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Article

Antimicrobial activity of *Pterogyne nitens* Tul., Fabaceae, against opportunistic fungi

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RESUMO: "Atividade antimicrobiana de *Pterogyne nitens* Tul., Fabaceae, contra fungos oportunistas". No contexto de nossas pesquisas por novos agentes antifúngicos obtidos da flora brasileira, oito extratos e doze frações de *Pterogyne nitens* Tul., Fabaceae, foram submetidos ao ensaio antifúngico pelo método de microdiluição, contra quatro espécies de fungos oportunistas, *Candida albicans, Candida krusei, Candida parapsilosis* e *Cryptococcus neoformans*. Este trabalho revelou que os extratos e frações de *P. nitens* foram mais ativos contra *C. krusei* e *C. parapsilosis* quando comparados a *C. neoformans,* sendo que o crescimento de *C. albicans* foi moderadamente afetado por todos os extratos e frações. As atividades mais potentes foram observadas para as frações *n*-butanólica dos galhos (CIM = 15,6 µg/mL) e raízes (CIM = 31,2 µg/mL) contra *C. krusei*. Adicionalmente, a fração *n*-butanólica dos galhos foi submetida ao fracionamento cromatográfico, resultando no isolamento de quatro alcaloides guanidínicos, sendo *N-1,N-2,N-3*-tri-isopentenilguanidina (**1**), descrito pela primeira vez em espécies da família Fabaceae e nitensidinas **A-C** (**2-4**), os quais apresentaram atividade antifúngica moderada contra *C. krusei* (CIM = 62,5 µg/mL) e *C. parapsilosis* (CIM = 31,2 µg/mL).

Unitermos: *Pterogyne nitens,* alcaloides guanidínicos, antifúngico, fungos oportunistas, *Candida* spp., *Cryptococcus neoformans.*

ABSTRACT: As part of our ongoing research on antifungal agents from Brazilian flora, eight extracts and twelve fractions from *Pterogyne nitens* Tul., Fabaceae, were screened for antimicrobial activity against four opportunistic fungi species (*Candida albicans, Candida krusei, Candida parapsilosis* and *Cryptococcus neoformans*) using a broth microdilution method. The present investigation reveals that *P. nitens* extracts and fractions were more effective against *C. krusei* and *C. parapsilosis* than against *C. neoformans*. The growth of *C. albicans* was moderately affected by all tested extracts and fractions. The strongest effects were observed for *n*-butanol fractions from branches (MIC = 15.6 µg/mL) and roots (MIC = 31.2 µg/mL) against *C. krusei.* Additionally, the chromatographic fractionation of the *n*-butanol fraction from branches afforded four guanidine alkaloids; *N-1,N-2,N-3*-triisopentenylguanidine (**1**), described for the first time in the Fabaceae family, and nitensidines **A-C (2-4)**, which showed moderate activity towards *C. krusei* (MIC = 62.5 µg/mL) and *C. parapsilosis* (MIC = 31.2 µg/mL).

Keywords: *Pterogyne nitens,* guanidine alkaloids, antifungal, opportunistic fungi, *Candida* spp.*, Cryptococcus neoformans.*

INTRODUCTION

The clinical relevance of opportunistic fungal infections have increased at an alarming rate in the second half of the twentyth century, including individuals infected with HIV, transplant recipients and patients with cancer (Clark & Hajjeh, 2002; Hage et al., 2002). The most frequent causing agents of such infections are *Candida, Aspergillus* species, *Cryptococcus neoformans, Mucor* and some unusual genera such as *Fusarium, Trichosporon,*

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and *Scedosporium* (Guarro et al., 2006; Nucci & Anaissie, 2007). Furthermore, the most common treatment for these infectious diseases involves azoles and polyene macrolides, which are limited in their spectrum, and presenting severe side effects (Helmerhorst et al., 1999). For this reason, there is a need for new antifungal compounds which can serve as lead compounds for further development in antifungal chemotherapy. In this way, plants have been proven to be a rich source of antimicrobial agents (Vieira et al., 2005, Alves et al., 2006; Dias et al., 2006; Sena Filho et al., 2006; Oliveira et al., 2007; Batista-Júnior et al., 2008; Cardoso-Lopes et al., 2008; Lopes et al., 2008; Tempone et al., 2008; Bertucci et al., 2009; Costa et al., 2009; Regasini et al., 2009a; Silva-Júnior et al., 2009).

Pterogyne nitens Tul., Fabaceae, is popularly named in South America as "tipa", "yvi-raró", "cocal", "amendoinzeiro", "bálsamo" and "amendoim-bravo" according to the region where this plant grows. It is a sole beautiful legume tree from *Pterogyne* genus, which is distributed mainly in Brazil, Bolivia, Paraguay and Argentina, reaching 5-12 m height (Burkart, 1952; Lorenzi, 1998). Cold aqueous preparations using stem barks has been used as folk medicine for treating of ascaridiasis (Crivos, 2007). Previous chemical and biological studies have demonstrated the presence of guanidine alkaloids, which exhibited cytotoxic activity against to human cancer cell lines (Bolzani et al., 1995; Ferreira et al., 2009; Regasini et al., 2007, 2009b,c) and phenolics with myeloperoxidase inhibitory and radical scavenging activities (Fernandes et al., 2008; Regasini et al., 2008a,b,c; Souza et al., 2009; Souza et al., 2010).

Thus, the aim of this investigation was to evaluate the antimicrobial activity of extracts and fractions from branches, green fruits, roots and stem barks and isolated terpene guanidine alkaloids from *P. nitens* against four opportunistic human yeasts (*Candida albicans, C. krusei, C. parapsilosis* and *Cryptococcus neoformans*), by using broth microdilution test.

MATERIAL AND METHODS

Plant material

Roots, branches, green fruits and stem barks of *Pterogyne nitens* Tul., Fabaceae, were collected in the Botanic Garden of São Paulo (São Paulo, Brazil) by Ph.D. Maria C. M. Young in January 2005 and identified by Ph.D. Inês Cordeiro (IBt-SMA). A voucher specimen (SP 204319b) was deposited at the State Herbarium "Maria Eneida Kaufmann" of Institute of Botany (São Paulo, Brazil).

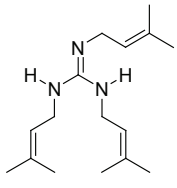
Extraction

The powdered shade-dried plant material was extracted three times (72 h) by maceration with hexane and, subsequently with ethanol. The solvent was removed under reduced pressure by rotary evaporation yielding a thick syrup. The crude ethanol extracts were further reconstituted in methanol-water (7:3) and then partitioned with ethyl acetate and *n*-butanol, successively. Samples from hexane and ethanol extracts, ethyl acetate, *n*-butanol and lyophilized hydroalcoholic fractions, were submitted to antimicrobial tests.

Isolation of antifungal compounds 1-4

The *n*-butanol fraction (2.5 g) from the ethanol extract of branches was subjected to gel permeation column chromatography using Sephadex LH-20, eluted with methanol to afford nine fractions (B1-B9), which were combined after comparison of their TLC profile [chloroform:methanol (9:1)] revealed with Dragendorff´s reagent. The alkaloidal fraction (B2, 587 mg) was subjected to reversed-phase C-18 silica gel CC eluted with acetonitrile:water gradient that afforded eight fractions (ALK1-ALK8). Purification of fraction ALK5 (121 mg) by repeated CC over silica gel (230-400 mesh) eluted with chloroform:methanol in a gradient ranging from 0 to 35% methanol, furnished *N-1,N-2,N-3*-triisopentenylguanidine (**1**, 7 mg) and nitensidine B (**3**, 12 mg). Fraction ALK-8 (88 mg) was subject to preparative TLC developed with ch loroform:methanol:triethylamine (87:12.5:0.5), developed three times to yield nitensidine A (**2**, 7 mg) and nitensidine C (**4**, 3 mg).

The molecular structures of these guanidine alkaloids were identified through ^{1}H NMR, ^{13}C NMR and MS data. The spectral data were in agreement with those published in the literature (Bolzani et al., 1995; Conegero et al., 2003).



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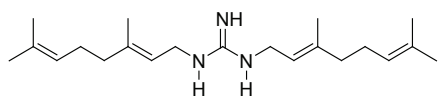

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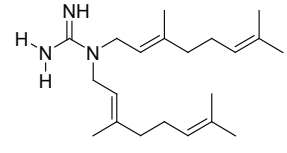

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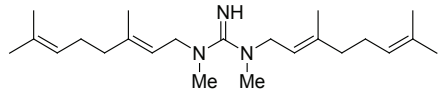

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Microorganisms and growth conditions

The test organisms *C. albicans* ATCC 90028, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019 and *C. neoformans* ATCC 90012 were originally obtained from the Mycology Laboratory of the Department of Clinical Analysis, School of Pharmaceutical Sciences at São Paulo State University (UNESP). The yeasts were grown and maintained on Sabouraud-dextrose agar for 24 to 48 h at room temperature.

Antimicrobial susceptibility testing

The antifungal activity tests were performed using broth microdilution method as described in the M27-A2 document of Clinical and Laboratory Standards Institute (CLSI) with modifications (Rodriguez-Tudela et al., 2003). The medium used was RPMI 1640 with L-glutamine buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS), supplemented with 2% glucose. Samples were diluted in DMSO and each well of a 96-well U-bottomed culture plate were added 100 µL culture together with 100 µL of 2-fold serial diluted test compound. The cell suspension was prepared in 0.85% saline with an optical density equivalent to McFarland 0.5 and diluted 1:100 in RPMI for the final concentration to be 1 x 10^5 to 5 x 10^5 CFU/mL. This suspension was inoculated on the microdilution plate previously prepared with the extracts, fractions and compounds **1-4** diluted at concentrations ranging from 1000 to 0.48 µg/mL. The plates were incubated with agitation at 37 °C for 24 h for *Candida* spp. and 48 h for *C. neoformans*. Amphotericin B and fluconazole were used as positive controls exhibiting a MIC value ranging from 2.00 to 0.06 and 64.00 to 2.00 µg/mL (Table 1).

The MIC values were calculated as the lowest concentration of the test samples which shows complete growth inhibition of the fungi strains. Results were analyzed visually and spectrophotometrically. All assays were performed in triplicate. The extracts and fractions showing a MIC lower than 100, ranging from 100-500, 500-1000 and over 1000 µg/mL were considered potent, moderated, weak and not active, respectively (Holetz et al., 2002).

Minimum fungicidal concentration (MFC)

All tested samples in antifungal study showing MIC<1000 µg/mL, were transferred to plates of Sabouraud-dextrose agar. The plates were incubated at 35 °C for 48 h (yeast). The MFC was defined as the lowest concentration of the extract that did not permit any visible fungal colony growth on the appropriate agar plate after the period of incubation. All assays were performed in triplicate.

RESULTS

In this work, eight extracts and twelve fractions as well as four guanidine alkaloids (**1-4**) were tested at concentrations ranging from 1000 to 0.48 µg/mL against four human opportunistic yeasts (*C. albicans, C. krusei, C. parapsilosis* and *C. neoformans*). These results were summarized in Table 1.

All extracts and fractions from *Pterogyne nitens* Tul., Fabaceae, were weakly active against the yeast *C. albicans* with MIC values ranging from 1000 to 250 µg/mL, which were considered moderate to weak according to Holetz et al. (2002). The hexane extract from roots was not active to *C. albicans* and *C. neoformans* (MIC>1000 µg/mL). The extracts and fractions from roots and green fruits demonstrated moderate activity against *C. krusei,* except the *n*-butanol fraction from roots, which presented strong activity (MIC 31.2 µg/mL) against this yeast. Additionally, branches and stem barks exhibited potent activity against *C. krusei* with MIC values ranging from 15.6 to 62.5 µg/mL and the best result was observed for the *n*-butanol fraction from branches (MIC = 15.6 µg/mL). Furthermore the hexane extracts and hydroalcoholic fractions from branches and stem barks showed moderate to weak activity against this yeast. All the samples had weak to moderate activity against *C. parapsilosis* and *C. neoformans*, except for the ethanol extract, ethyl acetate, and *n*-butanol fractions from stem barks, which exhibited potent inhibition of *C. parapsilosis* (MIC = 62.5 µg/mL).

Compounds **1-4** isolated from *n*-butanol fraction of branches from *P. nitens* inhibits the growth of opportunistic fungi with MIC values of 62.5 and 31.2 µg/mL against *C. krusei* and *C. parapsilosis*, respectively.

Additionally, minimal fungicidal concentration (MFC) of all extracts, fractions and guanidine alkaloids (**1-4**) were also evaluated against the four yeasts and showed MFC values higher than 1000 µg/mL, indicating a fungistatic behavior.

DISCUSSION

Several Fabaceae species have been evaluated for their antifungal properties (Scorzoni et al., 2007) among them *Pterogyne nitens* Tul., Fabaceae, a typical leguminous plant from Brazilian flora has been studied for antimicrobial activity. Previous biological studies have demonstrated that extracts from the barks of *P. nitens* did not show antibacterial activity (Salvat et al., 2001).

The bioactivity presented for compounds **1-4** indicated that antifungal activity observed in the extracts and fractions may be at least partially due to guanidine alkaloids. It was also observed that the strongest antifungal effects of the tested samples were on strains of *C. krusei* and *C. parapsilosis* when compared with *C. albicans* and *C. neoformans.*

P. nitens and isolated compounds displayed

Table 1. *In vitro* antifungal activity (MIC in µg/mL) of extracts, fractions and isolated compounds **1-4** from *Pterogyne nitens*.

Plant part or guanidine alkaloids	Type extract or Fraction tested	Ca[a]	Ck[b]	Cp[c]	Cn[d]
Roots					
	hexane	>1000	500	500	>1000
	ethanol	1000	250	1000	1000
	ethyl acetate	250	125	250	250
	n-butanol	250	31.2	250	250
	hydroalcoholic	500	500	500	500
Green fruits					
	hexane	500	250	250	500
	ethanol	250	250	125	250
	ethyl acetate	500	125	250	500
	n-butanol	1000	1000	500	1000
	hydroalcoholic	500	250	250	500
Branches					
	hexane	1000	500	500	1000
	ethanol	250	62.5	125	250
	ethyl acetate	250	62.5	125	250
	n-butanol	250	15.6	125	250
	hydroalcoholic	250	250	250	250
Stem barks					
	hexane	250	125	500	250
	ethanol	500	62.5	62.5	500
	ethyl acetate	250	62.5	62.5	250
	n-butanol	250	62.5	62.5	250
	hydroalcoholic	1000	125	500	1000
N-1,N-2,N-3-triisopentenylguanidine (1)	–	250	62.5	31.2	250
nitensidine A (2)	–	250	62.5	31.2	250
nitensidine B (3)	–	250	62.5	31.2	250
nitensidine C (4)	–	250	62.5	31.2	250
amphotericin B[e]		2.00	2.00	1.00	0.06
fluconazole[e]		2.00	64.0	8.00	4.00

[a] Ca = *Candida albicans*; [b] Ck = *Candida krusei*; [c] Cp = *Candida parapsilosis*; [d] Cn=*Cryptococcus neoformans*; [e] positive controls.

activity against *C. krusei,* which is very significant because this yeast has natural resistance against commercial drug fluconazole (Rex et al., 1995), suggesting its potential application for treating *C. krusei* infections, considering a possible mode of action different from those azole drugs.

The current study suggests that *P. nitens* have antimicrobial activity against opportunistic yeasts. Therefore, it could be an important source of antifungal compounds which can be useful for developing new lead compounds, including compound **1**, which was described for the first time in the Fabaceae family. Further chemical and pharmacological investigations are thus needed in order to isolate and identify additional constituents and establish the potential mechanisms of action.

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