UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

Screening of antifungal and antibiofilm activity of Brazilian plant extracts exhibiting selective activity against *Cryptococcus neoformans*

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Trabalho de Conclusão de Curso apresentado ao Curso de Farmácia da Universidade Federal do Rio Grande do Sul como requisito à obtenção do título de grau de Farmacêutico.

Orientador: Prof. Dra. Lucélia Santi

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Em primeiro lugar à minha família, especialmente minhas irmãs, que estiveram comigo durante todo o processo de escrita do trabalho e não mediram esforços para me ajudar nos momentos mais difíceis. Agradeço muito também à minha orientadora e todos meus colegas de laboratório por criarem um ambiente em que eu pude me sentir confortável e acolhida para trabalhar. Por fim, aos amigos que fiz durante todo o curso de Farmácia: sem eles, não teria sido tão divertido trilhar esse caminho.

APRESENTAÇÃO

Esse Trabalho de Conclusão de Curso foi redigido sob a forma de artigo ao qual foi elaborado segundo as normas da revista Brazilian Journal of Pharmaceutical Sciences, apresentadas em anexo.

Screening of antifungal and antibiofilm activity of Brazilian plant extracts exhibiting selective activity against *Cryptococcus neoformans*

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ABSTRACT

Extracts derived from plants have long been recognized as having potential antimicrobial activity and their use has contributed immensely to the pharmacotherapy of infectious diseases. In a biomedical context of increasing microbial resistance to traditional drugs and persistence due to biofilm, new bioactives with proven antifungal and antibiofilm activities emerge as resourceful options to get past these problems. In this work, we tested the capacity of 45 extracts derived from seeds of Amazonic and Southern Brazilian plants to inhibit the formation and/or to eradicate the preformed biofilm of *Cryptococcus neoformans* and *Candida albicans* - common pathogens of severe systemic infections. Minimal inhibitory concentration (MIC) was also carried out. Our study identified one selective extract with a rate of biofilm eradication superior to 80% and no activity against planktonic cells of *C. neoformans*. This may shed light to a plant-derived extract with no antifungal activity but with the ability to affect formed biofilm, which induces less resistance by the yeast.

Keywords:

biofilm eradication - *Cryptococcus neoformans* - *Candida albicans* - fungal resistance - plant derived extracts

INTRODUCTION

Natural products are specialized chemical compounds produced by any life form that interact with and influence cellular activity in crucial ways. Its medicinal use has been explored since Antiquity and still represents an important pharmaceutical asset to healthcare (Hug *et al.*, 2020). Several biological activities are being described for natural products and fungal infections are no exception to this applicability. The first antifungal drug commercialized, back in the 1950's decade - Amphotericin B - was obtained from a bacterial compound and is still largely used to treat systemic fungal infections nowadays. The echinocandins, a class of antifungal bioactives derived from fungi, were also introduced in medical practice in the last two decades (Heard *et al.*, 2021). However, the most used class of antifungal agents is the azoles - synthetic compounds that act on the fungal cell membrane and to which yeasts are starting to present less susceptibility as a consequence of overuse (Stott *et al.*, 2021).

Cryptococcus neoformans, one of the most common yeasts identified in systemic infections, has emerged as a life-threatening pathogen in the last decades, causing lung infection and meningoencephalitis, especially in patients with advanced HIV disease and solid-organ transplantation (Rathore *et al.*, 2022). *Candida albicans*, another opportunistic yeast, is part of the normal microbiota in about 50% of the population but, in many cases of immunosuppression, it can invade the bloodstream and compromise multiple organs (Talapko *et al*., 2021). Globally, it is estimated that fungal infections kill more than 1.5 million people per year, with *C. albicans* and *C. neoformans* being largely responsible for these numbers (Firacative, 2020).

With the rising resistance of fungal pathogens to conventional pharmaceutical drugs, there is an increasing urge to find new antifungal therapies that explore diverse and innovative mechanisms of action. An alternative to these drugs is the development of agents capable of inhibiting other virulence factors, such as the polysaccharide capsule and melanization process of *C. neoformans*, the expression of adhesin or yeast-to-hyphal transition of *C. albicans*, and, most importantly, the formation of biofilm on both yeasts. Many studies have already found synthetic and natural compounds able to inhibit the formation of biofilm of *C. albicans* (Abirami *et al.*, 2020; Puspitawati *et al*., 2019), but the search for

candidates against *C. neoformans* seems more difficult, especially in natural sources. Thus, research focusing on the discovery of novel therapeutic molecules capable of acting on different targets of the yeasts remains much in need.

Considering Brazil's biodiversity, therefore, our study aimed at exploring the plants of the Amazon and Southern regions through the investigation of new substances capable of inhibiting the formation and eradicating the formed biofilm of *C. neoformans*, as well as eradicating the formation of *C. albicans*' biofilm. Through the screening of different extracts, our goal was to find promising candidates with good activity against one of the most important virulence factors - the biofilm - which can, eventually, serve as a source to develop new antifungals used against resistant strains.

MATERIAL AND METHODS

Fungal Strain and Growth Conditions

Cryptococcus neoformans var. *neoformans* strain B3501, serotype D (ATCC 34873), and *Candida albicans* (ATCC 14053) were acquired from the American Type Culture Collection (Manassas, VA). The yeasts were stored as 15% (v/v) glycerol stocks at -80°C. Prior to each experiment, cells were subcultured from the stocks onto Sabouraud Dextrose Agar (SDA) (Becton Dickinson) at 37°C for 48 h.

Plant Material and Preparation of Extracts

The plant seeds used were collected from August 2008 to July 2009 in the state of Amazonas (Ibama authorization number 2008522) and from September 2018 to December 2018 in Rio Grande do Sul state (SISBio register numbers AFD4624 and A37FE07). The seeds were pulverized on a knife mill and 20 mL of distilled water was added to each 1 g of pulverized material. The solution was centrifuged at 10,000 rpm for 15 minutes, filtered through a Whatman nº 1 filter paper and lyophilized. The resulting powder was dissolved in distilled water at a concentration of 100 mg/mL and sterilized by filtration (0.22 µm; Whatman). A total of 45 extracts were prepared.

Biofilm Formation

C. neoformans and *C. albicans* cells were cultured in Sabouraud Dextrose Broth (Difco Laboratories, Detroit, MI) for 16-18 h in an incubator shaker (150 rpm) at 35°C. After this period, the cells were recovered by centrifugation at 10,000 rpm for 5 minutes and washed three times with sterile 0.9% saline solution. Subsequently, the pellet was resuspended in SDB and adjusted to the desired cellular density ($1x10^7$ cells/mL) by counting in a haemocytometer. This standardized suspension (200 µL) was added to the 96-well polystyrene plates and incubated at 37°C without shaking for 24 h (*C. albicans*) or 48 h (*C. neoformans*) for the biofilm formation. After incubation, the wells containing *C. neoformans* or *C. albicans* biofilms were washed three times with phosphate-buffered saline (PBS) to remove planktonic cells. The cells that still remained attached to the polystyrene surface were considered as true biofilm. All tests were performed in triplicates.

Evaluation of antibiofilm activity

a) Biofilm eradication

To evaluate the effect of biofilm eradication, 45 extracts in a concentration of 5 mg/mL were added on the preformed biofilm to each well (as previously described). The plates were incubated at 37°C for 24 h. After this time, the crystal violet assay was performed, and the optical density at 570 nm was read using a SpectraMax i3x microplate reader. The amount of biofilm eradication was calculated relative to the amount of biofilm grown in the absence of extracts (defined as 100%) and the media sterility control (defined as 0%). The experiments were made at least two times with 4 biological replicates each.

b) Biofilm formation

To evaluate the inhibition of biofilm formation, the selected extract was added to the wells containing the cells ($1x10^7$ cells/mL) at concentrations ranging from 5 mg/mL to 19 µg/mL. The microplate was incubated at 37°C for 24 h or 48 h, for *C. albicans* or *C. neoformans*, respectively. After this time, the wells were washed three times with sterile PBS and crystal violet dying was read at 570 nm using a SpectraMax i3x microplate reader.

Determination of Minimum Inhibitory Concentration (MIC)

The value of minimum inhibitory concentration (MIC) for *C. neoformans* was determined using the protocol established by the Clinical & Laboratory Standards Institute (CLSI), M27-A2 Reference Method. The selected extract was serially diluted in RPMI-1640 (pH 7.2; 2% glucose) to a concentration ranging from 5 mg/mL to 19 µg/mL. The microplates were incubated at 37°C for 48 h and the optical density was read using a SpectraMax i3x microplate reader at 600 nm.

RESULTS AND DISCUSSION

Invasive fungal diseases are increasing, especially in immunosuppressed patients, which commonly require prolonged use of catheters and shunts. Given the ability of both microorganisms focused on our study (*C. neoformans* and *C. albicans*) to form biofilms on these indwelling medical devices, the number of hospitalized patients with systemic infections caused and aggravated by these devices is continuously enlarging (Tits *et al*., 2020).

Biofilms are tridimensional complex structures of grouped microorganisms attached to a surface and wrapped by a self-produced extracellular matrix, which covers the cells and protects them from the action of medications or environment (Wu *et al.,* 2017). The difference between biofilm and planktonic (free-living) cells is not restricted simply to its architecture. There are important physiological and biochemical distinctions that involve higher tolerance to environmental stress and even immune activity (Martinez, Casadevall, 2007; Santi *et al.,* 2014). A dramatic variable response to antifungal treatments is also observed when comparing planktonic and biofilm cells (Martinez, Casadevall, 2007). It was reported that fungi with biofilms were up to 1000-fold more resistant to antifungal agents than planktonic cells (Ramage *et al*., 2001; Tre-Hardy *et al*., 2008).

There are a series of factors that contribute to fungal multiresistance and high associated morbimortality being a public health problem - from slow new drugs development in the last decades to fungal intrinsic characteristics. Despite acknowledging adaptive mechanisms of the isolated infective cells, the main source of resistance is the formation of biofilms. As a consequence, the infections

caused by fungi have become resistant to many first-line treatments. This leads to the use of alternative therapies, which can offer higher toxicity (Wall, Lopez-Ribot, 2020). At present, the abuse of antifungal drugs has not been completely controlled, and it is still happening in different regions and to varying degrees around the world. Thus, the difficult eradication of biofilms by traditional antifungal drugs urges the development of new therapeutic agents capable of acting on different targets of the fungi cell.

Antibiofilm and antifungal activities

Natural products have long been the base for development of important antifungal therapies (Newmann, Cragg, 2020). The initial screening was performed with 45 seed extracts to evaluate their capacity to eradicate the preformed biofilm of *C. neoformans* (Figure 1). Only four of them (8.9%) destroyed more than 25% of the biofilm in a concentration of 5 mg/mL. Two of them were prepared for a second round of experiments, showing a higher eradication percentage: SG extract with 80.67% inhibition, and JC with 33.09% biofilm inhibition (Figure 2).

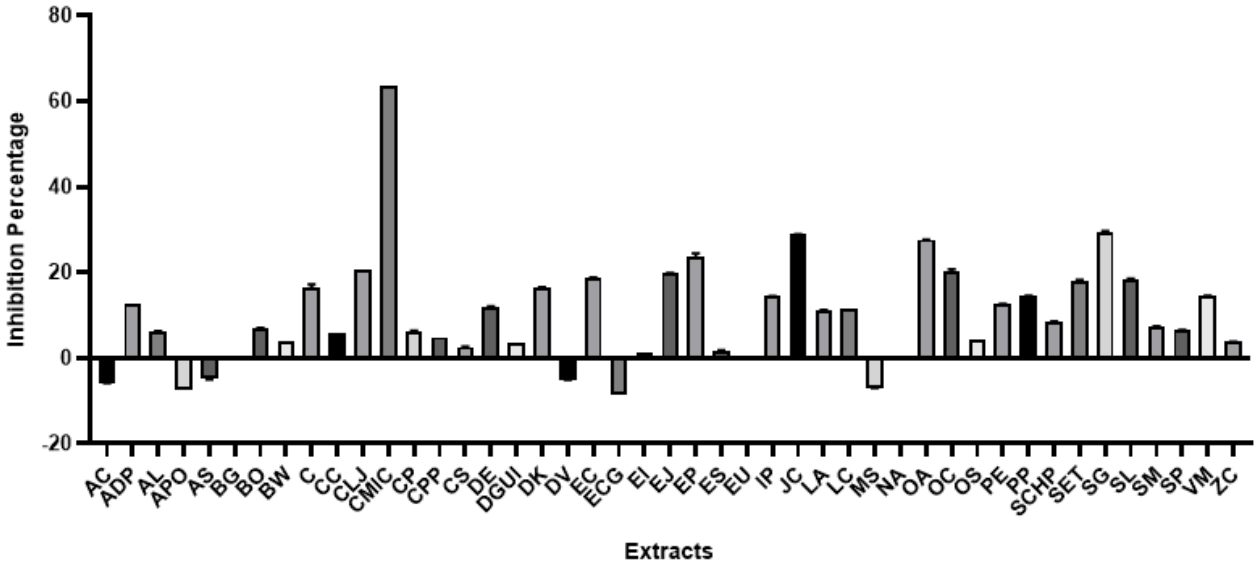

FIGURE 1 - Screening of Amazon and Southern Brazilian extracts (5 mg/mL) against the mature biofilm of *Cryptococcus neoformans* B3501.

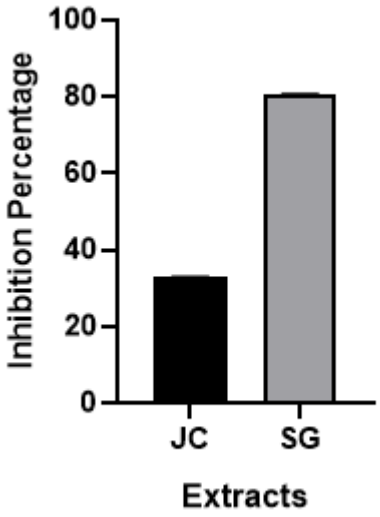

FIGURE 2 - Inhibition percentage of the two selected extracts (5 mg/mL) tested against the mature biofilm of *Cryptococcus neoformans* B3501.

Since SG presented the greatest antibiofilm activity, the minimal inhibitory concentration (MIC) of this extract was performed. Interestingly, no activity was found against the planktonic cells of *C. neoformans*. Also, the biofilm-inhibition assay was performed to evaluate if SG could prevent the biofilm formation of *C. neoformans*, but no significant activity was noticed.

Although unusual, the capacity of destroying biofilm but not killing the cells is interesting, since it possibly indicates a distinct mechanism of action than traditional antifungals, indicating its promising activity. Therefore, the discovery of a virulence inhibitor that does not present direct antifungal activity can be considered a promising alternative to traditional antifungal treatment since it is expected to eradicate the mature biofilm without increasing the risk of drug resistance and toxicity to the host (Fisher *et al*., 2022). Examples of this are the benzophenones xanthochymol and garcinol, isolated from *Garcinia xanthochymus* fruits. They are capable of inhibiting *C. albicans*' hyphal development and subsequent biofilm maturation without affecting the growth or viability of planktonic cells. Both compounds, therefore, have potential as adjuvants for the treatment of biofilm-related resistant infections (Jackson *et al.*, 2015). In addition, the effect of acetylcholine (ACh) against *C. albicans* has been tested in a *Galleria mellonella* infection model. The data suggest that ACh is not fungicidal at the concentrations used in the study. Instead, it can inhibit the ability of *C. albicans* to form biofilms *in vitro* besides downregulating the expression of *C. albicans* biofilm-associated genes *in vivo* (Rajendran *et al.*, 2015). Silva *et al*. (2017) also showed antivirulence properties of a natural compound - myricetin - against *Staphylococcus aureus* without inhibiting bacterial growth.

Selective antibiofilm activity

To evaluate the selective antibiofilm activity of SG extract, a biofilm eradication test was made against *C. albicans* biofilms. No inhibition of preformed biofilm was observed (data not shown). The distinct morphology of both species studied may explain this contrast. Biofilm formation occurs in several phases: adherence to the surface, cell proliferation, extracellular matrix accumulation and dispersion. However, since *C. albicans* can transition from yeast to hyphae, filamentation occurs after attachment and the cells can continue growing into filamentous hyphal forms (Talapko *et al.*, 2021). *C. neoformans'* biofilm formation, on the other hand, is initiated through the local release of capsular polysaccharide by attached cryptococcal cells, which creates an exopolymeric matrix (Martinez, Casadevall, 2005), in a flower-like structure (Lopes *et al.,* 2017).

Likewise, Da *et al*. (2019) presented Wogonin, a component obtained from the *Scutellaria* root extract, with antifungal activity towards filamentous fungus,

Trichophyton rubrum, *Trichophyton mentagrophytes*, and *Aspergillus fumigatus*, except *C. albicans*. According to the authors, the differences in cell wall chemical composition and biosynthesis between *C. albicans* and dermatophytes might be the cause of this selectivity. In agreement, *C. neoformans* has different chemical compounds in the cell wall, including the presence of exopolysaccharide capsule, melanin, chitosan and alpha-1,3 glucans (Garcia-Rubio *et al.,* 2020). Therefore, this might also explain the selective difference between *C. neoformans* and *C. albicans* biofilms. However, other studies are needed to confirm this hypothesis.

CONCLUSIONS

Our search for compounds that affect biofilm integrity resulted in the discovery of a possible candidate as a virulence factor inhibitor, acting on the preformed biofilm. In spite of the limited and slow development of antifungal agents in the last decades and the broad use of synthetic compounds, natural products still play an important role as it becomes more difficult to develop efficient drugs and they represent an underexplored source of biochemical targets. This illustrates the pivotal importance of natural product research in the development of translational medicines.

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