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Growth Inhibition Effect of Brazilian Cerrado Plant Extracts on *Candida* Species

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Abstract

Ethanol extracts from leaves of *Annona crassiflora* and *A. coriacea*, and the fruits of *Solanum lycocarpum* and *S. grandiflorum* were investigated against 52 strains of *Candida albicans*, four strains of *C. tropicalis* and three strains of *C. krusei* isolated from human immunodeficiency virus-infected individuals with oropharyngeal candidosis, using the agar dilution method. Among the extracts tested, leaves of *A. crassiflora* was active against all the microorganisms and showed the greatest antifungal activity based on the MIC values. It was observed that 57 strains (96%) were inhibited by the extract from *A. crassiflora* at a concentration of 64 µg/ml, whereas against 18 strains (30%), it exhibited MIC values as low as 0.5 µg/ml against 10^6 UFC/ml organisms. *Candida albicans* CBS 562, a reference strain used as a control, showed a similar inhibition pattern. The activities of fluconazole, itraconazole, and ketoconazole were also evaluated and afforded MIC values ≥ 32 µg/ml (19.5% of all strains), ≥ 64 µg/ml (13.6%), and ≥ 128 µg/ml (32.2%), respectively.

Keywords: *Annona coriacea*, *Annona crassiflora*, *Solanum grandiflorum*, *Solanum lycocarpum*, *Candida* species, ethanol extracts, antifungal activity.

Introduction

The importance of infections due to *Candida* species, particularly *Candida albicans* and *Candida tropicalis*, associated with acquired immune deficiency syndrome (AIDS) have increased dramatically in the last two decades (Greene, 1990). Unfortunately, despite the recent development of several new antifungal agents, treatment of immunocompromised patients, who frequently develop opportunistic systemic and superficial mycosis (Li et al., 1995), such as candidiasis and cryptococcosis, is generally unsatisfactory. Problems with toxicity, emergence of resistance strains, and lack of fungicidal activity frequently compromises effective therapy with existing antifungal agents (McCutchen et al., 1994; Nwosu & Okafor, 1995). For these reasons, it is important to have new chemotherapy approaches for the treatment of patients with common and rare fungal infectious diseases (Janssen & Cawenbergh, 1990). Extracts and natural products of plant origin offer a wide variety of bioactive compounds that could meet the aforesaid requirements (Shu, 1998). In our effort to screen antifungal extracts of native plants from Brazilian Cerrado, we evaluated the anticandidiasis activity of plants used in traditional medicine for several purposes including antimicrobial effects. The aim of this study was to test the antifungal activity of Brazilian Cerrado plants *Annona* and *Solanum* species against 52 strains of *Candida albicans*, four strains of *C. tropicalis* and three strains of *C. krusei* isolated from human immunodeficiency virus-infected individuals with oropharyngeal candidosis.

Materials and Methods

Plant material

Leaves of *Annona crassiflora* Mart. (Annonaceae) and *A. coriacea* Mart. were collected in the city of Paraúna, whereas fruits of *Solanum lycocarpum* St. Hil. (Solanaceae) were col-

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lected at the outskirts of the city of Goiânia in May 1997, and *Solanum grandiflorum* Ruiz et Pav. at National Ema's Park in December 1995, Goiás State, Brazil. Samples were authenticated by Professor Heleno D. Ferreira, and voucher specimens are deposited in the Herbarium of Universidade Federal de Goiás.

Preparation of extracts

Air-dried and powdered plant materials were extracted three times with 95% ethanol at room temperature. *Solanum* the fruits were previously subjected to a treatment with petrol ether in a Soxhlet apparatus, in order to remove lipids. The alcohol solutions were filtered and then concentrated under reduced pressure, below 40 °C, to dryness.

Microorganisms

All the 52 strains of *Candida albicans*, *C. tropicalis* (4 strains) and *C. krusei* (3 strains) used in this study were clinical isolates from human immunodeficiency virus-infected patients with oropharyngeal candidosis, at the Hospital of Tropical Diseases, Goiás State, Brazil. *Candida albicans* CBS 562 was kindly provided by Dr. Claudete R. de Paula, Department of Microbiology, University of São Paulo, and used as a reference strain with know sensitivity to various antifungal agents. The stock cultures were maintained on Sabouraud dextrose agar medium (Difco, USA) at 25 °C.

Drugs

Ketoconazole and itraconazole (Johnson & Johnson, USA) and fluconazole (Pfizer, Belgium) were suspended in sterile physiological Tris buffer (pH 7.4, 0.05 M) and included in assays as positive controls. All other chemicals were purchased from Sigma Chemical Co. (USA).

Antifungal testing

Candida strains were grown on Yeast Nitrogen Base Phosphate (YNBP) agar plates (Difco, USA). The Alves and Cury (1992) dilution method for MIC determination was employed. The inoculum suspensions were prepared from 6 h broth cultures diluted in 5 ml of sterile physiological Tris buffer (pH 7.4, 0.05 M) and adjusted to an OD (530 nm) of 0.05, equivalent to 1×10^6 UFC/ml. Susceptibility assays were performed initially with *C. albicans* (8 strains), *C. tropicalis* (2 strains) and *C. krusei* (2 strains), and each extract (100 mg) was dissolved in 1 ml of dimethylsulphoxide (DMSO) and serially 10-fold diluted in YNBP medium to obtain a concentration range of 62.5–1000 µg/ml. Fungal suspensions (3 µL) were inoculated on the surfaces using a steer's replicator, and plates (diameter: 25 cm) were incubated at 37 °C for 24 h. A second experiment was performed with all *Candida* strains on the concentration range of 0.25–128 µg/ml (Table 1). The MIC was defined as the lowest concentration able to inhibit any visible fungal growth (Table 2). Cultures containing only DMSO diluted in the same conditions, which did not influence fungal growth, were used as controls. The sensitivity of all strains to ketoconazole, itra-

Table 1. In vitro antifungal activity of *Annona crassiflora* leaf extract against 59 strains of *Candida* isolated from HIV-infected patients with oropharyngeal candidosis.

Minimal inhibitory concentration (µg/ml)	No. of strains	(%)
>128	1	1.7
128	1	1.7
64	7	11.9
32	3	5.0
16	2	3.4
8	4	6.8
4	4	6.8
2	10	17.0
1	9	15.2
0.5	11	18.6
0.25	7	11.9
15	*C. albicans* CBS 562	

Table 2. Minimal inhibitory concentration (µg/ml) of *Annona crassiflora* leaf extract and therapeutic drugs against 59 strains of *Candida* [*C. albicans* (52), *C. tropicalis* (4), *C. krusei* (3)] isolated from HIV-infected patients with oropharyngeal candidosis.

	MIC_{50}				MIC_{90}			
		Therapeutic drugs				Therapeutic drugs		
Candida species (strains)	*Annona crassiflora*	KT	FL	IT	*Annona crassiflora*	KT	FL	IT
C. albicans (52)	2.0	16.0	8.0	8.0	64.0	128.0	64.0	64.0
C. tropicalis (4)	0.25	8.0	128.0	8.0	64.0	8.0	128.0	8.0
C. krusei (3)	0.5	16.0	128.0	8.0	64.0	128.0	128.0	8.0

KT: Ketoconazole; FL: Fluconazole; IT: Itraconazole.

conazole and fluconazole was tested using the same techniques. All antifungal assays were tested in duplicate and the experiment performed four times on different days.

Results and Discussion

The initial antifungal screening afforded the *A. crassiflora* leaf extract as the only sample active against randomised 12 yeast strains of *Candida* isolates from mucosa surface of human HIV-infected patients. Despite the fact that the glycoalkaloids of *Solanum* fruits may act as effective antifungal defences against various plant pathogens (Cipollini & Levey, 1997; Mohamed et al., 1996), dermatophytes (Caceres et al., 1991) and opportunistic fungi (He et al., 1994), the fruits of *S. lycocarpum* and *S. grandiflorum* in addition of the leaves extract from *A. coriacea*, at concentrations up to 1000 µg/ml, did not show antifungal activity against any of the strains tested. In contrast, the ethanol extract of *A. crassiflora* leaves was active against nearly all leading the AIDS-related *Candida* species. At concentrations up to 62.5 µg/ml, the extract suppressed the yeast formation.

The above results prompted us to investigate the minimum inhibitory concentration (MIC) against all fungal strains (Table 1). It is important to note that 98.3% of *Candida* isolates were inhibited with this extract at concentration up to ≤ 128 µg/ml. A majority (62.7%) of fungal strains was inhibited at 2 µg/ml. Potency of the extract was stronger against 11.9% of the strains isolated. The sensitivity of the same fungal isolates to commonly used therapeutic drugs was tested. As shown in Table 2, 32.2% of the strains were highly resistant to ketoconazole, with MIC ≥ 128 µg/ml needed for inhibition, whereas 19.5% and 13.6% of the all strains were resistant to fluconazole and itraconale (MICs ≥ 32 µg/ml and ≥ 64 µg/ml, respectively). More impressively, the ethanol extract demonstrated better activity than the therapeutic drugs in all the *Candida* species. The antifungal screening revealed that *C. tropicalis* strains was more sensity than *C. krusei* and *C. albicans* (Table 2). Their MIC values for the ethanol extract were 0.25, 0.5 and 2.0 µg/ml, respectively, whereas the same MIC_{90} (64 µg/ml) as obtained for the *Candida* species.

Fungal infections are the primary cause of mortality in patients with severely impaired host defence mechanisms (Kulberg, 1997). The increase of the AIDS-related fungal opportunistic pathogens and the emergence of resistance strains in recent years has lent additional urgency to antifungal studies. For example, resistance to fluconazole is emerging in *C. albicans*, the major agent of oropharyngeal candidosis in AIDS patients, after long-term suppressive therapy (Hernáez et al., 1997). Botanical species with a long tradition of medical use have been an important source of new antimicrobial agents (Cordell, 1993; Freixa et al., 1998), but because the infecting microorganisms tested are rarely the same as those infecting higher animals, there are no compelling reasons to suppose that plant antiinfective agents would be active against human or veterinary pathogens. In the present study, we demonstrate that crude *A. crassiflora* leaf extract has antifungal activity against 59 strains of *Candida* clinical isolates. Previously, the essential oil from *A. crassiflora* was reported to possess antifungal activity against dermatophytes (Lima et al., 1992). It was found that the cytotoxic ethanolic extract from seeds of this same plant showed a non-specific inhibitory effect on drug-induced contractions of guinea-pig ileum (Weinber et al., 1993); in addition, the extract contains phenylalkylamines (Santos et al., 1996a) and polyketides with significant activities against human tumor cell lines (Santos et al., 1996b). Polyketides from the *Annona* genus have a broad range of potential biological roles, for example, cytotoxic, antitumor, pesticidal, antimicrobial and antiparasitic activities. Studies were mainly carried out with the seed, bark, and root, while little is know of the distribution in leaves (Zafra-Polo et al., 1998). Our preliminary chemical analysis has indicated a high concentration of polyphenols (proanthocyanidins) in *A. crassiflora* leaves, but not in *A. coriacea* leaf extract, indicating that the antifungal activity may be due to the presence of polyketides or polyphenols either individually, or in combination. Bioassay-directed fractionation of the crude extract is in progress to isolate and identify the compounds responsible for the antifungal activity.

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