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

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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.

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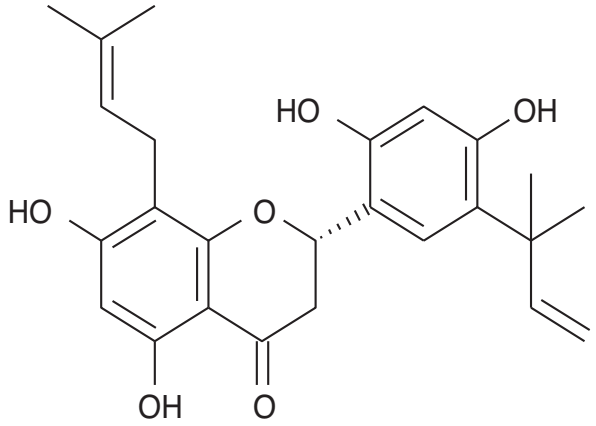

8PP (**1**)

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

7.2) (Pierce et al., 2008). Then, 100 μl of 1 × 10^7 cells/ml suspension in Yeast Peptone Dextrose (YPD) (Difco) were inoculated, and plates were incubated at 37 °C for 90 min to allow the attachment of cells to the surface. Non-adhered cells were removed by washing the wells with sterile PBS, and plates were incubated at 37 °C for 48 h without shaking to allow biofilm formation. Following growth, biofilms were washed with sterile PBS and incubated at 37 °C for 48 h with different concentrations of fluconazole (0.8–6.5 μM) and 8PP (1.5–100 μM), according to previous reports (Ramage and López-Rivot, 2005). After incubation, the supernatant was separated for extracellular oxidative stress assays (Angel Villegas et al., 2013). Plates were gently rinsed with distilled water and air dried for 24 h prior to CV staining. Adherent films of each well were stained with 200 μl per well of 1% (w/v) CV (5 min) and then washed with water to remove unbound dye. Afterward, CV was extracted with 200 μl of the bleaching solution: ethanol/glacial acetone (70:30), and biofilms were quantified by measuring the optical density (OD) at 595 nm using a microplate reader (Tecan Sunrise Model, TECAN, AUS).

Both strains were classified as biofilm producers according to the following classification: OD ≤ ODc = no biofilm producer; ODc < OD ≤ (2 × ODc) = weak biofilm producer, (2 × ODc) < OD ≤ (4 × ODc) = moderate biofilm producer and (4 × ODc) < OD = strong biofilm producer. The biofilm biomass unit (BBU) was arbitrarily defined with 0.1 OD_{595} equal to 1 BBU (Angel Villegas et al., 2013; Arce Miranda et al., 2011).

Antifungal activity

The minimal inhibitory concentration (MIC) for planktonic cells was determined following the guidelines of the M27-A3 document of the Clinical and Laboratory Standards Institute standard method (CLSI, 2008), and defined as the lowest drug concentration able to produce a growth inhibition higher than 90%. The sessile minimal inhibitory concentration (SMIC) was determined on formed biofilms as described below (Pierce et al., 2008). Briefly, 100 μl per well of antifungal solutions were added in order to obtain final concentrations ranging from 0.8 to 6.5 μM for fluconazole and 1.5 to 100 μM for 8PP. In addition, control wells without antifungal solution were used. After inoculation, the microtiter plate was incubated at 37 °C for 48 h, and absorbance was measured at 595 nm.

Sessile minimal inhibitory concentrations 50 and 80 (SMIC50 and SMIC80, respectively) were defined as the drug concentrations at 50 and 80% decreases in absorbance (Pierce et al., 2008).

After antifungal treatment, the supernatant was eliminated and 100 μl of sterile water was added to each well and sonicated (40 kHz, 60 s) in order to re-suspend the biofilm cells thoroughly. Then, the suspension was diluted 1000 times with sterile water and 100 μl of the suspension was then pipetted out and spread evenly by using a sterile plastic transferring loop on SDA (Difco), before being incubated at 37 °C for 24 h. Viable cells were determined by CFU/ml for correlation studies with BBU.

Assays for oxidative metabolites and antioxidative activity of biofilms

ROS production was detected in the supernatant (0.1 ml) by the reduction of nitro blue tetrazolium (NBT, Sigma-Aldrich) to form an insoluble dark blue diformazan precipitate (0.1 ml of NBT 1 mg/ml). This by-product of the assay is proportional to the generated ROS in biofilms and was measured at 540 nm, with the results being expressed as Abs_{540nm}/BBU (Angel Villegas et al., 2013; Arce Miranda et al., 2011).

The total SOD activity was assayed photochemically based on the inhibition of NBT reduction in 50 ml of the supernatant. Then, the ability of SOD to inhibit the reduction of NBT by the generated $O_2^{\cdot-}$, through the illumination of riboflavin in the presence of oxygen and the electron donor methionine, was evaluated in the samples, with the results being expressed as SOD activation (%)/BBU (Baronetti et al., 2013).

To quantify CAT activity, biofilms were treated with 50 μl of PBS, 40 μl of 0.2 M H_2O_2 and 200 μl of 0.2 M potassium dichromate ($K_2Cr_2O_7$) solution in glacial acetic acid, and a calibration curve was made with different concentrations of pure CAT plus the reagents mentioned above. The OD was determined at 570 nm, and the results were expressed as CAT (U)/BBU (Angel Villegas et al., 2013).

Total antioxidant capacity was evaluated by the ferric reducing antioxidant potency (FRAP) assay. Briefly, 10 μl of the sample were mixed with 300 μl of the following mixture (10:1:1): (a) 300 mM acetate buffer pH: 3.6, (b) 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl and (c) 20 mM $FeCl_3.6H_2O$. Then, the absorbance was measured at 593 nm after 4 min of incubation, with FRAP values being calculated using $FeSO_4$ calibration curve and expressed as μM/BBU (Baronetti et al., 2013).

Biofilm analysis by optical microscopy and confocal scanning laser microscopy (CSLM)

Biofilms were observed by CSLM as described below. Prior to imaging, biofilms were formed on small glass covers (12 mm) placed in the wells of a 24-well microtiter plate (Greiner Bio-One, Germany). Following biofilm formation and antifungal exposure, the supernatants were eliminated and disks were rinsed with sterile 10 mM PBS (pH 7.2; no autofluorescence detected). Disks were stained for 1 min with 30 μl of Calcofluor-White (0.05% [vol/vol]; Sigma-Aldrich), which was excited at 355 nm and which stains the fungal cell walls blue.

After being washed in PBS, disks were incubated with 50 μl of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Inc., Eugene, Oreg.) (10 μM) for 15 min in the dark at room temperature, and a 488 nm excitation source was used to visualize the sites of ROS production. DCFH-DA is a non-polar, non-fluorescent compound that readily diffuses across membranes, and is hydrolyzed within the cell by esterases to the polar, non-fluorescent, membrane-impermeable derivative 2′,7′-dichlorodihydrofluorescein (DCFH), which is rapidly oxidized by ROS to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) (Weissman et al., 2005).

After staining, disks were removed from the wells; air dried for 15 min in darkness conditions and placed inverted in 35-mm glass-bottom microwell dishes. Intact biofilms were examined using a Olympus FluoView FV1000 CSLM (Olympus Latin America, Miami, FL, USA) equipped with UPlanSApo 100X/1.40 oil UIS2 Olympus oil immersion lens. Optical sections were acquired at 0.5 μm intervals for the total thickness of biofilms. Then, for each sample, images from three randomly selected positions were obtained and analyzed independently by three investigators (MAP, MA da S and MGP).

Statistical analysis

All experiments were performed in triplicate, and numerical data were presented as means ± standard errors of the means (SEMs). The data were analyzed by using ANOVA followed by the Student–Newman–Keuls test for multiple comparisons, $^{*}p < 0.01$ and $^{**}p < 0.001$ were considered significant for comparisons with non-treated biofilms and $^{\#}p < 0.01$ and $^{\#\#}p < 0.001$ for comparisons between SCa and RCa.

Results

Fig. 2 shows SMIC 50 and SMIC 80 for fluconazole and 8PP in RCa and SCa biofilms ($^{**}p > 0.001$), where viable cells were determined by CFU/ml. It was observed that the cellular viability of biofilms decreased with increased concentration of the compounds assayed,

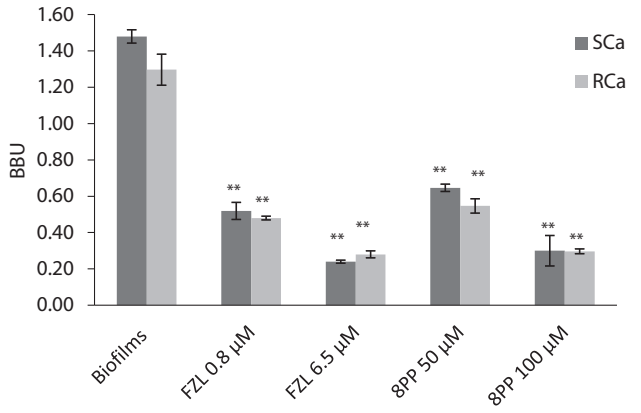

Fig. 2. Quantification of effect of fluconazole (FZL) and 8PP on RCa and SCa biofilms: by crystal violet (CV) staining expressed in biofilm biomass units (BBU). Error bars represent the standard deviations of the means of three independent experiments. ** denote statistical significance at $p < 0.01$ versus untreated biofilms.

with the results showing a correlation between the CV assay and CFU/ml (data not shown).

C. albicans biofilms produced detectable amounts of ROS when evaluated by the NBT assay. Fig. 3A shows the production of ROS expressed as the ratio between stress metabolites and the biofilm (ROS/BBU), where it can be observed that in biofilms treated with fluconazole, the ROS generation was greater for SCa than RCa ($^{\#\#}p > 0.001$). In addition, we found increases in oxidative stress at SMIC 50 and SMIC 80 (but smaller at SMIC 80) compared to the basal level. A small rise in ROS was observed in biofilms treated with 8PP ($^{**}p > 0.001$), but similar patterns in SCa and RCa at SMIC 80 and SMIC 50 were observed.

Another form of stress is termed nitrosative stress, with nitrite (NO_2^-) and nitrate (NO_3^-) being terminal electron acceptors under anaerobic conditions. Fig. 3B shows the reactive nitrogen intermediates (RNI) evaluated as nitrite using the Griess reagent. With fluconazole, both strains showed similar levels of nitrosative stress as the basal at SMIC 50, but in SCa a slight generation of RNI was observed in SMIC 80. SCa and RCa biofilms treated with 8PP at SMIC 80 showed important generation of RNI (23.55 and 20.41 RNI/BBU, respectively), 10-fold increased with respect to the controls (2.29 and 2.81 RNI/BBU for SCa and RCa biofilms, respectively) ($^{**}p > 0.001$).

Protective antioxidant mechanisms include enzymatic and non-enzymatic defenses that either scavenger or detoxify ROS. The SOD and CAT activities were studied in an attempt to correlate the enzymatic antioxidant activity with ROS production in biofilms under different antifungal treatments. For both compounds, SOD activity increased as a response to oxidative stress, being higher for fluconazole than for 8PP. Moreover, this increase was greater in SCa than in RCa at all concentrations assayed (Fig. 3C). A similar pattern was also observed in CAT activity for both strains (data not shown).

The main enzymes of this system that are involved in the detoxification of ROS, are SOD and CAT. In order to determine the biofilm's total antioxidant capacity that include enzymatic and non-enzymatic components, FRAP assay was performed. These are known as "reducing activity. The resistant strain showed a higher basal level of FRAP than the sensitive one (826.39 versus 256.30 FRAP/BBU). However for both strains, FRAP increased significantly in the presence of fluconazole and 8PP. In particular, in biofilms treated with fluconazole at SMIC50, this rise was greater in SCa than in RCa (3004.26 versus 2146.38 FRAP/BBU) (Fig. 3D).

Biofilms were grown on disks and examined by CSLM using Calcofluor-White, a UV-excitable dye that binds chitin and beta-glucan, which has long been used to highlight fungal cell walls. Fig. 4 (A and D) shows laser scanning fluorescence images for XY (top) and

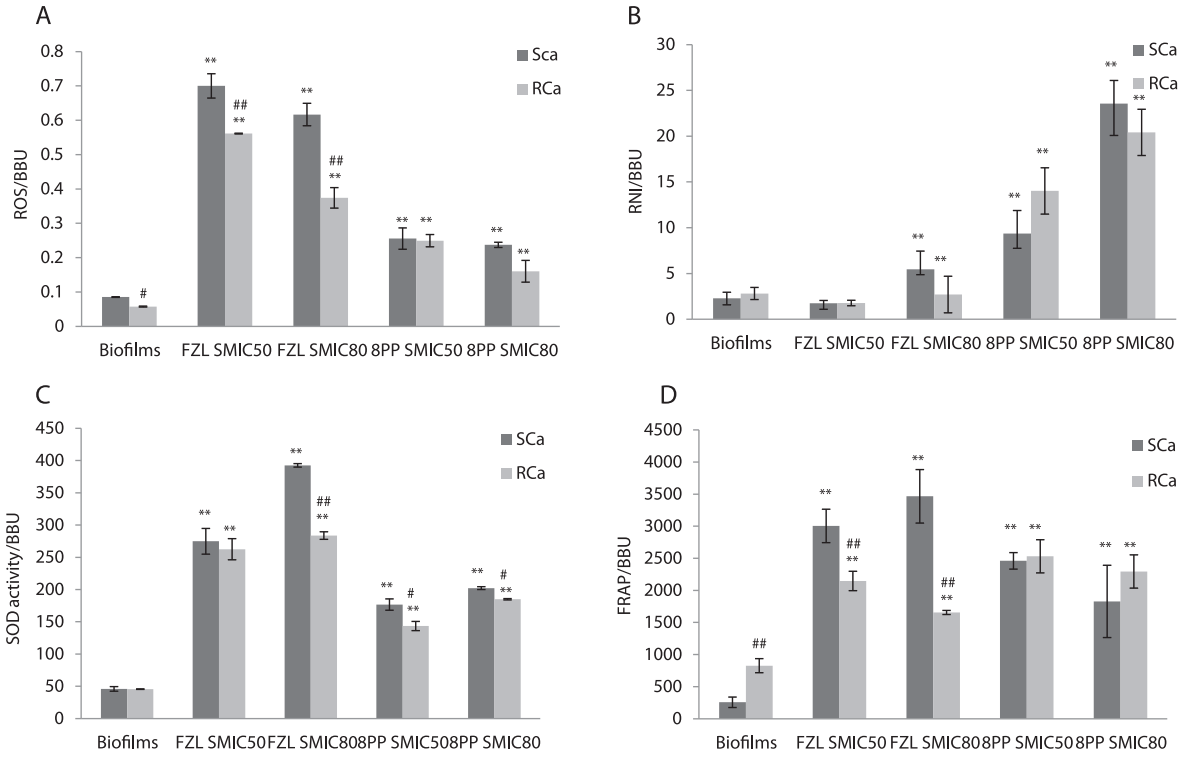

Fig. 3. Metabolites of cellular stress in *C. albicans* biofilms: (A) ROS/BBU ratio determined by NBT assay. (B) RNI /BBU ratio determined by Griess method. Antioxidant defenses in biofilms: (C) SOD activation (%)/BBU and (D) FRAP/BBU. Error bars represent the standard deviations of the means of three independent experiments. * and ** denote statistical significance at $p < 0.01$ and $p < 0.001$ respectively, when compared to untreated biofilms. # and ## indicate statistical significance at $p < 0.01$ and $p < 0.001$ respectively, when SCa and RCa were compared.

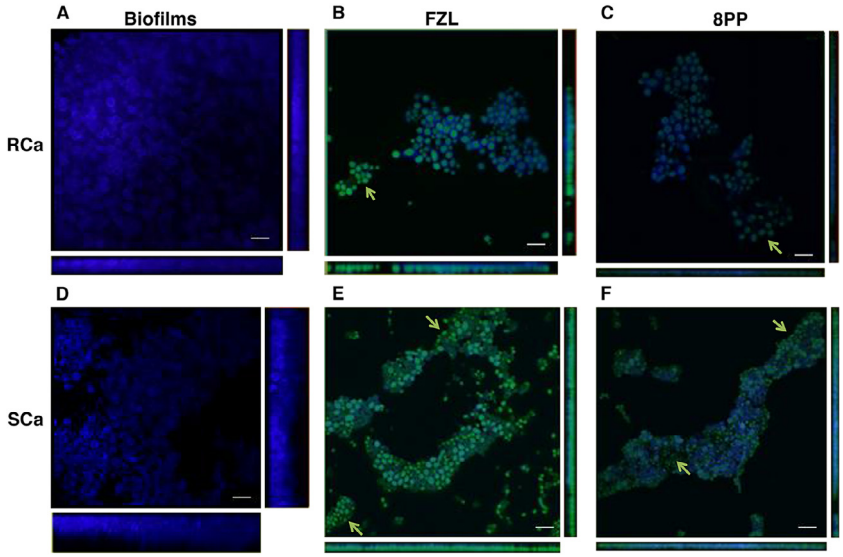

Fig. 4. CSLM images of growing RCa and SCa biofilms. Blue channel shows calcofluor in sessile cells walls, and green channel shows oxidation of the dye DCFH as an indicator of ROS production inside biofilms. (A) and (D) Untreated biofilm control. (B) and (E) FZL at SMIC 50. (C) and (F) 8PP at SMIC 50. The Z-stack image series was used to evaluate the size of biofilms. Magnification 60x. Arrows indicate the production of ROS within biofilms. Scale bar is 10 μm.

XZ (bottom) of sessile cells (blue) of *Candida* biofilms, which reveal that biofilm formation for RCa was less than that for SCa (16.5 μm versus 22.5 μm). The hazy biofilm appearance was due to diffuse staining of the extracellular material with Calcofluor-White, and implies that this material was composed of mainly cell-wall-like polysaccharides.

In the antifungal-treated *C. albicans* biofilm, most of *C. albicans* cells were present as blastospores (yeast forms) attached to the surface of the disk, which appeared as a haze-like film covering the fungal microcolonies. In the presence of 8PP the biofilms thickness was reduced to 4.5 μm for RCa and 6.5 μm for SCa, corresponding to reductions of 72.7% and 71.1% compared to their respective controls, with cells being fewer and of less density compared to those of the untreated control (Fig. 4C and F) ($p < 0.001$). Similar images were obtained with fluconazole (Fig. 4B and E).

In order to determine if cellular stress was implied in the mechanism of action of 8PP, we used the fluorogenic stain DCFH-DA by CSLM to monitor the generation of intracellular ROS inside *Candida* biofilms after antifungal treatment. A green fluorescence, resulting from oxidation of the dye DCFH-DA by superoxide radical (O_2^-) which yielded the fluorescent molecule DCF, was observed in sessile cells and indicated the presence of ROS (Fig. 4C and F). High-intensity DCF fluorescence was also detected inside fungal cells in the presence of fluconazole (Fig. 4B and E), with results showing a correlation between the NBT assay and DCFH-DA.

Discussion

Treatment of invasive *Candida* infections is often difficult due to the ability of *Candida* species to form biofilms that exhibit elevated resistance to various antifungal agents (Mah, 2012; Paraje et al., 2008). Compared to bacterial infections, few drugs are available to treat fungal infections. Clinical management of fungal diseases is being further compromised by the emergence of antifungal drug resistance, which limits the available drug classes that could be used as treatment options (Delattin et al., 2014). Moreover, the widespread use of antifungal drugs has led to the development of drug-resistant isolates, and resistance to azole antifungals continues to be a significant problem in the common fungal pathogen *C. albicans*.

Several molecular mechanisms that contribute to drug resistance have been identified, including increased mRNA levels for two types of efflux pump genes: the ATP binding cassette transporter CDRs (*CDR1* and *CDR2*) and the major facilitator MDR1 (Mah, 2012; White et al., 2002).

In the present study, the antifungal activity of the prenylated flavonoid 8PP against *C. albicans* biofilms was investigated. These results demonstrated that 8PP has similar pronounced antibiofilm effects against sensitive and resistant *C. albicans* strains. The biofilm formation was strongly inhibited (> 85%) by 8PP at 100 μM.

ROS generation may play a major role in tissue invasion and infection, but when the level of ROS exceeds the intracellular redox balance, the homeostasis is altered (Delattin et al., 2014; Maurya et al., 2011). In order to cope with an excess of free radicals, cells have developed sophisticated mechanisms to maintain redox homeostasis (Candiracci et al., 2012). Barraud et al. (2006) detected peroxynitrite ($ONOO^-$) inside microcolonies in *P. aeruginosa* biofilms with $ONOO^-$ being formed from nitric oxide (NO) oxidation, but only in the presence of ROS (Barraud et al., 2006). In another study, Schlag et al. (2007) provided evidence that nitrite-derived NO played a role in the inhibition of biofilm formation (Schlag et al., 2007). Although nitrosative stress in biofilms has been extensively studied with bacterial biofilms, comparatively little is known about fungal biofilm responses, with their basis still being unknown. In the present study, we observed that nitrosative stress could be produced inside biofilms treated with 8PP, consequently affecting their growth. The RNI could then accumulate in an extracellular medium and thus affect the matrix. However, in spite of other flavonoids such as 4-hydroxycordoin (Messier et al., 2011) and baicalein (Cao et al., 2008) having been reported as potent inhibitors of biofilm formation, no studies have been carried out related to the inhibition by flavonoids of established biofilms. To our knowledge, this is the first study that has attempted to correlate biofilm inhibition with the changes in the oxidative balance, in this case with ROS and RNI production by the flavonoid 8PP.

In our investigation, it was observed that biofilm treated with an antifungal was influenced by the production of oxidant metabolites and the levels of the antioxidant defenses, which varied with fluconazole or 8PP. It was found that the SOD and CAT levels were low in untreated biofilm, due to the levels of ROS also being low in these sessile cells. We suggest that when this balance was altered by fluconazole, an increase in the ROS production induced an overproduction of cellular stress, resulting in higher levels of SOD and

CAT in order to detoxify the oxidative stress metabolites generated. However, in biofilms treated with 8PP, the SOD and CAT levels were lower than after fluconazole treatment, due to the lower levels of ROS, with no differences being observed between sensitive and resistant strains. An elevated antioxidant capacity was observed by FRAP. Related to this, it should be pointed out that the ability of a microorganism to overcome cellular stress depends on its enzymatic and non-enzymatic antioxidant mechanisms, which minimize the generation of ROS and RNI. Although the basal level of FRAP was higher in RCa than SCa, similar levels were found in both strains biofilms after treatment with 8PP, indicating that a better activation of antioxidant defenses was achieved in SCa. Therefore, this concept could probably be extended in the future to include the possibility that strains with a low efflux can be resistant as a consequence of a high antioxidant capacity as one factor of defence against the increase in ROS and RNI provoked by 8PP.

The results obtained in the present work suggest that oxidative stress could be involved in the antifungal mechanism of action of 8PP on *C. albicans* biofilms. In fact, it was observed that the accumulation of ROS and RNI was strongly increased in sessile *Candida* cells treated with 8PP. The analysis ROS and RNI as two promoters of oxidative stress, demonstrated that the resistant strain showed low stress at SMIC 80, since the sensitive strain suffered high increases of ROS and NO. These findings are indicating that these oxidative metabolites may be responsible for the inhibition of biofilms of this compound. Our data therefore suggest that 8PP may be useful for the treatment of biofilm-related *Candida* infections, by producing nitrosative stress and an accumulation of endogenous ROS, as well as inducing an adaptive response that resulted in a coordinated increase of antioxidant defenses.

Conflict of interest

The authors declare that they have no conflict of interest.

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