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Original Research
Infection Control/
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Anadenanthera Colubrina vell Brenan: anti-*Candida* and antibiofilm activities, toxicity and therapeutical action

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Abstract: We evaluated the antifungal and antibiofilm potential of the hydroalcoholic extract of bark from *Anadenanthera colubrina* (vell.) Brenan, known as Angico, against *Candida* spp. Antifungal activity was evaluated using the microdilution technique through the Minimum Inhibitory and Fungicide Concentrations (MIC and MFC). The antibiofilm potential was tested in mature biofilms formed by *Candida* species and analyzed through the counting of CFU/mL and scanning electron micrograph (SEM). *In vivo* toxicity and therapeutic action was evaluated in the *Galleria mellonella* model. The treatment with the extract, in low doses, was able to reduce the growth of planktonic cells of *Candida* species. MIC values range between 19.5 and 39 µg/mL and MFC values range between 79 and 625 µg/mL. In addition was able to reduce the number of CFU/mL in biofilms and to cause structural alteration and cellular destruction, observed via SEM. *A. colubrina* showed low toxicity in the *in vivo* assay, having not affected the viability of the larvae at doses below 100mg/kg and high potential in the treatment of *C. albicans* infection. Considering its high antifungal potential, its low toxicity and potential to treatment of infections in *in vivo* model, *A. colubrina* extract is a strong candidate for development of a new agent for the treatment of oral candidiasis.

Keywords: Infection Control; Biofilms; *Candida*; Phytotherapy.

Introduction

Candidiasis is a fungal infection caused by *Candida* species and *Candida albicans* (*C. albicans*) is the variety most associated with it. Changes of normal conditions in the oral environment can favor the growth of *Candida* with a change of its commensal state into pathogenic. Most of the time, these changes are related to immunological imbalances in the host.[1,2,3] Candidiasis may be local (mucocutaneous) or systemic, and present in acute, chronic and in association with other types of lesions.[4] *C. albicans* is part of the oral microbiota and is isolated in approximately 80% of healthy individuals.[2,3] Candidiasis is one of the most frequent opportunistic infections in immunocompromised patients, especially Acquired Immunodeficiency Syndrome (AIDS) and cancer patients,[5] with a prevalence of 9 to 31% and 20%, respectively. In addition, it occurs in about

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5% to 7% of children in the first years of life and is quite frequent in users of the total dental prosthesis.[6,7]

In relation to the treatment of oral candidiasis, it is possible to mention drugs such as azole derivatives (fluconazole, miconazole and itraconazole) and polyene (nystatin), which despite having good therapeutic action, may exhibit undesirable, mainly dose-related toxicity.[8,9] The dose-dependent toxicity reported to antifungals is only one of the most reported limitations to these drugs. Long-term therapy may lead to more serious risks, including recurrent drug interactions, organic dysfunction, skin reactions and malignancies.[10,11] Thus, an ideal antifungal agent should have no toxicity or reduced toxicity to human cells.[12] From this perspective, medicinal plants represent a vast source of active compounds, with potential for the development of new and effective pharmaceutical forms.

Anadenanthera colubrina (vell) Brenan (*A. colubrina*), popularly known as Angico, represents a plant widely used in folk medicine for the treatment of inflammation, respiratory problems related to infection (cough, influenza, and bronchitis), diarrhea and toothache.[13,14] It is considered as a plant of therapeutic potential, but still little explored from the pharmacological point of view.[15] In the world, the species occurs to the South of the line of the Equator, and in Brazil, from the Northeastern to the Southeastern regions. It is a woody species typical of the Caatinga biome.[16] Caatinga is a biome that exists only in Brazil, composed of xenophilic vegetation adapted to the scarcity of water. Despite its exclusivity, it is currently the Brazilian biome with the smallest total area protected by conservation units compared to other biomes.[17] Caatinga has many species at risk of extinction and the scientific contribution evidencing its natural riches will assist in the preservation processes of this rich biome that is being invaded by cattle breeding and unsustainable extractivism.

Recent studies have shown that *A. colubrina* has promising therapeutic properties, such as antifungal,[18,19,20] anti-inflammatory and antinociceptive activities[21], the last two having been confirmed in an animal model. In a previous study, our group verified that *A. colubrina* has a strong antifungal potential against *C. albicans*, inhibiting biofilm formation with changes in its morphology.[19] These results have signaled this plant as a possible source of active compounds for the development of antifungal formulations for the treatment of oral candidiasis. In this study, we evaluate antifungal and antibiofilm action in different species of the genus *Candida*, as well as the toxicity level of the *A. colubrina* extract, in order for it to be considered as an alternative for treatment of oral candidiasis.

Methodology

Preparation of Hydroalcoholic extract

Anadenanthera colubrina (vell) Brenan was collected in September in the Queimadas city, geographically located in the Borborema Plateau, Paraíba State, Northeastern region of Brazil (7º 22' 25"S, 35º 59' 32" W), which is part of the caatinga biome. A voucher specimen was deposited in the Manuel de Arruda Câmara Herbarium of the Paraíba State University under nº 667/ACAM. The bark was dehydrated, moistened and immersed in 80% ethanol (100 g/250 mL) for 48h at room temperature. Then it was filtered and the residues were immersed in 80% ethyl alcohol. The three final extraction phases were concentrated in a rotary evaporator, freeze-dried and stored at -20ºC.

Microorganisms and susceptibilidy assay

Microorganisms used: *Candida albicans* MYA 2876, *Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019 e *Candida krusei* ATCC 6258. Two clinical isolates of *Candida albicans*, named 1 and 2, removed from oral candidiasis lesions of patients from the school clinic of the State University of Paraíba were also included, under the approval of the institution's ethics committee, under number: 51779315.7.0000.5187 (Dec 15th, 2015). The antimicrobial activity was identified via microdilution in broth, determining the Minimum Inhibitory Concentration (MIC) (CLSI, M27-A2)[22] and the Minimum Fungicidal Concentration (MFC). The test was performed on 96 well microplates containing using RPMI 1640 medium (Angus Buffers and Biochemicals, Niagara Falls, USA).

The extract was diluted in 20% DMSO and later in RMPI. The final DMSO concentration at wel was <1% which would not affect the viability of the yeasts. Serial dilutions were made, obtaining concentrations of 5000 and 4.882 µg/mL. Nystatin (Sigma-Aldrich) was used as positive control. An inoculum of $5x10^3$ cells/mL was considered, which is the well that fell to $2.5x10^3$ cells/mL, standardized in spectrophotometer. The plates were incubated at 37°C for 24 h. MIC was defined via visual method initially considering the change in color of the culture medium from pink to yellow. Then, 30 µL of resazurin 0.1% (Sigma-Aldrich, St. Louis, USA) was added to the wells. For determination of MFC, an aliquot (20 µL) of each well with concentrations equal to and greater than the MIC were seeded on a dextrose agar medium incubated at 37°C for 24 h.

Antibiofilm activity assay

Candida biofilm formation was induced in 96-well microplates. A inoculum (between 10^7 and 10^8 cells/mL) in a Yeast Nitrogen Base (YNB) culture medium (Sigma-Aldrich) supplemented with glucose, were distributed in each well and incubated at 37 °C for 24 h.[23] After this time, the supernatant was discarded and the biofilm washed 3 times with a 0.9% NaCl solution. The treatment was performed with *A. colubrina* extract at MIC-based concentrations (100 and 500 x MIC). The extract was diluted in 20% dimethylsulfoxide (DMSO) and then in a RPMI 1640 culture medium. After a dilution the concentration of DMSO dropped to less than 1% in the wells. Nystatin at concentration of 100 x MIC for each strain was used as positive control. The biofilm was treated for 24 h at 37°C. It was then removed, diluted in a 0.9% NaCl solution and seeded in a Petri dish containing Saboraud dextrose agar medium and incubated at 37 °C for 24 h for counting CFU/mL.

In vivo toxicity and treatment of systemic infection in Galleria mellonella (G. mellonella)

An *in vivo* model of *G. mellonella* was used to evaluate the acute toxicity of *A. colubrina* extract in a manner similar to that previously described, with some modifications.[24,25] Different doses of the extract were tested in increasing order to obtain LD 50. A random selection of 10 healthy-looking larvae weighing between 0.2 and 0.3 g was made for each group. A volume of 5 µL of the extract or control (20% v/v, DMSO) was injected into the left proleg of the hemocele using a 25 µL Hamilton Syringe (Hamilton, Reno, NV). We evaluate the therapeutic potential of *A. colubrina* extract to treatment of systemic *C. albicans* infection. A culture of *C. albicans* ATCC 90028 was grown overnight in BHI broth. The inoculum was diluted in 0.9% NaCl and standardized to $5x10^7$ cells in a spectrophotometer. Four groups of 10 larvae received 10 µl of inoculum injected in the last left proleg of each larva and then in the last right proleg the following treatments, after one hour: group 1 (*A. colubrina* 50x MIC - 975 µg/mL), grupo 2 (*A. colubrina* 100x MIC – 1,950 µg/mL) group 3 (A. (Amphotericin 100x MIC – 50 µg/mL) and Group 4 (0.9% NaCl solution).The larvae were incubated at 30°C and their survival was evaluated every 9 h until the maximum time of 48 h. The larvae with a high degree of melanization and lack of movement when touched were counted as dead.

Analysis of cell morphology

The biofilms were formed (Lab-Tek, Nunc, Naperville, USA), using the method described in item *Antibiofilm activity assay*. Thus we evaluated the following samples: biofilm of *C. albicans* 90028 treated with *A. colubrina* 100xMIC, treated with *A. colubrina* 500xMIC and negative control without treatment. They were treated with the extract at concentrations 100 x MIC and 500 x MIC for 24 h. The cells were fixed in 3% glutaraldehyde at room temperature for 12 h. The biofilm was then dehydrated in increasing concentrations of ethanol, metallized with gold and examined through Scanning Electron Microscopy (SEM) (SEM JEOL JSM 5600LV, JEOL Tokyo, Japan).

Statistical analysis

The assays were performed in triplicate and in three independent experiments. The statistical analyses for evaluation of biofilm reduction were applied at the follow conditions: the type of variable for UFC

quantification is quantitative continuous numerical; data are parametrical with normal distribution by means of Lilliefors test (BioEstat software) for all groups non-significant (ns) with $p < 0.05$ (α value). Subsequently, an analysis of variance and post-hoc analysis were used in accordance with the Tukey test, with a significance level of 5 % ($\alpha < 0.05$). For the *G. mellonella* model, Kaplan–Meier killing curves were plotted on GraphPad Prism 5.0 and estimations of differences in survival were compared using the log-rank test.

Results

A. colubrina extract inhibited the growth of all *Candida* species tested at 19.5 – 5000 µg/mL range concentrations (Table), and *C. albicans* exhibited the lowest values of MIC (19.5 µg/mL). However, the extract was fungicidal and in low concentrations only for the species *C. parapsilosis* and *C. krusei*, while for the other species the result was negligible.

A. colubrina extract showed activity against biofilm formed by species of *Candida*. The best results were observed in the species *C. parapsilosis* and *C. krusei* (biofilm treated with the extract at a concentration of 500xMIC = 19,500 µg/mL). Only for these species and concentrations the extract could totally kill all microorganisms. However, all biofilms species underwent alteration and decrease in the number of CFU/mL when treated with *A. colubrina* extract (Figure 1).

The microorganisms that exhibited a reduction in the quantity of viable cells, after treatment with *A. colubrina*, were submitted to microscopic analysis via SEM. As seen in Figure 2, the results were similar for the recorded effect typically found in *C. albicans* ATCC 90028 (Figure 2). The SEM showed the appearance of biofilm structural alterations such as cell destruction, holes and channels and decrease of the hyphatic form in relation to the control (Figure 2A to 2F).

About the toxicity of the *A. colubrina* extract, in the *G. mellonella in vivo* model, low acute systemic toxicity was observed. In the antimicrobial therapeutic doses, the extract did not affect the survival of the larvae until the 72 h period ($p > 0.05$). Only at high doses up to 5 g/kg (LD_{50}), the larvae died in the range from 50%–100% ($LD_{100} = 10$ g/kg) ($p < 0.0001$). The vehicle used to solubilize the extract (DMSO 1%), as well as doses of the extract below 100 mg/kg, did not affect the viability of the larvae ($p > 0.05$) (Figure 3a). Nystatin showed toxicity at concentrations above 1 g/kg. The nystatin concentration required to kill 50% of the larvae (LD_{50}) was 2.5 g/kg (Figure 3b).

A. colubrina extract at 100xMIC concentration was able to treat and protect 20% of the larvae. This value was similar to that of the systemic antifungal amphotericin, at 100xMIC concentration. *A. colubrina* 50x MIC prevented the death of 10% of the group. The group treated with the dilution vehicle had 100% death of the sample and the group without infection

Table. Distribution of minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of *A. colubrina* extract, for the *Candida* species.

Microorganisms	A. colubrina		Nystatin	
	MIC|MFC		MIC|MFC	
	µg|mL	Ratio	µg|mL	Ratio
Candida albicans MYA 2876	19.5|625	32	1|2	2
Candida albicans ATCC 90028	19.5|625	32	1|2	2
Candida albicans (clinical strain 1)	19.5|625	32	3|6	2
Candida albicans (clinical strain 2)	39|625	16	3|6	2
Candida parapsilosis ATCC 22019	39|79	2	1|2	2
Candida krusei ATCC 6258	39|39	1	1|2	2

Fungicide (MFC/MIC< 4) or fungistatic (MFC/MIC > 4).[23]

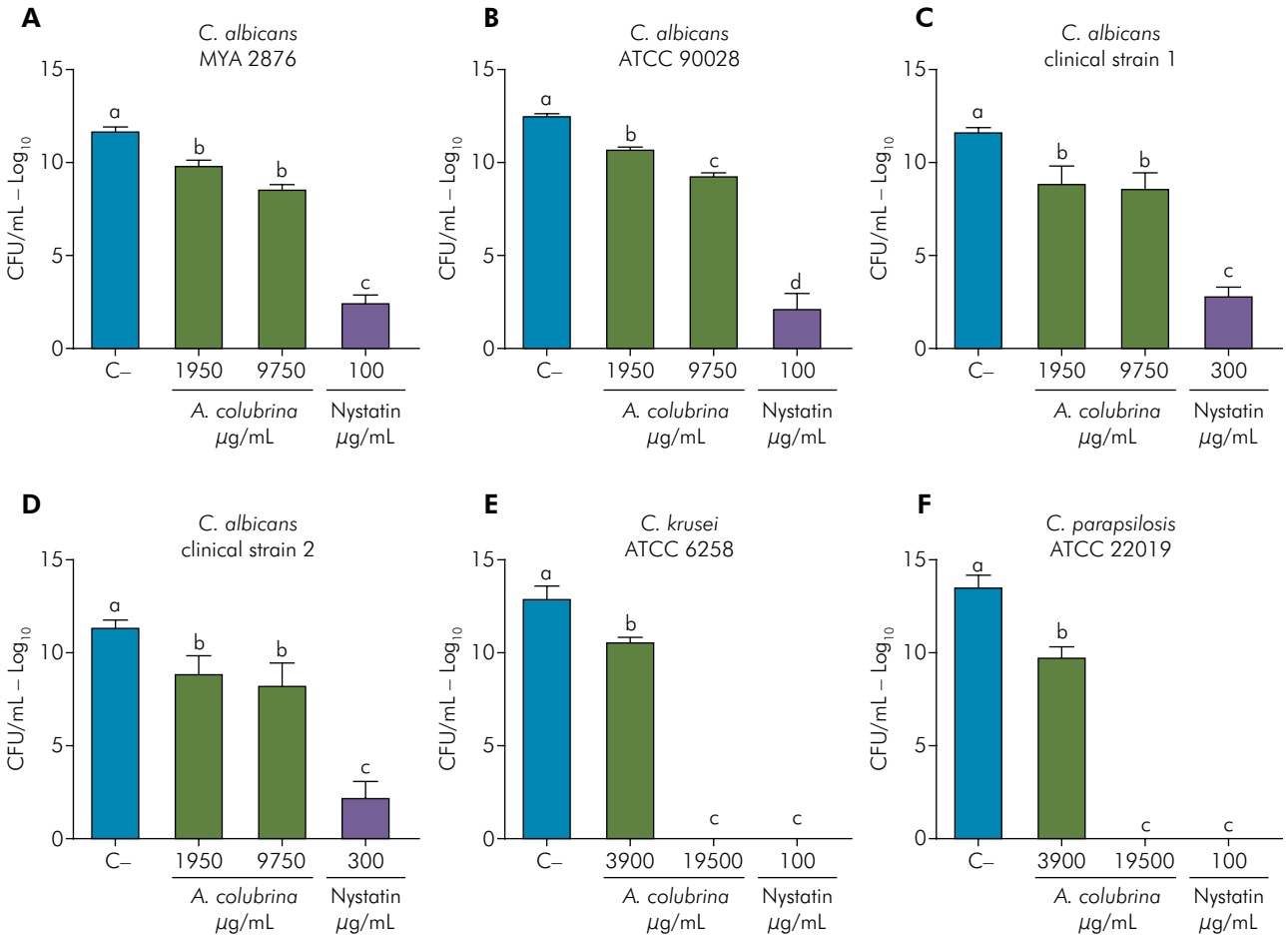

Figure 1. Antibiofilm activity of the *A. colubrina* extract evidencing a discrete reduction of cell viability (- Log) in *C. albicans* MYA 2876 (a), *C. albicans* ATCC 90028 (b) and *C. albicans* clinical strains 1 (c) and 2 (d), and reduction of cell viability in 100% for *C. krusei* (500x MIC) (e) and *C. parapsilosis* (500x MIC) (f), after treatment ($p < 0.005$, ANOVA with Tukey's pos-test).

and that received only the vehicle presented 100% viability (Figure 4).

Discussion

A. columbirna, a plant known as Angico and used by the people of the Brazilian caatinga region for the treatment of respiratory problems and inflammation, was previously flagged as a plant with strong growth-inhibitory capacity for the *C. albicans* specie.[19,20] We show here that this ability to inhibit growth also extends to other species of *Candida* non-*albicans*, such as *C. parapsilosis* and *C. krusei* (Table).

We found MIC values for *C. albicans*, which differ from those previously found[19,20] and our current results are more relevant. This difference may be related to the use of different strains between studies. Another factor that may have influenced this difference is the dilution vehicle of the extract. Previous studies used 40% alcohol and in this study, we used 1% DMSO. It is possible that DMSO may have solubilized the components of the extract better, potentializing its action. Here, we showed very low MIC values of *A. colubrina* (Table) against *C. albicans* strains (19 µg/mL for stock strains and one clinical strain and 39 µg/mL for another clinical strain). In addition, equally low values were found for non-*albicans* species, considered as emerging (39 µg/mL for *C. parapsilosis* and *C. krusei*). It has been considered that MIC values below 100 µg/mL are deemed as very promising for a phytocomposite.[26]

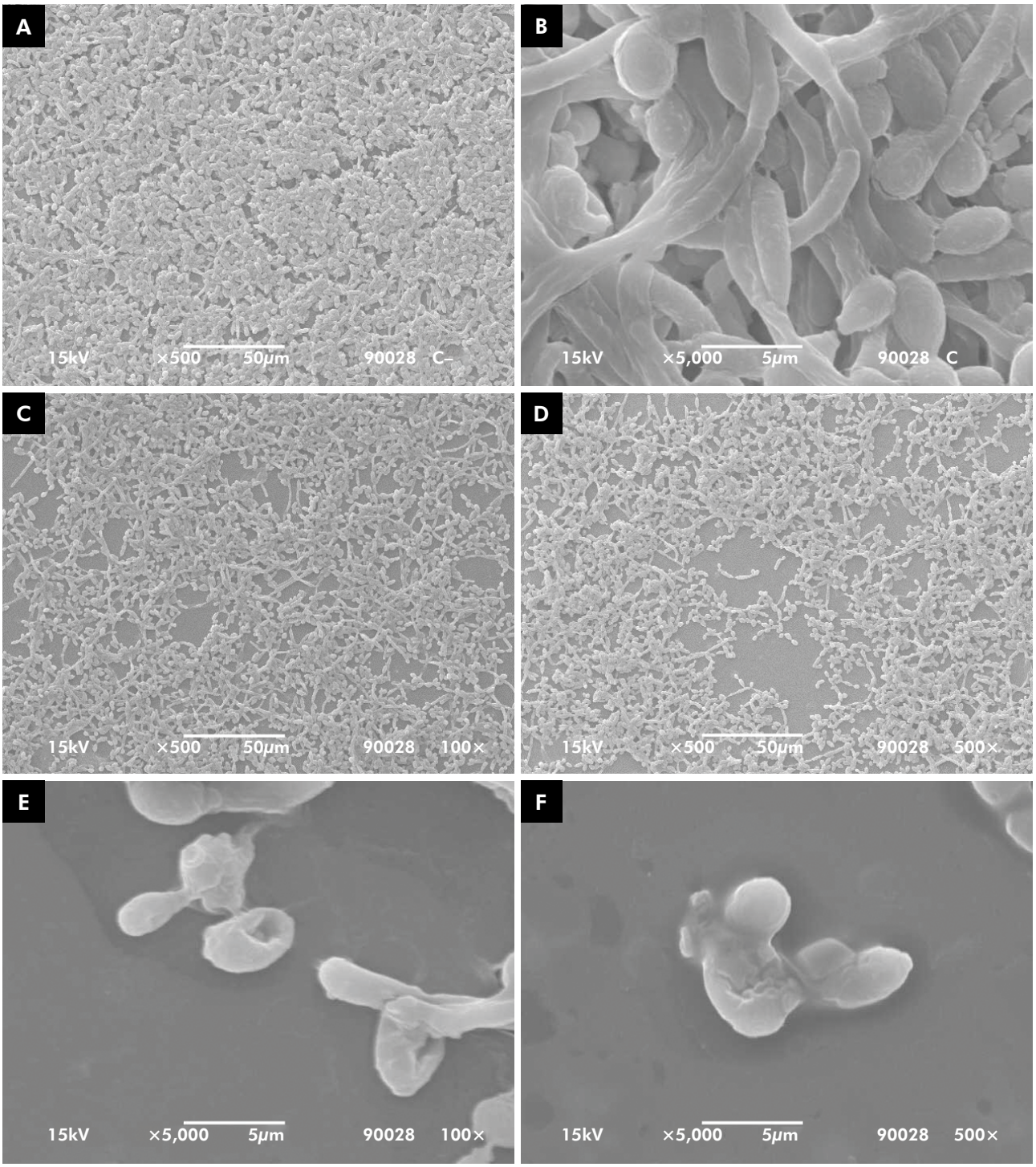

Figure 2. SEM of C. albicans ATCC 90028 showing the cellular morphology. In the first column: untreated biofilm (a), treated with A. colubrina 100x MIC = 1950 ug/mL (c) and biofilm treated with A. colubrina 500x MIC = 9750 ug/mL (e), in a 500x view. In the second column, the same findings may be observed at a 5,000x view .

C. albicans is the species associated with infections and also the most studied, and its clinical strains are more resistant than the corresponding collection.[27] For comparison purposes, we also evaluated the effect of *A. colubrina* on two clinical strains of *C. albicans;* in this case, one strain had the same result as that of

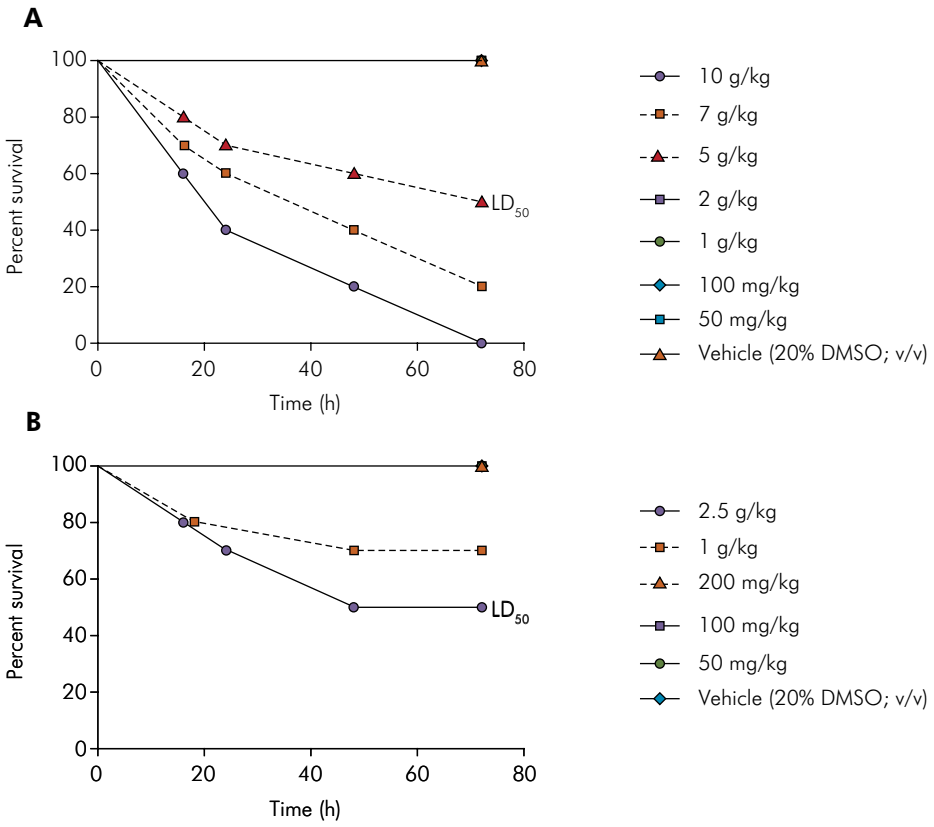

Figure 3. *In vivo* systemic toxicity of the *A.colubrina,* increasing doses from 50 mg/kg to 10 g/kg (A); and increasing nystatin doses from 5 mg/kg to 2.5 g/kg (B), using a *G. mellonella* modelo ($p > 0.05$, log-rank test).

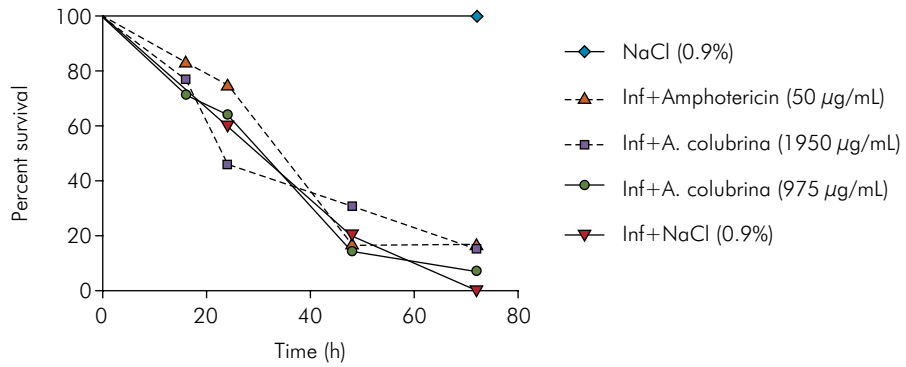

Figure 4. Treatment of systemic infection by *C. albicans* with amphotericin 100xMIC (50 μg/mL), *A. colubrina* 50xMIC (975 μg/mL), *A. colubrina* 100xMIC (1,950 μg/mL) e vehicle of dilution (NaCl 0.9%) in *G. mellonella* model ($p > 0.05$, log-rank test).

the collection while the other was a dilution above. This data shows that the *A. colubrina* extract is equally effective for inhibiting the growth of wild strains of *Candida* despite its greater virulence, evidencing the effectiveness of the compound and suggesting a potential for clinical efficacy.

The ability of the *Candida* species to develop as a biofilm reflects clinical consequences because microorganisms show resistance to antifungal therapy at usual therapeutic doses. Thus, biofilm formation is considered one of the main virulence factors associated with *C. albicans*, which contributes to increased

mortality in patients immunocompromised with candidiasis.[28,29] *A. colubrina* at concentrations higher than MIC can affect the biofilm of all *Candida* species. *C. albicans*, considered the most present specie in the oral cavity of humans,[30] was the most sensitive in the planktonic form and the least sensitive in the biofilm form. Indeed, *C. albicans* is reported to form quantitatively larger and qualitatively more complex biofilms than other *Candida* species.[31] However, there was a significant reduction in the number of CFU / mL in *C. albicans* biofilm treated with *A. colubrina*.

Although *C. albicans* is described as the main *Candida* specie related to candidiasis, non-*albicans* species demonstrate virulence factors previously attributed to *C. albicans*, reflecting its increase in antifungal resistance.[32] In addition, species of *Candida* non-*albicans* are increasingly reported in cases of fungemia.[33] Thus, the discovery of new antifungals with effects also on species of *Candida* non-*albicans* is necessary. The *A. colubrina* extract showed a promising ability to alter the biofilm of *Candida* non-*albicans*. The extract was able to cause severe decrease in cellular viability for the *C. krusei* specie at a 100x MIC concentration and 100% decrease at a 500x MIC concentration. The same occurred with *C. parapsilosis*. In view of that, *C. krusei* have an intrinsic resistance to fluconazole[34] and *C. parapsilosis* showed high MIC values for echinocandins, a recent antifungal class,[35] whereas *A. colubrina* shows itself to be a potent alternative source of active principles capable of preventing the growth of these species in low concentrations, as well as destroying their biofilms in higher concentrations. Concentrations 100 and 500 x the MIC may appear high, but, it is still below the concentration that was toxic in the in vivo model of *G. mellonella*.

Our findings have shown an antibiofilm activity superior to that shown by other studies with natural products.[36,37] Although we used higher concentrations, we proved that they are well below the dose considered toxic in our model in vivo.

In vitro studies have the limitation of not accurately reflecting the conditions of the oral environment, such as the presence of saliva, the host's immune component, and multispecies biofilms with different species of *Candida* and bacteria. However, the extract of *A. colubrina* was able to alter the integrity of the biofilms, significantly reducing the number of CFU/mL in all the concentrations tested for the biofilms of the *Candida* species analyzed in this study. This data shows that the extract has a remarkable antibiofilm activity *in vitro*, in biofilm formed by species of the genus *Candida*. There is evidence that most of the conditions resulting from fungal infections are associated with their ability to form biofilm.[38] Previously, we evaluate the phytochemical composition of the *A. colubrina* extract. The results showed a high total polyphenol content (53.18% gallic acid equivalents). Tannin content was 8.77% catechin equivalents and flavonoid content was 0.28% quercetin equivalents.[19] The biological activities evaluated here appear to be related to these compounds.

The SEM showed that the biofilm formed by *C. albicans* is affected after treatment with *A. colubrina*. Visually, there is little difference in relation to the two concentrations tested (100 and 500x MIC). This data is similar to that found in the CFU/mL count of this same strain, where the difference between the two concentrations is discrete. However, in relation to the control, a significant alteration of the biofilm is observed when treated with *A. colubrina* extract. It is possible to observe the appearance of structural alterations such as the presence of holes and channels in the biofilm, which may have been provoked by the cellular destruction in the regions where the extract was able to penetrate. In addition, hyphae decreased, indicating a possible decrease in virulence since the formation of hyphae is related to tissue invasion.

Data from the *in vivo G. mellonella* toxicity model suggest that *A. colubrina* shows a promising safety index. Only high doses of the *A. colubrina* extract (5, 7 and 10 g/kg) affected the viability of the larvae, higher than those tested in the antibiofilm assay. *G. mellonella* has become a widely used method for the primary evaluation of substance toxicity, drug efficacy and virulence of microorganisms.[39]

To avaliate the therapeutic potential of *A. colubrina* extract we conduced a *in vivo* assay to treatment of *Candida* infection in *G. mellonella*. the extract of *A. colubrina* was able to treat infection in larvae showing a similar result to amphotericin. As shown by the survival curve, *A. colubrina* not only prevented the death of 20% of the sample but provided the

most life time for the larvae that died in relation to amphotericin. For example, at 24 h and 48 h the group treated with *A. colubrina* had 50% and 30% of live larvae, respectively, while the group treated with amphotericin at the time 24 h had only 20% of live larvae. At the end of the experiment, both treatments kept 20% of the live larvae. Thus, considering that *A. colubrina* is a crude extract and amphotericin a monodrug, we can consider that *A. colubrina* has a high therapeutic potential in the treatment of *C. albicans* infection. Studies with larger animals need to be performed to confirm this initial data. However, our data serve as a basis for reducing the number of animals needed in future tests, since *G. mellonella* is an effective alternative model for this purpose.[39]

Thus, considering a possible topical use of the extract and its low toxicity in this model *in vivo*, these data support the idea that the extract of *A. colubrina* may have a high safety potential. It is noticeable that the crude extract of this plant has promising therapeutic potential. However, it is still little explored pharmacologically. Issues such as the mechanism of action, posology and therapeutic indications still need to be better studied.[15]

Based on the *in vivo* toxicity test results, the concentrations tested in the biofilm assays were extrapolated to 100 and 500x MIC. This is the first study showing the potential for systemic toxicity *in vivo* of the extract of *Anadenanthera colubrina* vell. Brenan and its antifungal activity and antibiofilm on different *Candida* species. It is important to remember that treatment with a monodrug, such as antifungal agents, is exposure to a specific molecule that may be in high concentrations (causing high dose-dependent toxicity). Although natural products also have dose-dependent toxicity, there is a big difference. In natural products the active principles are in low concentrations within the crude extract and may even be acting synergistically, as suggested by the work of Lima et al.,[19] who did not verify improvement of the action after fractionation of the *A. colubrina* extract. Small amounts of different molecules acting synergistically suggest a product with lower potential for toxicity and other adverse effects.

Ethnopharmacology mentions the therapeutic potential of *A. colubrina* and its use by the population for the treatment of conditions that affect health, such as inflammation, bronchitis, influenza, cough, diarrhea, and toothache.[13,14] Considering the anti-*Candida* potential and toxicity results, showed in our study, there may be a large margin of safety for the therapeutic use of this extract, especially in relation to oral candidiasis, since most cases of oral candidiasis are resolved with topical application of antifungal agents.[9]

Thus, considering the high anti-*Candida* potential and low toxicity potential of the *A. colubrina* extract, our findings stimulate further studies to confirm the hypothesis that the extract has efficacy in the treatment of candidiasis and large safety margin. The elucidation of the safety and therapeutic potential of this plant will add scientific value, ensuring its use in an effective and safe way for the treatment of oral candidiasis. Clinical studies with larger animals and humans are needed to define information such as posology and mode of use.

Conclusion

The results show that the extract of the shell of *A. colubrina* has antifungal and antibiofilm properties against different *Candida* species, low systemic toxicity *in vivo* and high potential in the treatment of *C. albicans* infection. This data which shows a potential safety for its possible use. In view of the increased resistance of the *Candida* species to antifungal agents currently used for the treatment of candidiasis, and the increasing appearance of fungemia caused by *albicans* and non-*albicans* species, *A. colubrina*, can be considered as a promising source of active principles for the treatment of candidiasis. Their compounds can be used in the development of a phytotherapic treatment or of allopathic drugs. Clinical studies are necessary to confirm its efficacy and safety in the treatment of oral candidiasis.

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