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Antifungal activity of medicinal plants from Northeastern Brazil

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The aim of this work was to find how medicinal plants play an important role as source of new bioactive molecules. To evaluate the antifungal activity of 10 medicinal plants from northeastern Brazil, traditionally used as anti-infective agents. The activity of 30 crude extracts (water; ethanol:water, 1:1; acetone:water, 1:1) against four standard species of *Candida* yeasts (*Candida albicans* ATCC 90028, *Candida dubliniensis* ATCC 7289, *Candida glabrata* ATCC 2001 and *Candida krusei* ATCC 6258) was investigated by the Minimal Inhibitory Concentration (MIC), using the microdilution method and the working range used was from 1.95 to 1000 µg/mL. Extracts from leaves of *Eugenia uniflora* (Myrtaceae), stem bark of *Caesalpinia ferrea* (Caesalpinaceae) and leaves of *Psidium guajava* (Myrtaceae) showed significant activity against all yeasts evaluated. The best antifungal activities were achieved against *C. glabrata* and *C. krusei* by *E. uniflora* extract (MIC = 15.62) and followed by extracts from *C. ferrea* and *P. guajava* (MIC ranged from 15.62 to 250 µg/mL). *E. uniflora* also showed fungicidal properties against all yeasts, especially against *Candida dubliniensis*. This study identified plant species that may be candidates for the development of alternative treatments for candidiasis.

Key words: Antifungal activity, candidiasis, medicinal plants, crude extracts, fungicide, northeastern Brazil.

INTRODUCTION

Species of *Candida* are harmless saprophyte yeasts, a normal component of the human microbiota in the gastrointestinal tract and the oral and vaginal mucosae. These yeasts often cause superficial infections such as vaginitis; however, if the immune defenses of the host become compromised by anticancer therapy, human immunodeficiency virus infection, organ transplantation, or therapy with broad-spectrum antibiotics, they can cause severe systemic infections (Cruz et al., 2002; Morschauser, 2002).

Due to the lack of new classes of drugs or different molecular targets, drug combinations play a major role as therapeutic strategy, given the multiplicity of fungal targets against which current agents are ineffective (Mukherjee et al., 2005).

This situation makes it imperative to develop antifungal strategies based on new classes of molecules, which act through new sites and/or mechanisms (Wisplinghoff et al., 2004; Venkatesan et al., 2005; Gálvan and Mariscal, 2006). The discovery of new antifungal agents remains an important challenge for the scientific community and traditional medicinal plants may furnish promising

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Table 1. Plant species collected for antifungal screening.

Botanical name	Family	Part used	Locality	Voucher number
Eugenia uniflora Linn.	Myrtaceae	leaves	Natal (RN)	11763 (UFRN)
Caesalpinia ferrea Mart.	Caesalpiniaceae	stem bark	Garanhuns (PE)	86678 (IPA)
Psidium guajava Linn.	Myrtaceae	leaves	Natal (RN)	8214 (UFRN)
Persea americana Mill.	Lauraceae	leaves	Olinda (PE)	52805 (UFPE)
Piptadenia colubrina (Vell.) Benth.	Mimosaceae	stem bark	Recife (PE)	38384 (IPA)
Schinus terebinthifolius Raddi	Anacardiaceae	stem bark	Recife (PE)	8758 (IPA)
Mimosa ophthalmocentra Mart. ex Benth.	Mimosaceae	stem bark	Cabaceiras (PB)	83114 (UFPE)
Parapiptadenia rigida (Benth.) Brenan	Fabaceae	stem bark	Cabaceiras (PB)	83115 (UFPE)
Hymenaea stigonocarpa Mart. ex Hayne	Caesalpiniaceae	stem bark	Camocim de São Félix (PE)	83653 (UFPE)
Mimosa tenuiflora (Willd.) Poir.	Mimosaceae	stem bark	Cabaceiras (PB)	83113 (UFPE)

material for the development of antifungal drugs (Cruz et al., 2007; Souza et al., 2010).

Although the number of reports on species distributed by regions or biomes remains relatively small, interest in medicinal plants from north-eastern Brazil has been increasing, especially in plants from the semi-arid Caatinga biome (Albuquerque et al., 2007a). Several studies have described this region's rich diversity of plants used for medicinal purposes, widely employed by the traditional communities until the present day (Almeida et al., 2005; Albuquerque et al., 2007a,b; Agra et al., 2007, 2008; Cruz et al., 2007; Silva et al., 2009).

Some of these species used in local traditional medicine and investigated for antimicrobial activity have shown fewer side effects, with a wider spectrum of action and for this reason may play an important role as sources of new antifungal compounds (Mesa et al., 2004; Gatto et al., 2011; Talibi et al., 2011).

Because vast numbers of plant species from northeastern Brazil have not yet been phyto-chemically or biologically evaluated, this study screened 10 medicinal plant extracts for *in vitro* antifungal activity against four reference yeast species of the genus *Candida* (*Candida albicans*, *Candida dubliniensis*, *Candida glabrata* and *Candida krusei*).

MATERIALS AND METHODS

Plant material

The botanical material from 10 species, native of the Brazilian Northeast region were collected from several localities in three states (Pernambuco, PE; Paraíba, PB; or Rio Grande do Norte, RN) (Table 1). The species were identified by members of the Department of Botany at the Universidade Federal de Pernambuco (UFPE), the Department of Botany at the Universidade Federal do Rio Grande do Norte (UFRN), or the Agronomy Institute of Pernambuco (IPA).

Preparation of crude extracts

The extract solutions were obtained by reflux for 15 min (10% (w/v) of dried and ground raw material) using water, ethanol:water (1:1), or acetone:water (1:1). The extracts were filtered and concentrated under reduced pressure in an evaporator for complete elimination of the organic solvent.

Finally, the extracts were lyophilized to yield a crude extract (CE). After lyophilization, the CEs were weighed into eppendorfs (identified) and diluted with 950 µL of water and 50 µL of dimethylsulfoxide (DMSO) to yield the final concentration of 2000 µg/mL, which is the stock solution. The working range used for the MIC determination was from 1.95 to 1000 µg/mL.

Screening for antifungal activities

Fungal strains

The microorganisms and strains used for the biological evaluation, obtained from the American Type Culture Collection (ATCC), were: *C. albicans* (90028), *C. dubliniensis (7289), C. glabrata (2001) and C. krusei (6258).*

Broth microdilution method

The susceptibility assays were carried out through the method of microdilution in broth and fluconazole (Pfizer Inc., New York, NY, USA) was used as reference antimicotic control. Minimal Inhibitory Concentration (MIC) was determined based on the Clinical and Laboratory Standards Institute (CLSI, 2008) for fluconazole (FLU). The same methodology with some adaptations for natural products was employed for plants extracts.

To prepare the inoculum, yeast cells were suspended in sterile saline solution (SSS); concentration was adjusted at 90% transmittance in 530 nm spectrophotometer

(Bausch and Lomb) so that 100 µL of this suspension added to the antifungal already distributed in the plate, contained 0.5 and 2.5 x 10^3 Colony-forming unit by milliliter (CFU/mL).

The tests were made in sterile plastic microplates (Nunclon, Delta) containing 96 wells arranged in eight series from A to H, each one with 12 wells numbered 1 through 12. Each line corresponded to a strain of yeast and received 100 µL of the corresponding inoculum.

The extracts were diluted on DMSO (final concentration 5%, v/v; Synth) and were prepared more concentrated than the final concentration of the test (2000 µg/mL) and then diluted in the medium used (1:2).

In wells of columns 2 to 11, 100 µL of medium Muller Hinton Broth (MHB; Difco) was added and in column 12, 200 µL was added. 100 µL of drug (crude extracts) was added in the wells of columns 1 and 2. With the multichannel, a serial dilution was performed starting from the second column, homogenizing and removing 100 µL of the mixture (medium and drug) and adding to the well of the next column, the procedure was repeated the same way up the column 10, discarding the remaining 100 µL. Thus natural extracts suffered serial dilution at a ratio of 2 until dilutions of 1:1024. In other words, from the well 1 through 10, extracts concentrations ranged from 1.95 to 1000 µg/mL.

For each yeast the following controls were included: negative (only MHB); positive (MHB plus inoculum, without antifungal addition) and diluent (DMSO and inoculum). In each plate a strain of *Candida parapsilosis* (ATCC 22019) was included as reference yeast. Microplates mounted were incubated in stove at 35°C for 48 to 72 h with daily monitoring. After 48 h the reading of the FLU test was performed in microplate reader (Asys Hitech GmbH, Eugendorf/Áustria) and after 72 h that of the plant extracts was made through visual comparison, by mirror reflex (Shinobu-Mesquita et al., 2011).

The Minimum Inhibitory Concentration (MIC) was considered as the smallest concentration of the extract capable of inhibiting the growth each of yeast substantially (50%) with reference to its respective positive control (n = 3).

Minimum fungicidal concentration (MFC)

Aliquots of the MIC wells were transferred to Sabouraud Dextrose Agar (SDA) plates without the drug. The plates were incubated at 37°C for 48 h. The MFC was defined as the smallest concentration of the crude extract that was capable of preventing growth of yeasts.

RESULTS

Thirty crude extracts belonging to 10 plant species were assayed against four different yeasts (*Candida* spp.) and by measuring the inhibitory and fungicide activities. This preliminary evaluation was conducted with the aim of selecting plants whose extracts showed activity against *Candida* spp. of the 10 species tested against *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. krusei*.

In this study, almost all the extracts showed inhibition activity against at least one of the fungal strains evaluated. The crude extracts of *Eugenia uniflora*, *Caesalpinia ferrea*, *Psidium guajava* and *Persea americana* showed the highest antifungal efficiency, inhibiting the growth of all yeasts studied. These extracts showed better inhibitory activity against the non-*albicans* *Candida* species.

Concerning the 30 crude extracts, the most effective were whose obtained from *E. uniflora,* with MIC values ranging from 31.25 to 62.5 µg/mL against *C. albicans*, from 15.62 to 31.25 µg/mL against *C. dubliniensis*, and 15.62 µg/mL for both *C. glabrata* and *C. krusei*. Similar antifungal performance was observed for acetone:water extracts from both *C. ferrea* and *P. guajava* and *P. americana*. The results for this extract obtained from these three vegetal species showed MICs from 31.25 to 62.5 µg/mL against *C. albicans*. The MICs observed against *C. dubliniensis*, *C. glabrata* and *C. krusei* ranged between 15.62 and 62.5 µg/mL, suggesting the effectiveness of these plants against non-*albicans* species.

Extracts from *Piptadenia colubrina, Mimosa ophthalmocentra* and *Parapiptadenia rigida* showed an inhibitory activity highly variable, depending on the type of extract and also of *Candida* spp specie. However, the extracts (ethanol:water and acetone:water) of *P. colubrina* showed significant activity against *C. glabrata* and *C. krusei*. All extracts from *Schinus terebinthifolius* were effective only against *C. albicans* and *C. dubliniensis.* Finally, the extracts from *Hymenaea stigonocarpa* showed no important activity against any of the yeasts.

Using the FLU as reference drug, the minimum inhibitory concentration was determined in relation to strains studied, obtained as values 1, 2, 32 and 64 µg/mL, to *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. krusei*, respectively. Thus, compared to control strain used (*C. parapsilosis* ATCC 22019), the MIC of this strain always remained within the range recommended by CLSI document (2 µg/mL).

Important antifungal performance could be observed against both *C. glabrata* and *C. krusei*. The MICs for both non-*albicans Candida* species ranged from 15.62 to 62.5 µg/mL. Extracts (aqueous, ethanol:water and acetone:water) from leaves of *E. uniflora* were able to inhibit both yeasts at MIC of 15.62 µg/mL and MFC against *C. krusei* of 62.5 µg/mL, for the aqueous extract.

The extracts from *Mimosa tenuiflora* did not inhibit the growth of *C. albicans* at the highest concentration tested (1000 µg/mL). Therefore, the extracts from *M. tenuiflora* were omitted from further analyses with the other *Candida* spp. strains (Table 2).

Regarding fungicide activity, the results demonstrated, in general high values of MFC, apparently *C. albicans* and *C. glabrata* required the largest concentration of most of the crude extracts to die and not only inhibit. An exception was the extracts from *E. uniflora*, which showed MFCs between 125 and 500 µg/mL for *C. albicans*. The antifungal properties against *C. dubliniensis*, the extracts from *E. uniflora*, *C. ferrea*, and *P. guajava* showed MFCs between 62.5 and 125, 250 and 500, and 250 and 500 µg/mL, respectively. Finally, the aqueous extracts from *E. uniflora* performed best

Table 2. Minimal Inhibitory Concentration (µg/ml) of synthetic antifungal and active extracts against yeasts ATCC of *Candida* spp.

Specie	*C. albicans*			*C. dubliniensis*			*C. glabrata*			*C. krusei*		
	Aq	Et	Ac	Aq	Et	Ac	Aq	Et	Ac	Aq	Et	Ac
E. uniflora	31.25	62.50	31.25	31.25	31.25	15.62	15.62	15.62	15.62	15.62	15.62	15.62
C. ferrea	125.00	62.50	62.50	125.00	62.50	31.25	31.25	62.50	31.25	62.50	15.62	62.50
P. guajava	250.00	62.50	31.25	125.00	62.50	31.25	62.50	15.62	15.62	62.50	62.50	31.25
P. americana	125.00	62.50	31.25	62.50	250.00	15.62	62.50	62.50	31.25	62.50	62.50	62.50
P. colubrina	31.25	250.00	500.00	250.00	125.00	125.00	31.25	31.25	31.25	*	31.25	31.25
S. terebinthifolius	62.50	31.25	62.50	125.00	31.25	62.50	*	*	*	NA	NA	NA
M. ophthalmocentra	250.00	62.50	125.00	*	125.00	125.00	NA	125.00	*	NA	62.50	NA
P. rigida	1000.00	250.00	31.25	*	250.00	*	NA	62.50	NA	NA	62.50	NA
H. stigonocarpa	125.00	250.00	1000.00	250.00	125.00	*	*	125.00	NA	NA	*	NA
M. tenuiflora	*	*	*	NA	NA	NA	NA	NA	NA	NA	NA	NA
Fluconazole (FLU)		1.00			2.00			32.00			64.00	

Crude Extract: Aq - Aqueous, Et – Ethanol:water, Ac – Acetone:water; NA: Not Analyzed; *No inhibition.

Table 3. Minimal Fungicidal Concentration (µg/mL) of active extracts against yeasts ATCC of *Candida* spp.

Specie	*C. albicans*			*C. dubliniensis*			*C. glabrata*			*C. krusei*		
	Aq	Et	Ac	Aq	Et	Ac	Aq	Et	Ac	Aq	Et	Ac
E. uniflora	250	500	125	125	125	62.5	500	1000	500	62.5	125	250
C. ferrea	>1000	1000	>1000	500	250	500	>1000	>1000	>1000	250	1000	1000
P. guajava	>1000	>1000	>1000	500	250	250	>1000	1000	1000	1000	1000	250
P. americana	>1000	>1000	1000	>1000	500	250	>1000	500	250	>1000	>1000	>1000
P. colubrina	>1000	>1000	>1000	>1000	1000	>1000	1000	>1000	>1000	>1000	500	250
S. terebinthifolius	>1000	>1000	>1000	1000	500	>1000	>1000	>1000	>1000	NA	NA	NA
M. ophthalmocentra	>1000	>1000	>1000	>1000	500	>1000	>1000	1000	>1000	NA	500	NA
P. rigida	>1000	>1000	>1000	>1000	1000	>1000	NA	>1000	NA	NA	1000	NA
H. stigonocarpa	>1000	>1000	>1000	>1000	500	1000	NA	>1000	NA	NA	NA	NA
M. tenuiflora	>1000	>1000	>1000	NA	NA	NA	NA	NA	NA	NA	NA	NA

Crude Extract: Aq - Aqueous, Et – Ethanol:water, Ac – Acetone:water; NA: Not Analyzed.

against *C. krusei*, with an MFC of 62.5 µg/mL (Table 3).

DISCUSSION

Candida albicans is the predominant cause of invasive fungal infections from yeasts (Ruhnke, 2006; Horn et al., 2009). This is the species most frequently isolated from fungal infections. Its higher pathogenicity can be attributed to several sources such as virulence factors, which can make the yeast more resistant to conventional antifungal agents (Colombo and Guimarães, 2003; Magee and Magee, 2005; Tiraschi et al., 2007).

The epidemiology of yeast infections is rapidly evolving and non-*albicans Candida* species and other rare yeasts have emerged as major

opportunistic pathogens (Miceli et al., 2011). In recent years, the investigation of non-*albicans Candida* species has received special attention. Several species of this group are commonly associated with oral mucosa and are identified as commensals for a minority of healthy individuals (McManus et al., 2008).

The successful management of new and emerging resistant fungal pathogens of humans in the present and future depends on the discovery and/or development of new antifungal agents. The plant secondary metabolism is a natural source of a wide range of classes of substances with antimicrobial and antifungal properties, fostered by the intense pressure exerted by microbial pathogens in the environment (Silva et al., 2009).

In the past two decades, these species have become significantly more common agents of human infections and the clinical isolates are less sensitive to fluconazole and amphotericin B (Pemán et al., 2004; Barchieesi et al., 2005; Hakki et al., 2006; Li et al., 2007; Pfaller et al., 2008). Overall, the results of the assay for both *C. glabrata* and *C. krusei* were promising and may help to develop new strategies against both yeasts which are resistant to fluconazole.

According to the literature, the inhibitory activity of compounds or products can be classified as strong, moderate or weak, for MICs (µg/mL) up to 500; between 500, 1500 and above 1500, respectively (Aligiannis et al., 2001). End point criteria adopted in this study are different but it is possible to affirm that aqueous extract of *E. uniflora* was strong to die and not only inhibit all *Candida* species evaluated. This result is relevant, since fluconazole, which is a reference antifungal drug for candidiasis treatment is fungistatic and fungicide.

In spite of the lack of reports of antifungal activity of some species such as *C. ferrea* and *P. americana*, the same cannot be observed for *E. uniflora*, which has been the object of several studies for this biological activity. Regarding the extracts from *E. uniflora*, the differences among antifungal assay procedures as well as the extraction parameters such as the solvent (aqueous or hydroalcoholic mixtures) and/or extraction procedures (infusion, decoction or maceration) may explain the better antifungal performance found in this study, in comparison with previous studies (Schapoval et al., 1994; Holetz et al., 2002). As well know, the extractive conditions (procedure e solvents) plays an important role on the extract chemical spectrums, in special the polyphenolic compounds from leaves of *E. uniflora* such as hydrolysable tannins (Lee et al., 1997, 2000) and/or quercetin/ myricetin-glicosyl derivates (Rattman et al., 2012).

In addition to the above-mentioned differences in experimental conditions, several studies used the agar diffusion method, because of its simplicity and low cost. However, the dilution method is the recommended procedure for the quantitative assessment of these properties (Eloff, 1998; CLSI, 2008; Langfield et al., 2004). This study used the broth microdilution method, for a quantitative assessment of antimicrobial activity based on the value of MIC (CLSI, 2008).

The increases in the drug resistance as well as the several side effects from current antifungal therapy reinforce the importance and the potential of plant extracts and their secondary metabolites as alternatives for new, more effective and less toxic treatments. The natural products represent more safety to the consumer, because they have been considered at low risk for resistance development by pathogenic fungi and they represent a rich source of potential bioactive compounds (Tripathi et al., 2008).

Moreover, it is believed that it is difficult for the pathogens to develop resistance to such a mixture of plant extracts components with apparently different modes of action (Daferera et al., 2003).

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