IN VITRO ANTIFUNGAL ACTIVITY AND CYTOTOXICITY SCREENING OF DRY CRUDE EXTRACTS FROM BRAZILIAN AMAZONIA PLANTS

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Abstract

Background: Antifungal multidrug resistance has been reported worldwide and has stimulated investigations of plant species for the treatment of candidiasis. In particular, in vitro antifungal activities and cytotoxicity of dry extracts from Ceasalpinia ferrea (tul.) Martius, Brosimum acutifolium Huber, and Salacia impressifolia (Miers) A.C. Smith were evaluated.

Materials and Methods: Minimum inhibitory concentrations (MIC) and minimum fungicide (MFC) values were established according to the protocol M27-A2 of the Clinical and Laboratory Standards Institute (CLSI). Subsequent evaluations were performed using strains of Candida albicans from the American Type Culture Collection (ATCC) 10231, clinical isolated Candida albicans, Candida glabrata (CCT) 0728, Candida krusei (FTI) CCT 1517, and Candida guilliermondii (CCT) 1890. Morphological changes were evaluated using scanning electron microscopy (SEM), and cytotoxicity was evaluated in murine L929 fibroblast cells after treatment with plant extracts.

Results: MIC values indicated antifungal potential of all three extracts against the main fungi that causes candidiasis.

Conclusion: In particular, C. ferrea showed promising antimicrobial potential against all strains. Hence, future studies are warranted to investigate pharmacologically active compounds from this extract that could be used as prototypes for drug development and/or as a source of raw pharmaceutical materials for the treatment of candidiasis.

Keywords: Candidiasis; Natural products; Toxicity

Introduction

Candidiasis is one of the most prevalent and important fungal infections of the oral cavity and is caused by various Candida species, particularly Candida albicans. However, 50% of these infections are caused by non-albicans species, such as Candida tropicalis, Candida parapsilosis, Candida glabrata, Candida guilliermondii, and Candida krusei (Forastiero et al., 2015). Bensadoun, Patton, Lalla and Epstein (2011) described oropharyngeal candidiasis as the principal cause of morbidity in patients with malignancies in hospitals, and if not treated correctly, these infections can reach the bloodstream and the urinary tract. Moreover, these infections have been observed in surgical sites, producing challenging conditions for tertiary care globally. The incidence of candidemia in Brazilian public hospitals is about 2.5 cases per 1,000 admissions (Colombo et al., 2013), and is considered the most common manifestation of invasive candidiasis. However, more than 30% of blood cultures are negative, and Candida species are almost never considered contagious (Kauffman, 2016). Nonetheless, candidiasis is considered a significant public health problem in underdeveloped areas, and a dangerous infection in developing countries (Nucci et al., 2013).
Pharmacological treatments for *Candida* spp. infections include polyenes, azoles, and their derivatives (Mangueria, Mangueria and Melo Diniz, 2010). However, these agents lack specificity for *Candida* species and their efficacy varies between fungal species, hosts, and therapies (Arendrup, 2013). In addition, microorganisms of the *Candida* genus have generated resistance to a variety of antifungal drugs, leading to poor treatment effects and increasing disease severity. Hence, studies of new pharmacologically active compounds for the treatment of fungal conditions are warranted (Morais-Braga et al., 2016).

Innovations of natural product research remain scarce in developing countries. However, numbers of studies of plant species have increased exponentially in recent decades, and many of these have been translated into novel technologies and prospective treatments (Simonetti et al., 2016). Although the herbal medicines market accounts for less than 5% of all marketed medicines (Dutra et al. 2016), Brazil has considerable potential for the “green market,” which follows an average annual growth of around 8%–25%, well above the 4% increase in marketing of synthetic products (Febrafarma, 2007). Hence, increase in the global demand for green medicinal products and natural therapies, with fewer adverse effects is comparable to that of synthetic compounds in existing formulations (Dutra et al., 2016).

Brazilian biodiversity is considered the largest in the world, and is distributed in biomes including the Amazon, Caatinga, Pantanal, Cerrado, Pampas and Atlantic Forest (Araújo et al., 2014; Instituto Brasileiro de Geografia e Estatística [IBGE], 2016). Many plant species from Brazilian biomes are used as traditional medicines and many have demonstrated antimicrobial (Kuete et al., 2011; Oliveira et al.; 2015; Akca et al., 2016) and antifungal properties (Mollashahi et al., 2015; Aghasadeh et al., 2016). Among these, *Salacia impressifolia* (Miers) A.C. Smith is popularly known as mirarurá (Smithsonian Institution, 2015), and *salacia* species present antidiabetic (Ripardo Filho et al., 2016), antimicrobial, antifungal, and antioxidant activities (Martins et al., 2016), and hepatoprotective, cytotoxic, anti-inflammatory, antiobesity, hypotensive, and antiulcerogenic effects (Prata, 2007), warranting their use in traditional medicines and as phytomedications. In addition, the Brazilian species *Caesalpinia ferrea* tul. Martius is popularly referred to as ironwood or jucá, and pharmaceutical applications of shells, seeds, roots, and fruits from this species are traditionally used to heal wounds and bruises (Oliveira et al., 2014a). Accordingly, anti-inflammatory (Oliveira et al., 2014b), antifungal (Bariani et al., 2012), antihistaminic, antiallergic, anticoagulant, and larvicidal activities (Cavalcante et al., 2009), and antiproliferative, cytoprotective, and antimutagenic effects have been shown (Silva et al., 2015). More, *Brosimum acutifolium* Huber, a genus widely known in the Amazon under various denominations (mururé, congona, mapuré-pagé, moruré) (Lima, Silva and Veiga-Júnior, 2013), stands out as a traditional medicine, with its roots, and stems, and efficacy in the treatment of rheumatism, muscular and bone pain, and as a diuretic, anti-allergic, immunomodulatory, antioxidant, provascular, antitumor, and antimicrobial agent (Lima, Silva and Veiga-Júnior, 2013).

Herein, we evaluated antifungal activities of dried extracts of *Salacia impressifolia* (Miers) A.C. Smith, *Caesalpinia ferrea* tul. Martius, and *Brosimum acutifolium* Huber against the predominant strains of the genus *Candida*, and the effect on the viability of mouse L929 fibroblast cells.

**Materials and Methods**

**Plant Species**

Peels of *Brosimum acutifolium* Huber, *Salacia impressifolia* (Miers) A.C. Smith, and seeds of *Caesalpinia ferrea* (Tul.) Martius were acquired at the “Ver-O-Peso” market in Belem, Pará, Brazil (Latitude 01270210 0S and Longitude 48300160 OW). Extracts were obtained by macerating 25-g specimens of specified plant parts in 70% (v/v) ethanol for 72 h in the dark. Extracts were filtered under low pressure (Rotavapor® R-210, Buchi, Switzerland), lyophilized (ALPHA 1-4 ID plus, Christ, Germany) under 1.8-mBar pressure at −14°C, and then maintained at room temperature in the dark, until use in *in vitro* assays.

**Fungal species**

Microbiological analyses were performed using standard *C. albicans*, *American Type Culture Collection* (ATCC) 10231; *C. glabrata* (Taniwaki, M.H.), *Collection of Tropical Cultures* (CCT) 0728; *C. krusei*, (FTI) CCT 1517; *C. guilliermondii* (CCT), 1890 from the Foundation André Tosello (Campinas, São Paulo, Brazil); and *C. albicans* from clinical specimens, which were a gift from the Laboratory Murilo Baldi of the academicals hospital of the Federal University of Juiz de Fora, Minas Gerais, Brazil.

**Antifungal activity**

MIC and MFC values were established according to the protocol M27-A2 of the Clinical and Laboratory Standards Institute [CLSI] (2002). Briefly, fungal suspensions were obtained from respective strains using sterile saline solution [0.9 % (v/v)] after 48 h of growth at 35 ± 2°C. Densities of suspensions were adjusted to 89%–90% transmittance in a spectrophotometer (Multiskan GO Microplate Spectrophotometer, Toronto, Ontario, Canada) at 530 nm. Samples were then diluted in buffered Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Aldrich Chemistry, Burlington, Massachusetts, USA) with 3(N-morpholino) propanesulfonic (MOPS) (JTBaker®, Phillipsburg, NJ, USA) to obtain 5–25 × 10^5 CFU and pH was adjusted to 7.0 ± 0.1 using 0.5-M sodium hydroxide.
Assays were performed in triplicate and plant extracts were diluted to 4.8–5,000 µg/mL in RPMI-1640 medium containing MOPS buffer and 2 µl of 70% (v/v) ethanol solution.

Sterile polypropylene microplates with 96 wells and level depths were purchased from Sarstedt AG and Co (Germany), and 100-µL aliquots of respective dilutions of vegetable extracts were added with 100 µL aliquots of standardized fungal suspensions. Control wells contained 100 µL of the same inoculated culture medium containing 2 µL of 70% ethanol and 98 µL of RPMI 1640 medium containing MOPS. The negative control contained 200 µL of medium. Amphotericin B (Cristália, Cristal Pharma, Contagem, MG, Brazil) and nystatin (Cavalieri handling Pharmacy, Juiz de Fora, MG, Brazil) were used as reference drugs at 0.0313–16.0 µg/mL.

After inoculation, microplates were incubated at 35 ± 2ºC for 48 h, and MIC values were established as the lowest concentration in which no fungal growth was observed. MFC values were determined using the microdilution method. Briefly, 10-µL aliquots were withdrawn from wells in which no growth was observed in the MIC procedure, were transferred to new wells containing 1 mL of Sabouraud dextrose broth (SDB), and were incubated at 35 ± 2ºC for 48 h. Subsequently, concentrations with no fungal growth were classified as MFC values.

### Scanning electron microscopy

Samples for SEM analyses were prepared from untreated fungal colonies (negative control) and from colonies exposed to reference drugs or dried extracts. Fungal suspensions were standardized at 89%–90% transmittance using a spectrophotometer at a fixed wavelength of 530 nm. All samples, containing dried extracts or reference drugs were diluted to their respective MIC values in MOPS buffered RPMI 1640 medium. Subsequently, fungal suspensions containing test-substances were added to microplates and were incubated at 35 ± 2ºC for 48 h. Samples were then washed in 0.1-M phosphate buffer, were centrifuged at 1,301 × g, and were fixed to glass cover slips with 2.5% (v/v) glutaraldehyde solution. Samples were then stored for 12 h at 8ºC prior to dehydration using an ethanol sequence of 50%, 70%, 90%, and then 100% over 15 min. Dehydrated samples were then adhered to coverslips using poli-d-lisina (Sigma®), were fixed in aluminum stubs that were metallized with a thin layer of gold (Balzers FL-9496/Furstentum Liechtenstein), and were analyzed using SEM with a JSM-5310, JEOL instrument at 25-kV power and a working distance of 17 nm. All electronmicrographs were captured in the Center for Scanning Electron Microscopy at the Museum National of the University Federal of Rio de Janeiro (UFRJ).

### Evaluation of cellular viability using MTT assays

Cell viability of L929 fibroblasts was evaluated after culture in DMEM (Nutricell®, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (SFB) (Invitrogen, CA, USA), 100-U/mL penicillin, 100-U/mL streptomycin, and 10-mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES) (Mosmann, 1983). Cells were cultured in sterile 96-well plates with level depths (Sarstedt, Germany) at a density of 5 × 10^4 cells/well and were then incubated in an oven at 37 ± 2ºC in an atmosphere containing 5% CO<sub>2</sub> for 48 h. Subsequently, culture media were replaced with sample solutions (reference drugs or dry extracts) at concentrations of 7.81–1,000 µg/mL. Plates were then incubated at 37 ± 2ºC in an atmosphere containing 5% CO<sub>2</sub> for 48 h. Controls were not inoculated and contained 2 µl of 70% (v/v) ethanol. After 48 h, culture media were removed and 100-µL aliquots of 10% [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT, 5 mg/mL) in DMEM were added to all wells. Plates were immediately incubated at 37 ± 2ºC in an atmosphere containing 5% CO<sub>2</sub> for 3 h. Finally, the resulting formazan crystals were dissolved in DMSO and absorbance was evaluated using a spectrophotometer (Multiskan GO, Thermo Scientific, USA) at 540 nm (Twente and Luscombe, 1987).

### Statistical analysis

Data are expressed as means ± standard errors of the mean (SEM) and are representative of five replicates. Differences were identified using analyses of variance (ANOVA) followed by Bonferroni’s test (Graphpad Prism versão 6 e IBM SPSS Statistics 21) and were considered significant when p < 0.05.

### Results and Discussion

#### Antifungal activity

Dry extracts were tested in the range of 4.8–5,000 µg/mL, and antifungal actions against the standard C. albicans strain (MIC = 9.7 µg/mL) were observed for C. ferrea (MFC = 1,250 µg/mL) and S. impressifolia (MFC = 2,500 µg/mL). Regarding non-albicans species, fungistatic actions of C. ferrea were observed against all strains with MIC values of 19 µg/mL for C. glabrata, 78 µg/mL for C. krusei, and 4.8 µg/mL for C. guilliermondii. Despite fungicidal the actions of S. impressifolia against all non-albicans strains, MFC values were considered high (2,500–5,000 µg/mL). MIC and MFC values are summarized in Table 1.

According to the classifications of antifungal activity employed by Simonetti et al. (2016) extracts with MIC < 100 µg/mL are considered promising antimicrobials, whereas those with MIC values of 100–500, 500–1,000, and >1,000 µg/mL are considered moderate, weak, and inactive against pathogens, respectively. Accordingly, the species S. impressifolia presented moderate activity against clinically isolated C. albicans (MIC, 312 µg/mL). Furthermore, dry
extracts of *C. ferrea* showed promising antimicrobial potential against all *albicans* and non-*albicans* species. For Mendes et al. (2011) there is no consensus on the level of acceptable inhibition by plant extracts when compared with reference drugs, likely reflecting general differences in antimicrobial actions of drugs between distinct microbial cultures (Kuete et al., 2011).

**Table 1: Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of reference drugs and dry extracts of the plant species of interest.**

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Nystatin</th>
<th>Amphotericin B</th>
<th><em>Salacia impressifolia</em> Miers Smith</th>
<th><em>Caesalpinia ferrea</em> [Tul] Martius</th>
<th><em>Brosimum acutifolium</em> Huber</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
</tr>
<tr>
<td><em>C. albicans</em> ATCC 10231</td>
<td>0.050</td>
<td>0.5</td>
<td>0.125</td>
<td>0.5</td>
<td>9.7</td>
</tr>
<tr>
<td><em>C. albicans</em> clinical isolated</td>
<td>0.0125</td>
<td>0.125</td>
<td>0.078</td>
<td>0.312</td>
<td>312</td>
</tr>
<tr>
<td><em>Candida glabrata</em> CCT 0728</td>
<td>0.1</td>
<td>0.4</td>
<td>0.25</td>
<td>0.5</td>
<td>5,000</td>
</tr>
<tr>
<td><em>Candida krusei</em> CCT 1517</td>
<td>0.4</td>
<td>0.4</td>
<td>2</td>
<td>2</td>
<td>2,500</td>
</tr>
<tr>
<td><em>Candida guilliermondii</em> CCT 1890</td>
<td>0.2</td>
<td>1.6</td>
<td>0.0312</td>
<td>0.0312</td>
<td>2,500</td>
</tr>
</tbody>
</table>

Results of MIC and MFC were expressed in µg/mL.

The widely used pharmacological treatments nystatin and fluconazole are still recommended as the first line of therapy for candidiasis (Mangueira, Mangueira and Melo Diniz, 2010). However, these agents are merely fungistatic against *candida spp.* (Bensadoun et al., 2011; Arendrup, 2013). Hence, the antifungal multiresistance that has been reported in several countries, especially for non-*albicans* species, has led scientists to determine patterns of susceptibility of this pathogen to conventional antifungal agents with the purpose of ensuring the most effective therapeutic strategy (Kuete et al., 2011; Bensadoun et al., 2011; Arendrup, 2013; Morais-Braga et al., 2016).

Furthermore, natural products have been evaluated alone or in combination with other drugs in the search for new components that can neutralize mechanisms of resistance in yeasts of the genus *Candida* (Morais-Braga et al., 2016).

*S. impressifolia* has varied botanical distribution and contains various secondary metabolites of interest (Prata, 2007; Ripardo-Filho et al., 2016; ). However, no previous reports demonstrate antifungal activities of *S. impressifolia* against various *Candida* strains. In contrast, antifungal activities of fruits, stem, and leaves of *Caesalpinia* species are widely disseminated in the literature (Freitas, 2012; Araújo et al., 2014; Oliveira et al., 2014a; Silva, 2015), although few studies have reported antifungal activities of dry *C. ferrea* seed extracts (Cavalheiro et al., 2009; Bariani et al., 2012).

Possible fungicidal actions of trypsin inhibitors in the seeds of this leguminous plant against phytopathogenic fungi show promising actions against natural fungicide, leading to decreased mycelial growth and sporulation (Bariani et al., 2012). In contrast, aqueous extracts of *C. ferrea* seeds did not inhibit the growth of *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium pallidoroseum*, *Mucor sp.*, *Neurospora sp.*, *Penicillium hurguei*, or *Phomopsis sp.* (Cavalheiro et al., 2009).

Scientific evidence of the actions of *C. ferrea* remain deficient, warranting studies of therapeutic effects at the cellular level, and studies of parts of the plant with specific activities (Oliveira et al. 2014a; Silva et al. 2015). Herein, we show that the effects of *B. acutifolium* against *candida* strains were similar to those described previously (Oliveira et al., 2015). In particular, antifungal activities of crude extracts from this plant species against *C. albicans* and *C. krusei* in disc diffusion tests led to average inhibition halos of 9.0 and 7.0, respectively, although no inhibition of *C. tropicalis* was observed (Brito-Costa et al. 2013). Previously, crude extracts of this species had an average MIC value of 64 µg/mL in 50% of studied microbial species, with a MIC of 32 µg/mL against the standard *C. albicans* strain (Kuete et al. 2011).

**Scanning electron microscopy (SEM)**

The electronmicrographs (Figure 1) showed morphological changes that were compatible with decreases and loss of viability of fungal cells after exposure to extracts of *S. impressifolia, C. ferrea, B. acutifolium*, and the reference drugs nystatin and amphotericin B. Exposures to these extracts and drugs caused similar changes in all yeasts, and resulted in dehydrated and compacted appearances of fungal cells. In particular, cell compression and adhesion, cell growth were frequently observed, and shear zones or fissures and areas of structural deformation with overflow of cellular material caused loss of the original morphology. These changes likely reflect cell membrane permeability to these drugs (Bensadoun et al., 2011; Arendrup, 2013).

Several authors suggest that the presence of triterpenes, catechins, sterols, and tannins in shells of *S. impressifolia* confer potential therapeutic actions (Ripardo Filho et al., 2016; Martins, Caneschi, Vieira, Barbosa and
Similarly, the presence of catechins (Araújo et al., 2014), terpenoids (Ximenes, 2009), polyphenols, hydroxy-phenols, or methoxylated compounds (Pereira et al., 2016) in C. ferrea may confer analgesic, anti-inflammatory, and antibiotic effects of the ensuing extracts.

Figure 1: Electromyographies of Candida albicans American Type Culture Collection (ATCC) 10231 (A), Candida glabrata (Taniwaki, M.H.) CCT 0728 (B), Candida guilliermondii CCT 1890 (C) and Candida krusei (FTI) CCT 1517 (D). Yeasts not exposed to pharmacological treatments (1); exposed to nystatin (2); to anphotericin B (3); to Salacia impressifolia Miers Smith (4); to Caesalpinia ferrea (Tul) Martius (5) and Brosimum acutifolium Huber (6) evidencing cell compression and adhesion in (A2;B2;B5;B6;D2 and D3); shear zones or fissures of hyphae (White index: A5; G6) and areas of structural deformation (White index: B3;D4;D5) with extravasation of cellular material (White index: C4;C5). Magnification: 5000x. Scale bar: 5µm.

Alternatively, ethanolic extracts of C. ferrea fruits contain tannins and phenols that can promote hemolysis by oxidizing hemoglobin (Almeida, Lima e Silva, Amorim, Maia and Albuquerque, 2005). Moreover, legume seeds are abundant in protein (10% overall), and the ensuing agglutination or precipitation of glycoconjugates reportedly leads to inhibition of cell growth and mitochondrial respiration (Ximenes, 2009; Bariani et al., 2012). Proteolytic enzymes from c. ferrea string bean lectins can also inhibit c. albicans growth, and have antifungal potential (Ximenes, 2009).

Other studies indicate that alkaloid and flavonoid phytoconstituents in B. acutifolium species are responsible for antifungal activity. Reported activities of these phytochemicals include blockade of free radical chain reactions, which donate oxygen atoms and cause cell lysis (Moraes, 2011; Kuete et al., 2011; Lima, Silva and Veiga-Júnior., 2013; Fonseca, 2014; Oliveira, 2015). Anti-inflammatory activities of this plant species were also shown using the isolated bas1 flavonoid (4'-hydroxy, 7, 8 - (2', 2''-dimethyl-pyran)-flavan) (Moraes, 2011).

Evaluation of cellular viability using MTT assays

The toxicity of dry extracts on the viability of mouse L929 fibroblast cells were investigated using MTT assays (Figure 2). These data revealed significant differences (p < 0.05) among the control (Dulbecco's Modified Eagle Medium - DMEM) and treatments with three plant species, with 80% inhibition of cell viability in the presence of higher concentrations (1,000; 500, and 250 µg/mL). However, at concentrations of 7.81–125 µg/mL, no cytotoxic effects were observed. According to Zieger (2007), lower required concentrations of extracts from plant species with cytotoxic and/or cytoprotective actions correspond with greater potential for the treatment of humans.

In the present study, C. ferrea, B. acutifolium and S. impressifolia extracts did not present cytotoxic effects (cell viability, 110.92%), at concentrations bellow of 250 µg/mL and/or 125 µg/mL. In agreement, Moraes (2011)
showed cytotoxic effects of \textit{B. acutifolium} extracts only at high concentrations (100 µM) in cell viability assay evaluated by the MTT in stimulated murine macrophages.

In the work of Oliveira and Marreiro (2014), the \textit{C. ferrea} sheath hydroalcoholic extract present in a formulation of the mouthwash, \textit{in vitro} citotoxicity assay, showed only 8% cell viability against murine fibroblasts. In another study, low cytotoxicity of bioactive lectins from \textit{C. ferrea} sheath hydroalcoholic extracts was demonstrated in tumor cells, suggesting biological activities and safety of these extracts (Freitas, 2012). Moreover, the crude extract of the seeds of \textit{C. ferrea} did not show acute toxicity in mice, even when administered at the maximum dose (0.3 mL/10 g body weight) (Cavalheiro et al., 2009). Numerous studies with plant species show its efficacy as a source of new drugs with therapeutic applications. In general, the indiscriminate use of plant preparations and products of unknown composition lead to health risks in the absence of rigid standards (Araújo et al., 2014; Oliveira et al., 2015).

Therefore, further studies of natural products with greater pharmacological activities, lower toxicity and lower production costs will lead to discoveries of new bioactive molecules, since few of them have been rigorously validated so far (Morais-Braga et al., 2016).
Conclusion

In conclusion, the present data demonstrate antifungal activities of three plant extracts. *C. ferrea* demonstrated the most promising antimicrobial potential against all *C. albicans* and non-*albicans* strains. Moreover, the electronmicrographs demonstrated significant structural deformations in all strains tested. At concentrations of 7.81–125 µg/mL, *C. ferrea* extract did not affect cell viability of L929 fibroblasts. These results indicate antifungal potential against the main fungi that causes oropharyngeal candidiasis, and warrant future studies of pharmacologically active compounds. Future studies may lead to consideration of these plant materials as prototypes for drug development and/or as sources of pharmaceutical raw materials for the treatment of candidiasis.

Conflict of Interest: The authors declare no conflict of interest.

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