza et al., 2008); moreover, their interaction is characterized as an antagonistic fungal–bacterial interaction (McAlester et al., 2008; Williams and Cámara, 2009). *C. albicans* and *Staphylococcus aureus* have also been coisolated from various mucosal surfaces, including vaginal and oral mucosa in a biofilm mode of growth (Klotz et al., 2007). Interestingly, the combined effect of *C. albicans* and *S. aureus* results in a synergistic interaction (Carlson and Johnson, 1985; Carlson, 1988).

Most previous studies examining the interactions between *Candida* and bacteria in mixed biofilms have focused on *C. albicans*, and a few studies have reported on biofilms not containing *C. albicans* in a mixed species environment (Bandara et al., 2010). Previously, *Candida glabrata* was considered a relatively nonpathogenic

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saprophyte of the normal flora of healthy humans, and, as such, it was not readily associated with serious infection. In fact, this species can rapidly disseminate throughout the body, and currently, *C. glabrata* infection is associated with a high mortality rate (Silva et al., 2011).

Analyses of mixed-species infections are limited, and observational studies are confounded by the fact that patients with polymicrobial infections may have other risk factors that correlate with a poor clinical outcome, such as greater severity of illness or inadequate therapy against either or both infecting organisms (Peleg et al., 2010; Morales and Hogan, 2010). In this context, steps should be taken to discover new compounds that are able to prevent or eradicate these biofilms. Natural plant compounds have emerged as potential biotechnological tools in the search for new antimicrobial drugs (Schachter, 2003). Moreover, some studies show that diterpenoids are often associated with the antimicrobial activity of natural extracts (Habibi et al., 2000; Velikova et al., 2000). In particular, the compound 1,4-dihydroxy-2E,6E,12E-trien-5-one-casbane diterpene was isolated previously from *Croton nepetaefolius* (Santos et al., 2008) and was recently shown to have antimicrobial activity against bacteria and yeast pathogenicity (Carneiro et al., 2011; Sá et al., 2012). The aim of the present study was to evaluate the effect of 1,4-dihydroxy-2E,6E,12E-trien-5-one-casbane diterpene on planktonic growth, biofilm formation and preformed biofilms of *C. albicans*, *C. glabrata*, *S. aureus* and *Pseudomonas aeruginosa*, as single species or in yeast–bacteria combinations.

2. Materials and methods

2.1. Plant material

Stalks from *C. nepetaefolius* were collected in Caucaia–Ceará (Brazil) in May 2004. The material was identified by Dr. Edson Paula Nunes at the Herbário Prisco Bezerra (EAC), Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, CE, Brazil, where the voucher specimens (No. 33.582) were deposited.

2.2. Isolation of casbane diterpene

Stalks (5.0 kg) of *C. nepetaefolius* were ground to powder and extracted with ethanol (EtOH) at room temperature. The solvent was removed under reduced pressure to obtain the EtOH extract. Such extract (58.6 g) was fractionated by silica gel chromatography followed by elution with hexane (fractions 1–15), hexane/ethylacetate (EtOAc) 1:1 v/v (fractions 16–25), EtOAc (fractions 26–40) and EtOH (fractions 41–48), resulting in 48 fractions of 100 mL each. The hexane fractions (fractions 1–15; 22.5 g) obtained in the previous step were pooled and re-chromatographed in a silica gel column resulting in new fractions (F′), which were eluted with hexane (F′ 1–10), hexane/EtOAc 1:1 v/v (F′ 11–16), EtOAc (F′ 17–21) and EtOH (F′ 22–25). The hexane/EtOAc fractions (F′ 11–16; 14 g) obtained in the previous step were pooled and re-chromatographed in a silica gel column resulting in new fractions (F″), which were eluted with hexane (F″ 1), hexane/EtOAc 9:1 v/v (F″ 2–5), hexane/EtOAc 8:2 v/v (F″ 6–15), hexane/EtOAc 7:3 v/v (F″ 16–32) and EtOAc (F″ 33). Fractions F″ 10–13, obtained with hexane/EtOAc 8:2 v/v, yielded a diterpene named 1,4-dihydroxy-2E,6E,12E-trien-5-one-casbane (CD1CN) (Fig. 1).

2.3. Microorganisms

The microorganisms used in this study included *S. aureus* JKD 6008, *P. aeruginosa* ATCC10145, *C. albicans* ATCC90028 and *C. glabrata* ATCC2001.

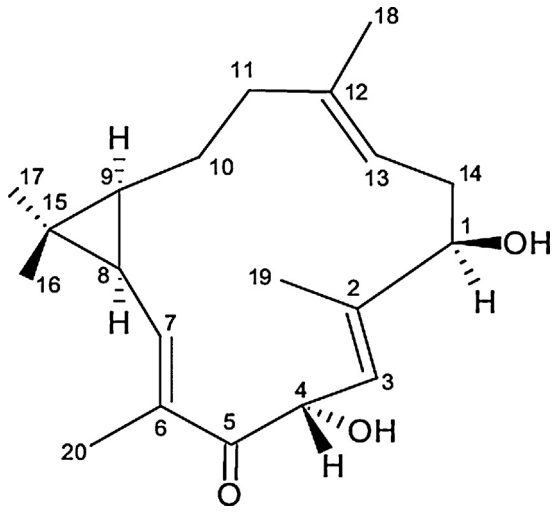

Fig. 1. Structure of 1,4-dihydroxy-2E,6E,12E-trien-5-one-casbane diterpene (CD1CN) isolated from the stalks of *C. nepetaefolius*.

2.4. Culture conditions

Bacteria and yeasts were grown in Trypticase Soy Agar (TSA) and Sabouraud Dextrose Agar (SDA) (both from Liofilchem, Italy), respectively, and incubated at 37 °C for 24 h. An isolated colony was then removed and inoculated into 10 mL of Trypticase Soy Broth (TSB) and Sabouraud Dextrose Broth (SDB) (both from Liofilchem, Italy) and incubated for 18 h at 37 °C under constant agitation. Prior to use, cells were harvested by centrifugation at 5000 × *g* for 10 min at 4 °C and washed using phosphate buffered saline (PBS; pH 7; 100 mM). Thereafter, the final concentration of bacterial inoculum was adjusted to 10^6 cells/mL in Nutrient Broth (NB) (Liofilchem, Italy) using a spectrophotometer (620 nm) and calibration curves previously determined for each bacterium. For yeasts, culture conditions were the same as those described above; however, the concentration of each yeast inoculum was adjusted to 10^6 cells/mL using a Neubauer chamber.

2.5. Minimum inhibitory concentration determination

The antimicrobial potential of CD1CN was assessed through a microdilution test in 96-well "round-bottom" polystyrene plates. CD1CN was diluted in NB with 4% Dimethyl sulfoxide (DMSO) v/v at concentrations ranging from 31.25 to 500 μg/mL and was incubated with 10^6 cells/mL (final concentration of CD1CN and cells). The plates were then incubated at 37 °C for 24 h under constant agitation. The optical density of the contents of each well was recorded at 640 nm (OD_{640}) using an automated Elisa Reader (Synergy TM HT Multi-Detection Microtiter Reader), and the minimum inhibitory concentration (MIC) was determined as the lowest concentration of CD1CN at which there was complete inhibition of the visible growth of the organism. The assays were performed with single- and dual-species (bacteria with yeasts).

2.6. Effect of CD1CN on biofilms

CD1CN activity was tested during biofilm formation, but it was also applied to preformed, mature biofilms. The methodology used to grow biofilms was based on the microtiter plate test developed by Stepanovic et al. (2000) with some modifications. The assays were performed with single and dual-species biofilms (bacteria–yeasts).

Assays to evaluate the formation of biofilms in the presence of CD1CN were performed in sterile 96-well polypropylene plates