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RESEARCH ARTICLE

Antifungal potential of *Sideroxylon obtusifolium* and *Syzygium cumini* and their mode of action against *Candida albicans*

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ABSTRACT

Context The emergence of resistant pathogens and toxicity of antifungals have encouraged an active search for novel candidates to manage *Candida* biofilms.

Objective In this study, the little known species *Sideroxylon obtusifolium* T.D. Penn (Sapotacea) and *Syzygium cumini* (L.) Skeels (Myrtaceae), from the Caatinga biome in Brazil were chemically characterized and explored for their antifungal potential against *C. albicans*.

Materials and methods We determined the effects of hydroalcoholic extracts/fractions upon fungal growth (minimum inhibitory and fungicidal concentrations, MIC/MFC), biofilm morphology (scanning electron microscopy) and viability (confocal laser scanning microscopy), proposed their mode of action (sorbitol and ergosterol assays), and finally investigated their effects against macrophage and keratinocyte cells in a cell-based assay. Data were analysed using one-way analysis of variance with Tukey-Kramer post-test ($\alpha = 0.05$).

Results The *n*-butanol (Nb) fraction from *S. obtusifolium* and *S. cumini* extract (Sc) showed flavonoids (39.11 ± 6.62 mg/g) and saponins (820.35 ± 225.38 mg/g), respectively, in their chemical composition and demonstrated antifungal activity, with MICs of 62.5 and 125 μg/mL, respectively. Nb and Sc may complex with ergosterol as there was a 4–16-fold increase in MICs in the presence of exogenous ergosterol, leading to disrupted permeability of cell membrane. Deleterious effects were observed on morphology and viability of treated biofilms from concentrations as low as their MICs and higher. Sc was not toxic to macrophages and keratinocytes at these concentrations ($p > 0.05$), unlike Nb.

Conclusions Nb and Sc demonstrated considerable antifungal activity and should be further investigated as potential alternative candidates to treat *Candida* biofilms.

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Introduction

Oral candidiasis is a common superficial and opportunistic infection caused by *Candida* spp., mainly *C. albicans*, when the host immunity becomes compromised (Dangi et al. 2010). It is considered a global health issue affecting a wide portion of individuals in the population, such as newborns (Ali et al. 2012), oral cancer patients (Alnuaimi et al. 2015), denture wearers (Dar-Odeh & Shehabi 2003), among others, presenting different clinical features. Recently, oral candidiasis has also been reported to be a risk factor for disseminated bacterial infections, which constitute a critical and life-threatening trait, particularly in immunocompromised individuals (Kong et al. 2015).

There are still a limited number of antifungal classes commercially available, many of which have the same mechanism of action, as a result of the few targets so far known in the fungal cell (Pierce et al. 2013). Not surprisingly, this non-diverse arsenal is sometimes ineffective upon microbial resistance (Ramage et al. 2012) leading to either persistent or recurrent fungal infections in the clinical setting. In addition, some of synthetic antifungals are potentially toxic for the host cells due to the similarity in both eukaryotic fungal and human cell components, such as sterols (Berman & Sudbery 2002). Biochemical engineering has been used to modify standard antifungals or even develop novel low-toxicity drugs (Boros-Majewska et al. 2014; Peng et al. 2015), but most drugs have not been tested in humans. Taken together, all these aspects have encouraged an active search for novel candidates to manage oral candidiasis in susceptible patients.

With this purpose, naturally-occurring agents are considered a promising source of bioactive molecules with antifungal properties (Arif et al. 2009; Negri et al. 2014). Over the last century, research on medicinal plant species has assessed their viability, sustainability and affordability to be used as natural drugs (Iwu 2000; Negri et al. 2014) or to prototype the development of synthetic compounds. Previous studies have suggested that the little known species *Sideroxylon obtusifolium* T.D. Penn (Sapotacea) (Albuquerque et al. 2011), native to northeastern Brazil, and *Syzygium cumini* (L.) Skeels (Myrtaceae) (Oliveira et al. 2007), common in tropical Asia, could be promising antifungal agents.

S. obtusifolium, known as *'quixabeira'*, *'quixaba'* or *'rompe-gibão'*, is a medicinal plant species native to the Brazilian *Caatinga*, which is a unique biome that has aroused attention for its exuberant and unexplored biodiversity. It is threatened with extinction mainly by the extraction of its bark in popular

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medicine to treat pain and inflammation (Araújo-Neto et al. 2010). However, other parts of this plant seem to have an unexplored potential against oral microorganisms of clinical relevance, such as streptococci and yeasts.

S. cumini, commonly known as jambolan, is a large evergreen and foliaceous tree whose seeds, bark, fruit and leaves have been used in traditional medicine. It is reported to have antioxidant, antinociceptive, hypoglycemic, anti-*Leishmania* and antimicrobial properties (Eshwarappa et al. 2014; Quintans et al. 2014; Rodrigues et al. 2015).

Interestingly, the antifungal properties of *S. obtusifolium* and *S. cumini* have never been comprehensively studied despite their use in folk medicine as antimicrobial (Albuquerque et al. 2011), which could open avenues for discovery of novel bioactive molecules and drug development and also aggregate value to the ethnopharmacological knowledge of the communities living in the *Caatinga* biome (28 million people as of 2013, according to the Brazilian Institute of Geography and Statistics - IBGE).

In this pioneering study, the antifungal activity of extracts and/or fractions from the leaves of *S. obtusifolium* and *S. cumini* from the Caatinga biome was determined on planktonic cells of *C. albicans*. Their effects on biofilm morphology and viability and mode of action were further established. With the perspective of future clinical use, the cytotoxic effects of these extracts were investigated upon murine and human cells. Further scientific exploration of these species could provide new opportunities for the development of needed novel antifungals to manage oral candidiasis.

Materials and methods

Plant material

Sideroxylon obtusifolium and *Syzygium cumini* leaves were collected in April and August 2013, respectively, in the semi-arid region in the countryside area of Campina Grande, Paraíba State, Brazil (7° 22′ 25″ S, 35° 59′ 32″ W), under permission of the Brazilian Ministry of Environment (Council for the Administration and Management of Genetic Patrimony - CGEN) via the National Council for Scientific and Technological Development (CNPq) [010637/2014-1]. Botanical specimens were identified by Prof. Maria Regina de Vasconcellos Barbosa, curator of Lauro Pires Xavier Herbarium (http://inct.florabrasil.net/participantes/herbarios-curadores/jpb/) at the Department of Molecular Biology, Federal University of Paraíba, João Pessoa, Paraíba, under voucher numbers JPB 57.985 and JPB 58.543, respectively.

After cleaning processing, the leaves were dried in an air-circulating oven at 40 °C until stabilization of final weight, and then ground in a Wiley mill (SL 30 Solab, Piracicaba, SP, Brazil). Hydroalcoholic extracts of the leaves were prepared by maceration following the proportion 200 g of ground plant to 1 L of 70% hydroalcoholic solution. The extracts were subjected to evaporation under reduced pressure, lyophilized at −20 to −40 °C and stored in glass containers, protected from light and under refrigeration.

Extract fractionation

S. obtusifolium extract was subjected to liquid–liquid fractionation (Cunha et al. 2013) using organic solvents in order of increasing polarity: hexane, dichloromethane, ethyl acetate and *n*-butanol (Nb). The final portion corresponded to the aqueous phase. Our preliminary experiments (not shown) indicated that the dichloromethane and Nb fractions had the best antimicrobial activity and therefore were selected for further testing.

Phytochemical analysis

The extract(s)/fraction(s) with the best activity were quantified for their content of total polyphenols, total flavonoids, condensed tannins and saponins. The method described by Chandra and Mejía (2004) was used to determine the content of total polyphenols in equivalent milligrams of gallic acid, with absorbance reading at 757 nm. The content of total flavonoids, expressed in equivalent milligrams of quercetin, was determined according to Meda et al. (2005) with absorbance reading at 420 nm. The content of condensed tannins, expressed as milligrams of catechin equivalents, was quantified using the method proposed by Makkar and Becker (1993) with absorbance reading at 500 nm. Finally, the content of saponins in milligrams of diosgenin was determined according to Makkar et al. (2007) with absorbance reading at 544 nm.

Microorganism and growth conditions

In all microbiological assays a reference strain of *Candida albicans* ATCC 10231, obtained from the American Type Culture Collection, was used. Cultures were kept in a glycerol stock solution at −80 °C and grown in Sabouraud dextrose broth and agar (HIMEDIA Laboratories Pvt. Ltd., Mumbai, India) at 35 °C for 24 h in aerobic atmosphere.

Inhibitory and lethal effects of the plant extracts on C. albicans cells

This study investigated the antifungal susceptibility of *C. albicans* to the action of *S. obtusifolium* extract and fractions and *S. cumini* extract (Sc). Their minimum inhibitory concentration (MIC) was established using 96-well U-bottom tissue culture microplates. Fresh solutions of the extracts and fractions were prepared using 10% (v/v) ethanol as vehicle. Then, aliquots of 100 µL of the samples were used to perform serial dilutions in RPMI-1640 medium with 0.165 M MOPS (Sigma-Aldrich, St. Louis, MO). Nystatin, caspofungin diacetate (Sigma-Aldrich, St. Louis, MO) and amphotericin B (União Química Farmacêutica Nacional, SP, Brazil) were used as positive controls, and the vehicle as the negative control. Sterility of the culture medium and samples and culture viability were also monitored. Fungal inoculum was adjusted at 530 nm to 2.5×10^3 CFU/mL in the wells totaling a volume of 200 µL. Plates were incubated at 35 °C for 24 h. The MIC was defined as the lowest concentration of the sample that inhibited visible microbial growth (CLSI 2002).

The lethal effects of the samples were determined through their minimum fungicidal concentrations (MFC) by subculturing on Sabouraud dextrose agar plates an aliquot from each well having a concentration equal to or higher than the MIC. The plates were incubated at 35 °C for 48 h. The MFC was defined as the lowest concentration of the extract or bioactive fraction that showed no visible growth on the agar plate (Manohar et al. 2001).

Finally, the MFC/MIC ratio was calculated to determine if the extracts and their bioactive fractions have fungistatic (MFC/MIC ≥ 4) or fungicidal (MFC/MIC < 4) properties (Siddiqui et al. 2013).

Antifungal mode of action

These assays aimed to elucidate the antifungal mechanisms through which the extracts and bioactive fractions interact with the fungal cells. Two major mechanisms were investigated: direct

interaction with the cell wall biosynthesis (sorbitol assay) or membrane ionic permeability (ergosterol assay).

Effects on cell wall biosynthesis

The MIC of the extracts or bioactive fractions was determined in the presence of an osmotic protector through an adaptation of the microdilution technique proposed by the CLSI (2002). Culture medium (RPMI-1640) was added to each well of the microplate and serial dilutions of the samples and controls were carried out. Finally, 100 µL of inoculum (2.5×10^5 CFU/mL) prepared with RPMI-1640 supplemented with 0.8 M sorbitol (final concentration) (Sigma-Aldrich, St. Louis, MO) was transferred to the wells. Caspofungin diacetate (Sigma-Aldrich, St. Louis, MO) was used as positive control. Yeast growth, media sterility and inertness of vehicle (negative control) were also monitored. Plates were incubated at 35 °C and read after 2 and 7 d (Lima et al. 2013; Freires et al. 2014).

Effects on membrane permeability

The MIC of the extracts or bioactive fractions was determined in absence and presence of exogenous ergosterol (Sigma-Aldrich, St. Louis, MO) at concentrations of 100, 200 and 400 µg/mL through an adaptation of the microdilution technique (CLSI 2002). Amphotericin B, which highly binds to ergosterol, was used as positive control. Yeast viability in presence of different concentrations of ergosterol and of vehicle was checked as a negative control. Plates were read after 24 h of incubation at 35 °C (Lima et al. 2013; Freires et al. 2014).

Scanning electron microscopy (SEM)

Biofilm cultures (2.5×10^5 CFU/mL) were grown in chambered glass slides (BD Falcon™, Bedford, MA) containing RPMI-1640 and were treated with the extract/bioactive fraction at different concentrations (MIC, MFC, 5 x MIC and 10 x MIC) or with nystatin (MIC). The chambered slides were kept shaking at 100 rpm, 35 °C for 72 h to form consistent biofilms. After that, the samples were washed twice to remove planktonic cells and kept in a 3% glutaraldehyde solution (pH 7.4) for 12 h at room temperature, followed by serial dehydration with ethanol (50, 70 and 90%) for 10 min and drying for 45 min under laminar flow. At last, the chambers were separated from the slides and the latter were coated with a gold pellicle in a metallizer device to be observed under scanning electron microscopy (Jeon JSM 5600LV microscope, Tokyo, Japan) (Freires et al. 2014).

Confocal laser scanning microscopy (CLSM)

C. albicans cultures were started in yeast nitrogen base (YNB) medium (Himedia®, Mumbai, India) supplemented with 50 mM glucose for 24 h at 35 °C. Cells were harvested (1200 rpm, 10 min, 10 °C), washed twice, resuspended in PBS (0.15 M, pH 7.2, Ca^{2+}- and Mg^2-free) and adjusted to 5×10^6 CFU/mL (530 nm, $\lambda = 0.08$–0.1). Final inocula concentration in the wells was 2.5×10^5 CFU/mL. For preparation of *C. albicans* biofilms, glass slides (324 mm^2 surface area) were immersed in 12-well flat-bottom plates (TPP, Trasadingen, Switzerland) containing fetal bovine serum (FBS) (Vitrocell Embriolife, Campinas, SP, Brazil), followed by incubation for 24 h at 35 °C. Then the slides were washed with PBS to remove residual FBS and transferred to new plates containing the *C. albicans* suspension, followed by incubation for 90 min at 35 °C. Subsequently, the slides were gently washed to ensure the removal of non-adhered cells before immersion in YNB medium containing the extract/bioactive fraction, vehicle and nystatin. The plates were incubated at 35 °C for 48 h for biofilm formation (adapted from Kuhn et al. 2002; Freires et al. 2015). 72 h biofilm formation was not possible using this method as the biofilm pellicle on the slide turns to be thick and detaches from the surface after 48 h.

For the CLSM analysis, the biofilms were placed in wells containing 10 µM FUN-1 (F-7030, Life Technologies, Carlsbad, CA) and 25 µg/mL Concanavalin A, Alexa Fluor® 488 Conjugate (C11252, Life Technologies, Carlsbad, CA) in 3 mL of PBS. The plates were then incubated for 45 min at 35 °C and the slides were subsequently visualized on a Leica TCS-SP5 confocal microscope (Mannheim, Germany) equipped with an HCX-PL-APO 63x oil immersion objective lens. Each biofilm sample was scanned at five randomly selected points and representative images were selected. 2D images were generated and processed using LAS X Suite for Windows®.

Cytotoxic activity on murine and human cell cultures

Murine macrophage RAW264.7 were grown in RPMI medium and human keratinocyte HaCat were cultured in DMEM medium, both supplemented with 10% fetal bovine serum (Gibco BR, Gaithersburg, MD). A viability assay was carried out in which RAW264.7 and HaCat cells were seeded onto 96-well plates at a density of 1×10^5 and 1×10^4 cells/well, respectively, and allowed to adhere for 30 h. After that, the adhered cells were exposed to the extracts/bioactive fraction at the concentrations of 200, 100, 20 and 10 µg/mL in dimethyl sulfoxide (DMSO)/medium and vehicle control. Final DMSO concentration (1%) did not affect cell viability. After 24 h of incubation, viability was determined by the addition of 200 µL of MTT solution (0.3 mg/mL). The precipitated formazan crystals were solubilized in ethanol, and the absorbance was determined at 570 nm using a microplate reader (Mosmann 1983).

Statistical analysis

All the tests in this study were carried out in triplicate in three independent experiments. Descriptive and inferential statistics were used to analyse the data on GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). One-way analysis of variance followed by Tukey-Kramer post-test was used to analyse the intra- and inter-group differences in the cytotoxicity assays, with type I error set as 0.05.

Results

Extracts and fractions yields

The extracts and fractions yields, expressed in relation to dry weight of plant material (%, w/w) were as follows: *S. obtusifolium* extract (3.64%) – dichloromethane fraction (13.1% in relation to crude extract)/ Nb fraction (11.8%, in relation to crude extract) – and Sc (6.25%).

Phytochemical analysis

The extract and/or fraction with the best antifungal activity were chemically characterized. Table 1 shows the phytochemical

makers of the Nb fraction from *S. obtusifolium* extract and Sc. The results showed a predominance of total flavonoids in the Nb fraction and saponins in the Sc.

Antifungal activity and mode of action

The inhibitory and lethal concentrations of the extracts, bioactive fractions and standard drugs against *C. albicans* can be found in Table 2. The Nb fraction from *S. obtusifolium* hydroalcoholic extract was shown to concentrate the most active constituents of *S. obtusifolium* with MIC/MFC values lower than those of the crude extract and dichloromethane fraction. Therefore, the Nb fraction was selected to be further tested as to mode of action, effects on biofilm morphology and viability and cytotoxicity. The crude extract of *S. cumini* was also tested in this study, which was also proven to have considerable antifungal activity against planktonic *C. albicans* cells.

Considering the promising antifungal effects of the Nb fraction and Sc, we next studied their modes of action upon yeast cells. The findings showed that Nb fraction and Sc do not interfere with cell wall biosynthesis pathways as does Caspofungin (used as a control), because the antifungal susceptibility of yeasts to them was unaltered either in the presence or absence of an osmotic protector (Table 3). Instead, their MIC values increased between 4 and 16 times with the addition of exogenous ergosterol, which indicates that they seem to complex with membrane ergosterol and this could potentially lead to increased membrane ionic permeability, cell leakage and death (Table 4).

Effects on biofilm morphology and viability

SEM analysis was initially carried out to evaluate the effects of the extract and bioactive fraction on the morphology and integrity of *C. albicans* biofilms.

We observed that the Nb fraction from *S. obtusifolium* severely affected the biofilm cell structures even at low inhibitory concentrations (62.5 µg/mL), and that at higher concentrations the damages were lethal to the cells. Marked structural changes with membrane rupture and possible leakage of cellular contents were observed. Therefore, the Nb fraction was shown to exert antifungal effects on *C. albicans* biofilms (Figure 1).

Similarly, Sc also affected *C. albicans* biofilm cells, with deleterious effects at concentrations as low as the MIC (125 µg/mL) and evident destruction from 500 µg/mL (MFC). Nystatin, used as positive control, was effective in disrupting biofilm integrity; and the vehicle did not affect the cells (Figure 2).

The inhibitory effects of the Nb fraction and Sc on the viability of *C. albicans* biofilms were visualized by CLSM (Figure 3). Viability of fungal cells upon the action of the tested substances could be observed through Concanavalin A (Alexa Fluor® 488 conjugate) and FUN® 1 cell staining. Both the fraction and the extract affected the viability of the biofilm cells when compared to the vehicle.

Cytotoxic activity

Cytotoxicity assays were carried out to provide preliminary evidence on whether these antifungal agents would affect or not host cells in a future clinical setting. Figure 4 shows that Sc did not show toxicity on murine macrophage cells, with no significant difference at any tested concentration (10–200 µg/mL) when compared to the vehicle. However, the Nb fraction from *S. obtusifolium* extract showed toxicity on macrophages at concentrations close to its MIC/MFC. On human keratinocytes, the toxicity threshold (limitrophe concentration that does not affect cell viability) for Sc and Nb fraction from *S. obtusifolium* extract was found to be 100 and 20 µg/mL, respectively, again lower than their MIC/MFC values.

Discussion

This study describes the antifungal potential and mode of action of *S. obtusifolium* and *S. cumini* against *C. albicans*. The plant material was collected in a semi-arid biome in northeastern Brazil termed '*Caatinga*'. It is a unique biogeographic region and considered one of the largest intermittently dry tropical forests in America (Penninghton et al. 2009). This biodiverse ecological setting is a shelter for 4 478 native plants species (Siqueira-Filho et al. 2012), including *S. obtusifolium* and exotic species, such as *S. cumini*, which are mostly explored by the local communities in folk medicine and for commercial purposes.

Due to the presence of major compounds in the leaves of *S. obtusifolium* and *S. cumini* that are known to have antifungal activity, such as total flavonoids (Onsare & Arora 2014; Yixi et al. 2015) and saponins (Sparg et al. 2004; Coleman et al. 2010), respectively, and in view of the potential sustainability of their use

Table 2. Antifungal activity of *Sideroxylon obtusifolium* extract and its fractions, *Syzygium cumini* extract, and standard drugs on *Candida albicans* ATCC 10231 (values are expressed as µg/mL).

Group	MIC	MFC	MFC/MIC ratio[a]
S. obtusifolium			
Crude extract	1000	>1000	>1
Dichloromethane fraction	1000	1000	1
Nb fraction	62.5	250	4
S. cumini			
Crude extract	125	500	4
Monodrugs			
Nystatin	0.97	1.95	2
Amphotericin B	0.07	0.14	2
Caspofungin	0.48	0.97	2

[a]Determines a fungistatic (MFC/MIC ≥ 4) or fungicidal (MFC/MIC < 4) activity.

Table 3. Effects of the Nb fraction from *Sideroxylon obtusifolium* extract, and Sc on *Candida albicans* (ATCC 10231) cell wall biosynthesis (*sorbitol assay*). Values are expressed as µg/mL.

Group	Without sorbitol	With sorbitol
S. obtusifolium, Nb fraction from extract	250	250
Sc	500	500
Caspofungin	0.97	62.5

Table 1. Phytochemical markers of the Nb fraction from *Sideroxylon obtusifolium* hydroalcoholic extract and *Syzygium cumini* hydroalcoholic extract.

Group	Condensed tannins	Total flavonoids	Saponins	Polyphenols[a]
S. obtusifolium, Nb fraction	5.5 ± 0.98	39.11 ± 6.62	–	*
Sc	54.26 ± 0.39	23.04 ± 3.02	820.35 ± 225.38	100.10 ± 39.97

Content values are expressed as mean ± SD (mg/g). (–) not detected; (*) not analysed.
[a]Note that the column 'polyphenols' refers to a general measurement of all polyphenolic compounds in the sample, including, for instance, flavonoids. In this case, the same compound might have been quantified in both 'total flavonoids' and 'polyphenols' analyses.

Table 4. Effect of different concentrations of exogenous ergosterol (100–400 μg/mL) on the antifungal activity of the Nb fraction from *Sideroxylon obtusifolium* extract; *Syzygium cumini* extract; and Amphotericin B on *Candida albicans* ATCC10231.

		MIC in the presence of different concentrations of ergosterol		
Group	MIC in the absence of ergosterol	100 μg/mL	200 μg/mL	400 μg/mL
S. obtusifolium, Nb fraction from extract	62.5	500	500	500
Sc	125	500	1000	2000
Amphotericin B	0.07	4.88	9.76	19.53
Yeast growth in presence of ergosterol	+	+	+	+
Vehicle	+	+	+	+

+, fungal growth.

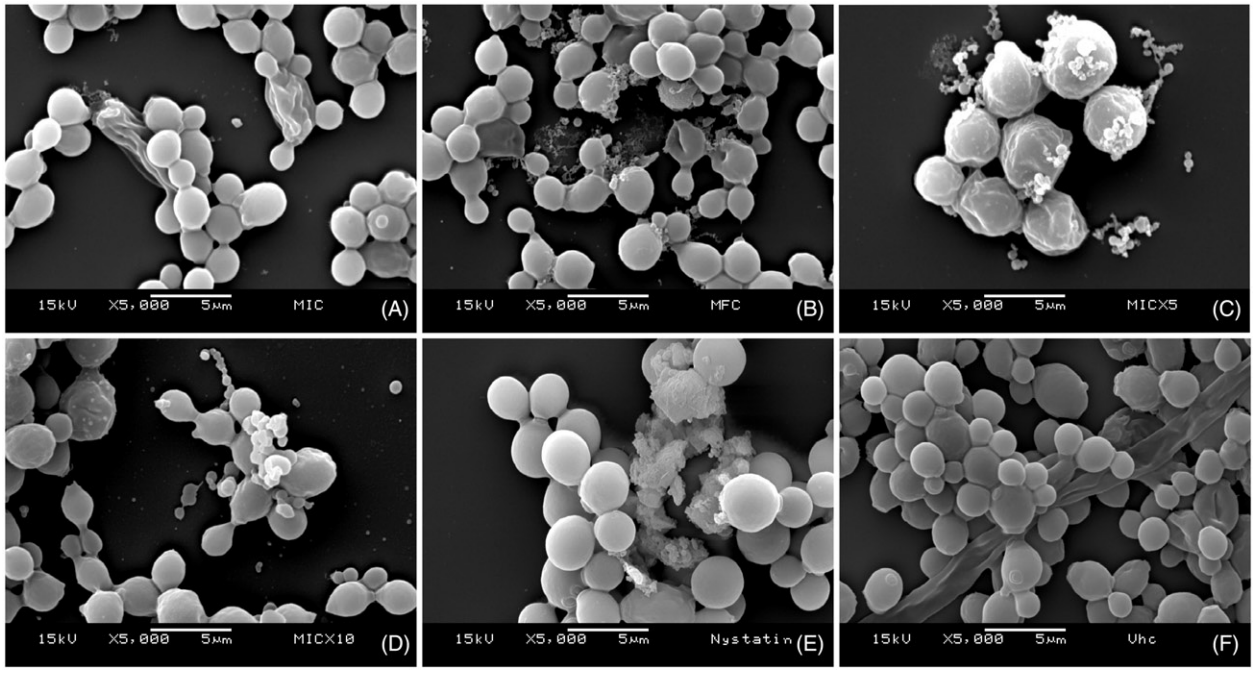

Figure 1. Effects of the Nb fraction from *Sideroxylon obtusifolium* extract **on** biofilm morphology/integrity. SEM photomicrographs (5000x) showing *Candida albicans* biofilm cells treated with different concentrations of the Nb fraction (A) 62.5 μg/mL (MIC), (B) 250 μg/mL (MFC), (C) 312.5 μg/mL (5xMIC), (D) 625 μg/mL (10xMIC); as well as (E) 0.97 μg/mL nystatin and (F) vehicle. The Nb fraction affected cell structures even at low concentrations (MIC). Nystatin was used as positive control, and the vehicle did not affect the biofilm cells.

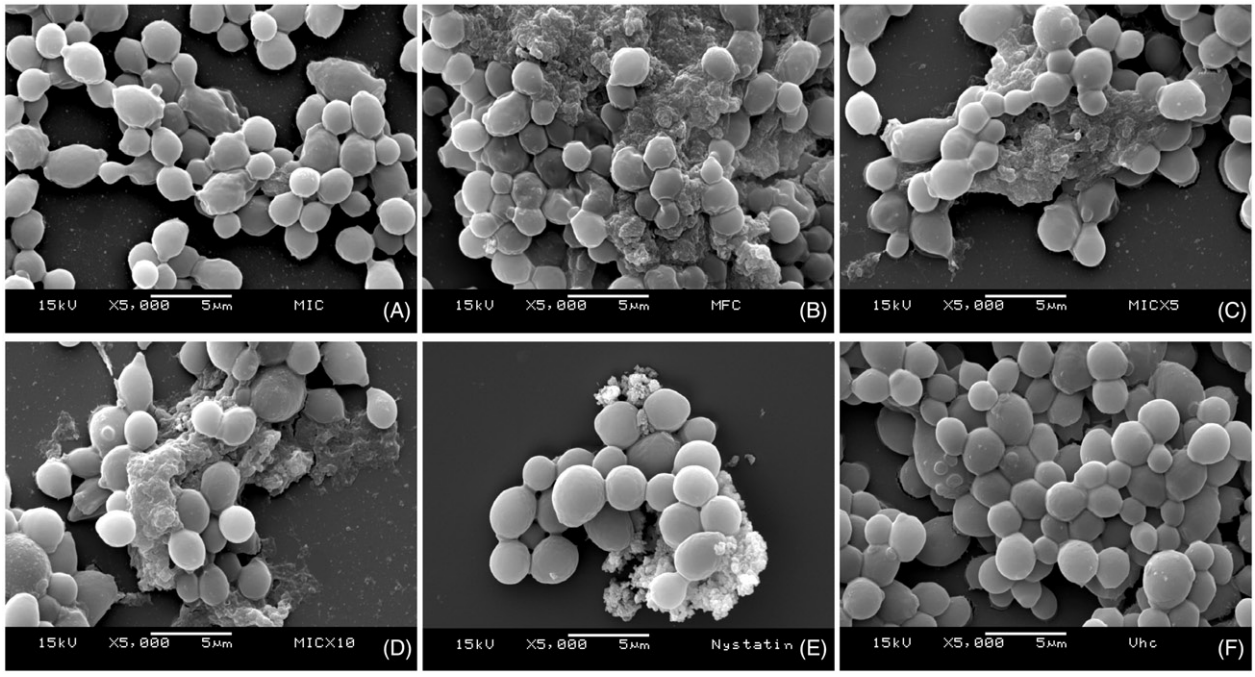

Figure 2. Effects of the *Syzygium cumini* extract on biofilm morphology/integrity. SEM photomicrographs (5000x) showing *Candida albicans* biofilm cells treated with different concentrations of Sc: (A) 125 μg/mL (MIC), (B) 500 μg/mL (MFC), (C) 625 μg/mL (5xMIC), (D) 1250 μg/mL (10xMIC); as well as (E) 0.97 μg/mL nystatin and (F) vehicle. It can be noted that Sc affected cell structures at concentrations as low as 125 μg/mL. Nystatin was used as positive control, and the vehicle did not affect the biofilm cells.

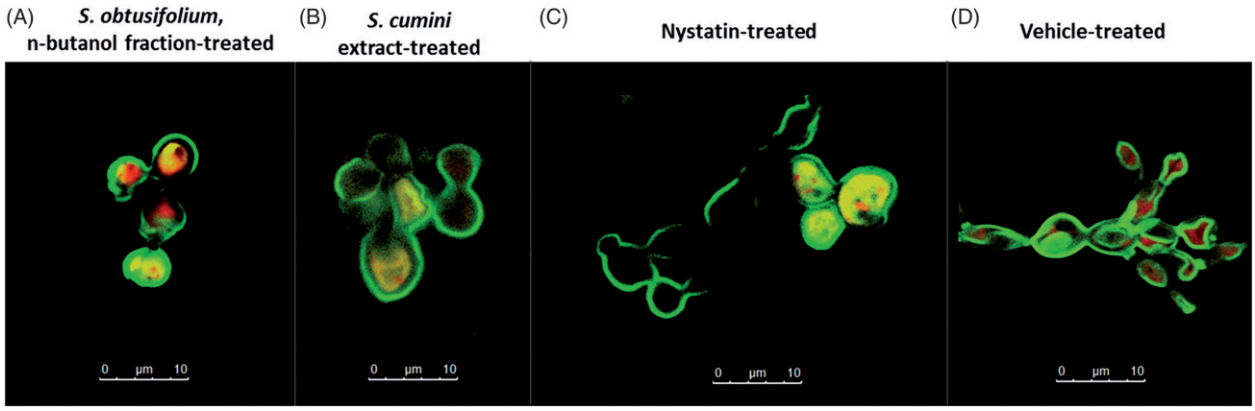

Figure 3. Effects on biofilm viability. This is a representative 2-D confocal imaging of *Candida albicans* biofilms treated with (A) Nb fraction from *S. obtusifolium* extract (5xMIC: 312.5 µg/mL); (B) *S. cumini* hydroalcoholic extract (5xMIC: 625 µg/mL); (C) Nystatin (MIC: 0.97 µg/mL) [positive control]; and (D) Vehicle (negative control). The structures depicted in green (Concanavalin A, Alexa Fluor® 488 Conjugate) represent the yeast cell wall, and those depicted in yellow (FUN® 1 Cell Stain) are nonviable cells, metabolically inactive. The viable cells, in turn, convert the dye FUN-1 to red fluorescent aggregates (63x optical magnitude, 2.85 zoomed-in). Concanavalin A selectively binds to polysaccharides, including alpha-mannopyranosyl and alpha-gluco-pyranosyl residues, and gives a green fluorescence. FUN-1 is a fluorescent dye taken up by yeast cells; in the presence of metabolic viability it is converted from a diffuse yellow cytoplasmic stain to red. It can be noted that both Sc and Nb fraction affected the viability of *C. albicans* cells when compared to the vehicle.

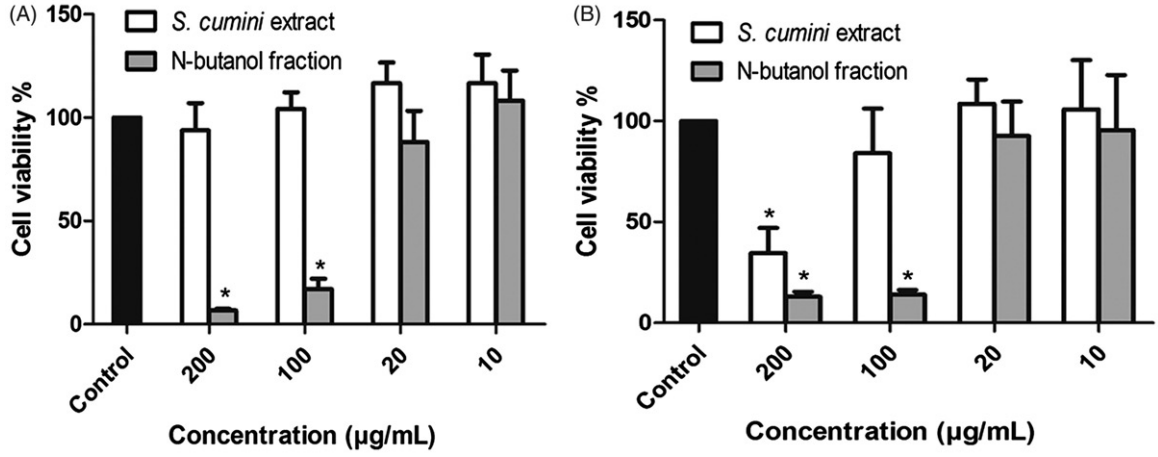

Figure 4. Cytotoxicity of Sc and Nb fraction from *S. obtusifolium* extract on murine macrophage cells (A) and human keratinocytes (B). Each concentration marked with '*' differs significantly from the vehicle ($p < 0.05$, One-way ANOVA followed by Tukey-Kramer).

for therapeutic purposes, this study was carried out to elucidate the antifungal mode of action of these species and their effects upon yeast cell morphology and viability in biofilm cultures.

In agreement with other studies, the extract of *S. obtusifolium* showed weak antifungal activity (Cruz et al. 2007; Almeida et al. 2012), as well as its dichloromethane fraction. However, its Nb fraction showed better activity (lower MIC/MFC values) than the crude extract, indicating that the fractionation scheme adopted in this study (Jeon et al. 2011) was successful. Likely, the predominance of flavonoids in the Nb fraction may have favored its anti-*Candida* properties. Flavonoids extracted from different plant species were found to have strong inhibitory effects on the formation and metabolic activity of *C. albicans* biofilms or planktonic cells (Onsare & Arora 2014), which means that this phytochemical class could be a marker for antifungal activity.

The crude extract of *S. cumini* was also tested in this study, which showed fungistatic activity. Other studies in the literature have reported on the antifungal effects of *S. cumini* on *C. albicans* (Oliveira et al. 2007; Höfling et al. 2010; Santos et al. 2012), but have not further described its mode of action and effects on biofilm morphology and viability (see further in the discussion). As to the chemical profile, the major compounds found in the extract of the leaves in this study were saponins. Coleman et al. (2010) found that two saponin family members disrupted *C. albicans* hyphae and biofilm formation; thus, it is assumed that saponins are possibly the active compounds in the Sc.

The next step in this bioprospection study was to elucidate the mode of action of the fraction or extract with the best antimicrobial activity determined by MIC/MFC. Herein, two possible chief mechanisms were investigated for Nb and Sc antifungal effects on yeasts: (i) disrupting cell wall biosynthesis or (ii) increasing membrane ionic permeability (Freires et al. 2014). The findings showed that both the Nb fraction from *S. obtusifolium* extract and Sc consistently bind to membrane ergosterol, leading to increased cell membrane permeability due to the formation of pores in the membrane. When at higher concentrations (250–500 µg/mL), their effects on the yeast cells are lethal, because ergosterol is crucial to maintain cell integrity, viability and normal growth (Ghannoum & Rice 1999). It is worth noting that other cell targets might also be involved in the antifungal activity of Nb fraction and Sc, warranting further investigation. In this study, the plant extract/fraction were found to be fungistatic (MFC/MIC = 4) and amphotericin B, fungicidal (MFC/MIC = 2), although both showed similar modes of action. This could be explained by other aspects that play a role in the major properties of antifungal drugs, such as drug affinity to ergosterol and chemical stability of

the molecules determining the biological effects (Milhaud et al. 2002).

The study of the antimicrobial properties of natural products should prioritize biofilm growth models, in order to provide greater reliability of data and closer approach to clinically relevant conditions (Freires et al. 2014). In this study, scanning electron and confocal laser microscopy were used to visualize *C. albicans* biofilms grown on abiotic surfaces and treated with the Nb bioactive fraction and Sc. It was found that both products disrupted the structure and viability of biofilm cells. The association between biofilm formation and antimicrobial resistance remains to be a serious health issue in the current days (Simões 2011), which encourages the search for active molecules able to disrupt biofilm integrity. Hence, the harmful effects of the extract/fraction upon biofilm assembly seen in this study could contribute to render the biofilm more susceptible to other antifungal drugs. In addition to oral candidiasis, this result may also be useful for studies addressing yeast biofilm formation on the surfaces of medical devices, such as central venous catheters and endotracheal tubes. Further studies should also perform a quantitative analysis of the effects of the extract and fraction on the biofilm composition (e.g., estimation of extracellular polysaccharides and other components of the biofilm matrix), biovolume (e.g., number of cells vs. exposure time, biofilm inhibitory concentration) and viability (e.g., XTT assay).

Following validation of antifungal activity, the experimental samples were finally checked for their cytotoxic activity on murine and human cell cultures, in order to provide evidence for future clinical use. Overall, cytotoxic activity was detected on both cell lines, particularly the Nb fraction, as the cytotoxicity values found were lower than those of the MIC/MFC of the extract/fraction. As expected, Sc showed a less cytotoxic profile when compared to the Nb fraction, given that the fractionation process may concentrate toxic compounds in the bioactive fractions. Nevertheless, it is a preliminary cell-based assay with 24 h exposure of cells to the test substances, which would be rather different from the clinical use. Ribeiro et al. (2014) reported that *S. cumini* hexanic extract has low toxicity on murine macrophages, with IC_{50} of 31.64 µg/mL. There have been no reports on the toxicity of *S. obtusifolium in vitro*.

An *in vivo* study by Ruela et al. (2011) evaluated the acute toxicity in mice of the ethyl acetate fraction from the stem bark of *S. obtusifolium*. The authors found that such fraction, rich in polyphenols, has very low cytotoxicity with LD_{50} of 777 mg/kg by intraperitoneal route. With regard to *S. cumini*, an *in vivo* study by Silva et al. (2012) showed that the hydroalcoholic extract of *S. cumini* leaves did not exert acute or chronic toxic effects on rodents by oral route, with LD_{50} of 489 mg/kg. More sophisticated toxicological assays are now encouraged to further determine whether the Nb fraction and Sc would pose a risk or not against the host cells.

The clinical management of oral candidiasis in most patients includes the administration of topical or systemic antifungal drugs as well as disinfection of dentures in patients presenting denture-related atrophic stomatitis (Gendreau & Loewy 2011). Hence, the Nb fraction and Sc may be of interest in both approaches, with particular focus on the denture disinfection protocol due to their potential toxicity toward host cells. In addition, several studies have also suggested the combination of synthetic drugs with natural products in order to improve the efficacy of both drugs and manage toxicity upon clinical use (Hemaiswaryaa et al. 2008; Castro et al. 2015).

Taken altogether, the findings of this bioprospection study support the view that these plant materials warrant further exploration of their biological properties and toxicity in future non-clinical studies and clinical trials. In summary, the Nb fraction from *S. obtusifolium* extract, and Sc, which are species from the unique *Caatinga* biome, showed antifungal activity against *C. albicans* biofilm, probably due to the predominance of flavonoids and saponins, respectively, in their chemical composition. Their ability to bind to ergosterol and thus increase cell membrane permeability may explain the deleterious effects observed on biofilm morphology and viability. The Nb fraction and Sc showed relative cytotoxic effects on murine and human cells. Other toxicological assays and *in vivo* studies of efficacy are now encouraged to further investigate the antifungal activity of the Nb fraction from *S. obtusifolium* and Sc as alternative candidates for the treatment of oral candidiasis.

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Disclosure statement

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