Accepted Manuscript

The antioxidant activity of a prenyl flavonoid alters its antifungal toxicity on *Candida albicans* biofilms

Mariana Andrea Peralta, María Gabriela Ortega, José Luis Cabrera, María Gabriela Paraje
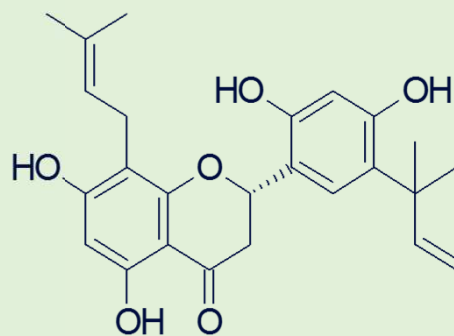
Please cite this article as: Peralta, M.A., Ortega, Marí.Gabriela., Cabrera, José.Luis., Paraje, Marí.Gabriela., The antioxidant activity of a prenyl flavonoid alters its antifungal toxicity on *Candida albicans* biofilms, *Food and Chemical Toxicology* (2018), doi: 10.1016/j.fct.2018.02.042.
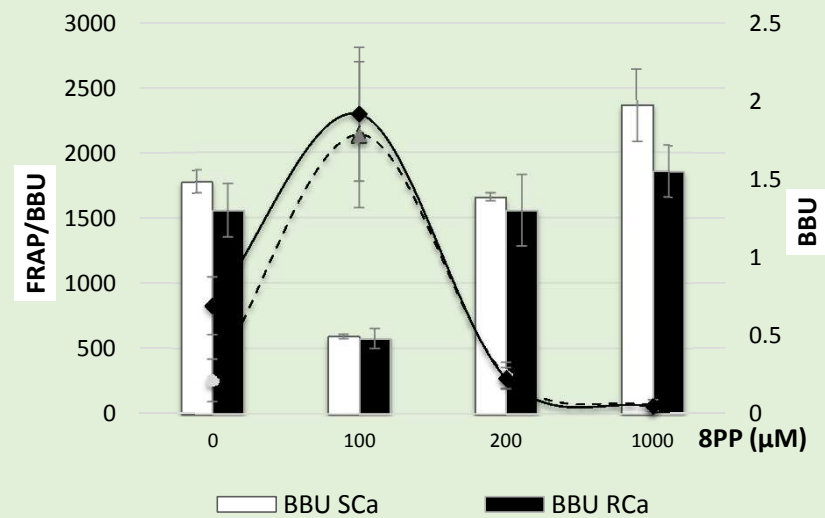
Candida albicans biofilms
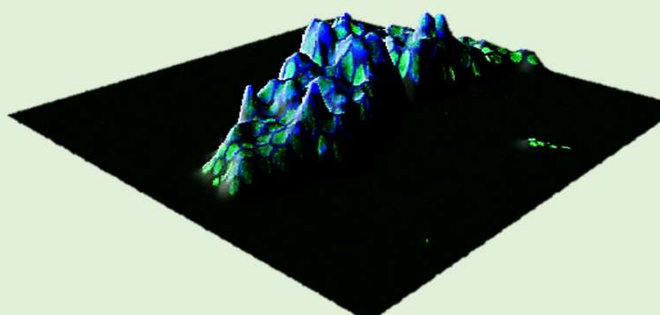
Prenyl flavonoid 8PP

Antifungal toxicity
(prooxidant/antioxidant dual action)

TITLE:

The antioxidant activity of a prenyl flavonoid alters its antifungal toxicity on *Candida albicans* biofilms

AUTHORS: Mariana Andrea Peralta[a], María Gabriela Ortega[a], José Luis Cabrera[a], María Gabriela Paraje[b*]

INSTITUTION:

[a] Universidad Nacional de Córdoba (UNC), Facultad de Ciencias Químicas, Departamento de Ciencias Farmacéuticas. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); Instituto Multidisciplinario de Biología Vegetal (IMBIV). Haya de la Torre y Medina Allende, 5000. Córdoba, Argentina.

[b] Universidad Nacional de Córdoba (UNC), Facultad de Ciencias Exactas Físicas y Naturales, Cátedra de Microbiología. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); Instituto Multidisciplinario de Biología Vegetal (IMBIV). Av. Vélez Sarsfield 299, Córdoba, Argentina.

* **CORRESPONDING AUTHOR:**

Cátedra de Microbiología, Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 299, Córdoba (5000), Argentina.

Tel: +54 351 5353800   int. 29731 Fax: +54 351 5332097

e-mail: gparaje@unc.edu.ar or gabrielaparaje@gmail.com

1

Graphical abstract
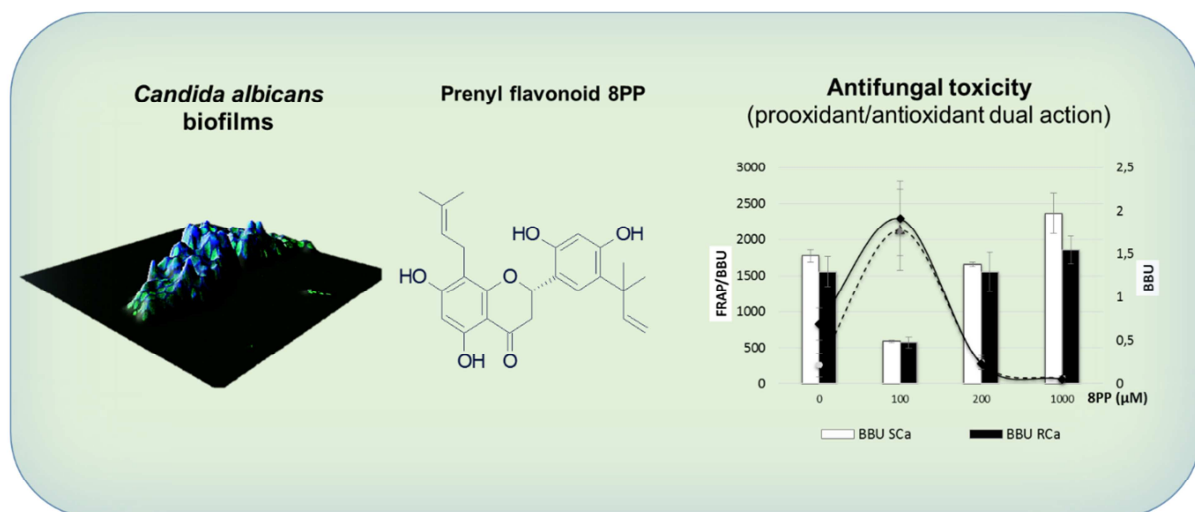
Highlights

- The antioxidant effect of 2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl)-8-prenylpinocembrin (8PP) was studied.

- 8PP displayed concentration-dependent antifungal activity on *C. albicans* biofilms, with dual prooxidant-antioxidant effect.

- The antioxidant effect, at high concentrations, was mainly mediated by the action of non-enzymatic system.

- At high concentrations, biofilms were more compact with little voids, pores and channels.

- Architecture and surface topography showing different diffusion distances could change the flow inside of biofilms.

Abstract

The antioxidant effect of 8PP, a prenylflavonoid from *Dalea elegans* on *Candida albicans* biofilms, was investigated. We previously reported that sensitive (SCa) and resistant *C. albicans* (RCa) biofilms were strongly inhibited by this compound, in a dose-depending manner (50 µM-100 µM), with a prooxidant effect leading to accumulation of endogenous oxidative metabolites and increased antioxidant defenses. In this work, the antifungal activity of high concentrations of 8PP (200-1000 µM), the cellular stress imbalance and the architecture of biofilms were evaluated. Biofilms were studied by crystal violet and confocal scanning laser microscopy (CSLM) with COMSTAT analysis. Superoxide anion radical, the activity of the superoxide dismutase and the total antioxidant capacity were measured. Intracellular ROS were detected by a DCFH-DA and visualized by CSLM; reactive nitrogen intermediates by Griess.

An antioxidant effect was detected at 1000 µM and levels of oxidant metabolites remained low, with major changes in the SCa. COMSTAT analysis showed that biofilms treated with higher concentrations exhibited different diffusion distances with altered topographic surface architectures, voids, channels and pores that could change the flow inside the matrix of biofilms. We demonstrate for first time, a concentration-dependent antioxidant action of 8PP, which can alter its antifungal activity on biofilms.

Keywords: *Dalea elegans*; prenyl flavonoid; *Candida albicans* biofilms; prooxidant/antioxidant action; free radicals; confocal scanning laser microscopy.

3

Abbreviations:

2',4'-dihydroxy-5'-(1''', 1'''-dimethylallyl)-8-prenylpinocembrin (8PP)

2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA)

Acquired immunodeficiency syndrome (AIDS)

Biofilm Biomass Unit (BBU)

Candida albicans strain azole-resistant (RCa)

Candida albicans strain fluconazole sensitive (SCa)

Confocal scanning laser microscopy (CSLM)

Colony forming units (CFU)

Crystal violet (CV)

Dimethyl sulfoxide (DMSO)

Ferrous reduction antioxidant potency assay (FRAP)

Fetal bovine serum (FBS)

Hydrogen peroxide (H_2O_2)

Hydroxyl radical (OH·)

Nitric oxide (NO)

Nitro blue tetrazolium (NBT)

Optical density (OD)

Phosphate-buffered saline (PBS)

Reactive nitrogen intermediates (RNI)

Reactive oxygen species (ROS)

Sabouraud dextrose broth (SDB)

Sabouraud dextrose agar (SDA)

Sessile minimum inhibitory concentration 80 (SMIC 80)

Superoxide dismutase (SOD)

Superoxide anion radical ($O_2^{\cdot-}$)

1. Introduction

Reactive oxygen species (ROS) are generated continuously during aerobic metabolism. The mitochondrial electron transport chain is the main source of ATP and is capable of generating ROS. The term ROS includes all highly reactive and unstable metabolites of molecular oxygen such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), alkyl peroxyl free radicals ($ROO^{\cdot}$) and non-radical molecules like hydrogen peroxide (H_2O_2) (Krumova and Cosa, 2016). The first step in ROS production is the reduction of molecular oxygen (O_2) to $O_2^{\cdot-}$. Even though $O_2^{\cdot-}$ is not a strong oxidant, it is a precursor of most other ROS, and it becomes involved in the propagation of oxidative chain reactions. In addition, $O_2^{\cdot-}$ may react with other radicals including nitric oxide (NO) in a reaction controlled by the rate of diffusion of both radicals (Beckhauser et al., 2016).

To ensure a proper balance of ROS, cells are equipped with intracellular antioxidant system and, if the level of ROS exceeds the intracellular defenses, homeostasis is altered. Their accumulation can lead to damage, such as protein cross-linking leading to protein inactivation, protein and lipid oxidation, breakage of DNA strands and cell death (Baronetti et al., 2011; Dickinson and Chang, 2011). The protective mechanisms either scavenge or detoxify ROS and include enzymatic and non-enzymatic antioxidant defenses. Among the endogenous antioxidant systems, the superoxide dismutase (SOD) enzyme is the first line of defense (Dantas et al., 2015).

Candida albicans is one of the most important fungal species causing disease in humans, especially in immunocompromised individuals such as those with immunosuppressive therapies, acquired immunodeficiency syndrome (AIDS), and patients with implanted medical devices (Kullberg et al., 2002; Nobile and Johnson, 2015). *C. albicans* biofilms are intrinsically resistant to conventional antifungal therapeutics and

6

confer protection from host immune defenses, which carry important clinical repercussions (Hirota et al., 2016). The inherent resistance of *C. albicans* biofilms to antifungal agents is due to the presence of the extracellular matrix, presence of recalcitrant persisters cells and upregulation of efflux pumps, between other factors. In *C. albicans*, two major classes of efflux pumps modulate drug exportation: the ATP-binding cassette transporter superfamily (including *CDR1* and *CDR2*) and the major facilitator class (including *MDR1*) (Ramage et al., 2002). Currently, there are only few antimycotics, such as azoles (miconazole), polyenes (amphotericin B) and echinocandins which are also effective against biofilms; interestingly, all increase ROS in fungal planktonic and sessile cells (Delattin et al., 2014).

Flavonoids have been widely investigated as antioxidants that prevent injury caused by free radicals by different mechanisms including direct scavenging of ROS and activation of antioxidant enzymes, among others (Procházková et al., 2011). Flavonoids are able to scavenge free radicals directly, resulting in the formation of a free radical phenoxyl by donating hydrogen, stabilized by delocalization of the unpaired electron in the aromatic ring (Valko et al., 2007).

Prenyl flavonoids are structures having substituents formed by chains of five, ten or fifteen carbon atoms (C5, C10 or C15), with a variety of structural arrangements linked to different positions in flavonoids rings (Rice-Evans et al., 1996). These compounds display a series of interesting biological activities due to the substitution of the flavonoid ring system with prenyl groups; these structural features increases the lipophilicity and confer to the molecule a strong affinity to biological membranes (Chen et al., 2014; Yang et al., 2015).

In previous articles, we reported the antimicrobial activity of a prenylated flavanone 2', 4'-dihydroxy-5'-(1''', 1'''-dimethylallyl)-8-prenylpinocembrin (8PP) (Ortega et al.,

7

1996; Pérez et al., 2003) and demonstrated its capacity to inhibit an azole resistant *C. albicans* strain (RCa) which presents an overexpression of CDR1, CDR2 and MDR1 genes (Peralta et al., 2012). Recently we informed the antibiofilm action of this compound against RCa and described that *C. albicans* biofilms were strongly inhibited by 8PP in concentration-dependent with a sessile minimum inhibitory concentration 80 (SMIC 80) at 100 µM concentration. We demonstrated that the cellular stress affected biofilm growth, through an accumulation of endogenous ROS and reactive nitrogen intermediates (RNI) that can induce an adaptive response based on an increase in antioxidant defenses (Peralta et al., 2015). Besides, the antioxidant activity of 8PP was also investigated demonstrating that this compound inhibited the enzymatic lipid peroxidation, exhibited scavenging and antioxidant activity in rat liver microsomes (Elingold et al., 2008).

The aim of this work was to investigate the antioxidant activity of 8PP, and whether this effect alters its antifungal activity on sessile cells and the architecture and surface topography of the biofilms in antioxidant redox status. Thus, cellular stress metabolites, the enzymatic and no-enzymatic antioxidant responses were studied in sessile cells of *C. albicans* biofilms in fluconazole sensitive (SCa) and RCa strains at high concentrations of 8PP. This is the first study that correlates the antifungal activity of 8PP with the redox imbalance provoked in dose-dependent manner and the architecture alteration inside of biofilms.

2. Material and methods

2.1. Reagents

Yeast Peptone Dextrose broth (1% yeast extract, 2% peptone, 2% dextrose); Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) (Difco). Fetal Bovine

8

Serum (FBS, Greiner Bio-One). Phosphate Buffer Solution (PBS) and dimethyl sulfoxide (DMSO, Merck). Fluconazole; Calcofluor-White and 2,7-dichlorofluorescein diacetate (DCFH-DA- Molecular Probes, Nitro Blue Tetrazolium (NBT); $NaNO_2$ and 2,4,6-tripyridyl-s-triazine (Sigma-Aldrich). $FeCl_3.6H_2O$ and $FeSO_4$ (Cicarelli); Crystal Violet (CV), sulfanilamide; HCl; ethanol; glacial acetone and H_2O_2 (Anedra). The solvents were distilled before use. Distilled or ultrapure water was used when was necessary.

2.2. Plant material, extraction and isolation

Dalea elegans was collected in province of Córdoba, Argentina (GPS coordinates: latitude: 31° 24´ 04.62" south; longitude: 64° 34´ 19.21" west; height: 763 m). A representative voucher specimen is on deposit as CORD Peralta 2 in the herbarium at the Botanical Museum (UNC, Argentina).

The prenyl flavanone 8PP (Fig. 1S) was purified from roots of *D. elegans* (Cafaratti et al., 1994) and its structure was characterized by means of spectroscopic and spectrophotometric methods and comparison with the data previously reported (Peralta et al., 2014). The purity of 8PP was determined as 96% by high performance liquid chromatography according to Peralta et al. (2015).

2.3. Fungal strains and growth conditions

Two well-characterized strains of *C. albicans* were isolated from the oral cavity of immunocompromised hosts (AIDS). The RCa (12-99) overexpresses the transporter genes CDR1, CDR2 and MDR1, and SCa (2-76) has a basal expression of these genes (White et al., 2002). Both strains were growth in Yeast Peptone Dextrose broth, both from a single colony grown on SDA and incubated 18 h at 37 °C. For long-term storage, yeasts stocks were kept from a glycerol 15% at -70°C (CLSI, 2002).

9

2.4. Assay for formation and quantification of biofilms

Quantification of biofilm formation was performed using 96-wells polystyrene microtitre plates (Greiner Bio-One) according to the method of O'Toole & Kolter (1996) with modification (Messier et al., 2011; Peralta et al., 2015). Briefly, microtitre plates were pre-treated with 50% FBS, were inoculated with 100 µl of 1×10^{7} cells/ml suspension in SDB and incubated at 37 °C for 90 min (Pierce et al., 2008; Peralta et al., 2015; Peralta et al., 2017). Each strain was assayed in four replicate wells. Non-adhered cells were removed and plates were incubated at 37 ºC for 48 h. Following growth, biofilms on mature phase of growth, were washed with sterile PBS and incubated at 37 ºC for 48 h with different concentrations of 8PP (1.5 to 1000 µM) from a 20 mM stock solution dissolved in DMSO (1% v/v final assay concentration). Fluconazole at concentration ranging from 0.8 to 6.5 µM was used as reference antifungal (positive control) from a 6.5 mM aqueous stock solution as previously described by Peralta at al., 2015. Untreated (controls) biofilms with DMSO (1% v/v) were also performed (Peralta et al., 2015; Ramage et al., 2012).

After incubation, the supernatant was carefully pipetted and into a separated plate in order to perform extracellular oxidative stress assays (Arce Miranda et al., 2011; Peralta et al., 2015; Peralta et al., 2017). The biofilms were rinsed three times with PBS, stained with 1% (w/v) CV for 5 min, and rinsed again with PBS. Afterwards, CV was dissolved with ethanol/glacial acetone (70:30). Finally, the optical density (OD) at 595 nm was determined using a microplate reader (Tecan Sunrise Model, TECAN, AUS). The amount of biofilms formed is reported as the Biofilm Biomass Unit (BBU), which corresponds to a simplified

expression used in previous studies and defined with 0.1 OD_{595} equal to 1 BBU (Arce Miranda et al., 2011; Marioni et al., 2016; Peralta et al., 2015).

2.5. Antifungal susceptibility testing on C. albicans biofilms

The SMIC 80 was defined as the concentration at which the BBU decreased 80% (Peralta et al., 2015; Marioni et al., 2017; Peralta et al., 2017). Antifungal solutions (100 µl per well) were added to a final volume of 200 µL per well, in order to obtain final concentrations ranging from 200 to 1000 µM for 8PP. Negative controls were included, containing SDB alone or SDB with 1% DMSO. After inoculation, the microtiter plate was incubated at 37 °C for 48 h, and OD was measured at 595 nm. To ensure accurate correlation between OD and cells concentration, a 100 µl sample of the SDB containing biofilms was sonicated for 3 s at 20% intensity to separate the sessile cells (TestLab Ultrasonic Cleaner, TestLab S.R.L, Buenos Aires, Argentina) before culturing on an agar plate for CFU counting. The cell 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. Then the plates were incubated at 37 ºC for 24 h (Peralta et al, 2015; Marioni et al., 2017; Peralta et al., 2017).

2.6. Non-enzymatic and enzymatic antioxidant activity

The total antioxidant capacity (enzymatic and non-enzymatic) of biofilms was determined by the ferric reducing antioxidant potency (FRAP) assay. The sample (10 µl) of were mixed with 300 µl of the mixture (10:1:1) of acetate buffer (300 mM, pH: 3.6), 2,4,6-tripyridyl-s-triazine (10 mM) in HCl (40 mM) and $FeCl_3.6H_2O$ (20 mM). The OD was

measured at 593 nm after 4 min of incubation. FRAP values were calculated using a $FeSO_4$ calibration curve and results were expressed as FRAP/BBU (Peralta et al., 2015; Peralta et al., 2017).

The total SOD activity was assayed through its ability to inhibit the photochemical reduction of NBT by $O_2^{\cdot-}$ to blue colored formazan measured at 560 nm. SOD inhibits the reduction of NBT by superoxide radical, generated through the illumination of riboflavin in the presence of oxygen and the electron donor methionine. The results were expressed as SOD activity (%)/BBU (Arce Miranda et al., 2011; Marioni et al., 2017; Peralta et al, 2015; Peralta et al., 2017).

2.7. Oxidative metabolites assay in biofilms

Nitric oxide was measured using Griess's reagent and $NaNO_2$ as standard. Supernatant (100 µl) and mixed with 200 µl of 1.5% sulfanilamide in 1 N HCl and 50 µL of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride aqueous solution were added and was measured 15 min later by spectrophotometry at 540 nm. Results were expressed respect to BBU (RNI/BBU) (Angel Villegas et al., 2013; Arce Miranda et al., 2011; Baronetti et al., 2011).

Extracellular $O_2^{\cdot-}$, were detected by the reduction of NBT (0.1 ml of NBT 1 mg ml^{-1}) and measured in the supernatant of biofilms under different conditions at 540 nm. The results were expressed as $O_2^{\cdot-}$/BBU. H_2O_2 treated samples were used as positive control (Arce Miranda et al., 2011; Marioni et al., 2017; Peralta et al, 2015; Peralta et al., 2017).

Intracellular ROS production was determined by Confocal scanning laser microscopy (CSLM) using DCFH-DA. Biofilms were formed on small glass covers (12 mm) placed in the wells of a 24-well microtiter plate (Greiner Bio-One, Germany). At first, disks were stained for 1 min with 30 µl of Calcofluor-White (0.05 % v/v), a

12

fluorescent dye that stains fungal cell walls blue (Peralta et al., 2015; Peralta et al., 2017). After being washed in PBS, disks were incubated with 50 µl DCFH-DA (10 µM) for 15 min in darkness at room temperature. DCFH-DA is a non-fluorescent compound that diffuses across membranes, and is hydrolyzed by intracellular esterases to a membrane-impermeable derivative 2',7'-dichlorodihydrofluorescein (DCFH), which is rapidly oxidized by ROS to the highly fluorescent 2',7'-dichlorofluorescein (DCF) which is trapped within the cell (Bergamo et al., 2015). After staining, intact biofilms over disks were removed from the wells, placed inverted in 35-mm glass-bottom micro well dishes and examined by using a Fluoview FV1000 Spectral Olympus CSLM (Olympus Latin America, Miami, FL, USA) equipped with PLAPON 60X O NA:1.42 Olympus oil immersion lens (Peralta et al, 2015; Marioni et al., 2017; Peralta et al., 2017).

2.8. Image acquisition and analysis by COMSTAT

For the analysis of the biofilms, two independent experiments were performed.

In both experiments images were acquired at 1 µm intervals down through the biofilms. Therefore, the number of images in each stack varied according to the thickness of the biofilms in each different condition (untreated biofilms and treated with 8PP at 1000 µM). Images from randomly selected positions were obtained and analyzed independently and optical sections were acquired at 1.0 µm intervals for the total thickness of biofilms. The structure of *C. albicans* biofilms was assessed using COMSTAT software by selecting the following parameters: Biomass ($µm^3/µm^2$), Mean thickness (µm), Maximum thickness (µm), Area occupied by cells (%), Surface-to-volume ratio ($µm^2/µm^3$), Maximum/Mean diffusion distance (µm) (Heydorn et al., 2000; Vorregaard, 2008). The analysis of the

13

images of the biofilms was performed by means of the NIH-ImageJ (Marioni et al., 2016; Peralta et al., 2017).

2.9. Statistical Analysis

Statistical analysis was performed using ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. Differences were considered significant for comparisons with non-treated biofilms if [*]$p < 0.01$ and [**]$p < 0.001$; and for comparisons between different concentrations of 8PP [#]$p < 0.01$ and [##]$p < 0.001$. All experiments were performed in triplicate and data are represented by the mean ± SD.

3. Results

Non-enzymatic and enzymatic antioxidant activity

Previously we determined the SMIC80 at 6.5 µM for Fluconazole and 100 µM for 8PP in RCa and SCa biofilms. We demonstrated that oxidative and nitrosative metabolites were increased, and the antioxidant defenses were activated in the presence of this compound at such concentration in both strains (Peralta et al., 2015).

According to these previous results, we evaluated the effect of higher concentrations of 8PP (200 to 1000 µM) on mature biofilms to determine whether affects its toxic activity on RCa and SCa sessile cells of biofilms. Fig.1 shows the relationship between the total antioxidant defenses and the BBU in presence of different concentrations of 8PP. At 200 µM and 1000 µM of this compound, the antioxidant defenses in biofilms determined by FRAP, decreased 8 and 40-fold respectively with respect to SMIC80 ([##]$p < 0.001$), concentration of 8PP that shown prooxidant activity in RCa (-○-), as was demonstrated in our previous investigation (Peralta et al., 2015). Also, SCa biofilms presented an important decrease in FRAP levels when were compared to those achieved by SMIC80 of 8PP (100

14

μM) (## p < 0.001). Furthermore, at 1000 μM the antioxidant capacity decreased fifteen-fold with respect to untreated RCa biofilms (**p < 0.001). This difference was not so remarkable in SCa (-◊-) at 200 μM of 8PP, which had similar FRAP values to the untreated. BBU had shown similar values at 200 μM of 8PP, than untreated samples in both strains. At this concentration, no inhibitory activity was detected. A correlation between CV assay and CFU/ ml was observed (data not shown).
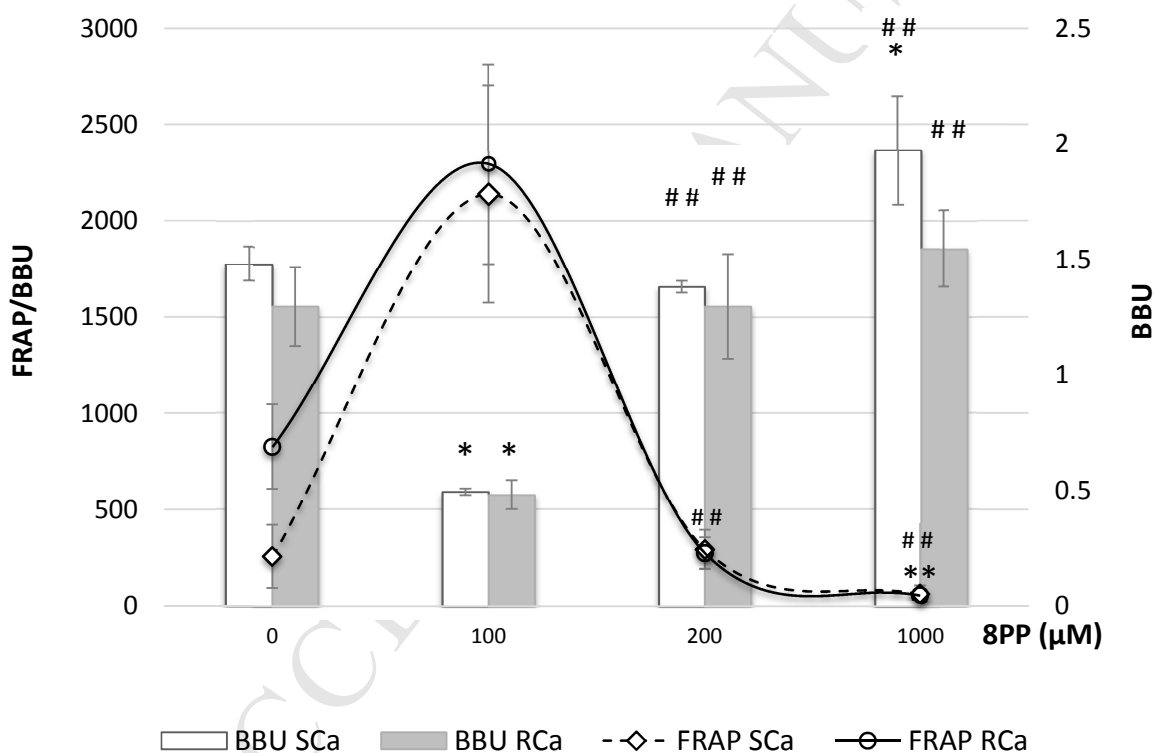

Fig. 1 Relationship between antioxidant defences determinate by ferrous reduction antioxidant potency assay (FRAP) and biofilm formation of RCa and SCa by crystal violet

(CV) staining expressed as biofilm biomass units (BBU). Error bars represent the standard deviations of the means of three independent experiments. * and **denote statistical significance $p < 0.01$ and $p < 0.001$ respectively, when were compared to untreated biofilms. [##] indicate statistical significance at $p < 0.001$ with respect to SMIC 80 (prooxidant activity).

The main enzyme involved in $O_2^{\cdot-}$ detoxification is the SOD. The activity of this enzyme was studied to correlate the enzymatic antioxidant activity with $O_2^{\cdot-}$ production, in biofilms under different concentration of 8PP. In SCa biofilms a little increase of SOD activity was detected at 200 µM 8PP, while at 1000 µM the enzymatic activity was lower than at 200 µM of 8PP, and no significant difference was observed with respect to the untreated basal level (*$p < 0.01$) (Fig. 2). SOD activity was not modified in the presence of 8PP at both concentrations in RCa biofilms. The no-activation of the SOD enzyme and the significant decrease of the total antioxidant system could be due to the antioxidant effect of 8PP at high concentrations that keeps anti-oxidized the entire structure of the biofilms, mainly by the effect of non-enzymatic system.
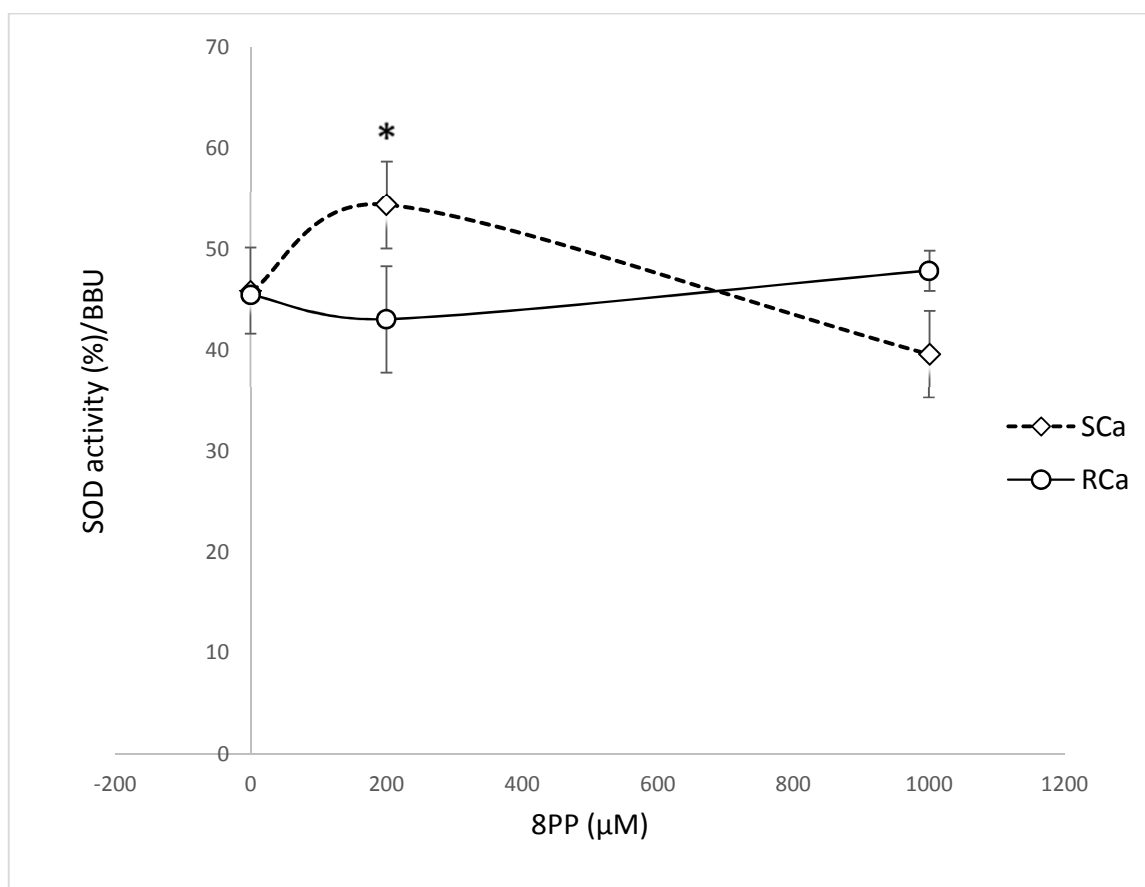
Fig. 2 Enzymatic activity of superoxide dismutase (SOD) (% SOD activation /BBU) in RCa and SCa biofilms under 200 and 1000 µM of 8PP.

Oxidative metabolites in C. albicans biofilms

In order to correlate the oxidant metabolites of biofilms with their antioxidant capacity, $O_2^{\cdot-}$ and RNI were assayed in biofilms of both strains and were expressed as increase of oxidative metabolites with respect to the basal values measured for the untreated biofilms (Fig. 3). At 200 µM a little rise in $O_2^{\cdot-}$ values of both strains was detected (* $p < 0.01$), while at 1000 µM $O_2^{\cdot-}$ were like the untreated samples and no differences were found between strains (Table 1). At high concentrations of 8PP, the

17

system would be highly antioxidized so that there are no differences between strains and levels of metabolites remain low with no intervention of the non-enzymatic system.

The presence of 8PP affects RNI levels showing differences between strains (Fig. 3 and Table 1). In fact, RNI values also were increased at 200 µM, however, this increase was more important in SCa biofilms ([**] $p < 0.001$). At 1000 µM of 8PP RNI levels were twice increased with respect to the untreated samples ([*] $p < 0.01$). The differences observed in total antioxidant defenses at 200 µM could be related to differences in NO levels.
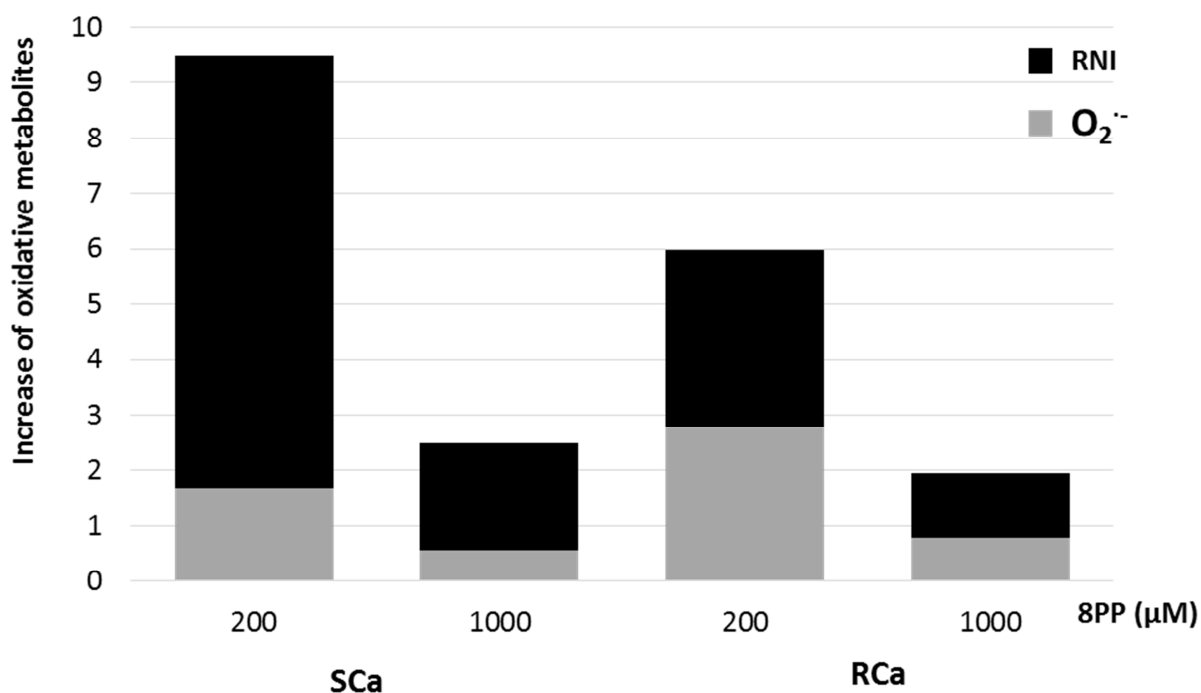
Fig. 3 Increase of superoxide anion radical ($O_2^{\cdot-}$) and reactive nitrogen intermediates (RNI) with respect to control (untreated) in RCa and SCa *C. albicans* biofilms.

8PP (μM)	SCa		RCa	
	$O_2^{\cdot-}$/BBU	RNI/BBU	$O_2^{\cdot-}$/BBU	RNI/BBU
0	0.09 ± 0.01	2.29 ± 0.66	0.06 ± 0.01	2.81 ± 0.71
200	0.14 ± 0.03*	17.86 ± 2.50**	0.16 ± 0.07*	8.95 ± 1.41**
1000	0.05 ± 0.01	4.39 ± 1.50*	0.04 ± 0.01	3.30 ± 0.45*

Table 1. Metabolites of cellular stress ($O_2^{\cdot-}$/BBU and RNI/BBU) in azole-resistant (RCa) and azole-sensitive (SCa) *Candida albicans* biofilms. Standard deviations of the

19

means of three independent experiments. * and ** denote statistical significance at $p < 0.01$ and $p < 0.001$ respectively, when compared to untreated biofilms.

Confocal scanning laser microscopy biofilms image and COMSTAT analysis

Intracellular ROS production was assessed inside sessile cells at 200 µM by CLSM with DCFH-DA which is hydrolyzed by intracellular esterases, resulting in green high-intensity DCF fluorescence (Fig. 4 A-D). Blue channel shows Calcofluor-White in sessile cells walls. Fig. 4D shows NIH-Image J quantitative analysis of DCF (green) fluorescence intensities for SCa and RCa. Increased fluorescence values were quantified in treated biofilms (8PP 200 µM) compared to the untreated ones. In fact, the values obtained were 0.25 ± 0.05 for untreated and 0.18 ± 0.02 for 8PP 200 µM in SCa ($^{*}p < 0.01$). However, this increase was more important in RCa biofilms, with values of 0.70 ± 0.03 for untreated and 0.50 ± 0.16 for 8PP 200 µM (**$p > 0.001$).

A RCa Biofilms

B Rca Biofilms + 8PP 200 µM

C SCa Biofilms

D Sca Biofilms + 8PP 200 µM
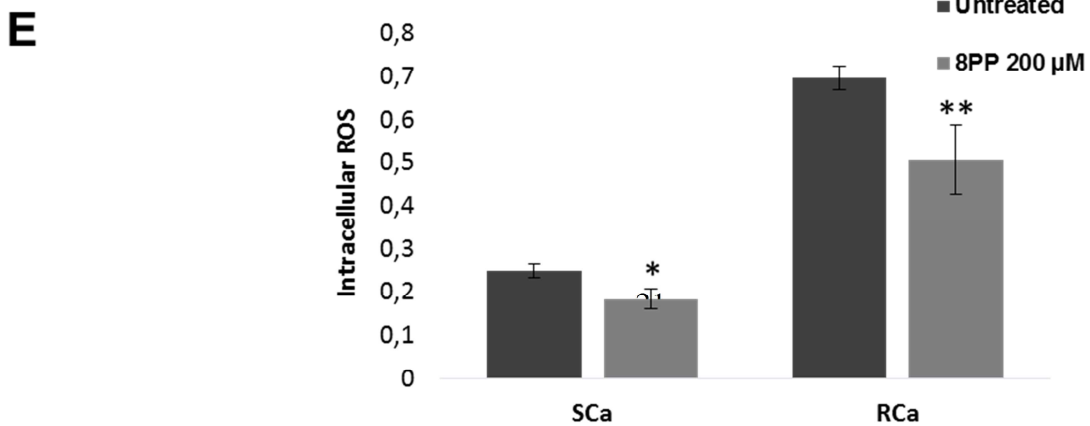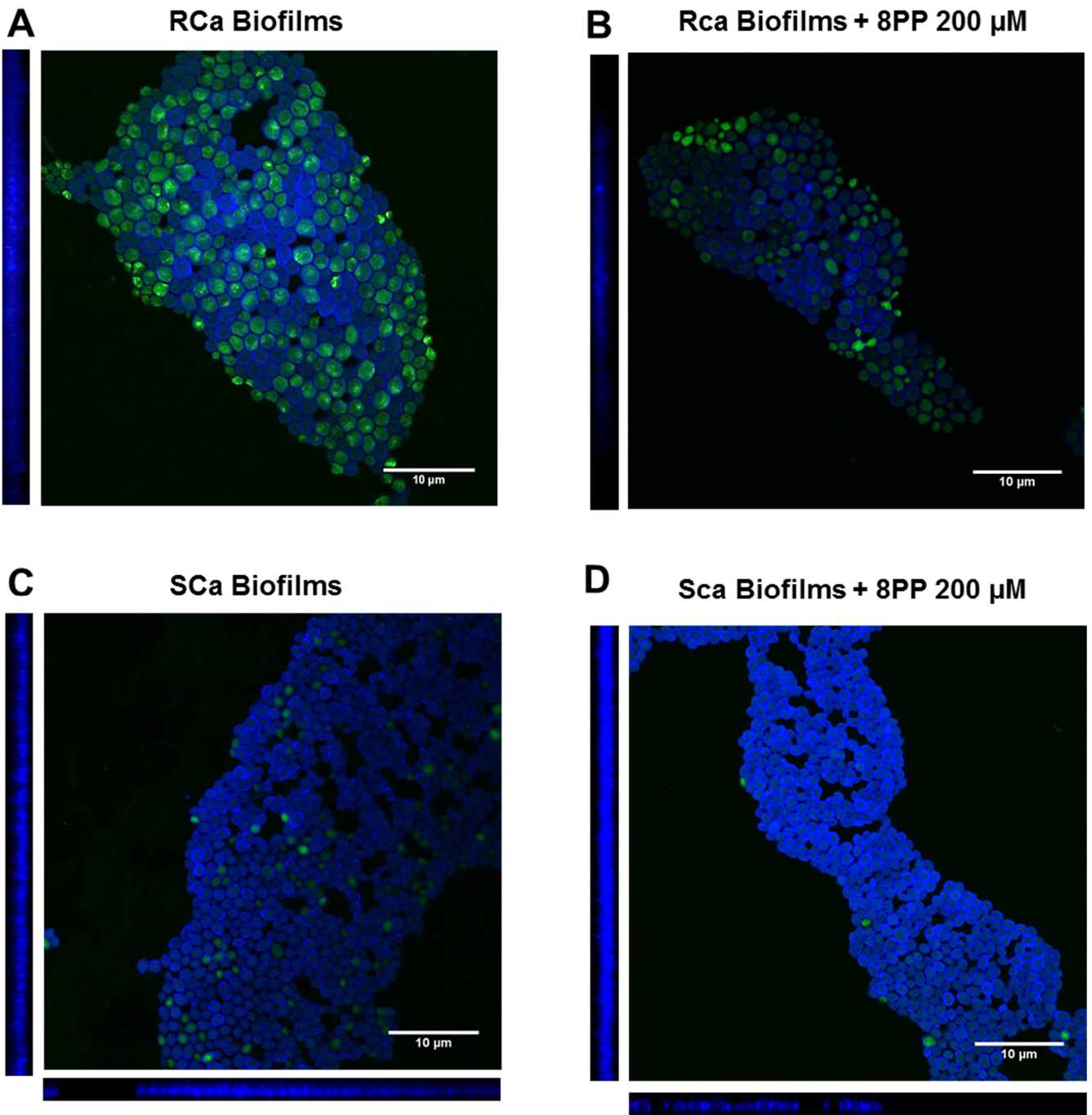
E

■ Untreated
■ 8PP 200 µM

Fig. 4: Confocal scanning laser microscopy. Images of RCa (A and B) and SCa (C and D) biofilms. Untreated biofilm control (A and C). Treatment with 8PP at 200 µM (B and D). Blue channel shows Calcofluor-White in sessile cells walls, and green channel shows oxidation of the dye DCFH as an indicator of reactive oxygen species (ROS) production inside of biofilms. Magnification 60x and scale bar 10 µm. D. Intracellular ROS in sessile cells of SCa and RCa (untreated and 8PP 200 µM) quantified as fluorescence of DCFH probe with NIH-Image J. Error bars represent the standard deviations of the means of three independent experiments. * and **denote statistical significance $p < 0.01$ and $p < 0.001$ respectively, when were compared to untreated biofilms.

Different parameters of biofilm architecture under different treatment conditions were performed with COMSTAT software (Table 2). Correlating with the results obtained for BBU, Biomass ($\mu m^3/\mu m^2$), Mean and Maximum thickness (µm) similar values were observed between 200 µM 8PP concentration and untreated samples. The Area occupied by cells (%) and Surface-to-volume ratio ($\mu m^2/\mu m^3$) founded in treated biofilms were different compared to untreated for RCa but not in SCa. Besides, biofilms of both strains at 200 µM of 8PP exhibited higher diffusion distances (Mean diffusion distance) in their structures when were compared to untreated samples for SCa and RCa. Visualization of the different topographic surface architectures of biofilms obtained by 3D image, showing different voids, channels, and pores that could change the flow inside the matrix of biofilms (Fig 5). The architecture and surface topography showing different diffusion distances could change the flow inside of biofilms in antioxidant effect at high concentrations of 8PP.

22

Table 2. COMSTAT analysis of architectural parameters of azole-resistant (RCa) and azole-sensitive (SCa) *C. albicans* biofilms in presence of 200 µM 8PP.

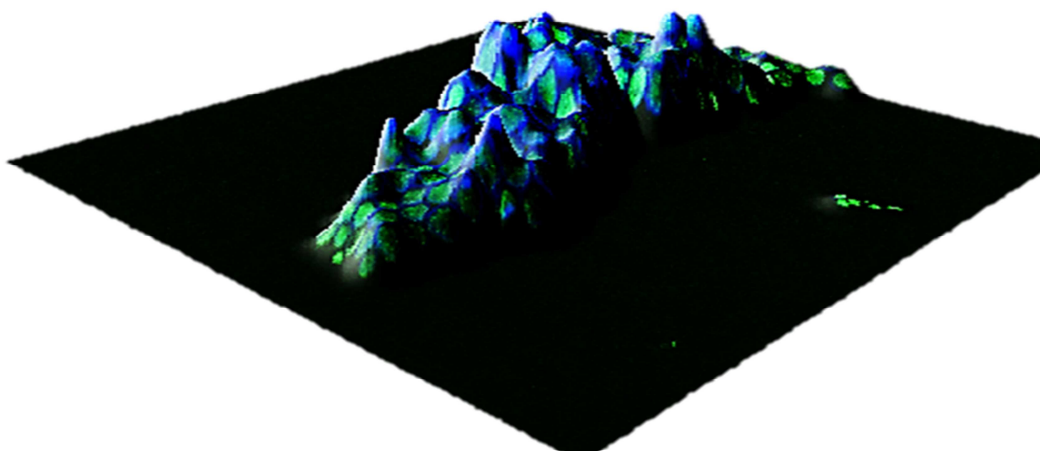|	SCa		RCa	
	Untreated	200 µM	Untreated	200 µM
Biomass ($\mu m^3/\mu m^2$)	2.86	2.39	3.64	3.45
Mean thickness (µm)	2.75	2.25	3.50	3.00
Maximum thickness (µm)	5.50	5.00	7.00	6.00
Area occuped by cells (%)	49.0	49.9	41.2	49.1
Surface-to-volume ratio ($\mu m^2/\mu m^3$)	20.4	20.6	15.8	14.1
Mean diffusi				0.62

A
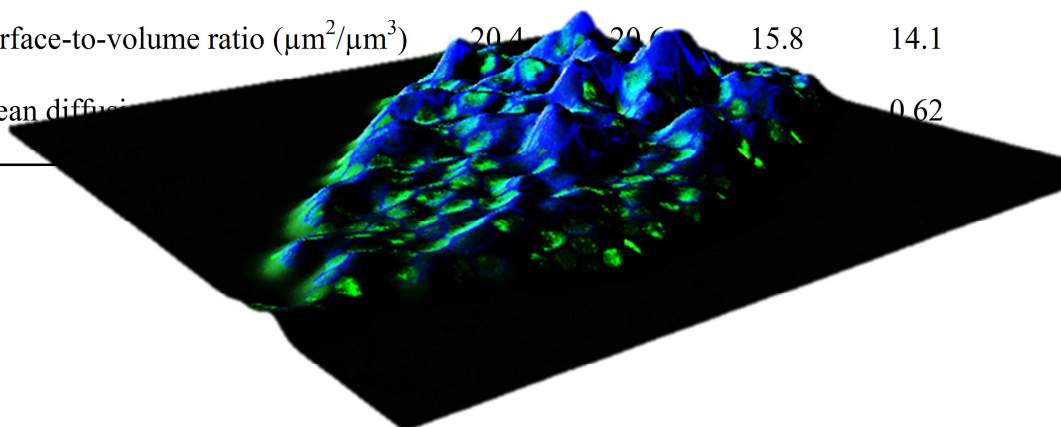
B

Fig. 5 A and B. Confocal scanning laser microscopy 3D image simulation of untreated and treated RCa biofilms, respectively.

4. Discussion

The *in vitro* antioxidant activity of flavonoids depends on the arrangement of functional groups on its core structure and the configuration and total number of -OH a group substantially influences the mechanism of the antioxidant activity (Heim et al., 2002). The prenylated flavonoid 8PP has two -OH groups on B ring, chemical feature to which the antioxidant activity, revealed by our results (Fig. 1), could be attributed. Besides, its structure has a conjunction between the 4-oxo group of the C ring and the -OH group of C5 in ring A (Procházková et al., 2011). The extent to which flavonoids are able to act as anti- or prooxidants *in vivo* is still poorly understood and this topic clearly requires further studies.

Biofilms formed by *C. albicans* are associated with drastically enhanced resistance against most antifungal agents and contributes to the persistence of this fungus (Mukherjee et al., 2004; Nobile and Johnson, 2015). We previously reported that SCa and RCa biofilms were strongly inhibited by 8PP in a dose-depending manner (1.5 to 100 µM), having important inhibitory activity at concentrations ranging from 50 µM to 100 µM. An accumulation of oxidative metabolites (ROS and RNI) that could be inducing an adaptive response with increase in antioxidant defenses was reported (Peralta et al., 2015).

24

Oxidative stress-mediated damage, because of excessive accumulation of ROS/RNI–induced damage, and the effect of ROS-mediated cell loss also depends, in the sessile cells of biofilms, on the ability of to produce, release and/or accumulate ROS into the extracellular matrix. In this work we demonstrated that at high concentrations (1000 μM) 8PP do not inhibit the biofilms of *C. albicans*; quite the opposite, an increase of the formation of the biofilms was detected, in both strains, with lower levels of antioxidants system (Figs. 1 and 2). However, according to our previous findings (Peralta et al., 2015) a pro-oxidant exogenous activity of 8PP was determinate at SMIC80 (100 μM), which indicates that this compound could induce a "redox" activity that depend of its concentrations on the biofilms. The ability of sessile cells to overcome cellular stress depends on its enzymatic and non-enzymatic antioxidant mechanisms. The basal level of FRAP was higher in RCa than SCa; however, similar levels were found in both strains biofilms after high doses treatment with 8PP, indicating that a better activation of antioxidant defenses was achieved in SCa if we compare it with their respective basal levels. Therefore, SCa with a low efflux could be resistant in biofilms forming forms because of a high antioxidant capacity, which imply an important factor of defence against oxidative stress. The highlight relevance of oxidative stress (prooxidant-antioxidant imbalance) in biofilms could be more important than in planktonic cells, because affecting the architectures of biofilm. The efflux pumps modulate drug exportation as the ATP-binding cassette transporter superfamily (including CDR1 and CDR2) and the major facilitator classes (as well as MDR1) are typically upregulated in response to antifungal drugs. However, in biofilms, they become upregulated within the first few hours of surface contact and remain upregulated throughout biofilm development, which clearly contributes to the recalcitrance of biofilms to treatment with antifungal agents (Nobile et al., 2012).

25

The prooxidant activity of 8PP at lower concentrations was previously reported by us (Peralta et al., 2015). The increased of total antioxidant capacity in the presence of 8PP, could be as response to the generation of $O_2^{\cdot-}$ which could induce of an adaptive response based on an increase in antioxidant defenses. On the other hand, at higher concentrations of 8PP there is a greater number of -OH in the B ring that can act on the "redox" cycle of Fe, restoring Fe^{2+} in the system and cooperating in the low formation of ROS, antioxidant effect, mainly by action of non-enzymatic system (Fig. 2 and 3).

Taborga et al. (2017) recently reported geranylphenols having in their structures hydroxyl groups and a geranyl chain attached to an aromatic nucleus, like the configuration of B ring in 8PP structure, which exhibited high percentages of inhibitory activity against standardized and clinical isolates of yeasts belonging to *Candida* and *Cryptococcus* genera. The structure-antifungal activity relationships determined that free -OH are important for their activity (Taborga et al., 2017).

RNI were reported to be bacterial cytotoxic, depending on its concentration and on the redox status of its environment (Arce Miranda et al., 2011; Angel Villegas et al., 2013; Singh et al., 2015). Previously, we reported a dose-dependent increase in NO levels until 100 µM of 8PP caused an oxidative stress status (Peralta et al., 2015). However, at higher concentrations of 8PP, the NO level was low in both strains (Fig. 3 and Table 1). Nevertheless, how flavonoids inhibit induction of NO synthase and increase NO production is not clearly understood yet, but several explanations are argued.

The architecture of sessile cells of biofilms was evaluated using CSLM that provided information about the morphological composition, topographic surface and spatial localization of oxidative stress inside of the cells (Figure 4 and Table 2). Biomass parameter, Mean and Maximum thickness of treated biofilms are similar than the untreated

26

samples. However, these parameters are ignoring pores and voids inside the biofilms, that were clearly distinguished in topographic surface obtained with 3D image simulation reconstruction for RCa biofilms (Fig. 5) and that were considered in Area occupied by cells, Surface-to-volume ratio and diffusion distance values (Table 2). The bio-volume of the biofilms and occupied area reflects how efficiently the yeast colonized the substrate, in addition, the diffusion distance are different after treatment with 8PP. The images obtained by CLSM showed a more compact biofilms for RCa than SCa and showed different values, demonstrating heterogeneous forms in "antioxidant status". The architecture of the biofilms is important for governing liquid and nutrient transport, and the exopolysaccharide matrix determines this microenvironment by affecting density, water content, charge, sorption properties, hydrophobicity, and mechanical stability (Flemming and Wingender, 2010). This allows to evaluate how the diffusion within the biofilms is and to estimate a smaller circulation of nutrients and oxygen, as well as of an antifungal agent which could favor the resistance. This complex topography of biofilms allows at the possible presence of heterogeneities within the biofilms, which could favor persisters cells development (Stewart, 2012).

The interest in possible health benefits of flavonoids has increased owing to their potent antioxidant and free radical scavenging activities observed *in vitro*. Nevertheless, the antioxidant efficacy of flavonoids *in vivo* is less documented and their prooxidant properties have been actually described. Similar to 8PP, ascorbic acid has both antioxidant and prooxidant effects depending upon the dose (Rahal et al., 2014).

Few drugs are available to treat fungal infections and new classes of antimicrobial drug are urgently required and the flavonoids represent a novel set of leads. Clinical management of fungal diseases is being further compromised by the emergence of

27

antifungal drug resistance, which limits the available drug classes that could be used as treatment options. Future optimization of new compounds through doses effective studies may allow the development of a pharmacologically acceptable antimicrobial agent or group of agents (Kalidindi et al., 2015).

5. Conclusions

Cellular redox homeostasis is very important for microbial survival and situations that can cause an imbalance between the oxidative metabolites and the antioxidant defences levels could affect the sessile cells of biofilms. This study validates earlier reports of antioxidant potential of flavonoids and 8PP prooxidant properties seem to be concentration dependent, similar that reported for other flavonoids. We shows for the first time, that 8PP had concentration dependent manner antifungal activity on *C. albicans* biofilms, with dual prooxidant-antioxidant effect. The antioxidant effect, at high concentrations, was mainly by action of non-enzymatic system and altered the architecture and surface topography showing different diffusion distances that could change the flow inside of biofilms. Depending on the level and relation to free radicals and their metabolites and antioxidant level status of system, this prenyl flavonoid may leads to different responses and to be toxic or not, to the sessile cells of biofilms, with different clinical consequences.

Supporting information

(Fig. 1**A**) are available as Supporting Information.

Conflicts of interest.

28

The authors declare no conflict of interest.

References

Angel Villegas, N., Baronetti, J., Albesa, I., Polifroni, R., Parma, A., Etcheverría, A., Becerra, M., Padola, N., Paraje, M.G., 2013. Relevance of biofilms in the pathogenesis of shiga-toxin-producing *Escherichia coli* infection. Scientific World J. 2013, 607258.

Arce Miranda, J.E., Sotomayor, C.E., Albesa, I., Paraje, M.G., 2011. Oxidative and nitrosative stress in *Staphylococcus aureus* biofilm. FEMS Microbiol. Lett. 315, 23-29.

Baronetti, J.L., Angel Villegas, N., Aiassa V., Paraje, M.G., Albesa, I., 2013. Hemolysin from *Escherichia coli* induces oxidative stress in blood. Toxicon 70, 15-20.

Baronetti, J.L., Angel Villegas, N.A., Paraje, M.G., Albesa, I., 2011. Nitric oxide-mediated apoptosis in rat macrophages subjected to Shiga toxin 2 from *Escherichia coli*, Microbiol. Immunol. 55, 231-238.

Beckhauser, T.F., Francis-Oliveira, J., De Pasquale, R., 2016. Reactive Oxygen Species: Physiological and physiopathological effects on synaptic plasticity. J Exp Neurosci, 10, 23-48. DOI: 10.4137/JEN.S39887

Bergamo, V.Z., Balbueno, E.A., Hatwig, C., Pippi, B., Dalla Lana, D.F., Donato, R.K., Schrekker, H.S. and Fuentefria, A.M., 2015. 1-n-Hexadecyl-3-methylimidazolium methanesulfonate and chloride salts with effective activities against *Candida tropicalis* biofilms. Lett. Appl. Microbiol. 61: 504–510. DOI:10.1111/lam.12488.

Cafaratti, M., Ortega, M.G., Scarafia, M.E., Ariza Espinar, L., Juliani, H., 1994. Prenylated flavanones from *Dalea elegans*. Phytochemistry 36, 1083-1084.

Chandra, J., Kuhn, D.M., Mukherjee, P.K., Hoyer, L.L., McCormick, T., Ghannoum, M.A., 2001. Biofilm formation by the fungal pathogen *Candida albicans*: Development, architecture, and drug resistance. J Bacteriol. 2001183: 5385–5394.

Chandra, J., Mukherjee, P.K., Hoyer, L.L., Ghannoum, M.A., 2012. *Candida* biofilms associated with CVC and medical devices. Mycoses 55, 46-57.

Chen, X., Mukwaya, E., Wong, M., Zhang, Y., 2014. A systematic review on biological activities of prenylated flavonoids. Pharm Biol. 52, 655-660.

Clinical and Laboratory Standards Institute (CLSI). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard-Second Edition. CLSI document M27-A2 (ISBN 1-56238-469-4). Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA, 2002.

Dantas, A., Day, A., Ikeh, M., Kos, I., Achan, B., Quinn, J., 2015. Oxidative stress responses in the human fungal pathogen *Candida albicans*. Biomolecules 5, 142-165.

Delattin, N., Cammue, B.P., Thevissen, K., 2014. Reactive oxygen species-inducing antifungal agents and their activity against fungal biofilms. Future Med. Chem. 6, 77-90. DOI: 10.4155/fmc.13.189.

Dickinson, B.C., 2011. Chemistry and biology of reactive oxygen species in signaling or stress responses. Nat. Chem. Biol. 7, 504-511.

Elingold, I., Isollabella, M.P., Casanova M.B., Celentano A.M., Pérez, C., Cabrera, J.L., Diez, R.A., Dubin, M., 2008. Mitochondrial toxicity and antioxidant activity of a prenylated flavonoid isolated from *Dalea elegans* Chem. Biol. Interact. 171, 294-305.

Flemming, H.C, Wingender J. 2010. The biofilm matrix. Nat. Rev. Microbiol. 8, 623-633.

Heim, K. E., Tagliaferro, A. R., Bobilya, D. J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem. 13, 572-584.

Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersbøll, B.K., Molin, S., 2000. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 146, 2395-2407.

Hirota K, Yumoto H, Sapaar B, Matsuo T, Ichikawa T, Miyake Y. Pathogenic factors in *Candida* biofilm-related infectious diseases. J Appl Microbiol. 2016 21. doi: 10.1111/jam.13330.

Kalidindi, N., Nandeep, R., Swetha, S., Kalidindi, B., 2015. Antifungal and antioxidant activities of organic and aqueous extracts of *Annona squamosa* linn. Leaves. J. Food Drug Anal. 23,795-802.

Krumova, K., Cosa, G., 2016. Chapter 1: Overview of Reactive Oxygen Species. In:

Singlet Oxygen: Applications in Biosciences and Nanosciences 1, 1-21

DOI: 10.1039/9781782622208-00001.

Kullberg, B.J., Oude Lashof, A.M., 2002. Epidemiology of opportunistic invasive mycoses.

Eur. J. Med. Res.7, 183-91.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: food

sources and bioavailability. Am. J. Clin. Nutr. 79, 727-747.

Marioni, J., da Silva, M.A., Cabrera, J.L., Montoya, S.C., Paraje, M.G., 2016. The

anthraquinones rubiadin and its 1-methyl ether isolated from *Heterophyllaea*

pustulata reduces *Candida tropicalis* biofilms formation. Phytomedicine 23, 1321-

1328.

Marioni, J., Bresolí-Obach, R., Agut, M., Comini, L.R., Cabrera, J.L., Paraje. M.G., Nonell,

S., Núñez Montoya, S.C., 2017. On the mechanism of *Candida tropicalis* biofilm

reduction by the combined action of naturally-occurring anthraquinones and blue

light. PLoS One 12, e0181517.

Messier C., Epifano F., Genovese S., Grenier D., 2011. Inhibition of *Candida*

albicans biofilm formation and yeast-hyphal transition by 4-

hydroxycordoin. Phytomedicine 18, 380-383.

Mukherjee, P.K., Chandra, J., 2004. *Candida* biofilm resistance. Drug Resist. Updat. 7,

301-309.

Nobile, C.J, Fox, E.P., Nett, J.E., Sorrells, T.R, Mitrovich, Q.M., Hernday, A.D.,Tuch,

B.B., Andes, D.R., Alexander D. Johnson, A.D., 2012. A recently evolved

transcriptional network controls biofilm development in *Candida albicans*. Cell.

148, 126-138.

Nobile, C.J., Johnson, A.D., 2015. *Candida albicans* biofilms and human disease. Ann. Rev. Microbiol. 69, 71-92. DOI:10.1146/annurev-micro-091014-104330.

Ortega, M.G., Scarafia, M.E., Juliani H., 1996. Antimicrobial agents in *Dalea elegans*, Fitoterapia 67, 81.

O'Toole G.A. and Kolter R., Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis, *Mol. Microbiol.* 28, 1998, 449–461.

Peralta, M.A., Calise, M., Fornari, M.C., Ortega, M.G., Diez, R.A., Cabrera, J.L., Pérez C., 2012. A prenylated flavanone from *Dalea elegans* inhibits rhodamine 6 G efflux and reverses fluconazole-resistance in *Candida albicans*. Planta Med. 78, 981-987.

Peralta, M.A., Santi, M.D., Agnese, A.M., Cabrera, J.L., Ortega, M.G., 2014. Flavanoids from *Dalea elegans:* chemical reassignment and determination of kinetics parameters related to their anti-tyrosinase activity. Phytochem. Lett. 10, 260-267.

Peralta, M.A., da Silva, M.A, Ortega, M.G., Cabrera, J.L., Paraje, M.G., 2015. Antifungal activity of a prenylated flavonoid from *Dalea elegans* against *Candida albicans* biofilms. Phytomedicine 22, 975-980.

Peralta, M.A., da Silva, M.A., Ortega, M.G., Cabrera, J.L., Paraje, M.G., 2017. Usnic acid activity on oxidative and nitrosative stress of azole-resistant *Candida albicans* biofilm. Planta Med. 83, 326-333. doi: 10.1055/s-0042-116442.

Pérez C., Tiraboschi I.N., Ortega M.G., Agnese A.M., Cabrera J.L., 2003. Further antimicrobial studies of 2′,4′-dihidroxy-5′-(1‴dimethylallyl)-6-prenyl-pinocembrin from *Dalea elegans*, Pharm. Biol. 41, 71.

Pierce, C.G., Uppuluri, P., Tristan, A.R., Wormley, F.L., Mowat, E., Ramage, G., Lopez-Ribot, J.L., 2008. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing, Nat. Protoc. 3, 1494–1500.

Procházková, D., Boušová, I., Wilhelmová, N., 2011. Antioxidant and prooxidant properties of flavonoids. Fitoterapia 82, 513-523.

Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., Dhama, K., 2014. Oxidative stress, prooxidants, and antioxidants: the interplay. BioMed Res. Int. 761264.

Ramage, G., Bachmann, S., Patterson, T.F., Wickes, B.L., López-Ribot, J.L., 2002. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. J. Antimicrob. Chemother. 49, 973-980.

Ramage, G., Rajendran, R., Sherry, L., Williams, C., 2012. Fungal biofilm resistance. Int. J. Microbiol. 2012, 1-14. Article ID 528521.

Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20, 933-956.

Singh,V., Pal, A., Daroka M.P., 2015. A polyphenolic flavonoid glabridin: Oxidative stress response in multidrug-resistant *Staphylococcus aureus*. Free Radic. Biol. Med. 87, 48-57.

Stewart, P.S., 2012. Mini-review: convection around biofilms. Biofouling. 28, 187-198.

Taborga, L., Sortino, M., Carrasco, H., Butassi, E., Zacchino, S., Espinoza, L., 2017. Antifungal toxicity of linear geranylphenol. Influence of oxygenate substituents. Food Chem. Toxicol. DOI: 10.1016/j.fct.2017.05.027.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44-84.

Vorregaard, M., 2008. Comstat2 - a modern 3D image analysis environment for biofilms, in Informatics and Mathematical Modelling. Technical University of Denmark: Kongens Lyngby, Denmark.

White T., Holleman S., Dy F., Mirels L., Stevens D., 2002. Resistance mechanisms in clinical isolates of *Candida albicans*. Antimicrob. Agents Chemother. 46, 1704–1713.

Yang, X., Jiang, Y., Yang, J., He, J., Sun, J., Chen, F., Zhang, M., Yang, B., 2015. Prenylated flavonoids, promising nutraceuticals with impressive biological activities. Trends Food Sci Technol., 44, 93-104.