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Antifungal effect of a metabolite of *Pseudomonas aeruginosa* LV strain on azole-resistant *Candida albicans*

Efeito antifúngico de um metabólito de *Pseudomonas aeruginosa* cepa LV sobre *Candida albicans* resistente a azóis

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ABSTRACT

Candida albicans remains the most common agent of candidiasis worldwide. This yeast is generally sensitive to most antifungals, however, the emergence of azole-resistant *C. albicans* has been reported. In addition, this microorganism can form biofilms on various surfaces, making it difficult to treat infections. In this study, the effect of secondary metabolites of *Pseudomonas aeruginosa* strain LV on planktonic and sessile cells of *C. albicans*, with different genotypes and susceptibility profile to fluconazole and voriconazole, was evaluated. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the semi-purified fraction F4a ranged from 1.56 to 6.25 μg/mL and 6.25 to 25 μg/mL, respectively. Fluopsin C appears to be the antifungal component of F4a. The semi-purified fraction and fluopsin C showed fungicidal activity, dose and time dependent. F4a caused severe damage to the morphology and ultrastructure of planktonic fungal cells, and significantly reduced the viability of 24-hour biofilms, with MIC for sessile cells from 12.5 to 25.0 μg/mL. However, cytotoxicity was detected in mammalian cells for F4a and fluopsin C at concentrations that showed antifungal activity. These results indicate that fluopsin C may be a prototype for the development of new antifungals for *C. albicans*.

Keywords: Antibiofilm. Antimicrobial activity. Fluopsin C. Fungicide.

RESUMO

Candida albicans permanece como agente mais comum de candidíase em todo o mundo. Essa levedura é geralmente sensível à maioria dos antifúngicos, entretanto o surgimento de *C. albicans* resistentes aos azóis tem sido relatado. Além disso, esse microrganismo pode formar biofilmes em diversas superfícies, dificultando o tratamento das infecções. Neste estudo, avaliou-se o efeito de metabólitos secundários de *Pseudomonas aeruginosa* cepa LV em células planctônicas e sésseis de *C. albicans*, com diferentes genótipos e perfil de sensibilidade ao fluconazol e ao voriconazol. A concentração inibitória mínima (CIM) e concentração fungicida mínima (CFM) da fração semipurificada F4a variaram de 1,56 a 6,25 μg/mL e 6,25 a 25 μg/mL, respectivamente. Fluopsina C parece ser o componente antifúngico de F4a. A fração semipurificada e fluopsina C apresentaram atividade fungicida dose e tempo dependentes. F4a causou graves danos à morfologia e à ultraestrutura das células fúngicas planctônicas, e reduziu significativamente a viabilidade de biofilmes de 24 horas, com CIM para células sésseis de 12,5 a 25,0 μg/mL. Detectou-se, entretanto, citotoxicidade em células de mamíferos para F4a e fluopsina C em concentrações que apresentaram atividade antifúngica. Estes resultados indicam que a fluopsina C pode ser um protótipo para o desenvolvimento de novos antifúngicos para *C. albicans*.

Palavras-chave: Antibiofilme. Atividade antimicrobiana. Fluopsina C. Fungicida.

INTRODUCTION

Candida albicans can interact with warm-blooded hosts as a commensal member of the microbiota, often colonizing the skin and mucous membranes (Ward et al., 2018), or as an opportunistic pathogen, causing a wide range of clinical manifestations (Lopes & Lionakis, 2022). Importantly, invasive candidiasis is associated with high mortality rates (Bretagne et al., 2022), and *C. albicans* colonization represents a significant risk of infection in humans (Patel, 2022).

Fluconazole, a triazole antifungal, has a broad spectrum of action, water solubility, high bioavailability, and can be applied orally or intravenously, with high penetration into the central nervous system, and low side-effects (Shafiei, Peyton, Hashemzadeh & Foroumadi, 2020). Due to these characteristics, this antifungal has been recommended to treat several fungal infections, including those caused by *C. albicans* (Shafiei et al., 2020). However, the increased use of this antifungal over the last decades for prophylaxis and treatment of candidiasis has imposed a selection pressure that contributes to the emergence of resistant isolates (Sharifi et al., 2023).

In addition to the resistance of planktonic cells, the biofilms represent a challenge in the therapy of *C. albicans* infections, since this mode of growth exhibits reduced sensitivity to antifungal agents and to host defense mechanisms (Fan et al., 2022), leading to persistent and difficult-to-treat infections associated with high mortality rates (Atiencia-Carrera, Cabezas-Mera, Tejera & Machado, 2022). Given this scenario, the prioritization of *C. albicans* as a significant fungal pathogen by the World Health Organization underscores the urgent need for the advancement of novel control approaches (World Health Organization, 2022).

Historically, secondary metabolites produced by microorganisms have contributed to the discovery of new antibiotics (Bansal et al., 2021). Among these microorganisms, *Pseudomonas* spp. produce a diverse array of bioactive molecules with the potential to be used in medicine and agriculture (Gross & Lopes, 2009; Afonso et al., 2022).

Prior research has demonstrated that secondary metabolites derived from the cultivation of *Pseudomonas aeruginosa* LV strain exhibit strong antibacterial properties against plant pathogens (Afonso et al., 2022), as well as bacterial (Kerbauy et al., 2016; Navarro et al., 2019; Bartolomeu-Gonçalves et al., 2022), and fungal (Spoladori et al., 2023) human pathogens, including those multidrug-resistant strains.

In the present study, the antifungal potential of secondary metabolites obtained from *P. aeruginosa* LV strain on planktonic and sessile (biofilm) cells of *C. albicans* exhibiting different susceptibility to azoles was investigated. Furthermore, the effect of bacterial metabolites on *C. albicans* germ tube formation and

their toxicity to mammalian cells was also investigated.

MATERIALS AND METHODS

Microorganisms

P. aeruginosa LV strain was cultured in nutrient agar supplemented with 0.01% $CuCl_2.2H_2O$, pH 6.8 at 28 ºC for 48h. Sixteen *C. albicans* isolates recovered from the oral cavity of healthy (*n*=4), diabetic (*n*=4), and HIV-positive (*n*=3) individuals, vaginal discharge (*n* = 2), and peritoneal secretion (*n* = 3) were included in this study. These isolates were chosen randomly from the fungal collection of the Laboratory of Molecular Biology of Microorganisms, Londrina State University, Paraná, Brazil.

The reference strains *C. albicans* ATCC 10231 (fluconazole and voriconazole resistant, www.atcc.org>resources>brochures) and *C. albicans* ATCC 26790 (fluconazole and voriconazole resistant) (Eldesouky, Mayhoub, Hazbun & Seleem, 2018) were also included. All *C. albicans* were cultivated in Sabouraud dextrose (SD) agar at 37 °C for 24h.

Before the experiments, three to five fungal colonies were cultured in SD broth under the same conditions. A standard suspension was prepared by centrifuging yeast cells (10,000 x *g* for 1 min) and resuspending in saline to reach a 0.5 McFarland standard turbidity (DensiCHEK™ PLUS, bioMérieux). This suspension was then diluted in a culture medium to achieve assay inocula. Bacterial and yeast cells were stored at -80 ºC in nutrient broth (NB) and SD broth, respectively, containing 30% glycerol.

F4a and Fluopsin C purification

The fraction 4a (F4a) and fluopsin C (secondary metabolites) were obtained from the culture of *P. aeruginosa* LV strain, as described by Bedoya et al. (2019). Briefly, log-phase planktonic cells were cultivated in NB supplemented with 0.01% $CuCl_2.2H_2O$, pH 6.8 at 28 °C for 10 days. After this period, the cells were harvested by centrifugation (4.500 x g, 20 min). F4a was isolated from cell-free supernatant using dichloromethane extraction and purified by silica gel flash chromatography (dichloromethane:ethyl acetate 1:1, v/v).

Fluopsin C was purified from F4a using another silica gel chromatography (petroleum ether:dichloromethane:ethyl ether, 65:25:10) and semi-preparative high-performance liquid chromatography (HPLC). Identification of fluopsin C was based on mass spectra obtained with an ESI-MS Quattro LCZ (Micromass, Manchester, United Kingdom); ^{1}H and ^{13}C nuclear magnetic resonance spectra recorded in solution using a Bruker Avance III 400 MHz spectrometer; X-ray microanalysis (EDS) carried out using a FEI-Quanta 200 Scanning Electron Microscope with an accelerating voltage of 25 kV (Navarro et al., 2019). All products were prepared as 4.0 mg/mL solutions in dimethylsulfoxide (DMSO, Merck, USA).

Random amplified polymorphic DNA

The genetic relatedness among *C. albicans* isolates and reference strains was determined by random amplified polymorphic DNA (RAPD), using the CA1 and CA2 primers, according to Noumi et al. (2009). Genomic DNA was purified using the DNeasy Blood and Tissue kit (Qiagen, Brazil), following the manufacturer's recommendation. Banding patterns were categorized using the Dice coefficient of Gel J software (Heras et al., 2015), with the band tolerance set at 3% and the threshold cutoff value set at 95% (Morey et al., 2016).

Antifungal activity against planktonic cells

The minimum inhibitory concentration (MIC) of fluconazole (Merck, Brazil), voriconazole (Merck, Brazil), F4a, and fluopsin C was determined using the broth microdilution technique, according to the standards recommended by the Clinical and Laboratory Standards Institute (2017). The tests used Roswell Park Memorial Institute 1640 medium (RPMI, Sigma-Aldrich, Brazil), without sodium bicarbonate and buffered with 0.164 M morpholinopropanesulfonic acid (MOPS, Sigma-Aldrich, Brazil) (RPMI-MOPS) at pH 7.0 with yeast concentration of 0.5-2.5 x 10^3 CFU/mL. Compound concentrations ranged from 0.098 to 50.0 μg/mL for bacterial metabolites, 0.25 to 128.0 μg/mL for fluconazole, and 0.031 to 16.0 μg/mL for voriconazole. *Candida parapsilosis* ATCC 22019 was included in each experiment as quality control.

In every assay, two wells of the 96-well plates were used as growth (medium *plus* 1.25% DMSO *plus* inoculum) and sterility (medium *plus* 1.25% DMSO) controls. The susceptibility breakpoints for the azoles were those recommended by the Clinical and Laboratory Standards Institute (2022). To determine the minimum fungicidal concentration (MFC) of bacterial metabolites, an aliquot of 10 μL was removed from each well that did not show visible growth in the MIC test and inoculated onto the SD agar (Spoladori et al., 2023). MFC was established as the lowest concentration that did not lead to colony growth after 24h at 37 ºC. The experiments were carried out in duplicate on three different occasions. Based on azole susceptibility profiles, four clinical isolates (4L, 1S, 3S, and 82) and the reference strains were selected for further analyses.

Time-kill kinetics

To determine the growth kinetics of planktonic cells in the presence of F4a or fluopsin C, fungal cells (0.5 - 2.5 x 10^3 cells) were incubated with MIC and MFC values at 37 ºC. At specified time points (4, 8, 16, and 24h), 10 μL of each treatment was removed and serially diluted (1:10) in saline. Each dilution (10 μL) was inoculated onto SD agar, and the CFU counts were carried out after 24h-incubation at 37 °C (Barry et al., 1999). To assess the fungicidal effect, data was plotted as $\log_{10}$ CFU/mL vs time (h). A compound was classified as fungicidal if it caused a 3-$\log_{10}$ reduction (99.9%) in the initial fungal inoculum (CFU/mL) (Klepser, Ernst, Lewis, Ernst & Pfaller, 1998).

Candida albicans viability

Fungal cells (3 x 10^7 CFU/mL) were treated with F4a (MIC and MFC) in 4 mL of RPMI-MOPS, pH 7.0 at 37 ºC for 12h. The cells were harvested by centrifugation (10,000 x *g* for 1 min) and were incubated with a combination of the fluorescent dyes FUN-1® (10 μM) and Calcofluor® White M2R (25 μM) of the LIVE/DEAD® Yeast Viability Kit (ThermoFisher Scientific, Brazil), according to the manufacturer's instructions. Yeast cells were analyzed by fluorescence microscopy (Zeiss Axio Imager 2), using fluorescein filters with excitation of 480 *n*m and emission ≥ 530 *n*m.

Transmission electron microscopy of *Candida albicans* planktonic cells

Fungal cells were treated with F4a (MIC and MFC) at 37 °C for 12h. The cells were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature. Samples were fixed in a solution containing 0.2 M sodium cacodylate buffer (pH 7.2), 2% OsO_4, 1.6% potassium ferrocyanide, and 10 mM $CaCl_2$ for 30 min at room temperature. Yeast cells were dehydrated in acetone, embedded in EPON™ resin, sectioned thinly using a Leica ultramicrotome, stained with 5% uranyl acetate and lead citrate, and observed using a TECNAI12-FEI transmission electron microscope (Endo, Cortez,

Ueda-Nakamura, Nakamura & Dias Filho, 2010).

Germ tube formation

Fungal cells (2.5 x 10^3 CFU/mL) were incubated in SD broth supplemented with F4a or fluopsin C (0.5xMIC values) at 37 °C for 2h. Then, a 50-μL aliquot of this cell suspension was incubated in 500 μL of fetal bovine serum (FBS, Gibco) at 37 °C, for 3h, to induce the formation of germ tubes. An aliquot was transferred to a slide and was analyzed under a light microscope (Olympus BX53) at 400x magnification. Germ tube formation was measured by counting the number of individual cells showing a growth tube, without constriction at the emerging point, at least twice the length of the cell, and the result was expressed as a percentage of the total cell population (Endo et al., 2010).

Candida albicans biofilm formation and treatment

C. albicans biofilms were formed in 96-well polystyrene microplates (Techno Plastic Products, Switzerland). A 20-μL aliquot of the standard fungal suspension was placed in each well of the plate, containing 180 μL of SD broth. The microplates were incubated at 37°C for 24h. After this period, the biofilms were washed gently with sterile 100 mM phosphate buffer, 0.15 M NaCl, pH 7.2 (PBS) to remove non-adherent cells. After washing, an aliquot (200 μL) of SD broth containing different concentrations of F4a or fluopsin C (3.12 - 100 μg/mL) was added, and the biofilms were incubated for further 24h.

Afterward, the medium was removed, and the biofilm was washed twice with PBS. Untreated biofilms were used as a control (Bizerra et al., 2008). The metabolic activity of biofilms was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Merck, Brazil) reduction assay, according to the manufacturer's instructions. The metabolic activity of biofilms was expressed as a percentage according to the equation: $OD_{treated}$ biofilms/$OD_{untreated}$ control x 100, in which the OD of the untreated control represents 100% cell viability. The minimum inhibitory concentrations capable of reducing 50% and 90% of the metabolic activity of sessile cells were considered $SMIC_{50}$ and $SMIC_{90}$, respectively. The experiment was carried out in quintuplicate on two different occasions.

Effect of F4a and Fluopsin C in mammalian cells

The cytotoxicity of F4a or fluopsin C for HeLa (human cervix adenocarcinoma epithelial) cells line (Merck, Brazil) was analyzed as described by Bartolomeu-Gonçalves et al. (2022), with modifications. Briefly, cells were cultured in a flat-bottomed 96-well microtiter plate containing Dulbecco′s Modified Eagle′s medium (DMEM) supplemented, with 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 1% tylosin and 5% CO_2 at 37 °C for 48h. After that, the system was gently washed with sterile PBS to remove non-adherent cells. The medium, containing different concentrations of F4a or fluopsin C (0.062 to 2.0 μg/mL), was added, and the cells were incubated for further 24h. Cell viability was analyzed using the MTT reduction assay according to the manufacturer's recommendation. The concentration of the compounds needed to inhibit viable cells up to 50% was calculated by regression analysis, and the value was considered the 50% cytotoxic concentration (CC_{50}).

Statistical analysis

GRAPHPAD PRISM software version 8.0.0 (GraphPad Software, San Diego, California, USA) was used for statistical analysis. For the broth microdilution test, MIC and MFC values of fluconazole and voriconazole for susceptible and resistant groups were compared by the Mann-Whitney test. For the growth kinetics, Two-Way ANOVA was used, followed by Tukey's multiple comparison test. For the biofilms, the mean values were tested by One-Way ANOVA. *P*-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

F4a inhibits the planktonic growth of *Candida albicans* displaying different genotypes and susceptibility profiles to azole compounds, and the fluopsin C appears to be one of the active components

In the present study, we evaluated the antifungal effect of the F4a obtained from the cell-free supernatant of *P. aeruginosa* LV strain cultivated under copper stress. First, we analyzed the genetic relatedness of the *C. albicans* isolates. Visual observation of bands generated by RAPD using the CA1 and CA2 primers (Noumi et al., 2009) revealed a high genetic diversity among *C. albicans* isolates and reference strains. Using a cut-off value of 95% (Morey et al., 2016), all *C. albicans* had unique RAPD fingerprint profiles, indicating their low genetic relatedness. Three clusters (D, J, and K) were identified using a cut-off value of 70%. Four, three, and three isolates were grouped in clusters D, J and K, respectively. The other seven isolates and *C. albicans* ATCC 10231 had unique banding profiles (Figure 1).

Following, we determined the susceptibility of all *C. albicans* isolates to two azole antifungals. The MIC values of fluconazole and voriconazole ranged from 1 to > 128 μg/mL, and from 0.031 to > 16 μg/mL, respectively. According to the CLSI (2022) antifungal breakpoints, among 16 *C. albicans* isolates, 11 (68.8%) were resistant to fluconazole (MIC $\geq$ 8 μg/mL), four (25.0%) were sensitive (MIC $\leq$ 2 μg/mL), and one (6.3%) was susceptible dose-dependent (MIC = 4 μg/mL). Regarding voriconazole, seven (43.8%) were resistant to voriconazole (MIC $\geq$ 1 μg/mL), six (37.5%) were intermediate (MIC 0.25-0.5 μg/mL), and three (18.8%) were sensitive (MIC $\leq$ 0.125 μg/mL). The reference strains were resistant to both azole compounds, validating the results (Figure 1).

F4a inhibited the growth of all *C. albicans*. The MIC values ranged from 1.56 to 12.5 μg/mL, and the MFC ranged from 6.25 to 25.0 μg/mL. Except for isolate 12, whose MIC and MFC values were 12.5 μg/mL. For the other isolates and the reference strains, the MFC was at least twice as high as the MIC (Figure 1).

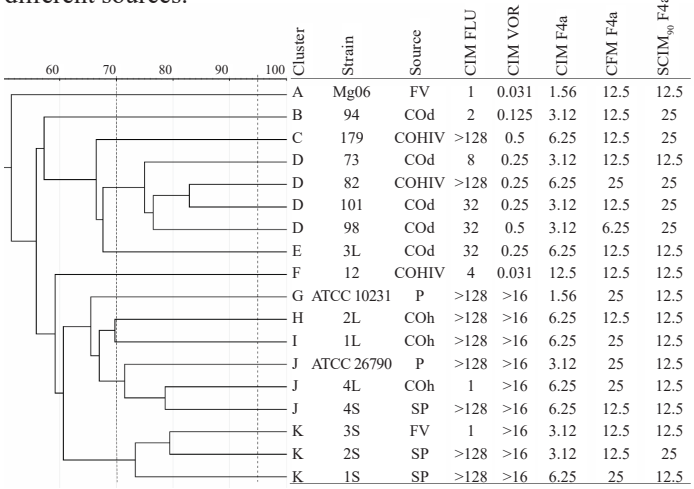
There was no significant difference (p>0.05) in the MIC and MFC values of F4a for *C. albicans* with different susceptibility profiles to azoles and genotypes. The MIC and MFC values of the F4a also showed statistically insignificant differences when comparing the anatomical site of isolation of *C. albicans* (p>0.05). Therefore, four clinical isolates (4L and 3S – resistant to voriconazole, 1S – resistant to both azoles, and 82 – resistant to fluconazole and intermediate to voriconazole) and the reference strains were selected for further analyses with planktonic cells.

Previous studies have shown that secondary metabolites from *P. aeruginosa* inhibit the planktonic growth of *C. albicans* (Egawa, Umino, Ito & Okuda, 1971; Kerr et al., 1999; Salvatori et al., 2020). F4a is composed of a mixture of phenazine-1-carboxyamide, phenazine-1-carboxylic acid, indolin-3-one, and the copper-containing compound fluopsin C (Bedoya et al., 2019; Navarro et al., 2019). Therefore, to investigate which compound was responsible for the antifungal activity, we tested each compound separately in planktonic cells of *C. albicans* reference strains. Only fluopsin C inhibited the growth of *C. albicans*, displaying MIC and MFC values of 1.56 μg/mL and 6.25 μg/mL (ATCC 10231), and 1.56 μg/mL and 3.12 μg/mL (ATCC 26790),

respectively, indicating that this copper-containing compound is the active antifungal component of F4a.

Figure 1

Genotypic characteristics and fluconazole, voriconazole and F4a susceptibility profiles for *Candida albicans* isolated from different sources.



Cluster	Strain	Source	CIM FLU	CIM VOR	CIM F4a	CFM F4a	$SCIM_{90}$ F4a
A	Mg06	FV	1	0.031	1.56	12.5	12.5
B	94	COd	2	0.125	3.12	12.5	25
C	179	COHIV	>128	0.5	6.25	12.5	25
D	73	COd	8	0.25	3.12	12.5	12.5
D	82	COHIV	>128	0.25	6.25	25	25
D	101	COd	32	0.25	3.12	12.5	25
D	98	COd	32	0.5	3.12	6.25	25
E	3L	COd	32	0.25	6.25	12.5	12.5
F	12	COHIV	4	0.031	12.5	12.5	12.5
G	ATCC 10231	P	>128	>16	1.56	25	12.5
H	2L	COh	>128	>16	6.25	12.5	12.5
I	1L	COh	>128	>16	6.25	25	12.5
J	ATCC 26790	P	>128	>16	3.12	25	12.5
J	4L	COh	1	>16	6.25	25	12.5
J	4S	SP	>128	>16	6.25	12.5	12.5
K	3S	FV	1	>16	3.12	12.5	12.5
K	2S	SP	>128	>16	3.12	12.5	25
K	1S	SP	>128	>16	6.25	25	12.5

Source: The authors.

Note. Cluster analysis of RAPD fingerprint patterns was performed using Dice coefficient of Gel J software (Heras et al., 2015), with band tolerance set at 3% and threshold cutoff value set at 95% (Morey et al., 2016). Minimum inhibitory concentration (MIC) of fluconazole (FLU), voriconazole (VOR) and F4a determined by broth microdilution method according to CLSI (CLSI, 2020). Minimum fungicidal concentration (MFC) of F4a determined by counting of colony-forming units in Sabouraud dextrose agar (Spoladori et al., 2023). Sessile cells minimum inhibitory concentration ($SMIC_{90}$) of F4a determined by the MTT-reduction assay (Bizerra et al., 2008). MIC, MFC, and SMIC values are expressed as μg/mL. In which, VF: vaginal fluid; OCd: oral cavity of individuals with *Diabetes mellitus*; OCHIV: oral cavity of HIV-positive individuals; OCh: oral cavity of healthy individuals; L: lung; PS: peritoneal secretion.

Fluopsin C was initially isolated and characterized from *Pseudomonas* MCRL 10107 strain growing in the presence of copper, and named as YC 73 antibiotic, as it inhibited the growth of several bacterial and fungal species, including *C. albicans* (MIC = 3.12 μg/mL) (Egawa, Umino, Awataguchi, Kawano & Okuda, 1970; Egawa et al., 1971). Subsequently, fluopsin C was isolated from *Pseudomonas fluorescens* KY 403 (first named the antibiotic as fluopsin C) (Itoh, Inuzuka & Suzuki, 1970), *Pseudomonas reptilivora* (Del Rio, Gorgé, Olivares & Mayor, 1972), *Streptomyces* sp. (antibiotic no. 4601) (Otsuka, Niwayama, Tanaka, Take & Uchiyama, 1971) and *P. aeruginosa* PAO1 (Patteson et al., 2021).

Similar to the study of Egawa et al. (1970), these authors reported the antimicrobial activity of fluopsin C on planktonic cells of several Gram-positive and Gram-negative bacteria, and the yeast *Saccharomyces cerevisiae*. Fluopsin C purified from *P. aeruginosa* LV strain also inhibited the growth of several antibiotic-resistant bacterial species, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VREfm), *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* (KPC-*K. pneumoniae*) (Kerbauy et al., 2016; Navarro et al., 2019; Bartolomeu-Gonçalves et al., 2022).

Recently, we reported the antifungal activity of F4a against *Candida auris* CBS 10913 (sensitive to fluconazole and amphotericin B) and *C. auris* CBS 12766 (resistant to fluconazole and amphotericin B). Only fluopsin C (MIC = 3.12 μg/mL and MFC = 6.25 μg/mL) and indolin-3-one (MIC = 100 μg/mL and MFC = 200 μg/mL) inhibited the growth of this yeast species. Furthermore, fluopsin C and indolin-3-one exhibited a synergistic antifungal interaction on planktonic cells of *C. auris* (Spoladori et al., 2023). Taken together, these results indicate the potential of fluopsin C as a starting point for the development of new compounds aimed at controlling antimicrobial-resistant microorganisms.

In contrast to our results, Kerr et al. (1999) reported the antifungal effect of 1-hydroxiphenazine obtained from *P. aeruginosa*, exhibiting MIC values of 25.0 μg/mL and 50.0 μg/mL for *C. albicans* and *Aspergillus fumigatus*, respectively. These results indicate that substituents in the C1-position of the phenazine molecule can affect the antifungal effect, while the presence of hydroxyl is more effective in inhibiting the growth of planktonic cells of *C. albicans*, compared to carbamoyl (phenazine-1-carboxyamide) and carboxylic acid (phenazine-1-carboxylic acid) groups. In turn, Salvatori et al. (2020) reported that phenazine compounds (pyocyanin and phenazine methosulfate) interfered in *C. albicans* filamentation and survival within macrophages.

F4a and fluopsin C display dose and time dependent fungicidal effect against planktonic cells

The growth kinetics of planktonic *C. albicans* cells in the presence of F4a or fluopsin C was monitored for 24 hours at 37 ºC to assess the nature and extent of the antifungal effect (Figure 2). Overall, using the MIC values of F4a, growth inhibition of *C. albicans* was observed over time, compared to untreated control cells. Differences of at least $4\log_{10}$UFC were observed in the CFU counts between untreated and treated cells after 24 hours for all *C. albicans* strains/isolates. On the other hand, in front of the MFC of F4a, CFU counts were zero after 24 hours (the reference strains) (Figures 2a and 2b), four hours (isolates 1S and 3S) (Figures 2c and 2d) and 16 hours (isolates 4L and 82) (Figures 2e and 2f).

We chose *C. albicans* ATCC 10231 reference strain to compare the time-kill kinetics of fluopsin C. Similar to F4a, fluopsin C exhibited a dose- and time-dependent fungicidal effect in planktonic cells. The CFU counts were zero after 24h-incubation (Figure 2g), corroborating that the antifungal effect of F4a is due to the presence of this copper-containing compound.

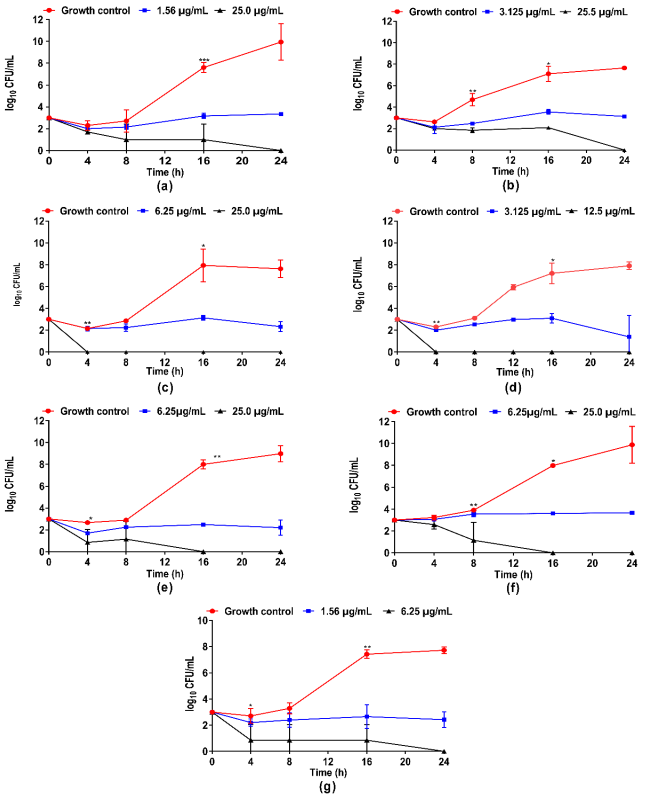
The fungicidal effect of F4a was visualized in greater detail after differential labeling, using the FUN-1™ and Calcofluor™ White M2R fluorescent probes, of *C. albicans* ATCC 10231, cultivated in the presence of F4a MIC and MFC. The images corroborated the dose-dependent character of the fungicidal activity of F4a. Figures 3a and 3b show untreated planktonic cells with typical yeast morphology, exhibiting reddish vacuolar structures, indicating intact membranes and preserved metabolic activity.

Similarly, from the treatment with the MIC of F4a (1.56 μg/mL), no morphological alteration was observed. The cells showed red-orange intravacuolar structures (Figures 3e and 3f), corroborating the fungistatic nature of the compound. On the other hand, cells treated with F4a MFC (25 μg/mL) exhibited diffuse green fluorescence (Figures 3i and 3j), indicating the absence of metabolic activity in these cells.

The morphological and ultrastructural changes of planktonic cells induced by F4a were also analyzed by TEM. The untreated control cells exhibited typical morphology, with a compact cell wall and intact cell membrane, with regular electron density (Figures 3c and 3d). Treatment with F4a with MIC caused disorganization of the cytoplasm and increase in the size of the vacuoles, as well as in Figures 3g and 3h. Intense cytoplasm disorganization, increased number of vacuoles, and plasma membrane detachment were observed in *C. albicans*

treated with F4a MFC (Figures 3k and 3l). Similar morphological and ultrastructural changes were also observed in *C. auris* after treatment with F4a (in MIC and MFC values) (Spoladori et al., 2023). Further studies are needed to elucidate the mode of action of fluopsin C in *Candida* species.

Figure 2
Antifungal activity of F4a against planktonic cells of *Candida albicans*.



Source: The authors.
Note. Time-kill curves of *C. albicans* ATCC 10231 (a) and *C. albicans* ATCC 26790 (b), and the clinical isolates: 1S (c), 3S (d), 4L (e), 82 (f) in presence of MIC and MFC of F4a for 24h at 37 ºC. Time-kill curve of *C. albicans* ATCC 10231 (g) in presence of MIC and MFC of fluopsin C for 24h at 37 ºC.The CFU counts were carried out at specified times. **p*-value<0.05, ***p*-value<0.01, ****p*<0.001 compared to untreated cells.

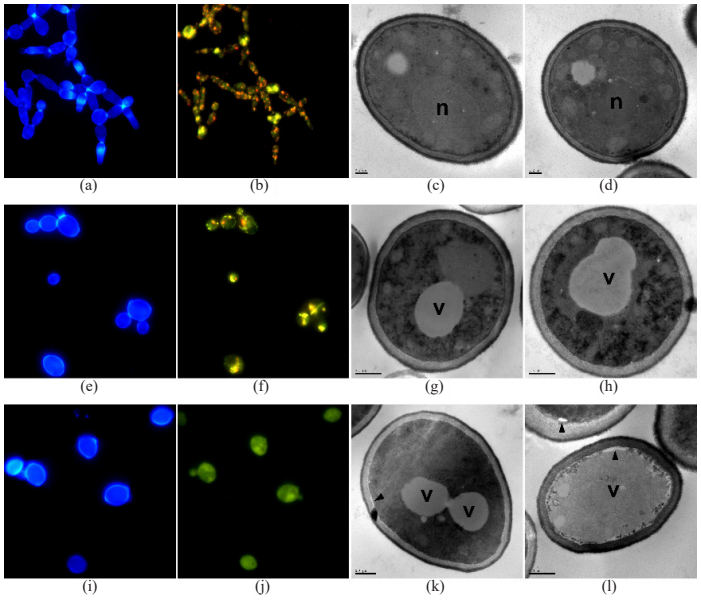
F4a and fluopsin C inhibit *Candida albicans* germ tube formation

The ability of *C. albicans* to switch between the two reversible morphological forms (yeast to filamentous structures) in response to various host environmental conditions is a well-established virulence trait (Noble, Gianetti & Witchley, 2017). Traditionally, the filamentous forms (pseudohyphae and hyphae) have been associated with the pathogenic lifestyle, whereas yeast forms were found as commensals (Moyes et al., 2010).

However, the transition of yeast and filamentous forms seems to contribute to disseminated candidiasis. Actually, previous studies have shown that *C. albicans* locked in the yeast or filamentous forms was strongly attenuated for virulence in a mouse model of disseminated candidiasis (Saville, Lazzell, Monteagudo & Lopez-Ribot, 2003). In this sense, yeast cells can disseminate through the bloodstream, while the filamentous forms can invade and damage internal organs (Saville et al., 2003). Environmental factors, such as temperature, pH, and culture medium, can induce the yeast-to-hyphae transition in *C. albicans* (Noble et al., 2017).

In this study, we evaluated the effect of F4a and fluopsin C on germ tube formation by *C. albicans* reference strains, using SD supplemented with FBS. Previous exposure of reference strains to subinhibitory concentrations (0.5xMIC) of F4a or fluopsin C for two hours significantly inhibited the germ tube formation by *C. albicans* reference strains (Figure 4). F4a inhibited around 90% and 70% of germ tube formation by ATCC 10231 and ATCC 26790, respectively.

Figure 3
Effect of F4a on cell viability, morphology, and ultrastructure of *Candida albicans* ATCC 10231.



Source: The authors.
Note. Viability of yeast cells were analyzed after differential labeling with FUN-1™ and Calcofluor White™ MR2 dyes. Planktonic cells were incubated without (a, b), and with MIC (e, f) and MFC (i, j) of F4a for 12h. Cells containing red fluorescent intravacuolar structures (b, f) represent metabolically active yeast, and blue fluorescence (a, e, i) indicates the cell wall of viable and non-viable cells. Cells with diffuse greenish-yellow fluorescence (j) characterize metabolically inactive cells. 400x magnification. Transmission electron microscopy images of untreated cells of *C. albicans* (c, d) exhibited typical spherical morphology showing a compact cell wall with regular electron density. The effect of F4a treatment with MIC (g, h) and MFC (k, l) concentrations for 12h revealed cytoplasmic disorganization, increased vacuoles, and cell membrane detachment (black arrowhead). In which, n: nucleus; v: vacuoles. Bars: 0.5 μm.

Similarly, fluopsin C inhibited 100% and 80% of this process in ATCC 10231 and ATCC 26790 (Figure 4a). Light microscopy images (Figures 4b to 4g) corroborate the inhibitory effect of F4a and fluopsin C on the germ tube formation in *C. albicans* reference strains. These results indicate that both compounds can affect the virulence of *C. albicans*, thus exhibiting "antivirulence" activity.

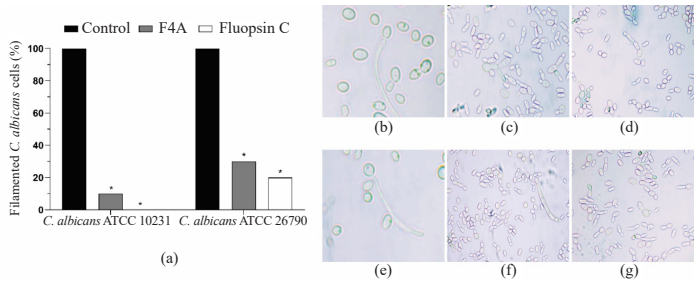
F4a and fluopsin C inhibit established biofilms of *Candida albicans*

In addition to their role in the pathogenesis of disseminated candidiasis, hyphae, along with yeasts and pseudohyphae, are present in biofilms formed by *C. albicans*, a feature of critical clinical importance (Fan et al., 2022). *C. albicans* biofilms are highly resistant to most available antifungal agents and defense molecules of the host immune system (Bizerra et al., 2008; Fan et al., 2022). The mechanisms of resistance of sessile cells are multifactorial and, in general, are related to the antifungal sequestration by biofilm matrix, the overexpression of resistance-encoded genes, the formation of polymicrobial biofilms, and the formation of extracellular vesicles (Bizerra et al., 2008; Fan et al., 2022).

In the present study, the treatment with different concentrations of F4a decreased the metabolic activity of *C.*

albicans sessile cells, as observed in Figure 5. For seven isolates and ATCC 26790 (44.4%), a significant reduction (p<0.05) of metabolic activity was observed in a concentration of 12.5 μg/mL. For three and ATCC 10231 (22.2%), three (16.7%), and three (16.7%) isolates, a significant decrease in metabolic activity was observed from 6.25 μg/mL, 3.125 μg/mL, and 25.0 μg/mL. The $SMIC_{90}$ of the isolates and reference strains ranged from 12.5 μg/mL to 25.0 μg/mL. For most isolates (66.7%), the $SMIC_{90}$ was equal to 12.5 μg/mL. For seven isolates (43.75%), the $SMIC_{90}$ was identical to the MFC (Figure 1).

Figure 4
Effect of F4a and fluopsin C on *Candida albicans* germ tube formation.



Source: The authors.
Note. (a) Graphical representation of germ tube formation in presence of F4a and fluopsin C at 0.5xMIC values. *p<0.001 compared to untreated yeast cells. Microscopy images of germ tube in *C. albicans* ATCC 10231 (b, c, d) and *C. albicans* ATCC 26790 (e, f, g). Untreated (b, e) and treated planktonic cells for 2h with 0.5xMIC values of F4a (c, f) and fluopsin C (d, g) were incubated in presence of fetal bovine serum at 37 °C for 3h. 400x magnification.

We also evaluated the effect of fluopsin C on 24h-biofilms of *C. albicans* ATCC 10231 (data not shown). A 60% reduction, approximately, was observed in metabolic activity of sessile cells after treatment with 3.12 μg/mL of fluopsin C. The $SMIC_{90}$ was equal to 6.25 μg/mL, slightly lower than that obtained for F4a ($SMIC_{90}$ = 12.5 μg/mL), corroborating that this compound is the active component of F4a.

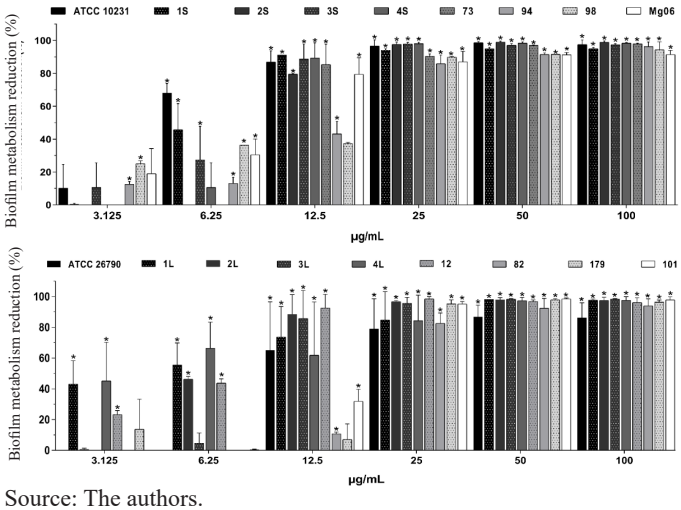
The inhibitory effect of secondary metabolites of *P. aeruginosa* strain LV on fungal biofilms has been little explored. As aforementioned, F4a inhibited the established (24h) biofilms of *C. auris*, displaying a dose-dependent inhibitory effect on the viability of sessile cells. The MIC capable of reducing 50% of CFU counts ($SMIC_{50}$) was 32.94 μg/mL and 47.02 μg/mL for *C. auris* CBS 10913 and *C. auris* CBS 12766, respectively (Spoladori et al., 2023). Compared to the $SMIC_{50}$ values for *C. albicans* (ranging from 3.12 to 12.5 μg/mL), this species seems more susceptible to F4a.

F4a and fluopsin C are toxic to HeLa cells

The effect of F4a and fluopsin C on human cervical adenocarcinoma cells (HeLa) was determined after 24 hours of treatment. The calculated CC_{50} values (the lowest cytotoxic concentration for 50% of the cells) were 0.86 μg/mL and 0.43 μg/mL for F4a and for fluopsin C, respectively. The CC_{50} values were lower than the MIC and SMIC values for *C. albicans* in this study, indicating greater toxicity to mammalian cells. In fact, previous studies reported time- and dose-dependent antitumor activity of fluopsin C on HeLa cells (Otsuka et al., 1971) and in human breast adenocarcinoma cells (Ma et al., 2013; Alves de Lima et al., 2022).

Specifically, in the MCF-7 cell line, fluopsin C induced cell membrane damage, cytoskeletal disorganization and accumulation of reactive oxygen species, decreased mitochondrial membrane potential and intracellular ATP level (Ma et al., 2013). In addition, it induced cell division arrest in the G1 phase, DNA damage, and reticular stress (Alves de Lima et al., 2022).

Figure 5
Antibiofilm activity of F4a on biofilms of *Candida albicans*.



Source: The authors.
Note. 24h-biofilms formed on polystyrene surface were treated with different concentrations of F4a for 24h at 37 °C. The viability of sessile cells was evaluated by the MTT-reduction assay. Values are mean ± standard deviation of two experiments in quintuplicate. The asterisks indicate a significant reduction of metabolically active sessile cells treated with F4a compared to untreated cells. *p<0.01.

Previous studies have also reported the toxicity of F4a and fluopsin C in primary cell lines under *in vitro* conditions. The CC_{50} of F4a (Bartolomeu-Gonçalves et al., 2022) and fluopsin C (Navarro et al., 2019) for LLC-MK2 cells (*Macaca mulatta* kidney epithelial cells) was equal to 3.44 μg/mL and 2.0 μg/mL, respectively. These values were also lower than the MIC and SMIC values for *C. albicans*. Furthermore, Ma et al. (2013) reported the toxicity of fluopsin C to human hepatocytes (HL7702) and mammary epithelial cells (HMLE). Finally, an LD_{50} (lethal dose capable of killing 50% of mice) of around 3.0 to 6.0 mg/kg via intraperitoneal (Egawa et al., 1970), and 4.0 mg/kg via intravenous (Navarro et al., 2019) was identified.

CONCLUSION

In this study, for the first time, we report the antifungal activity of F4a obtained from the LV strain of *P. aeruginosa* in planktonic and sessile cells of *C. albicans*, exhibiting different genotypes and sensitivity to fluconazole and voriconazole. The results indicate that this activity is related to the presence of fluopsin C. Additional studies are needed, with the aim of: a) reducing toxicity to mammalian cells, through modification of the chemical structure of the active components of F4a or the use of drug delivery systems; b) to evaluate the combined effect of F4a and its active components with other antifungal compounds, in an attempt to reduce MIC values.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: C. L. M., G. A., S. F. Y. O. *Formal analysis*: E. R. T., C. L. M. *Funding acquisition*: G. A., L. M. Y., S. F. Y. O. *Investigation*: C. L. M., G. B. G., G. S. R., A. S. S., E. R. T. *Methodology*: G. A., E. R. T., L. M. Y., S. F. Y. O. *Project administration*: G. A., S. F. Y. O. *Resources*: C. V. N., M. V. P. R. *Supervision*: S. F. Y. O. *Validation*: C. L. M., G. B. G., G. S. R., A. S. S., C. V. N., M. V. P. R., G. A., E. R. T., L. M. Y., S. F. Y. O. *Visualization*: C. L. M., G. B. G., S. F. Y. O. *Writing the initial draft*: C. L. M., S. F. Y. O. *Revision and editing of writing*: C. L. M., G. B. G., G. S. R., A. S. S., C. V. N., M. V. P. R., G. A., E. R. T., L. M. Y., S. F. Y. O.

PEER REVIEW

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