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Comparative study of disk diffusion and microdilution methods for evaluation of antifungal activity of natural compounds against medical yeasts Candida spp and Cryptococcus sp.

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ABSTRACT

Antifungal activity of natural products has been tested by adapting methods designed for synthetic drugs. In this study, two methods for the determination of antifungal activity of natural products, agar diffusion and broth microdilution, the CLSI reference methods for synthetic drugs, are compared and discussed. The microdilution method was more sensitive. The minimal inhibitory concentrations (MIC) of crude extracts, fractions and pure substances from different species of the plant families Piperaceae, Rubiaceae, Clusiaceae, Fabaceae and Lauraceae, from the Biota project, were determined. Antifungal activities against *Candida albicans*, *C.krusei*, *C.parapsilosis* and *Cryptococcus neoformans* were produced by several samples.

Keywords: natural products; antifungal activity; *Candida* sp; *Cryptococcus* sp.

INTRODUCTION

Medicinal plants are well-known natural sources of remedies, used in the treatment of innumerable diseases since antiquity. Plants are invaluable sources of pharmaceutical products and Brazil has supplied an incredible array of medicinal plants that has drawn the attention of ethnopharmacologists around the world. Many plants from various Brazilian biomes, such as the Cerrado (savannah-like), the Atlantic (uplands) and the Amazon (lowlands) rain-forests, have been used as natural medicines by the local population in the treatment of tropical diseases, including leishmaniasis, malaria, schistosomiasis, fungal and bacterial infections (Alves et al., 2000; Duarte et al., 2005).

Progress in methods of isolation and structure elucidation has led to an increase in the number of scientific publications dealing with the pharmacological examination of individual compounds of plant origin. Validation and selection of primary screening assays are pivotal to ensuring the sound selection of extracts or compounds with relevant pharmacological action and worth following up. Use of ethnopharmacological knowledge is one attractive way to reduce empiricism and enhance the probability of success in new drug-finding efforts (Patwardhan et al., 2005). Many articles on natural products claim to have discovered "exciting" antimicrobial activities, but show major failings in the methodologies.

Human fungal infections have increased at an alarming rate in the last 20 years, mainly among immunocompromised individuals (Perea & Patterson, 2002). New data indicate that the relative proportions of organisms causing nosocomial bloodstream infections have changed over the last decade, with *Candida* species now firmly established as one of the most frequent agents. Candidemia is not only associated with a high mortality but also extends the length of the hospital stay and increases the costs of medical care. Among human gastrointestinal tract isolates, 50-70% of total yeast isolates were identified as *Candida albicans*. Further frequent isolates are *C. tropicalis*, *C. parapsilosis*, and *C. glabrata*, while *C. kefyr* and *C. guilliermondii* are found occasionally (Barberino et al., 2006; Colombo et al., 2006).

Cryptococcus neoformans, encapsulated yeast, is the second most common cause of opportunistic fungal infection in patients with AIDS, but also can cause disease in normal hosts (Subramanian & Mathai, 2005). In fact, the clinical manifestations of this infection can range from an asymptomatic colonization of the respiratory tract to widespread dissemination, depending on the host immune response. When dissemination occurs, the central nervous system is commonly involved. Risk factors include: advanced HIV stage, use of corticosteroids, lymphomas, solid organ

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transplants and immunosuppressive diseases or drugs (Krick, 1981).

Routine antifungal drugs are essentially limited to the polyene amphotericin B (Wingard et al., 1999), and its various newer lipid complexes (Hiemenz & Walsh, 1996), the azole compounds such as fluconazole and itraconazole, and flucytosine (5-fluorocytosine). These agents suffer from a number of limitations that can lead to complications, for example, dose-limiting nephrotoxicity associated with amphotericin B, rapid development of resistance to flucytosine, drug-drug interactions, fungistatic but not fungicidal mode of action and resistance to the azoles. Thus, there is an urgent need for new antifungals with a broad fungicidal spectrum of action and with fewer dose-limiting side effects (Graybill, 1996; Maertens & Boogaerts, 2000).

The methods used to test natural compounds for antifungal activity are variable and each research group employs different types of test. The most commonly used are bio-autography (Cos et al., 2006), disk diffusion (Bartner et al., 1994; Gulluce et al., 2006), serial agar dilution (Passos et al., 2002; Souza et al., 2003; Cos et al., 2006) and serial dilution tests (Holetz et al., 2002). The general problems inherent in the antifungal screening of plant extracts have already been discussed by several authors (Rios et al., 1988; Hadacek & Greger, 2000). The antifungal tests are classified into three main groups, comprising diffusion, dilution and bio-autographic methods. Many laboratories have modified these methods for specific types of sample, such as essential oils and non-polar extracts, and these modifications make it impossible to compare results directly. Agar-based disk diffusion is widely used because of its simplicity and low cost. Absorbent disks or circular reservoirs containing various amounts of the substances to be examined are left in contact with an inoculated solid medium and the diameter of the clear zone around the disc or reservoir (inhibition diameter) is measured at the end of the incubation period and compared with standard drugs. There has been much research interest in agar-based testing of antifungal susceptibility *via* the disk-diffusion method, owing to its relative ease and the lack of need for specialized equipment (Rex et al., 2001).

The liquid-dilution method also allows determination of whether a compound or extract has fungicidal or fungistatic action at a particular concentration. The serial dilution test was reported to give the most reproducible results on the minimal inhibitory concentration (MIC) and was recommended as general standard methodology for the testing of natural products (Hadacek & Greger, 2000). Currently, the serial dilution methods recommended for testing commercial antifungal drugs for yeasts are M27-A2 (Clinical and Laboratory Standards Institute - CLSI, 2002) or EUCAST (European Committee on Antibiotic Susceptibility, 2002). Serial dilution techniques have been recommended for working with lipophilic compounds from natural products (Pauli & Kubeczka, 1996; Hadacek & Greger, 2000).

Microdilution technique is fully worked out, gives a direct comparison with the activity of antifungal drugs, and therefore appears to be appropriate for examining the anti-yeast properties of plant-derived compounds in general (Lee, 1999; Li et al., 2003; Larcher et al., 2004; Salgueiro et al., 2004). In our laboratory, the antifungal activity of a range of plant extracts and chromatographic fractions was evaluated by the disk-diffusion agar method and the microdilution method in accordance with M27-A2 (Clinical and Laboratory Standards Institute - CLSI, 2002) with modifications (European Committee on Antibiotic Susceptibility - EUCAST, 2002). In this study, comparative results between these two methodologies are reported.

MATERIALS AND METHODS

Plant material

Fractions from extracts of plants belonging to the families Apocynaceae and Verbenaceae were used to compare the two antifungals tests methods. Crude extracts, fractions and purified substances from plant species of the families Piperaceae, Rubiaceae, Clusiaceae, Fabaceae and Lauraceae from the FAPESP Biota project were then evaluated by the microdilution test. We used chromatographic procedures like high performance liquid chromatographic (HPLC) to isolate bioactive pure compounds or semipurified fractions.

Microorganisms used and growth conditions

The test organisms included *Candida albicans* ATCC 90028, *Candida krusei* ATCC 6258, *Candida parapsilosis* ATCC 22019 and *Cryptococcus neoformans* ATCC 90012. The microorganisms were obtained from the Mycology Laboratory of the Department of Clinical Analysis, Universidade Estadual Paulista (UNESP) at Araraquara (SP), Brazil. The yeasts were grown and maintained on Sabouraud-dextrose agar for 24h for *Candida* species and 48h for *Cryptococcus neoformans*, at room temperature.

Antimicrobial susceptibility testing

The antifungal activity tests were performed using agar diffusion and broth microdilution methods, described here.

Agar Diffusion Test

Agar diffusion was carried out with RPMI 1640 agar supplemented with glucose 2%. The inoculum was prepared using 24-hour plate cultures of *Candida* and *Cryptococcus* species. The colonies were suspended in 0.85% saline and the turbidity was compared with the 0.5 McFarland standard, to produce a yeast suspension of $1x10^6$ to $5x10^6$ cells/mL. The suspension was loaded on a sterile cotton swab that was rotated several times and press firmly against the

inside wall of the tube to remove excess inoculum from the swab. The dried surface of a RPMI agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated two more times, rotating the plate approximately 60^0 each time to ensure a uniform distribution of inoculum. Next, holes were bored in the agar, and the plant extracts were added at the concentration 250 g/mL, with a final volume of 50 µL. The plates were incubated at 35^0C for 24h and 48h, respectively, for *Candida* and *Cryptococcus* inocula. The zone diameter was read to the nearest whole millimeter at the point at which there is a sharp reduction in growth occurs (Clinical and Laboratory Standards Institute - CLSI, 2004). The control was amphotericin B at a concentration of 16 µg/ml.

Microdilution Test

The broth microdilution was performed as described in M27-A2 (CLSI) with modifications. The medium used was RPMI 1640 with L-glutamine buffered to pH 7.0 with 0.165 mol/L morpholinepropanesulfonic acid (MOPS), supplemented with 2% glucose.

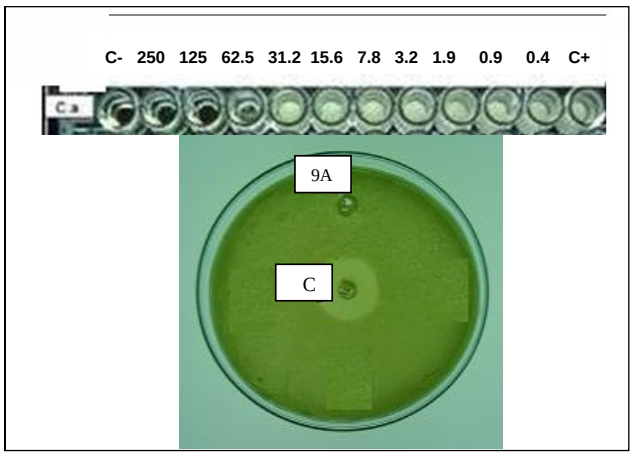
The plant extracts and fractions were prepared in DMSO and the correct volume was put in the first microplate well with RPMI medium, for the concentration of each natural compound to be 250µg/mL in that well. The cell suspension was prepared in 0.85% saline, with an optical density equivalent to 0.5 McFarland standard, and diluted 1:100 in RPMI to obtain a final concentration of 1×10^4 to 5×10^4 colony-forming units per milliliter (CFU/mL). This suspension was inoculated in each well of a microdilution plate previously prepared with the plant extracts and fractions to give concentrations from 250µg/mL down to 0.4µg/mL. The plates were incubated with agitation at 37°C for 24 h for *Candida* species and 48h for *Cryptococcus neoformans.*

The control drug for each ATCC strain was amphotericin B, diluted in DMSO; the concentrations tested ranged from 0.03 to 16 µg/mL. The minimum inhibitory concentration (MIC) for amphotericin B, determined by broth macrodilution, was defined as the lowest concentration of the drug completely inhibited the growth of the isolate. The MIC was defined as the lowest concentration at which the optical density (OD) was reduced to 90% of the OD in the growth control well as measured by spectrophotometer. For the plant extracts and fractions, the MIC was defined as the lowest concentration able to inhibit any visible fungal growth. Results were analyzed visually and spectrophotometrically.

Extracts displaying an MIC less than 75.0 µg/mL were considered to have strong antimicrobial activity, from 75.0 to 150.0 µg/mL, the antimicrobial activity was moderate, from 150.0 to 250.0 µg/mL, the antimicrobial activity was weak, and over 250.0 µg/mL, the extract was considered inactive.

RESULTS

The antifungal activity of the fractions of plants of the Apocynaceae and Verbenaceae families was evaluated by the disk diffusion agar and microdilution methods. Among the samples from Apocynaceae, fraction 9A alone showed activity and this only in the microdilution test, as shown in Figure 1.



C: Control with amphotericin B
C-: Negative control (Sterility control)
C+: Positive control (100% growth)

Figure 1. Comparative results of antifungal activity against *Candida albicans* of fraction 9A from family Apocynaceae by the agar diffusion and microdilution methods.

Additionally, seven fractions of Verbenaceae family showed moderate antifungal activity against *Candida krusei* and *Cryptococcus neoformans* by the microdilution test, but none of the fraction showed any activity up to 250 µg/mL by the diffusion method (Table 1). The diffusion agar method was thus less sensitive than the microdilution method in these antifungal tests.

Susceptibility tests, to determine the MIC to the *Candida* spp and *Cryptococcus neoformans*, were then made for 16 crude extracts, 27 fractions and 26 purified substances from plants of the families Piperaceae, Rubiaceae, Clusiaceae, Fabaceae and Lauraceae at concentrations starting from 250 µg/mL, which is the relevant range for research on substances for therapeutic purposes. Activity was demonstrated againt *Candida albicans*, in 43.7%, 40%, and 84.6%, respectively of the crude extracts, fractions and pure compounds. For *Candida krusei*, respectively 81.2%, 77.7% and 92.3% of the crude extracts, fractions and pure compounds showed antifungal activity. For *Candida parapsilosis*, 50%, 55% and 92.3% and for *Cryptococcus neoformans*, 93.7%, 74% and 88.4% of the crude extracts, fractions and pure compounds, respectively, were active. For *Candida albicans*, the best activity for crude extracts, with MIC values less than 75µg/mL, was found with the family Clusiaceae. An interesting results was obtained with Fabaceae plants: antifungal activity was higher after the chromatographic fractionation and pure substances presented activity to *C. albicans* (Table 2). For *Candida krusei*, the

crude extracts from family Clusiaceae, followed by Rubiaceae, showed the lowest MIC values for fractions. The family Fabaceae showed the lowest MIC for pure substances when tested against *C. krusei* (Table 3). On the other hand, crude extracts from Clusiaceae had the best activity against *Candida parapasilosis* followed by the Rubiaceae family. Once again, it is seen that for Fabaceae plants, the antifungal activity is higher with the pure substances (Table 4). Clusiaceae and Rubiaceae showed high percentages of crude extracts with activity against *Cryptococcus neoformans*. Fabaceae and Lauraceae pure substances also showed low MIC values with *Cryptococcus neoformans*. According to these data, the antifungal potentials of some of these fractions and crude extracts from plants are promising in the search for bioactive compounds (Table 5).

Table 1 - Comparative results of antifungal activity of seven fractions from plants of the Verbenaceae family against *Candida krusei* and *Cryptococcus neoformans* by the agar diffusion and microdilution methods.

Fraction of Extracts	*Candida krusei*		*Cryptococcus neoformans*	
	Agar diffusion µg/mL	Microdilution *MIC µg/mL	Agar diffusion µg/mL	Microdilution *MIC µg/mL
1V	**N.A.	125µg/mL	**N.A.	125µg/mL
2V	N.A.	125µg/mL	N.A.	125µg/mL
3V	N.A.	125µg/mL	N.A.	125µg/mL
4V	N.A.	62,5µg/mL	N.A.	125µg/mL
5V	N.A.	125µg/mL	N.A.	125µg/mL
6V	N.A.	125µg/mL	N.A.	125µg/mL
7V	N.A.	125µg/mL	N.A.	125µg/mL

*MIC: minimum inhibitory concentration
**N.A.: no activity at 250 µg/mL

Table 2 - MIC values in percentage of crude extracts, fractions and pure substances from plants of Piperaceae, Rubiaceae, Clusiaceae, Fabaceae and Lauraceae families against *Candida albicans*.

MIC*	**3.9-15.6 %**	**31.25-62.5 %**	**125-250 %**	**No activity %**
		Piperaceae¤ sp		
Crude extract (8)	-	12.5	12.5	75
Fraction of extract (17)	-	5.8	23.5	70
Pure substance (2)	-	-	-	100
		Rubiaceae¤ sp		
Crude extract (3)	-	-	66.6	33.4
Fraction of extract (6)	-	-	50	50
		Clusiaceae¤ sp		
Crude extract (4)	-	75	-	25%
		Fabaceae¤ sp		
Crude extract (1)	-	-		100
Fraction of extract (4)	-	-	75	25
Pure substance (4)	-	100	-	-
		Lauraceae¤ sp		
Pure substance(20)	-	-	90	10

¤ plant families
MIC*- minimum inhibitory concentration (µg/mL)

Table 3 - MIC values in percentage of crude extracts, fractions and pure substances from plants of Piperaceae, Rubiaceae, Clusiaceae, Fabaceae and Lauraceae families against *Candida krusei*.

MIC*	**3.9-15.6 %**	**31.25-62.5 %**	**125-250 %**	**No activity %**
		Piperaceae¤ sp		
Crude extract (8)	12.5	12.5	37.5	37.5
Fraction of extract (17)	11.7	11.7	52.9	23.5
Pure substance(2)	-	-	-	100
		Rubiaceae¤ sp		
Crude extract (3)	66.6	33.4	-	-
Fraction of extract (6)	33.3	16.6	33.3	16.6
		Clusiaceae¤ sp		
Crude extract (4)	75	-	25%	-
		Fabaceae¤ sp		
Crude extract (1)	-	-	100	-
Fraction of extract (4)	-	-	75	25
Pure substance (4)	-	50	50	-
		Lauraceae¤ sp		
Pure substance (20)	-	-	100	-

¤ plant families

MIC*- minimum inhibitory concentration (µg/mL)

Table 4 - MIC values in percentage of crude extracts, fractions and pure substances from plants of Piperaceae, Rubiaceae, Clusiaceae, Fabaceae and Lauraceae families against *Candida parapsilosis*.

MIC*	**3.9-15.6 %**	**31.25-62.5 %**	**125-250 %**	**No activity %**
		Piperaceae¤ sp		
Crude extract (8)	12.5	-	12.5	75
Fraction of extract (17)	-	11.7	58.8	29.4
Pure substance (2)	-	-	-	100
		Rubiaceae¤ sp		
Crude extract (3)	33.3	33.3	-	33.3
Fraction of extract (6)	16.6	33.3	-	49.9
		Clusiaceae¤ sp		
Crude extract (4)	50	25	25	-
		Fabaceae¤ sp		
Crude extract (1)	-	-	-	100
Fraction of extract (4)	-	-	-	100
Pure substance (4)	-	25	75	
		Lauraceae¤ sp		
Pure substance (20)	-	-	100	-

¤ plant families

MIC*- minimum inhibitory concentration (µg/mL)

Table 5 - MIC values in percentage of crude extracts, fractions and pure substances from plants of Piperaceae, Rubiaceae, Clusiaceae, Fabaceae and Clusiaceae families against *Cryptococcus neoformans*.

MIC*	3,9-15,6 %	31,25-62,5 %	125-250 %	No activity %
Piperaceae¤ sp				
Crude extract (8)	12.5	12.5	75	-
Fraction of extract (17)	11.7	11.7	47	29.6
Pure substance (2)	-	-	100	-
Rubiaceae¤ sp				
Crude extract (3)	66.6	33.3	-	-
Fraction of extract (6)	49.8	-	16.6	33.2
Clusiaceae¤ sp				
Crude extract (4)	75	-	-	25
Fabaceae¤ sp				
Crude extract (1)	-	100	-	-
Fraction of extract (4)	25	75	-	-
Pure substance (4)	75	25	-	-
Lauraceae¤ sp				
Pure substance (20)	85	-	-	15

¤ plant families

MIC*- minimum inhibitory concentration (µg/mL)

DISCUSSION

Historically, most of the drugs are derived from natural products. This country, due to its extensive territory, variety of climates and soils, is privileged in the biodiversity of native plants, as can be observed especially in the areas of the Atlantic Rain Forest and Brazilian Cerrado, affording conditions for great chemical diversity in the plants of these biomes. This study was conducted as part of our ongoing bioprospecting program, Biota-FAPESP, with the aim of discovering potential antifungal compounds that could be useful for the development of new tools for the control of infectious diseases.

Initially, in our laboratory, the antifungal activity of the extracts and fractions containing natural products was evaluated by the disk-diffusion agar method, which is appropriate because of its simplicity and low cost. The possibility of testing up to six extracts per plate against a single microrganism and the use of small sample volumes are specific advantages (Hadacek & Greger, 2000). The antimicrobial potency of different samples may not always be properly, mainly because of differences in physical properties, such as solubility, volatility and diffusion in agar. Compounds having a high diffusion coefficient or solubility and low antimicrobial activity may penetrate the agar rapidly, even in small amounts, and give zones like those of active compounds with poor penetration. This problem is encountered when zones of inhibition are compared for different classes of compounds. Additionally, size of inhibition zones might be influenced by volatilization of antimicrobial substances, disk size, amount of compound added to disk, adsorption by the disk, type of agar, agar strength, pH, volume of agar, and microbial strains used (Pauli, 2006). In our study, this method was not sufficiently sensitive for many extracts, in spite of the formation of the halo of inhibition with the control drug, amphotericin B.

In the dilution methods, each compound is mixed with an appropriate medium that has previously been inoculated with the fungal strain. In this method it is possible to determine the minimal inhibitory concentration (MIC), which is defined as the lowest concentration capable of inhibiting any visible fungal growth. Turbidity due to growth can be estimated visually or more accurately by measuring the optical density at 490nm. The liquid-dilution method can also be used to determine whether a compound or extract has a fungicidal or fungistatic action at a given concentration. This test yielded the most reproducible results for the MIC and was recommended as the general standard method for testing natural products (Hadacek & Greger, 2000). Currently, the serial dilution methods recommended for testing commercial antifungal drugs on yeasts are the M27-A2 (Clinical and Laboratory Standards Institute, 2002) and EUCAST (European Committee on Antibiotic Susceptibility, 2002) tests. The Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards [NCCLS]) originally developed a standardized broth macrodilution method M27-P (Clinical and Laboratory Standards Institute, 1992) to test *Candida* spp. and *Cryptococcus neoformans*, which greatly improved the reproducibility of antifungal susceptibility testing and serves as the "gold standard" reference method for all new methods. To increase the efficiency of testing large numbers of samples, a broth microdilution adaptation of the reference method was developed and evaluated and gives nearly identical results (Espinel-Ingroff et al., 1999; Arthington-Skaggs et al., 2002). In addition, the EUCAST (European Committee on Antibiotic Susceptibility, 2002) subcommittee has proposed the supplementation of the reference RPMI

1640 medium with 2% dextrose in order to obtain greater turbidity in the growth control well and thus shorten the MIC determination to 24 h (Espinel-Ingroff et al., 1999; Nguyen & Yu, 1999; Chryssanthou & Cuenca-Estrella, 2002; Morace et al., 2002). Subsequently, the CLSI approved a reference method for microdilution (M27-A- Clinical and Laboratory Standards Institute, 1997), updated in the current M27-A2 document (Clinical and Laboratory Standards Institute, 2002), for antifungal susceptibility testing of *Candida* spp. and *Cryptococcus* neoformans (Espinel-Ingroff et al., 2004), which provides 24 and 48 hours reference MICs for many agents. The methods for yeasts are appropriate for automation and use synthetic RPMI 1640 growth medium supplemented with 2% glucose, buffered to pH 7 with 3-(N-morpholino) propanesulfonic acid, an incubation time of 24 h, inoculum sizes of 0.5×10^5 to 2.5×10^5 colony-forming units per milliliter (CFU/mL) (Clinical and Laboratory Standards Institute, 2002) and/or 1-5 x 10^5 CFU/mL (European Committee On Antibiotic Susceptibility, 2002), flat-bottom microdilution trays, spectrophotometric reading, and dimethyl sulfoxide as solvent for hydrophobic compounds. However, several factors can affect both the run-to-run and the laboratory-to-laboratory variability of the test, including variations in the composition and pH of the test medium, variations in the preparation of antifungal drug dilutions, and the cell density of the inoculum (Revankar et al., 1998). Currently, the various laboratories that work with natural products use several methodologies, with several non-comparable parameters. There is a need to apply the same technique with the same parameters to achieve comparable conditions and results.

Plants of family Piperaceae afforded low activity against all the yeasts assayed. The antifungal activity of this family has been evaluated by several authors. Some fractions (in hexane, dichloromethane and ethyl acetate) and four pure compounds from *Piper solmsianum* were tested against 12 pathogenic fungi (De Campos et al., 2005). *Piper fulvescens* also had antifungal principles fractionated (Freixa et al., 2001). Novel benzoic acid derivatives from *Piper crassinervium, P. aduncum, P. hostmannianum* and *P. gaudichaudianum* were evaluated for antifungal activity against *Cladosporium cladosporioides* and *C. sphaerospermum* by bioautographic TLC assay and showed activity (Lago et al., 2004).

On the other hand, most of the crude and the fractionated extracts of Rubiaceae showed MIC values between 250 µg/mL and 3.9 µg/mL, indicating potential antifungal activity. The common madder *Rubia tinctorum*, exhibited antimicrobial activity against some Gram-positive and Gram-negative bacteria, yeasts, filamentous fungi and actinomycetes (Kalyoncu et al., 2006). Rubiaceae is a botanical family of great importance, to which more than 6,000 species belong. The bark of the tree *Hymenodictyon parvifolium* (Rubiaceae) is used in folk medicine as a remedy for skin diseases, venereal diseases and dysentery. This plant shows activity against *Trichophyton mentagrophytes, Trichophyton interdigitale, Microsporum gypseum, Epidermophyton floccosum* and *Candida albicans*. This activity supports the traditional use of this plant in the treatment of unspecified skin conditions (Kariba, 2002). Ethanol extracts of the coffee shrub, *Coffea arabica* (also a member of Rubiaceae) showed activity against *Candida albicans* isolates from vaginal candidiasis patients (Vaijayanthimala et al., 2000).

The Fabaceae extracts showed low activity to *Candida* spp, but appreciable activity to *Cryptococcus neoformans*, with MICs from 3.9 µg/mL to 15.6 µg/mL. Fabaceae is one of the biggest botanical families, also known as Leguminosae, of very wide geographic distribution. It contains approximately 18,000 species. Compounds from *Acacia mangium* and *A. auriculiformis* (Fabaceae) were examined regarding their antifungal activity, and *A. auriculiformis* showed better antifungal activity because it contained higher levels of flavonoids than *A. mangium* (Barry et al., 2005). Dried fruits of the neotropical shrub, *Caesalpinia pulcherrima* (Fabaceae) exhibited significant antifungal activity against *Candida albicans, Aspergillus niger* and *Rhizopus oligosporus* (Sudhakara et al., 2006).

Most of the species of the family Lauraceae showed antifungal activity. For *Candida* ssp, the MIC values were between 250 µg/mL and 125 µg/mL and for *Cryptococcus neoformans*, most of the MIC values were from 3.9 µg/mL up to 15.6 µg/mL. Lauraceae is an economically important family consisting mostly of trees. The genus *Cinnamomum* comprises about 250 species that are well distributed. The major constituent in *C. osmophloeum* leaves is eugenol. Leaf essential oils were analyzed for their chemical composition and antifungal activities, and most of the compounds assayed showed antifungal activity (Sen-Sung Chenga et al., 2006). The Lauraceae family comprises over 2500 species that occur within the subtropics and tropics of eastern Asia, South and North America. Most species possess aromatic roots, stems and fruits. The essential oils of *Aniba rosaeodora* (Amazon rosewood tree), *Laurus nobilis* (bay tree), Sassafras albidum and *Cinnamomum zeylanicum* (cinnamon) showed activity against *Aspergillus niger, A. flavus, A. terreus* (Simic et al., 2004).

The family Clusiaceae exhibited the best MIC values for *Candida krusei* and *Cryptococcus neoformans*, with values between 3.9 µg/mL and 15.6 µg/mL. Clusiaceae is a family of plants with more than a thousand species of trees and shrubs, of which about 400 belong to the St. John's wort genus, *Hypericum*. The essential oils of Hypericum scabrum, *Hypericum scabroides* and *Hypericum triquetrifolium* were studied for the first time for their antimicrobial activity against *Candida albicans* (Kizil G et al., 2004). The crude ethanol extract from the leaves of the Atlantic Forest plant *Tovomitopsis psychotriifolia* (Clusiaceae) exhibits antibacterial activity against *Bacillus cereus, Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Setzer et al., 1995).

Concluding this report, the microdilution method was more sensitive than disk diffusion and allowed us to

determine the MIC, the lowest concentration showing some microbial growth inhibition, crucial information for the research and production of new antifungal drugs.

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RESUMO

Estudo comparativo entre os métodos de difusão em disco e de microdiluição para avaliação de atividade antifúngica de produtos naturais contra as leveduras de interesse médico Candida *spp e* Cryptococcus *sp.*

Atividade antifúngica de produtos naturais foi determinada após algumas adaptações de métodos preconizados para fármacos sintéticos. Neste estudo foram comparados e discutidos os métodos para determinação de atividade antifúngica de produtos naturais por duas metodologias, difusão em ágar e microdiluição em caldo, segundo método preconizado pelo CLSI para fármacos sintéticos. A concentração mínima inibitória foi determinada de extratos brutos, frações e de substâncias puras de diferentes espécies de plantas das famílias Piperaceae, Rubiaceae, Clusiaceae, Fabaceae and Lauraceae do projeto Biota. Vários apresentaram atividade antifúngica para as leveduras *Candida albicans, C.krusei, C.parapsilosis* and *Cryptococcus neoformans*.

Palavras-Chave: produtos naturais; atividade antifúngica; *Candida* sp; *Cryptococcus* sp.

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