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Full Length Research Paper

Antifungal activity of bacterial strains from the rhizosphere of *Stachytarpheta crassifolia*

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This study evaluated the antifungal action of biomolecules produced from the secondary metabolism of bacterial strains found in the rhizosphere of semi arid plants against human pathogenic *Candida albicans.* Crude extracts were obtained using ethyl acetate as an organic solvent and the bioactivity was assessed with a bioautography technique. The results showed that bacterial strains, *Alcaligenes faecalis* MRbS12 and *Bacillus cereus* MRbS26, had compounds with antifungal bioactivity. The largest inhibition zones for both compounds were located on spots with Rf values below 0.500, indicating that the molecules possibly had polar characteristics. These results suggested that microorganisms found in the environment could have bioprospecting potential.

Key words: Biomolecules, bioautoghaphy, antifungal activity, *Alcaligenes faecalis*, *Bacillus cereus*, *Candida albicans*.

INTRODUCTION

In the last decade, increasingly invasive mycosis has become a public health problem. This increased incidence is related to human immunodeficiency virus (HIV), hematological malignancies, organ transplants and other immunosuppressive diseases. Fungal infections remain the major direct cause of death in patients who are treated for malignant diseases, where the emergence of fungal resistances is also a prominent issue. Immunocompromised patients are mainly infected by *Candida*, *Aspergillus, Cryptococcus* and other opportunistic fungi. *Candida albicans* is often associated with serious invasive fungal infections, but *Candida* species and yeast-like organisms (*Trichosporon*, *Blastoschizomyces* and *Malassezia*) have emerged as etiological agents in several mycoses (Anaissie, 1992; Carrillo-Muñoz et al., 2001; Ghannoum and Abu-Eltenn, 1990; Ghannoum and Rice, 1999; Kauffman, 2001)

Moreover, the treatment of fungal infections has been limited due to low drug efficacy, fungal resistance and pharmacological safety in regards to the existing pharmaceutical drugs in the market, such as amphotericin B, fluconazole and itraconazole, among others. These findings reinforce the growing need for bioprospection of new drugs with specific antifungal activity. Furthermore, increased demographic trends strongly suggest that the number of fungal infections are and will continue to increase, especially due to the aging of populations in developed countries (Barret, 2002; Carrillo-Muñoz, 2000; Odds, 2005; Anaissie, 1992).

It now appears that an enormous, relatively untapped source of microbial diversity is represented by microbes associated with plants. These microorganisms produce antimicrobial agents and seem to have unique genetic

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and biological systems that may have applications outside the host plants, in which they normally reside. Bioprospection of such organisms and their products represents a promising source in the discovery of new antifungal drugs, which could be applied in the pharmaceutical industry (Colwell, 1997; Demain, 2000; Strobel, 2003). This study was aimed to determine the antifungal activity against the human pathogen, *C. albicans* (CCMB 286), found in biomolecules obtained from bacterial strains of the rhizosphere of *Stachytarpheta crassifolia*, collected in Chapada Diamantina, Bahia, Brazil.

MATERIALS AND METHODS

Plants used and the rhizobacterial isolation

Two rhizobacterial strains, MRbS12 and MRbS26, were selected from a total of 32 strains previously isolated from *S. crassifolia* in Chapada Diamantina, Bahia, Brazil, (S13°00'06", W41°20'33.7"). These strains were subjected to *in vitro* screening to evaluate the antifungal activity of their secondary metabolites. The isolate was identified by sequencing the 16S rDNA region. Genomic DNA was extracted using the phenol-chloroform-isoamyl alcohol (25:24:1) protocol, as described by Sambrook et al. (2001). The following primers were used for PCR amplification of the 16S ribosomal DNA: fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg et al., 1991). The sequence obtained in this study was deposited to the GenBank nucleotide sequence database under the access number FJ959394 for *Alcaligenes faecalis* (MRbS12) and FJ959395 for *Bacillus cereus* (MRbS26).

Extraction procedure

To obtain the crude extract, an adapted protocol of Kilikian and Pessoa (2001) was used. The strains were inoculated in 1000 ml of nutrient broth, incubated at 37°C, at 180 rpm for 72 h and the supernatant was filter-sterilized by a vacuum filtration system (Sartorius of 0.45 µm milipore; Frankfurt, Germany). The filtrates were then liquid-liquid extracted in two-phase aqueous systems (SDFA) with the solvent ethyl acetate (PA, Vetec, Rio de Janeiro, Brazil) at a ratio of 1:1 (v/v). The organic phase was added to 5% anhydrous sodium sulphate (PA - Vetec, Rio de Janeiro, Brazil) and was concentrated in a rotary evaporator (Quimis, São Paulo, Brazil) at 45°C, under reduced pressure. The dried crude extract used as negative control, was obtained by extraction from the nutrient broth in ethyl acetate without the inoculum.

Antifungal activity

Thin-layer chromatography

This was performed to aid in the selection for the appropriate solvent system for the bioautography and also to provide relevant information of the chemical properties of the bacterial metabolites. Aluminum chromatoplate (TLC1, 5 x 8.0 in silica gel 60 F254-366, 0.25 mm thick - Merck, Darmstadt, Germany) was used during this procedure. Elution was performed with the use of solvent systems (mobile phase), ethyl acetate/hexane (7:3 v/v), and then were revealed under ultraviolet light (UV, 366 nm) for compounds, where visualization and resublimation was performed in an iodine chamber for qualitative analysis. The migration distance of the spots of the extracts was analyzed by calculating the retention rate (Rf).

Fungal test organisms

Initially, the experiment was performed with three fungal strains of *C. albicans* (CCMB242, CCMB 265 and CCMB286) for bioassay, however, the strain was selected and *C. albicans* CCMB286 devised its viability and performance. The fungal pathogens were obtained from the Culture Collection of Microorganisms of Bahia (CCMB), State University of the Feira de Santana, BA, Brazil. The strains were clinical isolates from the urine clinical sample and were maintained on Sabouraud dextrose agar (SAB, Himedia, Bombay, India).

Bioautography

The bioautography technique, using thin layer chromatography (TLC), was proposed by Romeiro (2001), who presented an adapted method to that of Hamburguer and Cordell (1987).

Petri dishes containing 2% water agar, were prepared and added to a chromatoplate with their extracts eluted. Then, Sabouraud dextrose agar was also added to the culture medium, which was composed of 0.8% 20 mg/ml^{-1} chloride 2,3,5-triphenyl-2H-tetrazolium (CTT) and 2.5 ml of the suspended cells from stain *C. albicans* CCMB 286, corresponding to the turbidity of 0.5 on the McFarland scale (equivalent to 1.5.106 CFU/ml) (McFarland, 1907), and scattered onto the chromatoplates. The plates were incubated at 37°C for 72 h. Zones of inhibition were then visualized by dehydrogenase activity detecting tetrazolium salt. Metabolically, fungal activity converts the tetrazolium salt into the corresponding intensely red colour. Thus, antifungal compounds appear as clear spots against a colored background. The results were obtained by bioautography, in conjunction with UV light visualization (wavelength, 366 nm), where the retention rate and the band width of the active compound inhibition zones (LB) were determined. All tests were performed in triplicate for each extract and the reference extract (crude extract of the medium without inoculum) was used as the negative control and Nystatin® was used as the standard control drugs for *C. albicans*.

RESULTS

The strains, MRbS12 and MRb26, demonstrated biomolecule production with antifungal activity against *C. albicans* CCMB286. Such strains were identified by partial sequencing of the 16S rRNA. Based on this gene fragment analysis, with approximately 1400 bp and similarity values of 99%, the following strains were identified: *A. faecalis* (MRbS12) and *B. cereus* (MRbS26).

Antifungal activity determination

The purpose of this research was to determine the antimicrobial biomolecule production, which presents biological control mechanisms against *C. albicans*. The presence of 11 spots was detected in the Ex12 extract, eluted in the ethyl acetate/hexane solvent system 9:1, and the Ex26 extract, eluted in the ethyl acetate/hexane 7:3 and with ten drops of methanol. All of them were visible under UV at 366 nm in the iodine chamber.

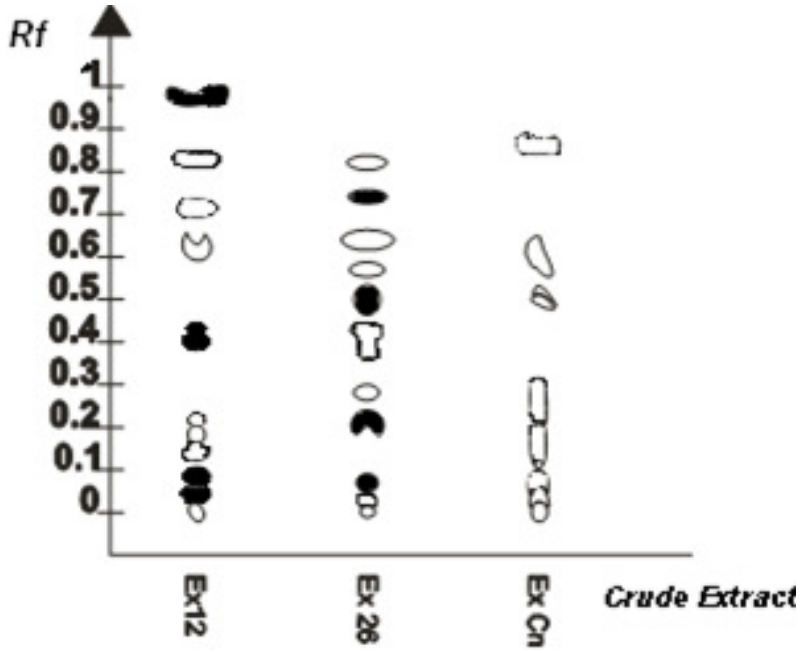

Figure 1. Summary of the distribution of compounds and fungal inhibition zones from the bacteria extracts applied to thin layer chromatography plates and developed in the extracts Ex12 (eluted in ethyl acetate/hexane 9:1), Ex18, Ex26 and ExCN (eluted in ethyl acetate/hexane 7:3 and 10 drops of methanol). Ultraviolet detection was at 366 nm. Compounds were indicated by circles at the Rf where they were detected. The active compounds against *C. albicans* are in black.

Antifugal spots analysis

Figure 1 shows the bioautography plate with inhibition zones and Figure 2 presents the summarized results of the TLC bioautography, where filled spots indicate the presence of antifungal compounds. The control extract from the non-inoculated NA showed no activity. In a chromatogram, the spots mean groups of the compounds are separated by differences in polarity. Spots next to the origin are the polar groups of the substances; whereas those closer to the end of the running eluent, are non-polar components (Burkhead et al., 1995; Aquino and Nunes, 2003).

In this study, the largest inhibition zones were on the spots below the Rf value of 0.500, indicating that the molecules with greater activity probably had polar characteristics and intermediate polarity. The inhibition zones of the extracts Ex12 of *A. faecalis* (MRbS12) were spots with Rf values of 0.043, 0.085 and 0.430; and the values of the Ex26 of *B. cereus* were 0.071, 0.214, 0.400 and 0.750 (Table 1). Only two antifungal compounds were detected above Rf 0.700. These compounds were present at Rf 0.970 for MRbS12, and at Rf 0.750 for MRbS26, according to the extraction conditions used, which was considered to have more nonpolar compound extracts. The control tests with solvent and extract from uninoculated nutrient broth showed no zones of inhibition. Although specific experiments related to the stability of the biomolecules have not been performed, it was observed that crude extract, when maintained by refrigeration (until approximately eight months at -4°C), kept its antifungal activity, demonstrating stability under these conditions. All antifungal compounds were associated with distinct spots, which had been detected under UV radiation.

DISCUSSION

Bioautography allows easy activity detection even in complex mixtures and is a method to detect antifungal activity on a chromatogram. This approach has wide application in the search for new antibiotics. The main purpose of the assay was to suspend microorganisms in a suitable broth to develop a TLC plate. Incubation in a humid atmosphere allows fungal inhibition zone growth that can then be viewed by dehydrogenase activity, using a tetrazolium salt reagent. Thus, antifungal compounds appear as clear spots against a colored background (Hamburguer and Cordell, 1987; Burkhead et al., 1995). In this study, such a technique was considered efficient and sensitive enough to determine the antifungal activity of the biomolecules obtained from rhizobacteria, collected from the *S. crassifolia* rizosphere. It was possible to observe the activity of the different substances separated

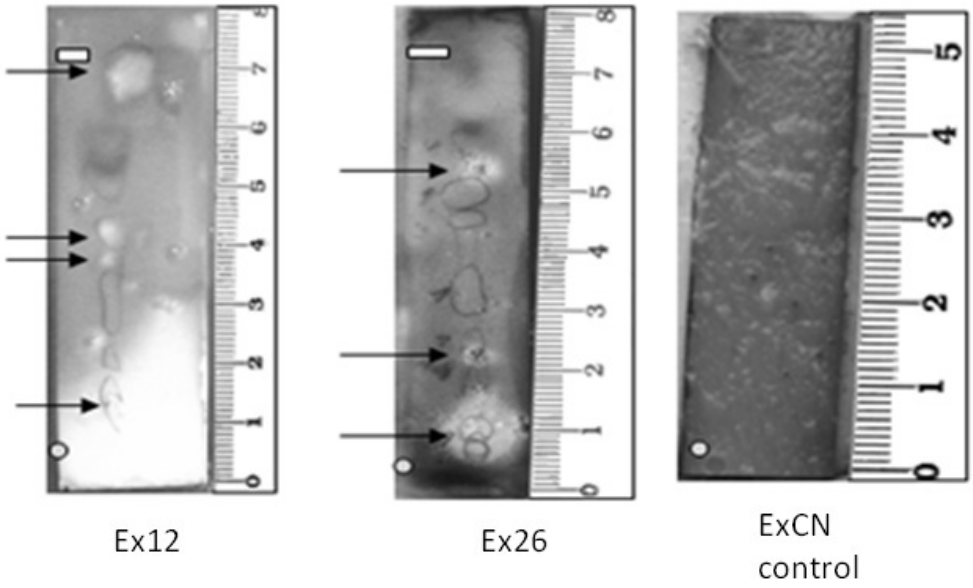

Figure 2. Section from bioautography plates of the extracts from the selected rhizobacteria to determine the antifungal activity. The clear areas correspond to the cell growth inhibition halos of *Candida albicans*.

Table 1. Crude extracts with antifungal activity, their Rfs and the extent of WA inhibition area in mm.

Crude extract	Rf with activity	WA (mm)	Concentration(mg/10 µl)
Ex12	0.043; 0.085*	8	4.75
	0.400	2	
	0.430	2	
	0.970	4	
Ex18	-	-	3.48
Ex26	0.071; 0.214*	10	4.18
	0.400	2	
	0.740	3	
ExCn	-	-	5.75

*Rfs comprising the same inhibition zone; (-) lack of inhibition; ExCn, negative control.

from the original extract on a single chromatographic plate, even with compounds of low concentration. As indicated in the research by Homans and Fuchs (1970), Hamburguer and Cordell (1987) and Magalhães et al. (2007), bioautography is a technique for easy application and fast detection of antifungal compounds obtained from secondary metabolites of microorganisms and plants, especially against *Aspergillus niger*, *Colletotrichum linde*, *Fusarium cingulata* and *C. albicans*.

According to the study by Bacic and Yoch (2008), filtrate from strains of *A. faecalis*, isolated from a swamp substrate, presented antimicrobial activity against both Gram-negative and positive bacteria. Honda et al. (1998) found antifungal activity against *Fusarium oxysporum* in the eluted *A. faecalis*, isolated from the rhizosphere, which corroborates with the findings of this study. Other authors such as Tripathi et al. (2000) and Melo (2002), showed that the strain of this species also produces compounds that can be of industrial use in the production of D-aminocyclases, semi-synthetic antibiotics (penicillin and cephalosporin), hormones (lutein), bioactive peptides and chemical pesticides. Also, the species can synthesize compounds with antagonistic action against plant pathogen nematodes (Tian et al., 2007). The research by Idris et al. (2007) demonstrated the antifungal acti-vities of *B. cereus* filtrate, obtained from the rhizosphere of

Sorghum bicolor, against *Fusarium oxysporum*. Milner et al. (1996) observed that strains of *Bacillus* have compounds with antifungal properties. Other authors reported that *B. cereus* significantly influences the microbial community of the rhizosphere by stimulating the growth of other bacteria that promote plant growth, as well as acting against plant pathogens. These species can be used as biological controls (Lambert et al., 1987; Kumar et al., 2002; Fortes et al., 2006). Kishore and Pande (2007) tested the antifungal activity of *B. cereus* against *Botrytis cinerea in vitro* and *in vivo*, using cellular suspension, and concluded that the compound exhibited antifungal activity. Later, the compound was recognized as chitinase, which degraded the fungal cell wall. The strains, *A. faecalis* (MRbS12) and *B. cereus* (MRbS26), isolated from the rhizosphere of *S. crassifolia*, were able to produce compounds with antifungal properties against *C. albicans* CCMB286. The results obtained in this study showed that secondary metabolites from rhizobacteria had antifungal potential and can be considered as promising sources of bioprospection to the pharmaceutical industry. Moreover, this work is the first scientific report for the secondary metabolites of bacterial strains associated with plants from the Brazilian semi-arid region.

REFERENCES

Anaissie E (1992). Opportunistic Mycoses in the Immunocompromised Host: Experience at a Cancer Center and Review. Clinic. Infec. Dis.14(1): 43-53.

Aquino Neto FR, Nunes DSS (2003). Cromatografia: Princípios Básicos e Técnicas Afins. Interc. Rio de Janeiro. p. 187.

Bacic MK, Yoch DC (2008). Antibiotic composition from Alcaligenes species and method for making and using the same. US Patent, p. 6224863.

Burkhead KD, Schisler DA, Slininger PJ (1995). Bioautography shows antibiotic production by soil bacterial isolates antagonistic to fungal dry rot of potatoes. S. Biol. Bioch. 27(12): 1611-1616.

Carrillo-Muñoz AJ, Brió S, Quindós G (2001). Una nueva generación de fármacos antifúngicos. Rev. Ibero. Micol. 18: 2-5.

Colwell RR (1997). Microbial diversity: the importance of exploration and conservation. J. Indust. Microbiol. Biotech. 18: 302-307.

Demain AL (2000). Small bugs, big business: The economic power of microbe. Biol. Adv. Oxford. 18: 499-514.

Fortes TO, Alviano DS, Tupinambá G, Padrón TS, Antoniolli AR, Alviano CS, Seldin L (2006). Production of an antimicrobial substance against Cryptococcus neoformans by Paenibacillus brasilensis Sa3 isolated from the rhizosphere of Kalanchoe brasiliensis. Microbiol. Res. 16: 1-8.

Ghannoum MA, Abu-Eltenn KH (1990). Pathogenicity determinants of Candida. Myc. 33: 265-282.

Ghannoum MA, Rice LB (1999). Antifungal Agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin. Microbi. Rev. 12(4): 501-517.

Hamburguer MO, Cordell GA (1987). A direct bioautographic TLC assay for compounds possessing antibacterial activity. J. Nat. Prod. 50(1): 19-22.

Homans AL, Fuchs A (1970). Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. J. Chromat. 51: 325-327.

Honda N, Hirai M, Ano T, Shoda M (1998). Antifungal effect of a heterotrophic nitrifier Alcaligenes faecalis. Biotech. Lett. 20(7): 703-705.

Idris HA, Labuschagne N, Korsten L (2007). Screening rhizobacterial for biological control of Fusarium root and crow rot of sorghum in Ethiopia. Biol. Cont. 40: 97-106.

Kauffman CA (2001). Fungal infections in older adults. Clinic. Infect. Dis. 33(4): 550-555.

Kilikian BV, Pessoa JR (2001). Purificação de Produtos biotecnológicos. In: Schmildell, W, Borzani W, Lima UA, Aquarone E. (Ed). Biotect. Ind. São Paulo: Edgar Blucher LTDA, p. 254.

Kishore GK, Pande S (2007). Chitin-supplemented foliar application of chitinolytic Bacillus cereus reduces severity of Botrytis gray mold disease in chickpea under controlled conditions. J. Lett. Appl. Microbiol. 44: 98-105.

Kumar NR, Thirumalai VA, Gunasekara P (2002). Genotypung of antifungal compounds procuving plant grow-promotion rhizobacteria, Pseudomonas fluorescens. Curr. Sci. 82: 12.

Lambet B, Leyns F, Van Rooyen L, Gossel F, Papon Y, Swings J (1987). Rhizobacteria of Maize and Their Antifungal Activities. App. Eviron. Microb. pp. 1866-1871.

Magalhães AF, Tozzi AMGA, Magalhães EG, Sannomiya M, Soriano MDPC, Perez MAF (2007). Flavonoids of Lonchocarpus montanus A.M.G. Azevedo and biological activity. An. Acad. Bras. Cienc. 79(3): 351-367.

McFarland J (1907). The nephelometer: an instrument for estimating the members of bacteria in suspension used for calculating the opsonic index and vaccines. J.A. Med. Assoc. 49: 1176.

Melo IS (2002). Recursos genéticos microbianos. In: Melo, IS; Valadare – Inglis, MC; Nass, LL; Valois, ACC. Recursos Genéticos e Melhoramento. Jaguariúna, EMBRAPA Meio Ambiente, p. 743.

Milner JL, Stohl EA, Handelsman E, Zwittermicin A (1996). Resistence gene from Bacillus cereus. J. Bacterial, 178(4): 4266-4272.

Odds FC (2005). Genomics, molecular targets and the discovery of antifungal drugs. Rev. Ibero. Micol. 22: 229-237.

Romeiro RS (2001). Métodos em bacteriologia de plantas. ed. UFV: Viçosa. p. 279.

Sambrook J, Maniatis T, Frisch EF (2001). Molecular cloning: A laboratory Manual. New York: Cold Spring Harbour. Lab. Press. p. 545.

Strobel G, Daisy B (2003). Bioprospecting for Microbial Endophytes and Their Natural Products. Microb. Mol. Biol. Rev. 67(4): 491-502.

Tian B, Yang J, Zhang KQ (2007). Bacteria used in the biological control of plant-parasitic nematodes: populations, mechanism of action, and future prospects. FEMS Microb. Ecol. 61: 197-213.

Tripathi CKM, Bihari V, Tyag RD (2000). Microbial production of D-amino acids. Proc. Bioch. 35: 1247-1251.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991). 16S Ribossomal DNA Amplication for Ohylogenetic Study. J. Bacterial. 173:2: 697-703.