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Usnic Acid Activity on Oxidative and Nitrosative Stress of Azole-Resistant *Candida albicans* Biofilm

Authors Mariana Andrea Peralta[1], María Angel da Silva[2], María Gabriela Ortega[1], José Luis Cabrera[1], María Gabriela Paraje[2]

Affiliations
[1] Instituto Multidisciplinario de Biología Vegetal (IMBIV) – Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina
[2] Instituto Multidisciplinario de Biología Vegetal (IMBIV) – Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Cátedra de Microbiología, Facultad de Ciencias Exactas Físicas y Naturales. Universidad Nacional de Córdoba, Argentina

Key words
- *Usnea amblyoclada*
- Usneaceae
- usnic acid
- *Candida albicans*
- biofilms
- prooxidant-antioxidant balance

Abstract

Several studies report that (+)-usnic acid, a lichen secondary metabolite, inhibits growth of different bacteria and fungi; however, the mechanism of its antimicrobial activity remains unknown. In this study, we explored the ability of usnic acid, obtained from *Usnea amblyoclada*, as an antibiofilm agent against azole-resistant and azole-sensitive Candida albicans strains by studying the cellular stress and antioxidant response in biofilms. The biofilm inhibitory concentration of usnic acid (4 µg/mL) exhibited a significant biofilm inhibition, 71.08% for azole-resistant and 87.84% for azole-sensitive C. albicans strains. Confocal scanning laser microscopy showed that the morphology of mature biofilm was altered (reduced the biomass and thickness) in the presence of usnic acid. The antifungal effect was mediated by an oxidative and nitrosative stress, with a significant accumulation of intracellular and extracellular reactive oxygen species detected by confocal scanning laser microscopy and by nitro blue tetrazolium, respectively. In fact, azole-resistant and azole-sensitive C. albicans biofilms treated at the biofilm inhibitory concentration of usnic acid presented 30-fold and 10-fold increased reactive oxygen species measurements compared to basal levels, respectively, and important nitric oxide generation, showing 25-fold and 60-fold increased reactive nitrogen intermediates levels with respect to the controls, respectively. Non-enzymatic and enzymatic antioxidant defenses were increased in both strains compared to biofilm basal levels as response to the increase of oxidant metabolites. The present study shows for the first time that usnic acid can alter the prooxidant-antioxidant balance, which may be the cause of the irreversible cell damage and lead to cell death. Our results suggest that usnic acid could be an alternative for the treatment of *Candida* infections, which deserves further investigation.

Abbreviations

Abs:	absorbance
BBU:	biofilm biomass unit
BIC:	biofilm inhibitory concentration
CFU:	colony-forming units
CLSI:	Clinical and Laboratory Standards Institute
CSLM:	confocal scanning laser microscopy
CV:	crystal violet
DCF:	2′,7′-dichlorofluorescein
DCFH:	2′,7′-dichlorodihydrofluorescein
DCFH-DA:	2′,7′-dichlorodihydrofluorescein diacetate
FLZ:	fluconazole
FRAP:	ferrous reduction antioxidant potency assay
MIC:	minimal inhibitory concentrations
NBT:	nitro blue tetrazolium
NO:	nitric oxide
OD:	optical density
RCa:	*Candida albicans* strain azole-resistant
RNI:	reactive nitrogen intermediates
ROS:	reactive oxygen species
SCa:	*Candida albicans* strain azole-sensitive
SOD:	enzyme superoxide dismutase
UA:	(+)-usnic acid

Supporting information available online at http://www.thieme-connect.de/products

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Correspondence
Prof. Dr. María Gabriela Paraje
Cátedra de Microbiología
Facultad de Ciencias Exactas Físicas y Naturales
Universidad Nacional de Córdoba
Av. Vélez Sarsfield 299
Córdoba 5000
Argentina
Phone: + 54 3 51-5 35 38 00 int. 2 97 31
Fax: + 54 3 51-4 33 41 98
gabrielaparaje@gmail.com

Introduction

The small number of drugs available for fungal treatment and the continued emergence of infections caused by antifungal resistant *Candida* stains encourage the search for new chemotherapeutics agents. In this sense, native flora is an important source of new bioactive chemical structures. It has been reported that natural products such as plant extracts [1] can influence the formation of microbial biofilms. Other studies informed that flavonoids such as 4-hydroxycordoin [2] and baicalein [3] are potent inhibitors of biofilm formation. However, few studies have been carried out related to the inhibition of established biofilms by natural compounds [4].

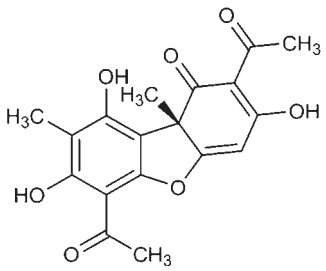
The *Usnea* genus (Usneaceae) is represented in Argentina by species known by the common name "barba de piedra" ("stone grass"), among which may be mentioned *U. amblyoclada* (Müll. Arg.) Zahlbr, *U. densirostra* Taylor, and *U. hieronymii* Kremp [5]. The genus is widely used in traditional medicine for its expectorant and antimicrobial activities [6]. UA [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzofurandione; **Fig. 1 A**] is a well-known metabolite widely distributed in lichen, mainly in *Usnea* genus. UA has been reported to possess antimicrobial, antifungal, antiprotozoal, and antiviral activities [7]. Several studies have confirmed that UA inhibits growth of different planctonic bacteria and fungi strains, including clinical isolates [8]. However, the antibiofilm activity of UA against various pathogens has been less investigated [9–11].

Biofilm-associated *Candida albicans* infections are clinically relevant due to their high levels of resistance to traditional antifungal agents. The over expression of certain drug transporter genes is one of the major sighted reasons for the multidrug-resistance, which results in drug extrusion mediated by active efflux of the antifungal outside the cell [12].

ROS formation has been suggested to be one of the antimicrobial mechanisms of action for some antimycotics such as miconazole, amphotericin B, and novel antifungal compounds [13–15]. ROS, RNI and their downstream derivatives are potentially dangerous. Certain ROS such as superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), which are present at low non-toxic concentrations, are formed either in the environment or as a by-product of aerobic metabolism, superoxide formation or dismutation, or as a product of oxidase activity. The incomplete reduction of O_2 during respiration produces $O_2^{\cdot-}$, which is enzymatically dismutated by SOD to H_2O_2 and can be eliminated by the activity of catalase. Both excessive H_2O_2 and its decomposition product hydroxyl radical ($OH^{\cdot}$), formed in a Fenton-type reaction, are harmful for most cell components. Therefore, for all aerobically living prokaryotic and eukaryotic cells, their rapid removal is essential. Oxidative stress is caused by an imbalance between the production of oxidants and the levels of antioxidant defenses.

The aim of this work was to evaluate the effects of UA, obtained from *U. amblyoclada*, on established biofilms of RCa and SCa by studying if there is a disturbance of the prooxidant-antioxidant balance. Therefore, the production of oxidative and nitrosative metabolites, the activation of the total antioxidant capacity of the system, and particularly the enzyme SOD were evaluated. The oxidative stress was the predominant effect, altering the prooxidant-antioxidant balance, which may be the cause of the irreversible cell injury in the biofilm. Our results showed that UA could be an alternative for the treatment of infections with resistant *Candida* biofilms, which deserves further investigation. To our knowledge, this is the first study that attempts to correlate biofilm reduction with alteration in ROS and RNI production by the action of UA.

A



B

BBU (% of biofilm inhibition)	Biofilm	UA (4 µg/mL)	FLZ (2 µg/mL)
SCa	1.48±0.07	0.18±0.04*** (87.84%)	0.24±0.02 *** (83.78%)
RCa	1.30±0.17	0.38±0.09** (71.08%)	0.33±0.04** (74.29%)

Fig. 1 **A** Chemical structure of (+)-usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzofurandione) isolated from *Usnea amblyoclada*. **B** Quantification of UA and FLZ effect over RCa and SCa biofilms expressed as BBU and % of biofilm inhibition. *Denote statistical significance at ***$p < 0.001$ and **$p < 0.01$ were considered significant for comparisons with control (non-treated biofilms).

Results

UA at different concentrations (2 to 128 µg/mL) was added on preformed biofilms. UA (4 µg/mL) was determined as the BIC because it was the lowest concentration that showed the maximum inhibition of biofilms in both strains: 87.84% for SCa (***$p > 0.001$) and 71.08% for and RCa (**$p > 0.01$). **Fig. 1 B** shows the BBU and the percentages of biofilm inhibition in the presence of UA or FLZ measured by CV assay. At higher concentrations the same inhibition was obtained. Similar inhibition values were obtained with 2 µg/mL of FLZ (BIC of FLZ). Viable cells were determined by enumerating colony-forming units per milliliter (CFU/mL) and the results showed a good correlation with the CV assay (data not shown).

In order to determine if cellular stress is implied on the mechanism of action of UA, ROS production was measured by NBT assay, allowing us to detect and quantify extracellular ROS levels in *C. albicans* biofilms. **Fig. 2 A** shows the production of ROS expressed as the ratio between this stress metabolite and BBU. In biofilms treated at BIC of UA (4 µg/mL), the ROS measurements were 1.40 and 0.43 showing a 30-fold and 10-fold increase compared to basal levels of RCa (***$p > 0.001$) and SCa (**$p > 0.01$), respectively. RCa biofilms treated at the BIC of FLZ (2 µg/mL) presented an augmented level of ROS compared with the basal level (**$p > 0.01$). Nevertheless, this increase was considerably smaller than that observed in presence of UA.

RNI was evaluated as nitrite using the Griess reagent in order to investigate NO production in the biofilms. **Fig. 2 B** shows that the basal levels of nitrosative stress of both strains were not sig-

nificantly modified when biofilms were treated with FLZ. However, SCa and RCa biofilms treated with UA at 4 µg/mL showed a notable NO generation (143.54 and 79.18 RNI/BBU, respectively), which marks a 60-fold and 25-fold increase compared with controls (2.29 and 2.81 RNI/BBU for SCa and RCa biofilms, respectively; $^{***}p > 0.001$).

Nonenzymatic and enzymatic defenses are protective antioxidant mechanisms that detoxify cellular stress. FRAP assay was performed with the aim to determine the total antioxidant capacity of biofilms, including both enzymatic and nonenzymatic antioxidant components. **Fig. 3A** shows that FRAP increased significantly in presence of UA and FLZ ($^{**}p > 0.01$). In SCa biofilms treated either with UA or FLZ this rise was greater than in RCa ($^{\#\#}p > 0.01$). In fact, UA treatment in SCa biofilm provoked a 10-fold increase of FRAP level compared to basal biofilms, while twice increased levels were observed for RCa biofilms (256.30 and 826.39 FRAP/BBU basal levels for SCa and RCa, respectively). The antioxidant capacity basal level was higher in RCa than SCa ($^{\#\#}p > 0.01$).

The main enzyme involved in ROS detoxification is SOD. The enzymatic activity of SOD was studied in order to correlate the enzymatic antioxidant activity with ROS production in biofilms under treatment with UA and FLZ. Increased SOD activity was observed for biofilms treated with both compounds compared to basal biofilms ($^{***}p > 0.001$). Moreover, this increase was greater in SCa than in RCa, with similar biofilms' basal levels (**Fig. 3B**) ($^{\#\#\#}p > 0.001$).

From the antifungal activity assay, the BIC of UA and FLZ were estimated (2 and 4 µg/mL, respectively).

CSLM confirmed the antibiofilm activity of UA (4 µg/ml) which was incubated at 37 °C for 48 h on pre-formed RCa biofilms. **Fig. 4** shows the CSLM images demonstrating the reduction in the thickness of pre-formed biofilms treated at BIC of UA (**Fig. 4B, D**) compared to the control (**Fig. 4A, C**). Similar values were obtained at the BIC of FLZ, which also reduced the biofilms (data not shown).

COMSTAT analysis (**Table 1**) shows that the biofilm biomass and thickness were reduced upon treatment with UA on comparison to the control. In the presence of UA (**Fig. 4B, D**) the biofilm's biomass was reduced to 4.91 $\mu m^3/\mu m^2$ for RCa, compared to the control (18.39 $\mu m^3/\mu m^2$) corresponding to an inhibition of 73.3%. The thickness distribution was reduced (76.8%) in the presence of UA.

Intracellular ROS production was observed inside sessile cells by CLSM with DCFH-DA which is hydrolyzed by intracellular esterases, resulting in high-intensity DCF fluorescence. **Fig. 5A** shows NIH-Image J quantitative analysis of DCF (green) fluorescence intensities. The values obtained were 0.78 ± 0.06 for biofilm treated with UA and 0.46 ± 0.05 for the control ($^{***}p > 0.001$). FLZ ($^{*}p > 0.05$) showed less intracellular stress and the results showed a correlation with the extracellular stress metabolite. CLSM images show the co-localization which is observed as turquoise color, merged of two different additive colors (blue and green). The biofilm treated with UA (**Fig. 5B, D**) shows the increased intracellular ROS compared to untreated (**Fig. 5C**).

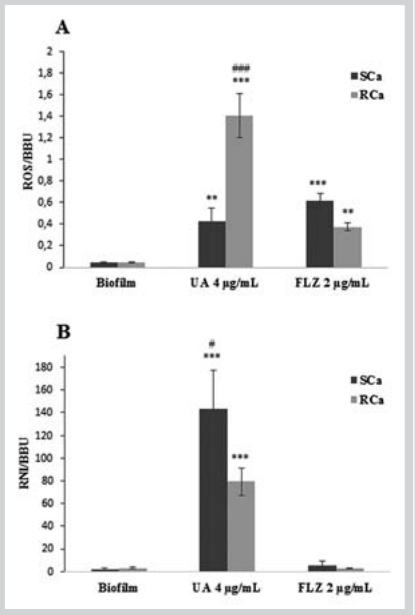


Fig. 2 Extracellular ROS and RNI generated by UA in *Candida albicans* biofilms. **A** ROS/BBU ratio determined by NBT assay. **B** RNI/BBU ratio determined by Griess method. Error bars represent the standard deviation (SD) of triplicates of three independent experiments. *Denote statistical significance at $^{**}p < 0.01$ and $^{***}p < 0.01$ were considered significant for comparisons with control (non-treated biofilms); and $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ for comparisons between RCa and SCa.

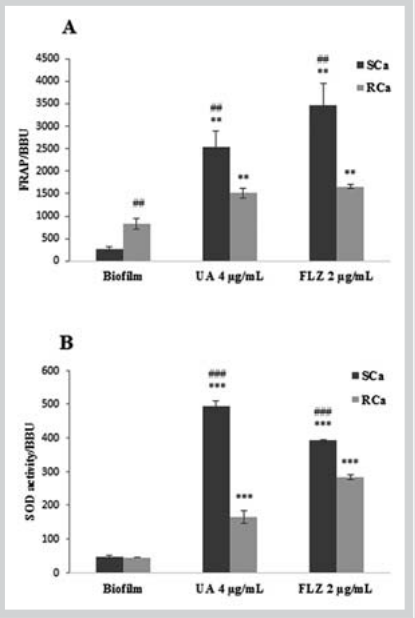


Fig. 3 Antioxidant system activated by UA in RCa and SCa biofilms. **A** FRAP/BBU and **B** Enzyme SOD activation (%)/BBU. Error bars represent the standard deviation (SD) of triplicates of three independent experiments. $^{**}P < 0.01$ and $^{***}p < 0.01$ were considered significant for comparisons with control (non-treated biofilms); $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ for comparisons between SCa and RCa.

Discussion

UA has been reported effective against biofilm formation of *Staphylococcus aureus* mainly by killing the attached cells [9]. The antibacterial activity against methicillin-resistant *S. aureus* (MRSA) is exerted by disruption of the cell membrane [16]; in *Pseudomonas aeruginosa*, possibly by interference with signaling pathways [9]. Its activity against *Candida* spp. was also recently reported [8]. Nithyanand et al. [10] described that UA at 100 µg/mL exhibited the maximum inhibition of biofilm (65%) and reduced the viability of the metabolically active cells in matured *C. albicans* biofilm. Also it prevented the property of adhesion and inhibited the yeast to hyphal switch [10]. According to these results we investigated another mechanism implicated on antibiofilm activity of this compound. We evaluated UA obtained from *U. amblyoclada* against both SCa and RCa biofilms and obtained that UA at 4 µg/mL reduced about 80% biofilms of *C. albicans* strains, even in RCa. As expected for a sensitive strain, the more significant inhibition of biofilms was observed in SCa. Also we investigated the implication of oxidative stress in this inhibition. Our results showed an increase of ROS (intra- and extracel-

lular) and antioxidant system, in response to oxidative stress in biofilms treated with UA. Similarly, RNI were stimulated in the presence of this compound. There is increasing evidence that the mechanism of cell death initiated by some antifungal involves the production of ROS [13,15], having been reported that miconazole induces the accumulation of endogenous ROS in *C. albicans* biofilms [17]. As well as Nithyanand et al. [10] we also observed by CLSM and further COMSTAT analysis that biofilm thickness was reduced upon treatment with UA. Based on these results, we deepened some mechanisms of action aspects of this compound. The increase of ROS and RNI production in the presence of UA suggests that it induces cellular stress produced inside biofilms. The ROS and RNI could then accumulate in the extracellular medium and thus affect the exopolisaccharide matrix [15, 18].

In the same way, it has been reported that FLZ treatment induces ROS generation and causes oxidative damage in DNA of *C. glabrata* [19]. However, we observed less intra- and extracellular stress metabolites than UA, maybe this is not the principal mechanism of action of FLZ. On the other hand, DNA damage caused by ROS can result in chemical base changes, structural alterations, single- and double-strand breaks and cross-linkage, allowing to alter mRNA [20].

Recently, it has been suggested that the primary effects of UA on *B. subtilis* and *S. aureus* are inhibition of RNA and DNA synthesis [21]. We hypothesized that a possible inhibition of RNA and DNA could be mediated by the increased levels of ROS and accumulation in the extracellular matrix induced by UA in *C. albicans* biofilms.

The ATP binding cassette transporters (CDR1 and CDR2) and MDR1 are two types of efflux pump genes involving in drug resistance of *Candida* by increased mRNA levels [22,23]. Despite mRNA inhibition mechanism has not been demonstrated for antifungal effects of UA in *C. albicans*, we suggest that a possible inhibition of mRNA exerted by UA could be involved in the strong inhibition observed against RCa which presents an overexpression of MDR genes.

Although both strains showed similar basal levels of oxidative and nitrosative stress, SCa biofilms presented a considerable activation of stress in the presence of UA, exerted principally by RNI. Páez et al. [24] informed that resistant strains show a higher FRAP level than sensitive ones while catalase and SOD do not show any differences between sensitive and resistant strains [24]. We observed that upon treatment with UA or FLZ, a better activation of antioxidant defenses was achieved in SCa. We hypothesized that strains with a low efflux can be resistant as a consequence of a high antioxidant capacity as a factor of defence against the increase in RNI and ROS provoked by UA or FLZ, because SOD was higher stimulated. SOD plays an important role in protecting *C. albicans* biofilms against high doses of miconazole through a fungal biofilm resistance mechanism [22]. As far as we know this is the first report about biofilms inhibition and its relationship with oxidative and nitrosative stress in the presence of UA.

As UA reduced the thickness and the biomass of biofilms, another possible mechanism that can be attributed is that UA acts on the polysaccharides by ROS and RNI accumulation. This observation is also supported by the findings reported by Nithyanand et al. [10] about the action of UA on the polysaccharides present in the exopolisaccharide layer. The oxidative metabolites may be responsible for the inhibition of biofilms by this compound.

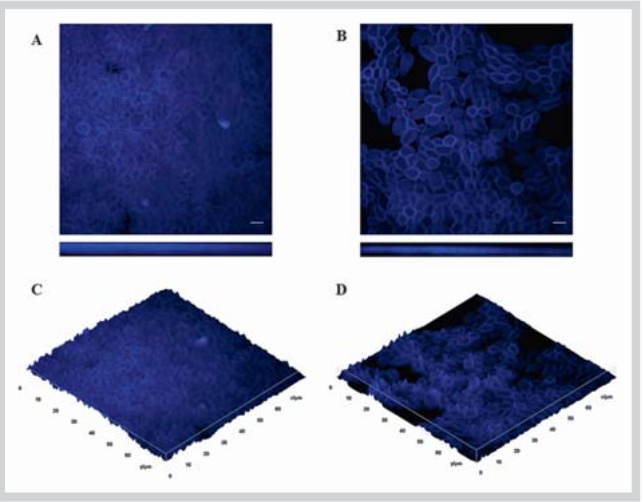


Fig. 4 CSLM images showing the antibiofilm activity of UA against matured RCa biofilms. XY (top) and XZ (bottom) of biofilms. Blue channel shows Calcofluor-White in sessile cells walls (**A**–**D**). **A** Control showing thickness biofilm formation. **B** RCa biofilm treated at BIC of UA. Magnification 60 × and scale bar is 5 µm. **C** and **D** Three-dimensional CLSM images of pre-formed biofilms control (**C**) and treated at BIC of UA (**D**). (Color figure available online only.)

Table 1 COMSTAT analysis of the effect of UA and FLZ on azole-resistant *C. albicans* (RCa) biofilms.

	Biomass ($\mu m^3/\mu m^2$)	Thickness distribution (µm)
Biofilm	18.39	20.91
UA (4 µg/mL)	4.91	4.85
FLZ (2 µg/mL)	4.25	4.17

The usage of *U. amblyoclada* in folk medicine and the extensive investigation of UA on antimicrobial effects encouraged this investigation that attempts to evaluate the potential use of this lichen as an antifungal source against *C. albicans* biofilms. The results enhance the importance of UA as an antifungal obtained from natural sources that could be used for *C. albicans* biofilms treatment and provides more evidence about the mechanisms of action of this compound.

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. 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*.

In our investigation, we observed that biofilm treated with an antifungal was influenced by the production of oxidant metabolites and levels of the antioxidant defenses. We observed ROS and RNI accumulation in sessile *Candida* cells treated with UA, inducing an adaptive response that resulted in an increase of antioxidant defenses. The oxidative stress could be involved in the antifungal mechanism of action of UA on *Candida albicans* biofilms. The *in vitro* studies support further efforts to determine whether UA can be used clinically to cure patients with *Candida* infections.

Materials and Methods

Chemicals and biochemicals

Fluconazole (purity ≥ 98%), Calcofluor-White, DCFH-DA, NBT, CV, $NaNO_2$, methionine, riboflavin, N-1-naphthyl ethylenediamide dihidrochloride, and 2,4,6-tripyridyl-s-triazine were purchased from Sigma-Aldrich. Fetal bovine serum was from Greiner Bio-One. Sabouraud dextrose agar broth was from Difco. Silica gel GF_{254} and dimethyl sulfoxide were from Merck. Sulfanilamide, HCl, benzene, $CHCl_3$, *n*-hexane and formic acid, ethanol, acetone and glycerol (Anedra). $FeSO_4$ and $FeCl_3{\cdot}6H_2O$ (Cicarelli).

Plant material

Usnea amblyoclada was collected in February 2014 in its natural habitat in Pampa de Achala (San Alberto Department, Province of Córdoba, Argentina, GPS coordinates: latitude: 31°40′57.4″ south; longitude: 64°50′13.9″ west; height: 2100 m). Plant material was identified by Dr. Juan Manuel Rodríguez (Universidad Nacional de Córdoba, Córdoba, Argentina). A representative voucher specimen has been deposited as Peralta & Cabrera 5 (CORDC 00005231) in the herbarium at the Botanical Museum (IMBIV, Universidad Nacional de Córdoba).

Extraction and isolation

Briefly, the air-dried parts (100 g) of *U. amblyoclada* were extracted with 200 mL of benzene using a Soxhlet apparatus to extract UA. The crude extract was filtered, reduced to the half of its original volume in a rotary evaporator, and stored at 4 °C for 24 h to precipitate UA. The UA precipitates were collected and subjected to preparative TLC carried out using 20 × 20 cm plates with 0.5 mm layer of silica gel GF_{254} (Merck) and eluted with $CHCl_3$:*n*-hexane (80 : 20). Spots were visualized by UV illumination (254 nm).

At the end of this process, 1150 mg of UA (95% purity) was obtained. Its chemical structure was determined by UV, ^{1}HNMR and ^{13}CNMR methods and confirmed by comparing with spectral data previously reported by Rashid et al. [25] (see Supporting Information).

Fungal strains and culture conditions

RCa and SCa strains were used. RCa strain (12–99) overexpresses the transporter genes CDR1, CDR2 and MDR1, whereas SCa strain (2–76) has a basal expression of these genes [15, 23]. For long-term storage, both strains were kept with 15% glycerol (v/v) at − 80 °C in a freezer. Prior to all the assays, *C. albicans* was grown onto Sabouraud dextrose agar to purity and viability control [26].

Formation and quantification of biofilms

The effect of UA on mature *C. albicans* biofilms was performed according to Peralta et al. [15]. Briefly, mature biofilms were prepared by adding 100 µL of a 1×10^7 cells/mL suspension in Sabouraud dextrose agar in flat-bottomed 96-well microplates (Greiner Bio-One) pre-treated with fetal bovine serum (50%) at 37 °C for 30 min and washed with 10 mM phosphate-buffered saline (pH 7.2) [15, 27]. Following cell adhesion (90 min), each well was washed with sterile phosphate-buffered saline and 200 µL of fresh broth was added. Negative control wells with the same pretreatment containing Sabouraud dextrose agar (pH = 7.2) were prepared. Then microplates were incubated at 37 °C for 48 h until formation of biofilms (BBU = 1.33 and 1.55 for SCa and RCa, respectively) [15].

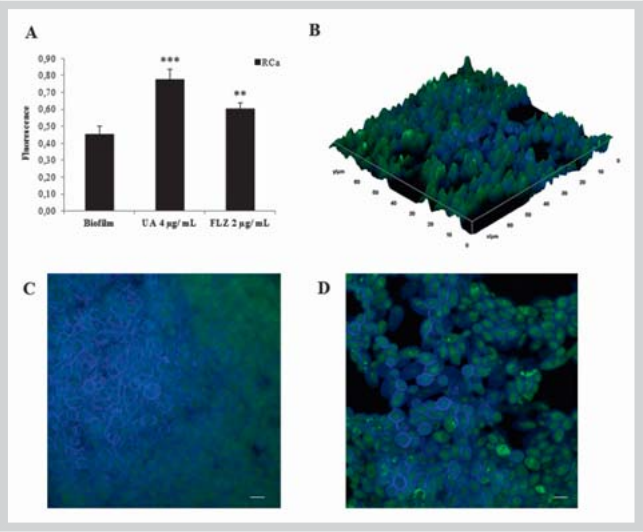


Fig. 5 **A** NIH-Image J quantitative analysis of DCF (green) fluorescence intensities as an indicator of intracellular ROS inside RCa biofilms. Values are expressed as the ratio of DCF (green) and Calcofluor-White (blue) intensities for control and treated biofilms with UA or FLZ. Error bars represent the standard deviation (SD) of triplicates of three independent experiments. * denote statistical significance at ***p < 0.001 and **p < 0.01 were considered significant for comparisons with control (non-treated biofilms). **B–D** Confocal scanning laser microscopy (CSLM) images showing two-color (blue and green) merged images. Magnification 60 × and scale bar is 5 µm. **C** Control biofilms ROS. **B** and **D** Three and two dimensional images showing increased intracellular ROS of RCa sessile cells treated with UA. (Color figure available online only.)

Different concentrations of UA (2 to 128 µg/mL) dissolved in dimethyl sulfoxide (final assay concentration: 1% v/v) or FLZ (0.25 to 2 µg/mL) [28] were added to each well containing mature biofilm. Dimethyl sulfoxide alone (1% v/v) was added as a control. After incubation at 37 °C for 48 h, the supernatant was separated for extracellular oxidative stress assays [15]. Then, plates were gently rinsed with distilled water and air dried for 24 h, prior to staining *C. albicans* biofilms with 200 µL per well with 1% CV solution (w/v) for 5 min. Subsequently, the dye was discarded and wells were rinsed twice with deionized water. Plates were allowed to dry before solubilizing CV with 200 µL of an ethanol/glacial acetone solution (70 : 30). Then, Abs was measured spectrophotometrically at 595 nm using a Multi Label Reader (Tecan Sunrise Model, TECAN). BBU was arbitrarily defined as 0.1 Abs595 equal to 1 BBU [15, 18].

The percentage of biofilm inhibition was calculated using the following formula [10]: Percentage of inhibition = [(control Abs595 nm – test Abs595 nm)/control Abs595 nm] × 100

The BIC of each compound was determined as the concentration which achieved the maximum biofilm inhibition [10].

C. albicans biofilms treated with UA were observed by CSLM as described below. Prior to imaging, biofilms were formed on small glass covers (12 mm in diameter) in a 24-well microtiter plate (Greiner Bio-One).

Following biofilm formation and antifungal exposure, supernatants were eliminated and disks were rinsed with sterile phosphate-buffered saline (no autofluorescence detected). Firstly, disks were stained for 15 min with 30 µL of Calcofluor-White (0.05% v/v), a carbohydrate-binding fluorescent dye that stains

fungal cell walls blue. Calcofluor-White was excited at 355 nm [15,29].

Biofilms were examined by using a Fluoview FV1000 Espectral Olympus CSLM (Olympus) equipped with UPlanSApo 60X/1.40 oil UIS2 oil immersion lens (Olympus). Optical sections were acquired at 1.2 µm intervals for the total thickness of biofilms. Then, for each sample, images from five randomly selected positions were obtained and analyzed independently.

The reduction in total biomass and maximum thickness of *C. albicans* biofilm was assessed using COMSTAT software [10,30].

Antifungal activity testing

The MIC for planktonic cells was determined following the guidelines of the M27-A3 document of the CLSI standard method and defined as the lowest drug concentration able to produce a growth inhibition higher than 90% [31]. The concentration of UA or FLZ that achieved maximum inhibition of the biofilm (BIC) [10] was determined on formed biofilms as described below [15,27]. Briefly, 100 µL per well of antifungal solutions were added in order to obtain final concentrations for FLZ (0.25 to 2 µg/mL) and AU (2 to 128 µg/mL). In addition, control wells without antifungal solution were used. Dimethyl sulfoxide alone (1% v/v) was added as a control which did not show any antifungal activity (data not shown). After inoculation with 100 µL of a 1×10^7 cells/mL suspension in Sabouraud dextrose agar, the microtiter plate was incubated at 37 °C for 48 h, and OD was measured at 595 nm.

After antifungal treatment, the supernatant was eliminated and 100 µL of sterile water was added to each well and the plate was sonicated (40 kHz, 60 s) in order to re-suspend the biofilm cells thoroughly [15,18]. Then, the suspension was diluted 1000 times with sterile water and 100 µL of the suspension was then pipetted out and spread evenly on Sabouraud dextrose agar by using a sterile plastic transferring loop, before being incubated at 37 °C for 24 h. Viable cells were determined by enumeration of CFU/mL for correlation studies with BBU [15].

ROS (intra and extracellular) production of biofilms

Extracellular ROS production was detected in the supernatant (0.1 mL) by the reduction of NBT 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 $OD_{540\,nm}$/BBU [18,29]. Intracellular ROS production was determined by CSLM using a probe of the non-fluorescent and cell-permeating compound (DCFH-DA). DCFH-DA (non-polar, non-fluorescent compound) diffuses across membranes, and is hydrolyzed by intracellular esterases to 2′,7′-dichlorodihydrofluorescein (DCFH) (polar, non-fluorescent, membrane-impermeable compound), which is rapidly oxidized by ROS to the highly fluorescent DCF which is trapped within the cell [32]. The quantitative analysis of DCF (green) fluorescence intensities was analyzed by means of the NIH-Image J.

RNI production of biofilms

RNI production was determined by a micro-plate assay using Griess reagent and $NaNO_2$ as standard. Supernatant (100 µL) was mixed with 200 µL of Griess reagent [sulfanilamide 1.5% in 1 N HCL and N-1-naphthyl ethylenediamide dihidrochloride 0.13% in sterile distilled water]. Absorbance was measured at 540 nm and the results were expressed as nitrite concentration value/BBU (RNI/BBU) [15,29].

Nonenzymatic and enzymatic antioxidant activity

The total antioxidant capacity was evaluated by a 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 $FeCl_3 \cdot 6H_2O$. Then, OD was measured at 595 nm after 4 min of incubation. Results were expressed as FRAP/BBU, using a $FeSO_4$ calibration curve [15].

The specific enzymatic activity (SOD) was assayed by inhibition of NBT. 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. Results were expressed as SOD activity/BBU [15].

Statistical analysis

All assays were performed in triplicate and in three independent experiments, and the averages and standard deviations were calculated for all of them. Data are presented as means ± standard deviation (SD). Data were analyzed by using ANOVA followed by the Student-Newman-Keuls test for multiple comparisons, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ were considered significant for comparisons with control biofilms (non-treated) and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ for comparisons between SCa and RCa.

Supporting information

UV, ^{1}HNMR and ^{13}CNMR spectral data and HMBC correlations for UA are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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