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Antifungal activity of a prenylated flavonoid from *Dalea elegans* against *Candida albicans* biofilms

Mariana Andrea Peralta[a], María Angel da Silva[b], María Gabriela Ortega[a], José Luis Cabrera[a], María Gabriela Paraje[b,*]

[a] *Departamento de Farmacia, Facultad de Ciencias Químicas, Instituto Multidisciplinario de Biología Vegetal (IMBIV) - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, 5000 Córdoba, Argentina*
[b] *Facultad de Ciencias Exactas Físicas y Naturales, Instituto Multidisciplinario de Biología Vegetal (IMBIV) - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); Cátedra de Microbiología, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 299, Córdoba, Argentina*

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ABSTRACT

Background: The continuing emergence of infections with antifungal resistant *Candida* strains requires a constant search for new antifungal drugs, with the plant kingdom being an important source of chemical structures.
Purpose: The present study investigated the antifungal effect of 2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl)-8-prenylpinocembrin (8PP, formerly 6PP), a natural prenylflavonoid, on *Candida albicans* biofilms, and compared this with an azole antifungal (fluconazole) by studying the cellular stress and antioxidant response.
Study design/methods: The fluconazole sensitive (SCa) and azole-resistant (RCa) *C. albicans* strains were used, with biofilm formation being studied using crystal violet (CV) and confocal scanning laser microscopy (CSLM). The minimal inhibitory concentration for sessile cells (SMIC) was defined as the concentration of antifungal that caused a 50% (SMIC 50) and 80% (SMIC 80) reduction of treated biofilms. The reactive oxygen species (ROS) were detected by the reduction of nitro blue tetrazolium (NBT), and reactive nitrogen intermediates (RNI) were determined by the Griess assay. The activities of the superoxide dismutase (SOD) and catalase (CAT) antioxidant enzymes and the total antioxidant capacity of the biofilms were measured by spectrophotometric methods. ROS accumulation was also detected inside biofilms by using the fluorogenic dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), which was visualized by CSLM.
Results: The SCa and RCa biofilms were strongly inhibited by 8PP at 100 μM (SMIC 80). We observed that cellular stress affected biofilms growth, resulting in an increase of ROS and also of reactive nitrogen intermediates (RNI), with SOD and CAT being increased significantly in the presence of 8PP. The basal level of the biofilm total antioxidant capacity was higher in RCa than SCa. Moreover, in SCa, the total antioxidant capacity rose considerably in the presence of both 8PP and fluconazole.
Conclusion: Our data suggest that 8PP may be useful for the treatment of biofilm-related *Candida* infections, through an accumulation of endogenous ROS and RNI that can induce an adaptive response based on a coordinated increase in antioxidant defenses. 8PP may also have a therapeutic potential in *C. albicans* infections.

Introduction

The formation of biofilms is an important virulence factor that allows *C. albicans* to cause many types of infections and is responsible for most cases of candidiasis at both mucosal and systemic sites (Garcia-Vidal et al., 2013). Biofilms are defined as highly structured communities of microorganisms that are either surface-associated or attached to one another, and are enclosed within a self-produced protective extracellular matrix (Fanning and Mitchell, 2012). It has been reported that *Candida* biofilms are 30–2000 times more resistant to several antifungal agents compared to their planktonic (or free-living) counterparts (Olsen, 2015). Moreover, the antifungal drugs

Abbreviations: BBU, biofilm biomass unit; RCa, *Candida albicans* strain azole-resistant; SCa, *Candida albicans* strain fluconazole sensitive; CAT, catalase; CSLM, confocal scanning laser microscopy; CV, crystal violet; CLSI, Clinical and Laboratory Standards Institute; SOD, enzyme superoxide dismutase; FRAP, ferrous reduction antioxidant potency assay; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; $OH^{\bullet}$, hydroxyl radical; MIC, minimal inhibitory concentration; NBT, nitro blue tetrazolium; OD, optical density; $ONOO^-$, peroxynitrite; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RNI, reactive nitrogen intermediates; SDA, sabouraud dextrose agar; SMIC, sessile minimal inhibitory concentration; $O_2^{\bullet-}$, superoxide radical; YPD, yeast peptone dextrose; 8PP, 2′,4′-dihydroxy-5′-(1‴, 1‴-dimethylallyl)-8-prenylpinocembrin; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DCFH, 2′,7′-dichlorodihydrofluorescein; DCF, 2′,7′-dichlorofluorescein.

* Corresponding author. Tel.: +54 351 4332090 int. 225; fax: +54 351 4334198.
E-mail address: paraje@efn.uncor.edu, gabrielaparaje@gmail.com (M.G. Paraje).

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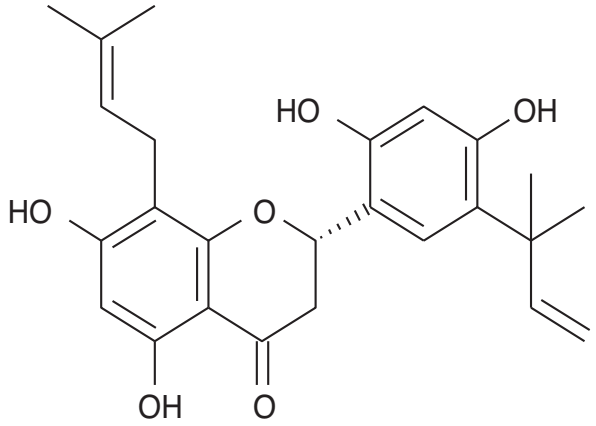

8PP (**1**)

Fig. 1. Structure of 2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl)-8-prenylpinocembrin (8PP, **1**) isolated from *Dalea elegans* Gillies ex Hook. et Arn. (*Fabaceae*).

available to successfully treat these infections are becoming increasingly limited, with polyenes, allylamines, azoles (e.g. fluconazole) and echinocandins being the current classes of molecules for the treatment of systemic and invasive candidiasis (Tobudic et al., 2012). Therefore, the development of novel approaches to inactivate *Candida* biofilms has a great clinical importance in treating candidiasis.

Oxidative stress is caused by an imbalance between the production of oxidants (such as free radicals) and the levels of antioxidant defenses. The incomplete reduction of O_2 during respiration produces O_2^-, which is enzymatically dismutated by superoxide dismutase (SOD) to H_2O_2 and can be eliminated by catalase (CAT) activity. Both excessive H_2O_2 and its decomposition product hydroxyl radical ($OH^•$), formed in a Fenton-type reaction, are harmful to most cell components. A disturbance in the prooxidant/antioxidant balance in favor of the overproduction of reactive oxygen species (ROS) can result in damage to the cellular components, including lipids, proteins and DNA. If this damage is not repaired, mutagenesis and cellular death can occur (Baronetti et al., 2011). The formation of ROS has been suggested to be one of the antimicrobial mechanisms (Delattin et al., 2014; Maurya et al., 2011), having been reported that miconazole induces the accumulation of endogenous ROS in *C. albicans* biofilms (Vandenbosch et al., 2010). In addition, a high ROS-detoxifying activity of SOD has been determined in miconazole-tolerant cells. These enzymes appear to play an important role in protecting *C. albicans* biofilms against high doses of antifungal miconazole through a fungal biofilm resistance mechanism (Mah, 2012).

The prenylated flavanone 2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl)-6-prenylpinocembrin (6PP) previously reported was isolated from the roots of *Dalea elegans* Gillies ex Hook. et Arn. (*Fabaceae*) by Cafaratti et al. (1994). Recently, this structure was revised according to new 2D NMR experimental data, thereby allowing the reassignment of this structure as 2′, 4′-dihydroxy-5′-(1‴, 1‴-dimethylallyl)-8-prenylpinocembrin (8PP) (Fig. 1) (Peralta et al., 2014).

In previous articles, between 1996 and 2012, we reported the antimicrobial activity of 6PP (hereafter 8PP) demonstrating its capacity to inhibit the membrane active transport in azole-resistant *C. albicans*, a strain which presents an overexpression of CDR1, CDR2 and MDR1 genes (Ortega et al., 1996; Peralta et al., 2012; Perez et al., 2003).

The present work was performed with the aim of knowing the effects of 8PP on *C. albicans* biofilms compared to those of fluconazole by studying the cellular stress production and the antioxidant response in biofilms. So, the oxidative metabolites ROS, the antioxidant enzymes SOD and CAT and the total antioxidant capacity were evaluated.

Material and methods

Plant material

D. elegans was collected in February 2011 during the flowering period, in its natural habitat in hills near Cabalango (Córdoba, Argentina, GPS coordinates: latitude: 31°24′ 04.62″ south; longitude: 64°34′ 19.21″ west; height: 763 m). Plant material was identified by Dr Gloria Barboza of the Botanical Museum, Universidad Nacional de Córdoba, Córdoba, Argentina (UNC). A representative voucher specimen has been deposited as CORD Peralta 2 in the herbarium at the Botanical Museum (IMBIV, UNC).

Extraction and isolation

The flavonoid 8PP (Fig. 1) was isolated and purified from roots of *D. elegans* (Cafaratti et al. 1994) and its structure was characterized by UV, NMR ^1H and ^{13}C and HRMS, according to Peralta et al. (2014). The purity of 8PP was determined as 95% by using a Varian (Palo Alto, CA) ProStar HPLC equipment which was coupled with a UV detector (Varian). The elution was carried out in a Varian C_{18} column (Ø 250 mm × 4.6 mm), in order to analyze the purity of 8PP, under two different conditions. Method 1: mobile phase: (A) H_2O, (B) MeOH; elution program: linear gradient from 50% B to 85% B in 60 min, followed by 100% B maintained for 10 min and finally linear gradient from 100% B to 50% B in 10 min. Method 2: mobile phase was composed of acetonitrile 1% acetic acid (A) and H_2O 1% acetic acid (B) with gradient elution system: 0–10 min, 10–40% A; 10–15 min, 40–50% A, maintained for 5 min; 20–25 min, 50–60% A; 25–30 min, 60–30% A; 30–35 min, 30–10% A, maintained for 5 min. Both methods were performed at a flow rate of 1.0 ml/min; detection wavelength: 290 nm; injection volume: 20 μl; temperature: 30 °C.

Chemicals

Fluconazole (purity ≥ 98%), Calcofluor-White and 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich Co, St Louis, MO, USA.

Yeast strains and growth conditions

Two well-characterized strains of *C. albicans* were isolated from the oral cavity of immunocompromised hosts (AIDS). These were generous gifts by Dr Theodore C. White, Seattle, WA. The azole-resistant strain (12-99, hereafter RCa) overexpresses the transporter genes CDR1, CDR2 and MDR1, whereas the sensitive strain (2-76, hereafter SCa) has a basal expression of these genes (White et al., 2002). Both strains were stored as frozen stocks with 15% glycerol at −80 °C. Before each experiment cells were subcultured from this stock onto Sabouraud dextrose agar (SDA) (Difco, Detroit, MI), and passaged to ensure purity and viability (CLSI, 2002).

Formation and quantification of biofilms

Biofilms were prepared in flat-bottomed 96-well microplates (Greiner Bio-One, Germany) adapted from a method of O'Toole & Kolter (1998), which is based on the ability of microorganisms to form biofilms on solid surfaces, and uses crystal violet (CV) to stain biofilms (Messier et al., 2011). Briefly, plates were pre-treated with 100 μl of 50% fetal bovine serum (FBS) (Sigma-Aldrich) at 37 °C for 30 min and washed twice with 10 mM phosphate-buffered saline (PBS pH