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In vitro Antifungal Activity of *Irlbachia purpurascens*, *Lantana macrophylla* and *Kielmeyera neglecta* Extracts Against *Candida* Isolates Collected from Patients with Vulvovaginal Candidiasis

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ABSTRACT

Vulvovaginal Candidiasis (VVC) is one of the major infections that affect the female genital system, causing discomfort. The most commonly used antifungals bring severe adverse reactions and exposure to them favors the establishment of the phenomenon of resistance. Therefore, it is necessary to search for new compounds with potential antifungal, lower toxicity and cost. The Brazilian ecosystems have been investigated as to their natural potential and the Atlantic Forest has great wealth to be exploited in the search for antimicrobial substances. The aim of this work was to evaluate the *in vitro* antifungal activity of the Atlantic Forest native species *Irlbachia purpurascens* (Gentianaceae), *Lantana macrophylla* (Verbenaceae) and *Kielmeyera neglecta* (Clusiaceae) ethanolic extracts, against yeast of the *Candida* genus, isolated from the cervicovaginal region of women with VVC. To evaluate the antifungal activity of the extracts, agar diffusion and broth microdilution techniques were used. By broth microdilution technique, the three ethanolic extracts presented fungistatic antifungal activity. The activity of *K. neglecta* extract against *C. krusei* isolates was considered statistically significant and the decreasing order of activity of extracts was: *K. neglecta, L. macrophylla* and *I. purpurascens*. The antifungal activity detected on these plants species showed potential applicability for medicinal drugs development.

Key words: Agar diffusion test, antimicrobial activity, broth microdilution

INTRODUCTION

Vulvovaginal Candidiasis (VVC) is an infection caused by yeast belonging to the genus *Candida*, which are part of the normal human (Galban and Mariscal, 2006). The imbalance of the yeast-host interaction can lead to the establishment of infection through increased proliferation of the microorganism and stimulation of its virulence factors (Ribeiro *et al*., 2004). *Candida albicans* is the main causative agent but other species are also reported, such as *Candida glabrata*, *Candida krusei* and *Candida parapsilosis* (Rad *et al*., 2011). VVC patients may exhibit symptoms such as dysuria, edema, pruritus, leukorrhea, hyperemia of the vulva and vagina

(De Holanda *et al.*, 2007; Andrioli *et al.*, 2009). This infection is the second most common complaint in gynecological offices (Boatto *et al.*, 2007; Phillips, 2005). It is estimated that out of every 100 women, 75 of them will present a VVC episode at least once. Of these, 50% will face new occurrences throughout their lives (Mohanty *et al.*, 2007; Richter *et al.*, 2005) and 5% will experience reoccurring cases of such discomfort (Neves *et al.*, 2005; Sobel *et al.*, 2004).

For VVC treatment, the most commonly used antifungals cause undesirable adverse reactions and too much exposure to them triggers resistance phenomena (Chen and Sorrell, 2007), in addition to being burdensome. These problems make it necessary to search for new natural compounds with antifungal potential, combined with lower toxicity and reduced costs (Almeida *et al.*, 2008).

Plants have great potential for the search of antimicrobial compounds (Sousa *et al.*, 2012). The Atlantic Forest has a high biological importance due of the richness of its species and is considered a concentrated center of diversity of various groups of plants (Martini *et al.*, 2007). *Irlbachia purpurascens* (Aubl.) Maas (Gentianaceae), *Lantana macrophylla* Schauer (Verbenaceae) and *Kielmeyera neglecta* Saddi (Clusiaceae) are some of the plants in this biome which are belonging to the same taxonomic genus and/or family of the plants that have already demonstrated antifungal activity in previous studies (Aguiar *et al.*, 2008; Lu *et al.*, 1999; Silva *et al.*, 2009). Thus, with aim at finding alternatives and to broaden the array of therapeutic approaches against candidiasis, particularly against VVC, crude ethanol extracts of *I. purpurascens*, *L. macrophylla* and *K. neglecta* were investigated with regards to their antifungal potential against yeasts belonging to *Candida* genus.

MATERIALS AND METHODS

Plant material and extraction: Leaves of *K. neglecta* were collected at Km 10 of the Valença/Guaibim highway, Bahia, Brazil, in 2001, they were dried spontaneously, sprayed and 1285.0 g this dried material was submitted to extraction by successive and exhaustive maceration using the ethanol solvent until the complete submersion of the material; the crude ethanol extract of *K. neglecta* (65.9 g) was obtained by filtration through qualitative filter paper and evaporation of the solvent by a rotary evaporator. The leaves of *L. macrophylla* were collected at Km 02 of the road connecting Ilhéus/Olivença, Bahia, Brazil, in 2006, they were dried in a ventilated oven at 50°C for 8 h, ground to fine particles and this dried material (201.9 g) was submitted to 12 successive extractions with 1 L of ethanol solvent/each; the crude ethanol extract of *L. macrophylla* (35.7 g) was obtained by filtration through qualitative filter paper and evaporation of the solvent by a rotary evaporator. Finally, the *I. purpurascens* plant was collected at Km 15 of the Ituberá/Gandu highway, Bahia, Brazil, in 2002 and the entire plant was dried in a ventilated oven at 50°C for 8 h, ground to fine particles and 990 g this material was submitted to six successive extractions with ethanol solvent until its complete submersion and the crude ethanol extract of *I. purpurascens* (145.5 g) was obtained by filtration through qualitative filter paper and evaporation of the solvent by a rotary evaporator. The time of maceration for obtaining the extracts ranged from 24-48 h. Voucher specimens were deposited in the Herbarium of the State University of Santa Cruz and the botanical identification was performed by Luiz Alberto Mattos Silva. The ethanol crude extracts were stored at 2-8°C and used to determine antimicrobial activity.

Microorganisms: The microorganisms used in the bioassays were obtained from samples collected from the cervical-vaginal area of women attended in clinics and Health Centers in the cities of

Ilhéus and Itabuna, Bahia, Brazil. Patients were informed about the study and signed a Free and Informed Consent (CNS Resolution 196/96). The project was approved by the Ethics Committee of the Universidade Estadual de Santa Cruz, Ilhéus, Bahia, Brazil (Protocol 119/07). The isolates were identified through the chromogenic media CHROMagar-Candida (Probac, Brazil) and by morphological and biochemical tests of fermentation and carbohydrate assimilation, as described by Andrioli *et al.* (2009). The isolates were kept in sterile distilled water, as pure cultures and during the time period of the analysis, a share of suspensions was collected, inoculated into Sabouraud Dextrose agar 4% (SDA; Acumedia, USA) and incubated at 36±1°C for 24-48 h (Carrillo-Munoz *et al.*, 2005). The number of tested microorganisms varied between 80 and 148 isolates, depending of the analysis (agar diffusion or broth microdilution) and of the extract. For quality control, *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used, according to the recommendations of Norm CLSI M27-A2 (NCCLS, 2002).

Agar diffusion method: Determination of antifungal activity was achieved by using the agar diffusion test, according to De Lima *et al.* (2006), with adjustments. The yeasts were cultured at 36±1°C for 24-48 h and suspended in saline solution (0.85% NaCl), in order to standardize cell density to equivalent to 0.5 of the McFarland scale (1×10^6 a 5×10^6 cells mL^{-1}), using the Vitek turbidimeter (Vitek Colorimeter-BioMérieux). A sterile swab was submerged in the suspension and the microorganisms were evenly spread on a Petri dish containing 20 mL of SDA.

The ethanol crude extracts, at a final concentration of 100 mg mL^{-1} (100 mg of extract+0.5 mL of dimethyl sulfoxide-DMSO (Vetec, Brazil) +0.5 mL sterile distilled water), were sterilized by filtration (membrane 0.22 μm diameter) and applied 20 μL in wells (4.6 mm diameter) perforated in the agar. The negative control was represented by DMSO (50%) and the positive control by amphotericin B (Cristália, Brazil) at 10 μg mL^{-1} in DMSO solution at 0.6%. After 24-48 h of incubation at 36±1°C, the size of the inhibition zone was measured in millimeters (mm) and presented as the arithmetic mean of the triplicate.

Broth microdilution method: The determination of the Minimum Inhibitory Concentration (MIC) of crude ethanol extracts was made by using the yeast susceptibility test, according to the description of Norm M27-A2 (NCCLS, 2002), with adjustments. Depending on the extracts, 80 or 90 isolates were tested, on 96-well microplates, using the RPMI 1640 medium, with glutamine and without bicarbonate (Cultilab, Brazil), buffered with MOPS (USB, USA) 0.165 mol L^{-1} pH 7.0. The final concentrations of extracts in the plates on the final of the test ranged of 0.01-5 mg mL^{-1}. After standardizing the inoculums (cell density equivalent to 0.5 of the McFarland scale), the suspension was diluted in RPMI medium to a final concentration of microorganisms between 0.5 and 2.5×10^3 UFC mL^{-1} and concentration of DMSO to = 2.5%. The microplates were incubated at 36±1°C for 24±2 h and the reading of growth inhibition was measured by the oxidation reduction indicator: 2,3,5-triphenyltetrazolium chloride -TTC at 5 mg mL^{-1} (Nuclear, Brazil). The MIC was considered as the lowest extract concentration in which inhibition equal or above 50% was observed, in comparison with the growth control (100 μL of RPMI with 2.5% DMSO+100 μL of inoculum). Sterility control of the medium was also performed. The profile of antifungal activity of the extract against the isolates was classified according to Holetz *et al.* (2002), with adjustments of the limits between categories. The assays were performed in duplicate.

Statistical analysis: Tables of frequency were used for the univariate analysis and t-test was used to compare the different means of growth inhibition zones observed for extracts and positive control. p-values of less than 5% were considered to be statistically significant.

RESULTS

With the agar diffusion technique, all 112 isolates tested with extract of *I. purpurascens* revealed no susceptibility. With regards to the extract of *L. macrophylla*, the same amount of isolates was assessed and four (3.6%) presented growth inhibition zones, all these being *C. tropicalis*. The means of the zones observed for extract and positive controls (amphotericin B 10 µg mL^{-1}) of these isolates were 7.20±0.2 and 8.05±0.35 mm, respectively. The *K. neglecta* extract showed the best activity by agar diffusion technique (Table 1), where 134 (90.5%) of the 148 isolates tested showed growth inhibition zones, presenting a reduction in growth, which could be characteristic of a fungistatic action. This occurred especially in the antifungal activity against *C. krusei* isolates (Table 1). A comparison between the means of growth inhibition zones for extracts of *K. neglecta* and of the Positive Control (PC) against *C. krusei* isolates were considered statistically significant, with a diameter mean of 8.93 mm *versus* 6.20 mm, respectively ($p<0.05$).

The susceptibility tests using the broth microdilution method were also performed on three crude ethanol extracts and the results showed that all of them presented antifungal behavior, however at different activity levels. The decreasing order of activity of the plant extracts was: *K. neglecta*, *L. macrophylla* and *I. purpurascens*.

The crude ethanol extract of *K. neglecta* presented the best antifungal activity, because all isolates, independently of the *Candida* species, revealed antifungal features, at concentrations of 0.01-0.625 mg mL^{-1}. From a qualitative perspective (Holetz *et al*., 2002), this activity varies from good to moderate (Table 2).

For crude ethanol extract of *L. macrophylla*, most of the isolates revealed MIC at 0.625 mg mL^{-1}, with activities, worth noting, against *C. albicans*, *C. tropicalis* and *C. krusei*. The reduced number of isolates tested for species *C. parapsilosis* and *C. glabrata* did not allow further conclusions on the inhibition profile for both species.

Our study has shown that crude ethanol extract of *I. purpurascens* revealed little inhibitory activity against *Candida* species. The percentages of unsatisfactory antifungal activity (starting at 2.5 mg mL^{-1}) against *C. albicans*, *C. parapsilosis* and *C. tropicalis* were of 80.4, 80 and 90%, respectively.

Table 1: Antifungal activity by agar diffusion technique (mm) of *K. neglecta* (KN) ethanolic extract and amphotericin B against 148 *Candida* spp. vaginal isolates

Yeasts species	N	Inhibition (%)	KN (mm)	PC (10 µg mL^{-1}) (mm)
C. albicans	76	66 (86.84)	6.50±0.63	9.49±0.63
C. parapsilosis	5	3 (60.00)	4.23±0.41	7.62±0.50
C. tropicalis	48	46 (95.80)	7.32±0.46	9.01±0.92
C. glabrata	1	1 (100)	8.99±0.91	7.94±0.00
C. krusei mean	18	18 (100)	8.93±0.57	6.20±0.75
			7.20±0.20	8.05±0.35
Total	148	134 (90.5)		

N: No. of isolates, KN: *Kielmeyera neglecta*, PC: Positive control (amphotericin B)

Table 2: Antifungal profile by MIC of *Candida* spp. vaginal isolates against the extracts *K. neglecta* (KN), *I. purpurascens* (IP) and *L. macrophylla* (LM), with concentrations between 0.01 and 5 mg mL^{-1}

Activity		Good					Mod		Weak	Inactive			
Yeasts	N	0.01	0.02	0.04	0.08	0.156	0.313	0.625	1.25	2.5	5	R	Average MIC# (mg mL^{-1})
C. albicans													
KN	54	3	2	5	19*	16*	8	1	0	0	0	0	0.10
IP	46	0	0	0	0	0	0	2	7	10*	5	22	2.50
LM	54	0	0	0	0	1	10	40*	1	1	0	1	0.60
C. krusei													
KN	18	0	0	7*	9*	1	1	0	0	0	0	0	0.08
IP	18	0	0	0	0	0	6*	2	0	3	6*	1	2.40
LM	18	3	3	0	0	0	8*	3	1	0	0	0	0.30
C. tropicalis													
KN	18	3	1	2	3	6*	3	0	0	0	0	0	0.10
IP	10	0	0	0	0	0	0	0	1	1	0	8	1.90
LM	18	3	0	0	0	0	3	11*	1	0	0	0	0.50
C. parapsilosis													
KN	5	0	0	2	1	1	1	0	0	0	0	0	0.10
IP	5	0	0	0	0	0	0	1	0	0	1	3	2.80
LM	5	0	0	0	0	0	0	0	2	3	0	0	2.00
C. glabrata													
KN	1	1	0	0	0	0	0	0	0	0	0	0	0.01
IP	1	0	0	0	0	0	1	0	0	0	0	0	0.30
LM	1	1	0	0	0	0	0	0	0	0	0	0	0.01

KN: *Kielmeyera neglecta*, IP: *Irlbachia purpurascens*, LM: *Lantana macrophylla*, MOD: Moderate, R: Resistant cases with absence of inhibition, N: Total no. of samples, *Ethanolic plant extract concentration that inhibited a high number of isolates, #Samples that presented MIC

DISCUSSION

The VVC is an infection that has worried the medicine for its high incidence (Boatto *et al*., 2007). The microbial scenario has revealed an increasing of pathogenic microorganisms that are resistant to available antibiotics and anti-yeasts compounds. Consequently, research has turned its attention and investments in search of natural plant substances that are effective against infectious diseases (Duarte, 2006). The intention is to use secondary metabolites derived from plants as raw materials for the production of new drugs.

The extract of *K. neglecta* showed average MIC ranging between 0.01 and 0.1 mg mL^{-1} (Table 2), values which are considered promising when compared with the results of other studies, such as Pozzatti *et al*. (2008) that tested some essential oils from plants against fluconazole-resistant and susceptible *Candida* species and found mean values of MIC between 0.34-3.2 mg mL^{-1} and (Dota *et al*., 2011) that found in their study most yeasts isolated from VVC patients inhibited by 0.55 mg mL^{-1} of total phenol content of propolis ethanol extract. Hofling *et al*. (2011) tested twelve extracts of six medicinal plants against oral isolates of *C. albicans* and only the methanol extract of *Arrabidaea chica* showed MIC less than 0.5 mg mL^{-1} for 4% of the isolates tested. The extract of *L. macrophylla* presented average MIC between 0.01 and 2.0 mg mL^{-1} (Table 2), better result than that found by Patel and Coogan (2008) that evaluated the antifungal activity of the extract of *Dodonaea viscosa* against *C. albicans* from HIV-infected patients and found mean values of MIC ranging between 6.25 and 25 mg mL^{-1}.

Plants of *Kielmeyera* genus are usually used to treat numerous diseases, such schistosomiasis, malaria, bacterial and fungal infections (Cortez *et al*., 2003). Secondary metabolites extracted with dichloromethane from the leaves and stems of *K. coriacea* presented antifungal activity against *Cladosporium cucumerinum* phytopathogen (xanthone and phenolic compounds) and against *C. albicans* (xanthones) (Cortez *et al*., 1998). Silva *et al*. (2009) evaluated the antifungal potential of 57 extracts of medicinal plants of the Brazilian cerrado against *Trichophyton rubrum* and *Candida albicans*. However, only four of these extracts were active against *C. albicans*, among which the dichloromethane extract of *Kielmeyera coriaceae* roots. It is possible that xanthones present in *K. neglecta* are conferring antifungal activity presented by the extracts of this plant Sousa *et al*. (2012).

Aguiar *et al*. (2008) found anti-*C. albicans* activity of the chloroform, acetone and ethanol extracts of *Lippia alba* (Verbenaceae) roots by the disk diffusion technique and antifungal activity by the MIC determination for chloroform and acetone extracts was 2 mg mL^{-1}. However, MIC equal or above 1 mg mL^{-1} are considered unsatisfactory (Holetz *et al*., 2002). In this study, satisfactory results were obtained by microdilution technique for ethanol extracts of *L. macrophylla*, a plant that belongs to the same family of *L. alba*.

The *Irlbachia alata* was studied by Lu *et al*. (1999), who isolated and characterized structurally irlbacholin, a new substance that revealed significant antifungal activity against *C. albicans* and *Cryptococcus neoformans*. This substance was synthesized in laboratory and maintained similar antifungal characteristics of natural irlbacholin.

Rodriguez *et al*. (1995), based on a scanning of 25 species of the Gentianaceae family, found by thin-layer autobiography that the ethanol and dichloromethane extracts of *Swertia calycina* presented antifungal activity against *Cladosporium cucumerinum* and *C. albicans*. Aqueous and ethyl acetate extracts of *Chironia baccifera* (also belonging to the Gentianaceae family) presented anti-*C. albicans* activity at 5 mg mL^{-1} by broth microdilution technique (Thring *et al*., 2007).

Analyzing the methods used, the results obtained by the agar diffusion showed correlation with the results found through the broth microdilution but the latter was more sensitive, as observed by Klancnik *et al*. (2010), detecting the antifungal activity of the extract of *I. purpurascens*, which was not identified by the diffusion method. Thus, the broth dilution method was more efficient in search of extracts with antifungal activity in this study, which may be used as a source of substances for the treatment against yeasts of clinical importance.

The search for bioactive compounds can be guided by previous studies of genera or families of plants, encouraging the continuation of studies in the search of antifungal activity in fractions of extracts of these plants and isolation of the substances responsible for this specific action. Furthermore, *in vivo* data can help to define the potential applicability of these plant substances in the treatment of fungal diseases, particularly candidiasis.

CONCLUSION

About the methods for detection of antifungal activity, the broth dilution demonstrated more sensitivity compared to diffusion in agar. In this study, was possible to demonstrate the effectiveness of chemotaxonomic approach to choice of plant species to biological activities studies. The *I. purpurascens*, *L. macrophylla* and *K. neglecta* extracts showed antifungal activity against *Candida* species, which the last one was more effective, especially against *C. krusei*. These results demonstrate the potential applications of these plants to medicinal drugs development.

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